CANCER IMMUNOTHERAPY BY DISRUPTING PD-l/PD-Ll SIGNALING

This application claims the benefit of U.S. Provisional Patent Application No. 61/647,442, filed May 15, 2012, and U.S. Provisional Patent Application No. 61/790,747, filed March 15, 2013, the contents of which are hereby incorporated herein by reference in their entirety.

Throughout this application, various other publications are referenced in parentheses by author name and date, or by Patent No. or Patent Publication No. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated in their entireties by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein. However, the citation of a reference herein should not be construed as an acknowledgement that such reference is prior art to the present invention.

FIELD OF THE INVENTION

This invention relates to methods for immunotherapy of a cancer patient comprising administering to the patient antibodies that disrupt the PD-l/PD-Ll signaling pathway. A biomarker may be used as part of this treatment for identifying suitable patients for immunotherapy and for predicting the efficacy of anti-PD-1 treatment.

BACKGROUND OF THE INVENTION

Human cancers harbor numerous genetic and epigenetic alterations, generating neoantigens potentially recognizable by the immune system (Sjoblom *et al.,* 2006). The adaptive immune system, comprised of T and B lymphocytes, has powerful anti-cancer potential, with a broad capacity and exquisite specificity to respond to diverse tumor antigens. Further, the immune system demonstrates considerable plasticity and a memory component. The successful harnessing of all these attributes of the adaptive immune system would make immunotherapy unique among all cancer treatment modalities. However, although an endogenous immune response to cancer is observed in preclinical models and patients, this response is ineffective, and established cancers are viewed as "self and tolerated by the immune system. Contributing to this state of tolerance, tumors may exploit several distinct mechanisms to actively subvert anti-tumor immunity. These mechanisms include dysfunctional T-cell signaling (Mizoguchi *etal,* 1992), suppressive regulatory cells (Facciabene *et al,* 2012), and the co-opting of endogenous "immune

checkpoints," which serve to down-modulate the intensity of adaptive immune responses and protect normal tissues from collateral damage, by tumors to evade immune destruction (Topalian *et al.,* 20\\; Mellman *et al.,* 201 1).

Until recently, cancer immunotherapy had focused substantial effort on approaches that enhance anti-tumor immune responses by adoptive-transfer of activated effector cells, immunization against relevant antigens, or providing non-specific immune-stimulatory agents such as cytokines. In the past decade, however, intensive efforts to develop specific immune checkpoint pathway inhibitors have begun to provide new immunotherapeutic approaches for treating cancer, including the development of an antibody (Ab), ipilimumab (YERVOY®), that binds to and inhibits Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) for the treatment of patients with advanced melanoma (Hodi *et al.,* 2010) and, as described herein, the development of Abs that block the inhibitory PD- 1 pathway.

Programmed Death- 1 (PD-1) is a key immune checkpoint receptor expressed by activated T and B cells and mediates immunosuppression. PD-1 is a member of the CD28 family of receptors, which includes CD28, CTLA-4, ICOS, PD-1, and BTLA. Two cell surface glycoprotein ligands for PD-1 have been identified, Programmed Death Ligand-1 (PD-L1) and Programmed Death Ligand-2 (PD-L2), that are expressed on antigen-presenting cells as well as many human cancers and have been shown to downregulate T cell activation and cytokine secretion upon binding to PD-1 (Freeman *et al.,* 2000; Latchman *et al,* 2001). Unlike CTLA-4, PD-1 primarily functions in peripheral tissues where activated T-cells may encounter the immunosuppressive PD-L1 (B7-H1) and PD-L2 (B7-DC) ligands expressed by tumor and/or stromal cells (Flies *et al,* 201 1; Topalian *et al.,* 2012a). Inhibition of the PD-l/PD-Ll interaction mediates potent antitumor activity in preclinical models (U.S. Patent Nos. 8,008,449 and 7,943,743), and the use of Ab inhibitors of the PD-l/PD-Ll interaction for treating cancer has entered clinical trials (Brahmer *et al.,* 2010; Topalian *et al.,* 2012b; Brahmer *et al.,* 2012; Flies *et al.,* 201 1; Pardoll, 2012; Hamid and Carvajal, 2013).

The promise of the emerging field of personalized medicine is that advances in pharmacogenomics will increasing be used to tailor therapeutics to defined sub-populations, and ultimately, individual patients in order to enhance efficacy and minimize adverse effects. Recent successes include, for example, the development of imatinib mesylate (GLEEVEC®), a protein tyrosine kinase inhibitor that inhibits the bcr-abl

tyrosine kinase, to treat Philadelphia chromosome-positive chronic myelogenous leukemia (CML); crizotinib (XALKORI®) to treat the 5% of patients with late-stage non-small cell lung cancers who express a mutant anaplastic lymphoma kinase (ALK) gene; and vemurafenib (ZELBORAF®), an inhibitor of mutated B-RAF protein (V600E-BRAF) which is expressed in around half of melanoma tumors. However, unlike the clinical development of small molecule agents that target discrete activating mutations found in select cancer populations, a particular challenge in cancer immunotherapy has been the identification of mechanism-based predictive biomarkers to enable patient selection and guide on-treatment management. Advances in validating PD-L1 expression as a biomarker for screening patients for anti-PD-1 immunotherapy are described herein.

SUMMARY OF THE INVENTION

The present disclosure provides a method for immunotherapy of a subject afflicted with cancer, which method comprises administering to the subject a composition comprising a therapeutically effective amount of an agent that disrupts, reduces or suppresses signaling from an inhibitory immunoregulator. In preferred embodiments, the agent is an Ab. In other preferred embodiments, the inhibitory immunoregulator is a component of the PD-1/PD-L1 signaling pathway. In further preferred embodiments, the Ab disrupts the interaction between PD-1 and PD-L1. In certain embodiments, the Ab is an anti-PD-1 Ab of the invention or an anti-PD-L1 Ab of the invention. In preferred embodiments, the anti-PD-1 Ab of the invention is nivolumab (BMS-936558) and the anti-PD-L1 Ab of the invention is BMS-936559. In certain embodiments, the subject has been pre-treated for the cancer. In other embodiments, the cancer is an advanced, metastatic and/or refractory cancer. In preferred embodiments, the administration of the Ab or antigen-binding portion to the subject thereof induces a durable clinical response in the subject.

This disclosure also provides a method for immunotherapy of a subject afflicted with cancer, which method comprises: (a) selecting a subject that is a suitable candidate for immunotherapy, the selecting comprising (i) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells, (ii) assessing the proportion of cells in the test tissue sample that express PD-L1 on the cell surface, and (iii) selecting the subject as a suitable candidate based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface exceeds a predetermined threshold level;

and (b) administering a composition comprising a therapeutically effective amount of an agent that disrupts signaling from the PD-1/PD-Ll pathway, for example, an anti-PD-1 or an anti-PD-Ll Ab, to the selected subject.

The disclosure further provides a method for treatment of a subject afflicted with cancer, which method comprises: (a) selecting a subject that is not suitable for immunotherapy with an agent that disrupts signaling from the PD-1/PD-L1 pathway, for example, an anti-PD-1 or an anti-PD-Ll Ab, the selecting comprising (i) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (ii) assessing the proportion of cells in the test tissue sample that express PD-Ll on the cell surface; and (iii) selecting the subject as not suitable for said immunotherapy based on an assessment that the proportion of cells in the test tissue sample that express PD-Ll on the cell surface is less than a predetermined threshold level; and (b) administering a standard-of-care therapeutic other than an agent that disrupts signaling from the PD-1/PD-L1 pathway to the selected subject.

In addition, the disclosure provides a method for selecting a cancer patient for immunotherapy with an agent that disrupts signaling from the PD-1/PD-L1 pathway, for example, an anti-PD-1 or an anti-PD-Ll Ab, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) assaying the test tissue sample to determine the proportion of cells therein that express PD-Ll on the cell surface; (c) comparing the proportion of cells that express PD-Ll on the cell surface with a predetermined threshold proportion; and (d) selecting the patient for immunotherapy based on an assessment that PD-Ll is expressed in cells of the test tissue sample.

This disclosure further provides a method for predicting the therapeutic effectiveness of an agent that disrupts signaling from the PD-1/PD-L1 pathway, for example, an anti-PD-1 or an anti-PD-Ll Ab, for treating a cancer patient, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) assaying the test tissue sample to determine the proportion of cells therein that express PD-Ll on the cell surface; (c) comparing the proportion of cells that express PD-Ll on the cell surface with a predetermined threshold value; and (d)

predicting the therapeutic effectiveness of the agent, wherein if the proportion of cells that express PD-L1 on the cell surface exceeds the threshold proportion the agent is predicted to be effective in treating the patient, and wherein if the proportion of cells that express PD-L1 on the cell surface is below the threshold proportion the agent is predicted to not be effective in treating the patient.

The present disclosure also provides a method for determining an immunotherapeutic regimen comprising an agent that disrupts signaling from the PD-1/PD-L1 pathway, for example, an anti-PD-1 or an anti-PD-L1 Ab, for treating a cancer patient, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) assaying the test tissue sample to determine the proportion of cells therein that express PD-L1 on the cell surface; (c) comparing the proportion of cells that express PD-L1 on the cell surface with a predetermined threshold proportion; and (d) determining an immunotherapeutic regimen comprising the agent based on the determination that the proportion of cells that express PD-L1 on the cell surface exceeds the predetermined threshold proportion.

In certain embodiments of the methods described herein, the test tissue sample is a formalin-fixed and paraffin-embedded (FFPE) sample. In certain other embodiments, assessing the proportion of cells in the test tissue sample that express PD-L1 on the cell surface is achieved by immunohistochemical (IHC) staining of the FFPE sample. In preferred embodiments, the mAb 28-8 or 5H1 is used in an automated IHC assay to bind to PD-L1 on the surface of cells in the test tissue sample. In preferred embodiments of any of the methods disclosed herein, the cancer is melanoma (MEL), renal cell carcinoma (RCC), squamous non-small cell lung cancer (NSCLC), non-squamous NSCLC, colorectal cancer (CRC), castration-resistant prostate cancer (CRPC), hepatocellular carcinoma (HCC), squamous cell carcinoma of the head and neck, carcinomas of the esophagus, ovary, gastrointestinal tract and breast, or a hematologic malignancy such as multiple myeloma, B-cell lymphoma, T-cell lymphoma, Hodgkin's lymphoma/primary mediastinal B-cell lymphoma, and chronic myelogenous leukemia.

This invention additionally provides a mAb or antigen-binding portion thereof that binds specifically to a cell surface-expressed human PD-L1 antigen in a FFPE tissue sample. In preferred embodiments, the mAb or antigen-binding portion thereof does not bind to a cytoplasmic PD-L1 antigen in the FFPE tissue sample. In other preferred

embodiments, the monoclonal Ab (mAb) is the rabbit mAb designated 28-8, 28-1, 28-12, 29-8 or 20-12, or the mouse mAb designated 5H1.

Other features and advantages of the instant invention will be apparent from the following detailed description and examples which should not be construed as limiting. The contents of all cited references, including scientific articles, newspaper reports, GenBank entries, patents and patent applications cited throughout this application are expressly incorporated herein by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C. Cross-competition between 5C4 and other HuMab anti-PD-1 mAbs for binding to human PD-1 (hPD-1) expressed on CHO cells. A, the 5C4 Fab fragment substantially blocked the binding of mAbs 5C4 itself, as well as the binding of 2D3 and 7D3; B, the 5C4 Fab fragment substantially blocked the binding of mAb 4H1; C, the 5C4 mAb substantially blocked the binding of mAb 17D8.

Figures 2A-2F. Cross-competition of FITC-conjugated human anti-hPD-Ll mAbs for binding to human PDF-LI (hPD-Ll) expressed on CHO cells. A, Binding of labeled 10H10 was partially blocked by 10A5, 11E6 and 13G4 and was significantly blocked by itself; B, Binding of labeled 3G10 was significantly blocked by each of the tested anti-PD-Ll Abs except 10H10; C, Binding of labeled 10A5 was significantly blocked by each of the tested anti-PD-Ll Abs except 10H10; D, Binding of labeled 11E6 was significantly blocked by each of the tested anti-PD-Ll Abs except 10H10; and E, Binding of labeled 12A4 was significantly blocked by each of the tested anti-PD-Ll Abs except 10H10; and F, Binding of labeled 13G4 was significantly blocked by each of the tested anti-PD-Ll Abs except 10H10.

Figure 3. Cross-competitive inhibition of binding of biotinylated mAb 12A4 to ES-2 cells by human anti-hPD-Ll mAbs. Fluorescence of bound biotin-12A4 is plotted against the concentration of unlabeled hPD-Ll HuMabs.

Figure 4. Spider plot showing activity of anti-PD-1 mAb in patients with treatment-refractory melanoma (MEL). A representative plot of changes in tumor burden over time demonstrates the time course of change in the sum of the longest diameters of target lesions, compared with baseline, in 27 MEL patients treated with 5C4 at a dose of 1.0 mg/kg. In the majority of patients who achieved an objective response (OR), responses were durable and evident by the end of cycle 3 (6 months) of treatment (vertical dashed line). Tumor regressions followed conventional as well as "immune-

related" patterns of response, such as prolonged reduction in tumor burden in the presence of new lesions.

Figure 5. Activity of anti-PD-1 mAb in patient with metastatic RCC. Partial regression of metastatic RCC in a 57-year-old patient treated with 5C4 at 1 mg/kg is illustrated. This patient had previously undergone radical surgery and had developed progressive disease after receiving sunitinib, temsirolimus, sorafenib, and pazopanib. Arrows show regression of recurrent tumor in the operative field.

Figures 6. Activity of anti-PD- 1 mAb in patient with metastatic MEL. A complete response of metastatic MEL is illustrated in a 62-year-old patient treated with 5C4 at 3 mg/kg, associated with vitiligo. (i) Pretreatment CT scan, inguinal lymph node metastasis (arrow); (ii) after 13 months of treatment. Numerous metastases in the subcutaneous tissue and retroperitoneum also regressed completely (not shown). Vitiligo developed after 6 months of treatment; photos taken at 9 months under visible light (iii) and ultraviolet light (iv). Skin biopsies with immunohistochemistry for microphthalmia-associated transcription factor (MITF) show melanocytes (arrows) at the epidermal-dermal junction in normal skin (v), and scarce (vi) or absent (vii) melanocytes in skin partially or fully affected by vitiligo.

Figure 7. Activity of anti-PD-1 mAb in patient with metastatic NSCLC. A partial response is illustrated in a patient with metastatic NSCLC (nonsquamous histology) treated with 5C4 at 10 mg/kg. Arrows show initial progression in pulmonary lesions followed by regression ("immune-related" pattern of response).

Figures 8A and 8B. Correlation between tumor PD-Ll expression and anti-PD-1 clinical response. Pretreatment tumor cell surface expression of PD-Ll, as determined by IHC on formalin-fixed paraffin-embedded specimens, correlates with OR to PD-1 blockade. Forty-two subjects with advanced cancers including melanoma, non-small cell lung cancer, colorectal cancer, renal cell cancer, and castration-resistant prostate cancer (n=18, 10, 7, 5, and 2, respectively) were studied. A, There was a significant correlation of tumor cell surface PD-Ll expression with objective clinical response. No patients with PD-Ll negative tumors experienced an OR. B, Examples of IHC analysis with the anti-PD-Ll mAb 5H1 are shown in a melanoma lymph node metastasis (top), a renal cell cancer nephrectomy specimen (middle), and a lung adenocarcinoma brain metastasis (bottom). All 400X original magnification. Arrows indicate one of many tumor cells in each specimen with surface membrane staining for PD-Ll. Asterisk indicates a normal

glomerulus in the nephrectomy specimen, which is negative for PD-L1 staining.

Figure 9. Graphical comparison of the binding of mAbs 28-8 and 5H1 to PD-L1 antigen in tumor tissues by histoscore analysis. The rabbit mAb 28-8 showed higher histoscores in 7 out of 10 samples tested.

Figures 10A-10D. Spider plot showing activity of anti-PD-L1 mAb in patients with treatment-refractory MEL and NSCLC. Representative plots demonstrate the time course of target lesion tumor burden over time in patients with MEL treated with BMS-936559 at doses of 1 (A), 3 (B), 10 mg/kg (C) and in patients with NSCLC treated at 10 mg/kg (D). In the majority of patients who achieved ORs, responses were durable and were evident by the end of cycle 2 (3 months) of treatment, irrespective of dose or tumor type. Tumor regressions followed conventional as well as "immune-related" patterns of response.

Figure 11. Complete response in a patient with melanoma treated with BMS-936559 at 3 mg/kg. Circles indicate an initial increase in the size of pulmonary nodules at 6 weeks and 3 months followed by complete regression at 10 months ("immune-related" pattern of response).

Figure 12. Complete response in a patient with melanoma treated with BMS-936559 at 1 mg/kg. This patient developed an isolated brain metastasis 3 months after initiation of treatment that was successfully treated with stereotactic radiosurgery. A partial response in abdominal disease (circled) was noted at 8 months, with no evidence of disease at 15 months.

Figure 13. Partial response in a patient with NSCLC (non-squamous histology) treated with BMS-936559 at 10 mg/kg. Note the response in disease in right lung pleura and liver.

Figures 14A and 14B. Clinical activity of MEL patients who received a concurrent regimen of nivolumab and ipilimumab. A, Representative spider plots show changes from baseline in the tumor burden, measured as the sum of products of perpendicular diameters of all target lesions, in patients who received the concurrent regimen of 1 mg/kg nivolumab + 3 mg/kg ipilimumab, the MTD. Triangles indicate the first occurrence of a new lesion. B, A representative waterfall plot shows maximum percentage response in baseline target lesions in patients who received the concurrent regimen.

Figure 15. Tumor regressions of a 52-year old MEL patient who received a

concurrent regimen of 1 mg/kg nivolumab + 3 mg/kg ipilimumab. This patient presented with extensive neck, mediastinal, axillary, abdominal and pelvic lymphadenopathy, bilateral pulmonary nodules, small bowel metastasis, peritoneal implants and diffuse subcutaneous nodules. Baseline lactate dehydrogenase (LDH) was 2.25 x upper limit of normal, hemoglobin was 9.7 g/dL, and symptoms included nausea and vomiting. Within 4 weeks of treatment the LDH normalized, symptoms improved (appetite increased, nausea decreased), and cutaneous lesions were regressing. At week- 12 scans, there was marked reduction in all areas of disease. Arrows denote location of metastatic disease.

Figures 16A-16C. Clinical activity of MEL patients who received various concurrent regimens of nivolumab and ipilimumab. Representative spider plots show changes from baseline in the tumor burden, measured as the sum of products of perpendicular diameters of all target lesions, in patients who received a concurrent regimen of 0.3 mg/kg nivolumab + 3 mg/kg ipilimumab (A), 3 mg/kg nivolumab + 1 mg/kg ipilimumab (B), or 3 mg/kg nivolumab + 3 mg/kg ipilimumab (C). Triangles indicate the first occurrence of a new lesion.

Figure 17. Tumor regressions of a 61-year old MEL patient who received a concurrent regimen of 0.3 mg/kg nivolumab + 3 mg/kg ipilimumab. The patient presented with stage IV (Mlc) MEL of an unknown primary site, metastatic to the stomach and the mesentery. Lactate dehydrogenase was 225 and hemoglobin was 9.6 g/dL after a recent transfusion. Twelve weeks after the initiation of study treatment, a CT scan showed an 86% reduction in the bulky disease burden.

Figures 18A-18C. Clinical activity of MEL patients who received a sequenced regimen of nivolumab and ipilimumab. Representative spider plots show changes from baseline in the tumor burden, measured as the sum of products of perpendicular diameters of all target lesions, in patients who received a sequenced regimen of 1 mg/kg nivolumab (A) or 3 mg/kg nivolumab (B) after prior ipilimumab therapy. Triangles indicate the first occurrence of a new lesion. C, A representative waterfall plot shows maximum percentage response in baseline target lesions in patients who received a sequenced regimen; "*" denotes patients who had radiographic progression with prior ipilimumab treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for immunotherapy of a subject afflicted with diseases such as cancer or an infectious disease, which methods comprise

administering to the subject a composition comprising a therapeutically effective amount of a compound or agent that potentiates an endogenous immune response, either stimulating the activation of the endogenous response or inhibiting the suppression of the endogenous response. More specifically, this disclosure provides methods for potentiating an endogenous immune response in a subject afflicted with cancer so as to thereby treat the patient, which method comprises administering to the subject a therapeutically effective amount of an agent, such as an Ab or an antigen-binding portion thereof, that disrupts or inhibits signaling from an inhibitory immunoregulator. In certain embodiments, the inhibitory immunoregulator is a component of the PD-1/PD-L1 signaling pathway. Accordingly, certain embodiments of the invention provide methods for immunotherapy of a subject afflicted with cancer, which methods comprise administering to the subject a therapeutically effective amount of an Ab or an antigen-binding portion thereof that disrupts the interaction between the PD-1 receptor and its ligand, PD-L1. In certain preferred embodiments, the Ab or antigen-binding portion thereof binds specifically to PD-1. In other preferred embodiments, the Ab or antigen-binding portion thereof binds specifically to PD-L1. Certain embodiments comprise the use of an anti-PD- 1 Ab is in combination with another anti-cancer agent, preferably an anti-CTLA-4 Ab, to treat cancer. In certain other embodiments, the subject is selected as suitable for immunotherapy in a method comprising measuring the surface expression of PD-L1 in a test tissue sample obtained from a patient with cancer of the tissue, for example, determining the proportion of cells in the test tissue sample that express PD-L1 on the cell surface, and selecting the patient for immunotherapy based on an assessment that PD-L1 is expressed on the surface of cells in the test tissue sample.

Terms

In order that the present disclosure may be more readily understood, certain terms are first defined. As used in this application, except as otherwise expressly provided herein, each of the following terms shall have the meaning set forth below. Additional definitions are set forth throughout the application.

"Administering" refers to the physical introduction of a composition comprising a therapeutic agent to a subject, using any of the various methods and delivery systems known to those skilled in the art. Preferred routes of administration for Abs of the invention include intravenous, intramuscular, subcutaneous, intraperitoneal, spinal or other parenteral routes of administration, for example by injection or infusion. The phrase

"parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intralymphatic, intralesional, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion, as well as *in vivo* electroporation. Alternatively, an Ab of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

An "adverse event" (AE) as used herein is any unfavorable and generally unintended or undesirable sign (including an abnormal laboratory finding), symptom, or disease associated with the use of a medical treatment. For example, an adverse event may be associated with activation of the immune system or expansion of immune system cells *(e.g.,* T cells) in response to a treatment. A medical treatment may have one or more associated AEs and each AE may have the same or different level of severity. Reference to methods capable of "altering adverse events" means a treatment regime that decreases the incidence and/or severity of one or more AEs associated with the use of a different treatment regime.

An "antibody" (Ab) shall include, without limitation, a glycoprotein immunoglobulin which binds specifically to an antigen and comprises at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen-binding portion thereof. Each H chain comprises a heavy chain variable region (abbreviated herein as v_H) and a heavy chain constant region. The heavy chain constant region comprises three constant domains, *Cm,* C_{H2} and *Cm-* Each light chain comprises a light chain variable region (abbreviated herein as Vz) and a light chain constant region. The light chain constant region is comprises one constant domain, *CL.* The V*H* and Vz regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and Vz comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of

the Abs may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system *(e.g.,* effector cells) and the first component (Clq) of the classical complement system.

Antibodies typically bind specifically to their cognate antigen with high affinity, reflected by a dissociation constant (K_D) of 10^{-5} to 10^{-11} M^{-1} or less. Any K_D greater than about 10^{-4} M^{-1} is generally considered to indicate nonspecific binding. As used herein, an Ab that "binds specifically" to an antigen refers to an Ab that binds to the antigen and substantially identical antigens with high affinity, which means having a ¾ of 10^{-7} M or less, preferably 10^{-8} M or less, even more preferably 5 x 10^{-9} M or less, and most preferably between 10^{-8} M and 10^{-10} M or less, but does not bind with high affinity to unrelated antigens. An antigen is "substantially identical" to a given antigen if it exhibits a high degree of sequence identity to the given antigen, for example, if it exhibits at least 80%, at least 90%, preferably at least 95%, more preferably at least 97%, or even more preferably at least 99% sequence identity to the sequence of the given antigen. By way of example, an Ab that binds specifically to human PD-1 may also have cross-reactivity with PD-1 antigens from certain primate species but may not cross-react with PD-1 antigens from certain rodent species or with an antigen other than PD-1, *e.g.,* a human PD-L1 antigen.

An immunoglobulin may derive from any of the commonly known isotypes, including but not limited to IgA, secretory IgA, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgGl, IgG2, IgG3 and IgG4. "Isotype" refers to the Ab class or subclass *(e.g.,* IgM or IgGl) that is encoded by the heavy chain constant region genes. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring Abs; monoclonal and polyclonal Abs; chimeric and humanized Abs; human or nonhuman Abs; wholly synthetic Abs; and single chain Abs. A nonhuman Ab may be humanized by recombinant methods to reduce its immunogenicity in man. Where not expressly stated, and unless the context indicates otherwise, the term "antibody" also includes an antigen-binding fragment or an antigen-binding portion of any of the aforementioned immunoglobulins, and includes a monovalent and a divalent fragment or portion, and a single chain Ab.

An "isolated antibody" refers to an Ab that is substantially free of other Abs having different antigenic specificities *(e.g.,* an isolated Ab that binds specifically to PD-1 is substantially free of Abs that bind specifically to antigens other than PD-1). An isolated

Ab that binds specifically to PD-1 may, however, have cross-reactivity to other antigens, such as PD-1 molecules from different species. Moreover, an isolated Ab may be substantially free of other cellular material and/or chemicals. By comparison, an "isolated" nucleic acid refers to a nucleic acid composition of matter that is markedly different, *i.e.,* has a distinctive chemical identity, nature and utility, from nucleic acids as they exist in nature. For example, an isolated DNA, unlike native DNA, is a free-standing portion of a native DNA and not an integral part of a larger structural complex, the chromosome, found in nature. Further, an isolated DNA, unlike native genomic DNA, can typically be used in applications or methods for which native genomic DNA is unsuited, *e.g.,* as a PCR primer or a hybridization probe for, among other things, measuring gene expression and detecting biomarker genes or mutations for diagnosing disease or assessing the efficacy of a therapeutic. An isolated nucleic acid may be purified so as to be substantially free of other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, using standard techniques well known in the art. Examples of isolated nucleic acids include fragments of genomic DNA, PCR-amplified DNA, cDNA and RNA.

The term "monoclonal antibody" ("mAb") refers to a preparation of Ab molecules of single molecular composition, *i.e.,* Ab molecules whose primary sequences are essentially identical, and which exhibits a single binding specificity and affinity for a particular epitope. A mAb is an example of an isolated Ab. MAbs may be produced by hybridoma, recombinant, transgenic or other techniques known to those skilled in the art.

A "human" antibody (HuMAb) refers to an Ab having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the Ab contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human Abs of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences *(e.g.,* mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo).* However, the term "human antibody," as used herein, is not intended to include Abs in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. The terms "human" Abs and "fully human" Abs and are used synonymously.

A "humanized" antibody refers to an Ab in which some, most or all of the amino

acids outside the CDR domains of a non-human Ab are replaced with corresponding amino acids derived from human immunoglobulins. In one embodiment of a humanized form of an Ab, some, most or all of the amino acids outside the CDR domains have been replaced with amino acids from human immunoglobulins, whereas some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they do not abrogate the ability of the Ab to bind to a particular antigen. A "humanized" Ab retains an antigenic specificity similar to that of the original Ab.

A "chimeric antibody" refers to an Ab in which the variable regions are derived from one species and the constant regions are derived from another species, such as an Ab in which the variable regions are derived from a mouse Ab and the constant regions are derived from a human Ab.

An "antigen-binding portion" of an Ab (also called an "antigen-binding fragment") refers to one or more fragments of an Ab that retain the ability to bind specifically to the antigen bound by the whole Ab.

A "cancer" refers a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth divide and grow results in the formation of malignant tumors that invade neighboring tissues and may also metastasize to distant parts of the body through the lymphatic system or bloodstream.

An "immune response" refers to the action of a cell of the immune system (for example, T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, eosinophils, mast cells, dendritic cells and neutrophils) and soluble macromolecules produced by any of these cells or the liver (including Abs, cytokines, and complement) that results in selective targeting, binding to, damage to, destruction of, and/or elimination from a vertebrate's body of invading pathogens, cells or tissues infected with pathogens, cancerous or other abnormal cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

An "immunoregulator" refers to a substance, an agent, a signaling pathway or a component thereof that regulates an immune response. "Regulating," "modifying" or "modulating" an immune response refers to any alteration in a cell of the immune system or in the activity of such cell. Such regulation includes stimulation or suppression of the immune system which may be manifested by an increase or decrease in the number of

various cell types, an increase or decrease in the activity of these cells, or any other changes which can occur within the immune system. Both inhibitory and stimulatory immunoregulators have been identified, some of which may have enhanced function in the cancer microenvironment.

The term "immunotherapy" refers to the treatment of a subject afflicted with, or at risk of contracting or suffering a recurrence of, a disease by a method comprising inducing, enhancing, suppressing or otherwise modifying an immune response. "Treatment" or "therapy" of a subject refers to any type of intervention or process performed on, or the administration of an active agent to, the subject with the objective of reversing, alleviating, ameliorating, inhibiting, slowing down or preventing the onset, progression, development, severity or recurrence of a symptom, complication, condition or biochemical indicia associated with a disease.

"Potentiating an endogenous immune response" means increasing the effectiveness or potency of an existing immune response in a subject. This increase in effectiveness and potency may be achieved, for example, by overcoming mechanisms that suppress the endogenous host immune response or by stimulating mechanisms that enhance the endogenous host immune response.

A "predetermined threshold value," relating to cell surface PD-Ll expression, refers to the proportion of cells in a test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells above which the sample is scored as being positive for cell surface PD-Ll expression. For cell surface expression assayed by IHC with the mAb 28-8, the predetermined threshold value for cells expressing PD-Ll on the cell surface ranges from at least about 0.01% to at least about 20% of the total number of cells. In preferred embodiments, the predetermined threshold value for cells expressing PD-Ll on the cell surface ranges from at least about 0.1% to at least about 10% of the total number of cells. More preferably, the predetermined threshold value is at least about 5%. Even more preferably, the predetermined threshold value is at least about 1%, or in the range of 1-5%.

The "Programmed Death- 1 (PD-1)" receptor refers to an immunoinhibitory receptor belonging to the CD28 family. PD-1 is expressed predominantly on previously activated T cells *in vivo,* and binds to two ligands, PD-Ll and PD-L2. The term "PD-1" as used herein includes human PD-1 (hPD-1), variants, isoforms, and species homologs of hPD-1, and analogs having at least one common epitope with hPD-1. The complete

hPD-1 sequence can be found under GenBank Accession No. U64863.

"Programmed Death Ligand-1 (PD-L1)" is one of two cell surface glycoprotein ligands for PD- 1 (the other being PD-L2) that downregulate T cell activation and cytokine secretion upon binding to PD-1. The term "PD-L1" as used herein includes human PD-L1 (hPD-Ll), variants, isoforms, and species homologs of hPD-Ll, and analogs having at least one common epitope with hPD-Ll. The complete hPD-Ll sequence can be found under GenBank Accession No. Q9NZQ7.

A "signal transduction pathway" or "signaling pathway" refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of the cell. A "cell surface receptor" includes, for example, molecules and complexes of molecules that are located on the surface of a cell and are capable of receiving a signal and transmitting such a signal across the plasma membrane of a cell. An example of a cell surface receptor of the present invention is the PD- 1 receptor, which is located on the surface of activated T cells, activated B cells and myeloid cells, and transmits a signal that results in a decrease in tumor-infiltrating lymphocytes and a decrease in T cell proliferation. An "inhibitor" of signaling refers to a compound or agent that antagonizes or reduces the initiation, reception or transmission of a signal, be that signal stimulatory or inhibitory, by any component of a signaling pathway such as a receptor or its ligand.

A "subject" includes any human or nonhuman animal. The term "nonhuman animal" includes, but is not limited to, vertebrates such as nonhuman primates, sheep, dogs, cats, rabbits and ferrets, rodents such as mice, rats and guinea pigs, avian species such as chickens, amphibians, and reptiles. In preferred embodiments, the subject is a mammal such as a nonhuman primate, sheep, dog, cat, rabbit, ferret or rodent. In more preferred embodiments, the subject is a human. The terms, "subject," "patient" and "individual" are used interchangeably herein.

A "therapeutically effective amount" or "therapeutically effective dosage" of a drug or therapeutic agent, such as an Ab of the invention, is any amount of the drug that, when used alone or in combination with another therapeutic agent, protects a subject against the onset of a disease or promotes disease regression evidenced by a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. The ability of a therapeutic agent to promote disease regression can be

evaluated using a variety of methods known to the skilled practitioner, such as in human subjects during clinical trials, in animal model systems predictive of efficacy in humans, or by assaying the activity of the agent in *in vitro* assays.

By way of example, an anti-cancer agent promotes cancer regression in a subject. In preferred embodiments, a therapeutically effective amount of the drug promotes cancer regression to the point of eliminating the cancer. "Promoting cancer regression" means that administering an effective amount of the drug, alone or in combination with an anti-neoplastic agent, results in a reduction in tumor growth or size, necrosis of the tumor, a decrease in severity of at least one disease symptom, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. In addition, the terms "effective" and "effectiveness" with regard to a treatment includes both pharmacological effectiveness and physiological safety. Pharmacological effectiveness refers to the ability of the drug to promote cancer regression in the patient. Physiological safety refers to the level of toxicity, or other adverse physiological effects at the cellular, organ and/or organism level (adverse effects) resulting from administration of the drug.

By way of example for the treatment of tumors, a therapeutically effective amount of the drug preferably inhibits cell growth or tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. In other preferred embodiments of the invention, tumor regression may be observed and continue for a period of at least about 20 days, more preferably at least about 40 days, or even more preferably at least about 60 days. Notwithstanding these ultimate measurements of therapeutic effectiveness, evaluation of immunotherapeutic drugs must also make allowance for "immune-related" response patterns.

An "immune-related" response pattern refers to a clinical response pattern often observed in cancer patients treated with immunotherapeutic agents that produce antitumor effects by inducing cancer-specific immune responses or by modifying native immune processes. This response pattern is characterized by a beneficial therapeutic effect that follows an initial increase in tumor burden or the appearance of new lesions, which in the evaluation of traditional chemotherapeutic agents would be classified as disease progression and would be synonymous with drug failure. Accordingly, proper evaluation of immunotherapeutic agents may require long-term monitoring of the effects of these

agents on the target disease.

A therapeutically effective amount of a drug includes a "prophylactically effective amount," which is any amount of the drug that, when administered alone or in combination with an anti-neoplastic agent to a subject at risk of developing a cancer (e.g., a subject having a pre-malignant condition) or of suffering a recurrence of cancer, inhibits the development or recurrence of the cancer. In preferred embodiments, the prophylactically effective amount prevents the development or recurrence of the cancer entirely. "Inhibiting" the development or recurrence of a cancer means either lessening the likelihood of the cancer's development or recurrence, or preventing the development or recurrence of the cancer entirely.

A "tumor-infiltrating inflammatory cell" is any type of cell that typically participates in an inflammatory response in a subject and which infiltrates tumor tissue. Such cells include tumor-infiltrating lymphocytes (TILs), macrophages, monocytes, eosinophils, histiocytes and dendritic cells.

The use of the alternative *(e.g.,* "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the indefinite articles "a" or "an" should be understood to refer to "one or more" of any recited or enumerated component.

The terms "about" or "comprising essentially of refer to a value or composition that is within an acceptable error range for the particular value or composition as determined by one of ordinary skill in the art, which will depend in part on how the value or composition is measured or determined, *i.e.,* the limitations of the measurement system. For example, "about" or "comprising essentially of can mean within 1 or more than 1 standard deviation per the practice in the art. Alternatively, "about" or "comprising essentially of can mean a range of up to 20%. Furthermore, particularly with respect to biological systems or processes, the terms can mean up to an order of magnitude or up to 5-fold of a value. When particular values or compositions are provided in the application and claims, unless otherwise stated, the meaning of "about" or "comprising essentially of should be assumed to be within an acceptable error range for that particular value or composition.

As described herein, any concentration range, percentage range, ratio range or integer range is to be understood to include the value of any integer within the recited

range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated.

Various aspects of the invention are described in further detail in the following subsections.

Antibodies of the Invention

Abs of the present invention include a variety of Abs having structural and functional properties described herein, including high-affinity binding to PD-1 or PD-Ll, respectively. These Abs may be used, for example, as therapeutic Abs to treat subjects afflicted with disease or as reagents in diagnostic assays to detect their cognate antigens. Human mAbs (HuMAbs) that bind specifically to PD-1 *(e.g.,* bind to human PD-1 and may cross-react with PD-1 from other species, such as cynomolgus monkey) with high affinity have been disclosed in U.S. Patent No. 8,008,449, and HuMAbs that bind specifically to PD-Ll with high affinity have been disclosed in U.S. Patent No. 7,943,743. The Abs of the invention include, but are not limited to, all of the anti-PD-1 and anti-PD-Ll Abs disclosed in U.S. Patent Nos. 8,008,449 and 7,943,743, respectively. Other anti-PD-1 mAbs have been described in, for example, U.S. Patent Nos. 6,808,710, 7,488,802 and 8,168,757, and PCT Publication No. WO 2012/145493, and anti-PD-Ll mAbs have been described in, for example, U.S. Patent Nos. 7,635,757 and 8,217,149, U.S. Publication No. 2009/03 17368, and PCT Publication Nos. WO 201 1/066389 and WO 2012/145493. To the extent these anti-PD-1 and anti-PD-Ll mAbs exhibit the structural and functional properties disclosed herein for antibodies of the invention, they too are included as antibodies of the invention.

Anti-PD-1 Antibodies of the Invention

Each of the anti-PD-1 HuMAbs disclosed in U.S. Patent No. 8,008,449 has been demonstrated to exhibit one or more of the following characteristics: (a) binds to human PD-1 with a K_D of 1×10^{-7} M or less, as determined by surface plasmon resonance using a Biacore biosensor system; (b) does not substantially bind to human CD28, CTLA-4 or ICOS; (c) increases T-cell proliferation in a Mixed Lymphocyte Reaction (MLR) assay; (d) increases interferon-γ production in an MLR assay; (e) increases IL-2 secretion in an MLR assay; (f) binds to human PD-1 and cynomolgus monkey PD-1; (g) inhibits the binding of PD-Ll and/or PD-L2 to PD-1; (h) stimulates antigen-specific memory responses; (i) stimulates Ab responses; and (j) inhibits tumor cell growth *in vivo.* Anti-PD-1 Abs of the present invention include mAbs that bind specifically to human PD-1

and exhibit at least one, preferably at least five, of the preceding characteristics.

U.S. Patent No. 8,008,449 exemplifies seven anti-PD-1 HuMAbs: 17D8, 2D3, 4H1, 5C4 (also referred to herein as nivolumab or BMS-936558), 4A1 1, 7D3 and 5F4. Isolated DNA molecules encoding the heavy and light chain variable regions of these Abs have been sequenced, from which the amino acid sequences of the variable regions were deduced. The V_H amino acid sequences of 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 and 5F4 are provided herein as SEQ ID NOs. 1, 2, 3, 4, 5, 6 and 7, respectively. The V_L amino acid sequences of 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 and 5F4 are provided herein as SEQ ID NOs. 8, 9, 10, 11, 12, 13 and 14, respectively.

Preferred anti-PD-1 Abs of the present invention include the anti-PD-1 HuMAbs 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 and 5F4. These preferred Abs bind specifically to human PD-1 and comprise: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 1 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 8; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 2 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 9; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 3 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 10; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 4 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 11; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 5 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 12; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 6 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 13; or (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 7 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 14.

Given that each of these Abs can bind to PD-1, the Y_H and Y_L sequences can be "mixed and matched" to create other anti-PD-1 Abs of the invention. PD-1 binding of such "mixed and matched" Abs can be tested using binding assays, *e.g.*, enzyme-linked immunosorbent assays (ELISAs), western blots, radioimmunoassays and Biacore analysis that are well known in the art *(see, e.g.,* U.S. Patent No. 8,008,449). Preferably, when Y_H and V_L chains are mixed and matched, a Y_H sequence from a particular *YHIYL* pairing is replaced with a structurally similar Y_H sequence. Likewise, preferably a Y_L sequence from a particular *YHIYL* pairing is replaced with a structurally similar Y_L sequence. Accordingly, anti-PD-1 Abs of the invention include an isolated mAb or antigen-binding portion thereof comprising: (a) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 1, 2, 3, 4, 5, 6 and 7, and (b) a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 8, 9, 10, 11, 12, 13 and 14, wherein the Ab specifically binds PD-1, preferably human PD-1.

The CDR domains of the above Abs have been delineated using the Kabat system, and these Abs may also be defined by combinations of their 3 heavy chain and 3 light chain CDRs *(see* U.S. Patent No. 8,008,449). Since each of these Abs can bind to PD-1 and antigen-binding specificity is provided primarily by the CDR1, CDR2, and CDR3 regions, the Y_H CDR1, CDR2, and CDR3 sequences and Y_K CDR1, CDR2, and CDR3 sequences can be "mixed and matched" *(i.e.,* CDRs from different Abs can be mixed and match, although each Ab must contain a Y_H CDR1, CDR2, and CDR3 and a Y_K CDR1, CDR2, and CDR3) to create other anti-PD-1 Abs that also constitute Abs of the invention. PD-1 binding of such "mixed and matched" Abs can be tested using the binding assays described above *(e.g.,* ELISAs, western blots, radioimmunoassays and Biacore analysis).

Abs of the invention also include isolated Abs that bind specifically to PD-1 and comprise a heavy chain variable region derived from a particular germline heavy chain immunoglobulin and/or a light chain variable region derived from a particular germline light chain immunoglobulin. Specifically, in certain embodiments, Abs of the invention include isolated Abs comprising: (a) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 3-33 or 4-39 germline sequence, and/or a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_K L6, or L15 germline sequence. The amino acid sequences of the Y_H and Y_K regions encoded by the Y_H 3-33,

V_H 4-39, V_KL6 and V_KL15 germline genes are provided in U.S. Patent No. 8,008,449.

As used herein, an Ab can be identified as comprising a heavy or a light chain variable region that is "derived from" a particular human germline immunoglobulin by comparing the amino acid sequence of the human Ab to the amino acid sequences encoded by human germline immunoglobulin genes, and selecting the human germline immunoglobulin sequence that is closest in sequence *(i.e.,* greatest percentage of sequence identity) to the sequence of the human Ab. A human Ab that is "derived from" a particular human germline immunoglobulin may contain amino acid differences as compared to the germline sequence, due to, for example, naturally-occurring somatic mutations or intentional introduction of site-directed mutation. However, a selected human Ab is generally at least 90% identical in amino acids sequence to an amino acid sequence encoded by a human germline immunoglobulin gene and contains amino acid residues that identify the human Ab as being human when compared to the germline immunoglobulin amino acid sequences of other species *(e.g.,* murine germline sequences). In certain cases, a human Ab may be at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene.

In certain embodiments, the sequence of a human Ab derived from a particular human germline sequence will display no more than 10 amino acid differences from the amino acid sequence encoded by the human germline immunoglobulin gene. In other embodiments, the human Ab may display no more than 5, or even no more than 4, 3, 2, or 1 amino acid differences from the amino acid sequence encoded by the germline immunoglobulin gene.

Preferred Abs of the invention also include isolated Abs or antigen-binding portions thereof comprising: (a) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_H 3-33 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_KL6 germline sequence; or (b) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_H 4-39 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_KL15 germline sequence. Examples of Abs having a V_H and a V_K derived from V#3-33 and V_KL6 germline sequences, respectively, include 17D8, 2D3, 4H1, 5C4, and

7D3. Examples of Abs having V_H and V_K regions derived from V_H 4-39 and V_KL15 germline sequences, respectively, include 4A1 1 and 5F4.

In yet other embodiments, anti-PD-1 Abs of the invention comprise heavy and light chain variable regions having amino acid sequences that are highly similar or homologous to the amino acid sequences of the preferred anti-PD-1 Abs described herein, wherein the Ab retains the functional properties of the preferred anti-PD-1 Abs of the invention. For example, Abs of the invention include mAbs comprising a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises consecutively linked amino acids having a sequence that is at least 80% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs. 1, 2, 3, 4, 5, 6 and 7, and the light chain variable region comprises consecutively linked amino acids having a sequence that is at least 80% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs. 8, 9, 10, 11, 12, 13 and 14. In other embodiments, the V_H and/or V_L amino acid sequences may exhibit at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to the sequences set forth above.

As used herein, the percent sequence identity (also referred to as the percent sequence homology) between two sequences (amino acid or nucleotide sequences) is a function of the number of identical positions shared by the sequences relative to the length of the sequences compared *(i.e.,* % identity = number of identical positions/total number of positions being compared x 100), taking into account the number of any gaps, and the length of each such gap, introduced to maximize the degree of sequence identity between the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using mathematical algorithms that are well know to those of ordinary skill in the art *(see, e.g.,* U.S. Patent No. 8,008,449).

Antibodies having very similar amino acid sequences are likely to have essentially the same functional properties where the sequence differences are conservative modifications. As used herein, "conservative sequence modifications" refer to amino acid modifications that do not significantly affect the binding characteristics of the Ab containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Conservative amino acid substitutions are substitutions in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Thus, for example, one or more amino acid residues within the CDR regions of an Ab of the invention can be replaced with other amino acid residues

from the same side chain family and the altered Ab can be tested for retained function using functional assays that are well known in the art. Accordingly, certain embodiments of the anti-PD-1 Abs of the invention comprise heavy and light chain variable regions each comprising CDR1, CDR2 and CDR3 domains, wherein one or more of these CDR domains comprise consecutively linked amino acids having sequences that are the same as the CDR sequences of the preferred anti-PD-1 Abs described herein *(e.g.,* 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 or 5F4), or conservative modifications thereof, and wherein the Abs retain the desired functional properties of the preferred anti-PD-1 Abs of the invention.

Further, it is well known in the art that the heavy chain CDR3 is the primary determinant of binding specificity and affinity of an Ab, and that multiple Abs can predictably be generated having the same binding characteristics based on a common CDR3 sequence *(see, e.g.,* Klimka *et al.,* 2000; Beiboer *et al.,* 2000; Rader *et al.,* 1998; Barbas *etal.,* 1994; Barbas *et al.,* 1995; Ditzel *etal.,* 1996; Berezov *etal.,* 2001; Igarashi *et al.,* 1995; Bourgeois *et al.,* 1998; Levi *et al.,* 1993; Polymenis and Stoller, 1994; and Xu and Davis, 2000). The foregoing publications demonstrate that, in general, once the heavy chain CDR3 sequence of a given Ab is defined, variability in the other five CDR sequences does not greatly affect the binding specificity of that Ab. Thus, Abs of the invention comprising 6 CDRs can be defined by specifying the sequence of the heavy chain CDR3 domain.

Anti-PD-1 Abs of the invention also include isolated Abs that bind specifically to human PD-1 and cross-compete for binding to human PD-1 with any of HuMAbs 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 and 5F4. Thus, anti-PD-1 Abs of the invention include isolated Abs or antigen-binding portions thereof that cross-compete for binding to PD-1 with a reference Ab or a reference antigen-binding portion thereof comprising: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 1 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 8; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 2 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 9; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 3 and a human light chain

variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 10; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 4 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 11; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 5 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 12; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 6 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 13; or (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 7 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 14.

The ability of Abs to cross-compete for binding to an antigen indicates that these Abs bind to the same epitope region *(i.e.,* the same or an overlapping or adjacent epitope) of the antigen and sterically hinder the binding of other cross-competing Abs to that particular epitope region. Thus, the ability of a test Ab to competitively inhibit the binding of, for example, 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 or 5F4, to human PD-1 demonstrates that the test Ab binds to the same epitope region of human PD-1 as 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 or 5F4, respectively. All isolated Abs that bind to the same epitope region of human PD-1 as does HuMAb 17D8, 2D3, 4H1, 5C4, 4A11, 7D3 or 5F4 are included among the Abs of the invention. These cross-competing Abs are expected to have very similar functional properties by virtue of their binding to the same epitope region of PD-1. For example, cross-competing anti-PD-1 mAbs 5C4, 2D3, 7D3, 4H1 and 17D8 have been shown to have similar functional properties *(see* U.S. Patent No. 8,008,449 at Examples 3-7). The higher the degree of cross-competition, the more similar will the functional properties be. Further, cross-competing Abs can be readily identified based on their ability to cross-compete with 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 or 5F4 in standard PD-1 binding assays. For example, Biacore analysis, ELISA assays or flow cytometry may be used to demonstrate cross-competition with the Abs of the invention *(see, e.g.,* Examples 1 and 2).

In certain embodiments, the Abs that cross-compete for binding to human PD-1

with, or bind to the same epitope region of human PD-1 as, 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 or 5F4 are mAbs. For administration to human patients, these cross-competing Abs are preferably chimeric Abs, or more preferably humanized or human Abs. Such human mAbs can be prepared and isolated as described in U.S. Patent No. 8,008,449. Data provided in Example 1 show that 5C4 or a Fab fragment thereof cross-competes with each of 2D3, 7D3, 4H1 or 17D8 for binding to hPD-1 expressed on the surface of a cell, indicating that all five anti-PD-1 mAbs bind to the same epitope region of hPD-1 (Figures 1A-1C).

An anti-PD-1 Ab of the invention further can be prepared using an Ab having one or more of the Y_H and/or Y_L sequences disclosed herein as starting material to engineer a modified Ab, which modified Ab may have altered properties from the starting Ab. An Ab can be engineered by modifying one or more residues within one or both variable regions *(i.e.,* Y_H and/or Y_L*)*, for example within one or more CDR regions and/or within one or more framework regions. Additionally or alternatively, an Ab can be engineered by modifying residues within the constant region(s), for example, to alter the effector function(s) of the Ab. Specific modifications to Abs include CDR grafting, site-specific mutation of amino acid residues within the Y_H and/or Y_K CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties *(e.g.,* affinity) of the Ab, site-specific mutation of amino acid residues within the Y_H and/or Y_K framework regions to decrease the immunogenicity of the Ab, modifications within the Fc region, typically to alter one or more functional properties of the Ab, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity, and chemical modification such as pegylation or alteration in glycosylation patterns to increase or decrease the biological *(e.g.,* serum) half life of the Ab. Specific examples of such modifications and methods of engineering Abs are described in detail in U.S. Patent No. 8,008,449. Anti-PD-1 Abs of the invention include all such engineered Abs that bind specifically to human PD- 1 and are obtained by modification of any of the above-described anti-PD-1 Abs.

Anti-PD-1 Abs of the invention also include antigen-binding portions of the above Abs. It has been amply demonstrated that the antigen-binding function of an Ab can be performed by fragments of a full-length Ab. Examples of binding fragments encompassed within the term "antigen-binding portion" of an Ab include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a

F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the Y_H and C_{H1} domains; and (iv) a Fv fragment consisting of the Y_L and Y_H domains of a single arm of an Ab.

These fragments, obtained initially through proteolysis with enzymes such as papain and pepsin, have been subsequently engineered into monovalent and multivalent antigen-binding fragments. For example, although the two domains of the Fv fragment, Y_L and Y_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker peptide that enables them to be made as a single protein chain in which the Y_L and Y_H regions pair to form monovalent molecules known as single chain variable fragments (scFv). Divalent or bivalent scFvs (di-scFvs or bi-scFvs) can be engineered by linking two scFvs in within a single peptide chain known as a tandem scFv which contains two Y_H and two VL regions. ScFv dimers and higher multimers can also be created using linker peptides of fewer than 10 amino acids that are too short for the two variable regions to fold together, which forces the scFvs to dimerize and produce diabodies or form other multimers. Diabodies have been shown to bind to their cognate antigen with much higher affinity than the corresponding scFvs, having dissociation constants up to 40-fold lower than the ¾ values for the scFvs. Very short linkers (< 3 amino acids) lead to the formation of trivalent triabodies or tetravalent tetrabodies that exhibit even higher affinities for to their antigens than diabodies. Other variants include minibodies, which are scFv-C_{H3} dimers, and larger scFv-Fc fragments (scFv-C_{H2}-C_{H3} dimers), and even an isolated CDR may exhibit antigen-binding function. These Ab fragments are engineered using conventional recombinant techniques known to those of skill in the art, and the fragments are screened for utility in the same manner as are intact Abs. All of the above proteolytic and engineered fragments of Abs and related variants (*see* Hollinger and Hudson, 2005; Olafsen and Wu, 2010, for further details) are intended to be encompassed within the term "antigen-binding portion" of an Ab.

Anti-PD-Ll Antibodies of the Invention

Each of the anti-PD-Ll HuMAbs disclosed in U.S. Patent No. 7,943,743 has been demonstrated to exhibit one or more of the following characteristics (a) binds to human PD-L1 with a ¾ of 1 x 10^{-7} M or less; (b) increases T-cell proliferation in a Mixed Lymphocyte Reaction (MLR) assay; (c) increase interferon-γ production in an MLR assay; (d) increase IL-2 secretion in an MLR assay; (e) stimulates Ab responses; (f) inhibits the binding of PD-L1 to PD-1; and (g) reverses the suppressive effect of T

regulatory cells on T cell effector cells and/or dendritic cells. Anti-PD-Ll Abs of the present invention include mAbs that bind specifically to human PD-L1 and exhibit at least one, preferably at least four, of the preceding characteristics.

U.S. Patent No. 7,943,743 exemplifies ten anti-PD-1 HuMAbs: 3G10, 12A4 (also referred to herein as BMS-936559), 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7, and 13G4. Isolated DNA molecules encoding the heavy and light chain variable regions of these Abs have been sequenced, from which the amino acid sequences of the variable regions were deduced. The V_H amino acid sequences of 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7, and 13G4 are shown in SEQ ID NOs. 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24, respectively, whereas their V_L amino acid sequences are shown in SEQ ID NOs. 25, 26, 27, 28, 29, 30, 31, 32, 33 and 34, respectively.

Preferred anti-PD-Ll Abs of the present invention include the anti-PD-Ll HuMAbs 3G10, 12A4, 10A5, 5F8, 10H10, IB 12, 7H1, 11E6, 12B7, and 13G4. These preferred Abs bind specifically to human PD-L1 and comprise: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 15 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 25; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 16 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 26; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 17 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 27; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 18 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 28; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 19 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 29; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 20 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 30; (g) a human heavy chain variable

region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 21 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 31; (h) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 22 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 32; (i) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 23 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 33; or (j) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 24 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 34.

Given that each of these Abs can bind to PD-L1, the *YH* and *YL* sequences can be "mixed and matched" to create other anti-PD-Ll Abs of the invention. PD-L1 binding of such "mixed and matched" Abs can be tested using binding assays *e.g.*, ELISAs, western blots, radioimmunoassays and Biacore analysis that are well known in the art *(see, e.g.*, U.S. Patent No. 7,943,743). Preferably, when Y_H and *YL* chains are mixed and matched, a *YH* sequence from a particular V_H/VL pairing is replaced with a structurally similar V*H* sequence. Likewise, preferably a Y_L sequence from a particular *YHIYL* pairing is replaced with a structurally similar Y_L sequence. Accordingly, Abs of the invention also include a mAb, or antigen binding portion thereof, comprising a heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in any of SEQ ID NOs. 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24, and a light chain variable region comprising consecutively linked amino acids having the sequence set forth in any of SEQ ID NOs. 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34, wherein the Ab binds specifically to PD-Ll, preferably human PD-L1.

The CDR domains of the above anti-PD-Ll HuMAbs have been delineated using the Kabat system, and these Abs may also be defined by combinations of their 3 heavy chain and 3 light chain CDRs *(see* U.S. Patent No. 7,943,743). Since each of these Abs can bind to PD-L1 and antigen-binding specificity is provided primarily by the CDR1, CDR2, and CDR3 regions, the Y_H CDR1, CDR2, and CDR3 sequences and Y_K CDR1, CDR2, and CDR3 sequences can be "mixed and matched" *(i.e.*, CDRs from different Abs

can be mixed and match, although each Ab must contain a Y_H CDR1, CDR2, and CDR3 and a Y_K CDR1, CDR2, and CDR3) to create other anti-PD-1 Abs that also constitute Abs of the invention. PD-L1 binding of such "mixed and matched" Abs can be tested using, for example, ELISAs, western blots, radioimmunoassays and Biacore analysis.

Antibodies of the invention also include Abs that bind specifically to PD-L1 and comprise a heavy chain variable region derived from a particular germline heavy chain immunoglobulin and/or a light chain variable region derived from a particular germline light chain immunoglobulin. Specifically, in certain embodiments, Abs of the invention include Abs comprising: (a) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-18, 1-69, 1-3 or 3-9 germline sequence, and/or a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL6, L15, A27 or L18 germline sequence. The amino acid sequences of the Y_H and Y_K regions encoded by the Y_H 1-18, Y_H 1-3, V_H 1-69, Y_H 3-9, Y_KL6, Y_KL15 and Y_KA27 germline genes are provided in U.S. Patent No. 7,943,743.

Preferred Abs of the invention include isolated Abs or antigen-binding portions thereof comprising: (a) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-18 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL6 germline sequence; (b) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-69 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL6 germline sequence; (c) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-3 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL15 germline sequence; (d) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-69 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KA27 germline sequence; (e) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 3-9 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a

sequence derived from a human V_KL15germline sequence; or (f) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_H 3-9 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_K L18germline sequence.

An example of an Ab having a V_H and a V_K derived from V_H 1-18 and V_KL6 germline sequences, respectively, is 3G10. Examples of Abs having V_H and V_K regions derived from V_H 1-69 and V_KL6 germline sequences, respectively, include 12A4, 1B12, 7H1 and 12B7. An example of an Ab having a V_H and a V_K derived from V_H 1-3 and V_K L15 germline sequences, respectively, is 10A5. Examples of Abs having V_H and V_K regions derived from V_H 1-69 and V_K*All* germline sequences, respectively, include 5F8, 11E6 and 1lE6a. An example of an Ab having a V_H and a V_K derived from V_H 3-9 and V_KL15 germline sequences, respectively, is 10H10. An example of an Ab having a V_H and a V_K derived from V_H 1-3 and V_KL15 germline sequences, respectively, is 10A5. An example of an Ab having a V_H and a V_K derived from V_H 3-9 and V_KL 18 germline sequences, respectively, is 13G4.

In certain embodiments, anti-PD-Ll Abs of the invention comprise heavy and light chain variable regions having amino acid sequences that are highly similar or homologous to the amino acid sequences of the preferred anti-PD-Ll Abs described herein, wherein the Ab retains the functional properties of the aforementioned anti-PD-Ll Abs of the invention. For example, Abs of the invention include mAbs comprising a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises consecutively linked amino acids having a sequence that is at least 80% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs. 15, 16, 17, 18, 19, 20, 21, 22, 23, and 24, and the light chain variable region comprises consecutively linked amino acids having a sequence that is at least 80% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs. 25, 26, 27, 28, 29, 30, 31, 32, 33, and 34. In other embodiments, the V_H and/or V_L amino acid sequences may exhibit at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to the sequences set forth above.

Certain embodiments of the anti-PD-Ll Abs of the invention comprise heavy and light chain variable regions each comprising CDR1, CDR2 and CDR3 domains, wherein one or more of these CDR domains comprise consecutively linked amino acids having

sequences that are the same as the CDR sequences of the preferred anti-PD-L1 Abs described herein *(e.g.,* 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 and 13G4), or conservative modifications thereof, and wherein the Abs retain the desired functional properties of the preferred anti-PD-L1 Abs of the invention.

On the basis of the evidence that the heavy chain CDR3 is the primary determinant of binding specificity and affinity of an Ab, it is generally true that once the heavy chain CDR3 sequence of a given Ab is defined, variability in the other five CDR sequences will not greatly affect the binding specificity of that Ab. Accordingly, anti-PD-L1 Abs of the invention include isolated Abs comprising 6 CDRs, wherein the Abs are defined by specifying the sequence of the heavy chain CDR3 domain.

Anti-PD-L1 Abs of the invention also include isolated Abs that bind specifically to human PD-L1 and cross-compete for binding to human PD-L1 with any of HuMAbs 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 and 13G4. Thus, anti-PD-L1 Abs of the invention include isolated Abs or antigen-binding portions thereof that cross-compete for binding to PD-L1 with a reference Ab or a reference antigen-binding portion thereof comprising: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 15 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 25; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 16 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 26; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 17 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 27; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 18 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 28; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 19 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 29; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 20 and a human light chain variable region

comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 30; (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 21 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 31; (h) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 22 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 32; (i) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 23 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 33; or (j) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 24 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 34.

The ability of an Ab to cross-compete with any of 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 and 13G4 for binding to human PD-Ll demonstrates that such Ab binds to the same epitope region of each of 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 and 13G4, respectively. All isolated Abs that bind to the same epitope region of human PD-Ll as does HuMAb 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 or 13G4 are included among the Abs of the invention. These cross-competing Abs are expected to have very similar functional properties by virtue of their binding to the same epitope region of PD-Ll. For example, cross-competing anti-PD-Ll mAbs 3G10, 1B12, 13G4, 12A4 (BMS-936559), 10A5, 12B7, 11E6 and 5F8 have been shown to have similar functional properties *(see* U.S. Patent No. 7,943,743 at Examples 3-11), whereas mAb 10H10, which binds to a different epitope region, behaves differently (U.S. Patent No. 7,943,743 at Example 11). The higher the degree of cross-competition, the more similar will the functional properties be. Further, cross-competing Abs can be identified in standard PD-Ll binding assays, *e.g.,* Biacore analysis, ELISA assays or flow cytometry, that are well known to persons skilled in the art. In preferred embodiments, the Abs that cross-compete for binding to human PD-1 with, or bind to the same epitope region of human PD-Ll as, 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7 or 13G4 are mAbs, preferably chimeric Abs, or more preferably humanized or human Abs.. Such human mAbs can be prepared and isolated as described in U.S.

Patent No. 7,943,743.

Data provided in Example 2 show that each of the anti-PD-L1 HuMAbs 5F8, 7H1, 1B12, 3G10, 10A5, 11E6, 12A4, 12B7 and 13G4, *i.e.,* all of the HuMAbs tested except 10H10, substantially blocked binding of mAbs 3G10, 10A5, 11E6, 12A4 and 13G4 to Chinese Hamster Ovary (CHO) cells expressing PD-L1 cells. HuMAb 10H10 substantially blocked the binding only of itself to CHO/PD-L1 cells. These data show that 3G10, 10A5, 11E6, 12A4 and 13G4 cross-compete with all of the HuMAbs tested, except for 10H10, for binding to the same epitope region of human PD-L1 (Figures 2A-F).

Data provided in Example 3 show that the binding of HuMAb 12A4 to ES-2 ovarian carcinoma cells expressing PD-L1 cells was substantially blocked by 12A4 itself and by 1B12 and 12B7, and was moderately to significantly blocked by mAbs 5F8, 10A5, 13G4 and 3G10, but was not blocked by mAb 10H10. These data, largely consistent with the data in Example 2, show that 12A4 itself, and 2 other HuMabs, 12B7 and 1B12, substantially cross-compete with 12A4 for binding to the same epitope region, possibly the same epitope, of human PD-L1; 5F8, 10A5, 13G4 and 3G10, exhibit a significant but lower level of cross-competition with 12A4, suggesting that these mAbs may bind to epitopes that overlap the 12A4 epitope; whereas 10H10 does not cross-compete at all with 12A4 (Figure 3), suggesting that this mAb binds to a different epitope region from 12A4.

Anti-PD-L1 Abs of the invention also include Abs engineered starting from Abs having one or more of the V_H and/or V_L sequences disclosed herein, which engineered Abs may have altered properties from the starting Abs. An anti-PD-L1 Ab can be engineered by a variety of modifications as described above for the engineering of modified anti-PD-1 Abs of the invention.

Anti-PD-L1 Abs of the invention also include isolated Abs selected for their ability to bind to PD-L1 in formalin-fixed, paraffin-embedded (FFPE) tissue specimens. The use of FFPE samples is essential for the long-term follow-up analysis of the correlation between PD-L1 expression in tumors and disease prognosis or progression. Yet, studies on measuring PD-L1 expression have often been conducted on frozen specimens because of the difficulty in isolating anti-human PD-L1 Abs that can be used to stain PD-L1 in FFPE specimens by IHC in general (Hamanishi *et ah,* 2007) and, in particular, Abs that bind specifically to membranous PD-L1 in these tissues. The use of different Abs to stain PD-L1 in frozen versus FFPE tissues, and the ability of certain Abs

to distinguish membranous and/or cytoplasmic forms of PD-L1, may account for some of the disparate data reported in the literature correlating PD-L1 expression with disease prognosis (Hamanishi *et al.*, 2007; Gadiot et al, 2011). This disclosure provides several rabbit mAbs that bind with high affinity specifically to membranous human PD-L1 in FFPE tissue samples comprising tumor cells and tumor-infiltrating inflammatory cells.

Rabbit and mouse anti-hPD-L1 mAbs were produced as described in Example 9. Out of almost 200 Ab multiclones screened, only ten rabbit multiclone Abs were found to specifically detect the membranous form of PD-L1, and the top five multiclones (designated Nos. 13, 20, 28, 29 and 49) were subsequently subcloned. The clone that produced the most robust detection specifically of membranous PD-L1, rabbit clone 28-8, was selected for the IHC assays. The sequences of the variable regions of mAb 28-8 are set forth in SEQ ID NOs. 35 and 36, respectively. The sequences of the heavy and light chain CDR domains of mAb 28-8, as delineated using the Kabat system, are set forth in SEQ ID NOs. 37-42. Rabbit clones 28-1, 28-12, 29-8 and 20-12 were the next best mAbs in terms of robust detection of membranous PD-L1 in FFPE tissues.

Anti-PD-L1 Abs of the invention also include antigen-binding portions of the above Abs, including Fab, $F(ab')_2$ Fd, Fv, and scFv, di-scFv or bi-scFv, and scFv-Fc fragments, diabodies, triabodies, tetrabodies, and isolated CDRs (*see* Hollinger and Hudson, 2005; Olafsen and Wu, 2010, for further details).

Nucleic Acid Molecules Encoding Antibodies of the Invention

Another aspect of the present disclosure pertains to isolated nucleic acid molecules that encode any of the Abs of the invention. These nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid of the invention can be, for example, DNA or RNA, and may or may not contain intronic sequences. In a preferred embodiment, the nucleic acid is a cDNA.

Nucleic acids of the invention can be obtained using standard molecular biology techniques. For Abs expressed by hybridomas (*e.g.*, hybridomas prepared from transgenic mice carrying human immunoglobulin genes as described further below), cDNAs encoding the light and heavy chains of the Ab made by the hybridoma can be obtained by standard PCR amplification or cDNA cloning techniques. Nucleic acids encoding Abs obtained from an immunoglobulin gene library (*e.g.*, using phage display techniques) can be recovered from the library.

Preferred nucleic acids molecules of the invention are those encoding the V*H* and

V_K sequences of the anti-PD-1 HuMAbs, 17D8, 2D3, 4H1, 5C4, 4A1 1, 7D3 and 5F4 (disclosed in U.S. Patent No. 8,008,449), and those encoding the V_H and V_K sequences of the anti-PD-Ll HuMAbs, 3G10, 12A4, 10A5, 5F8, 10H10, 1B12, 7H1, 11E6, 12B7, and 13G4 (disclosed in U.S. Patent No. 7,943,743). An isolated DNA encoding the V_H region can be converted to a full-length heavy chain gene by operatively linking the V_H-encoding DNA to another DNA molecule encoding heavy chain constant regions (Cm, C_{H2} and *Cm),* the sequences of which are known in the art and can be obtained by standard PCR amplification. The heavy chain constant region can be an IgGl, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but is preferably an IgGl or IgG4 constant region. Similarly, an isolated DNA encoding the V_L region can be converted to a full-length light chain gene by operatively linking the Vz-encoding DNA to another DNA molecule encoding the light chain constant region ((¾, the sequence of which is known in the art and can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

Pharmaceutical Compositions

Antibodies of the present invention may be constituted in a composition, *e.g.,* a pharmaceutical composition, containing one Ab or a combination of Abs, or an antigen-binding portion(s) thereof, and a pharmaceutically acceptable carrier. As used herein, a "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration *(e.g.,* by injection or infusion). A pharmaceutical composition of the invention may include one or more pharmaceutically acceptable salts, anti-oxidant, aqueous and nonaqueous carriers, and/or adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents.

Dosage regimens are adjusted to provide the optimum desired response, *e.g.,* a therapeutic response or minimal adverse effects. For administration of an anti-PD-1 or anti-PD-Ll Ab, the dosage ranges from about 0.0001 to about 100 mg/kg, usually from about 0.001 to about 20 mg/kg, and more usually from about 0.01 to about 10 mg/kg, of the subject's body weight. Preferably, the dosage is within the range of 0.1-10 mg/kg body weight. For example, dosages can be 0.1, 0.3, 1, 3, 5 or 10 mg/kg body weight, and

more preferably, 0.3, 1, 3, or 10 mg/kg body weight. The dosing schedule is typically designed to achieve exposures that result in sustained receptor occupancy (RO) based on typical pharmacokinetic properties of an Ab. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months. The dosage and scheduling may change during a course of treatment. For example, dosing schedule may comprise administering the Ab: (i) every two weeks in 6-week cycles; (ii) every four weeks for six dosages, then every three months; (iii) every three weeks; (iv) 3-10 mg/kg body weight once followed by 1 mg/kg body weight every 2-3 weeks. Considering that an IgG4 Ab typically has a half-life of 2-3 weeks, a preferred dosage regimen for an anti-PD-1 or anti-PD-L1 Ab of the invention comprises 0.3-10 mg/kg body weight, preferably 3-10 mg/kg body weight, more preferably 3 mg/kg body weight via intravenous administration, with the Ab being given every 14 days in up to 6-week or 12-week cycles until complete response or confirmed progressive disease.

In some methods, two or more mAbs with different binding specificities are administered simultaneously, in which case the dosage of each Ab administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be, for example, weekly, every 2 weeks, every 3 weeks, monthly, every three months or yearly. Intervals can also be irregular as indicated by measuring blood levels of Ab to the target antigen in the patient. In some methods, dosage is adjusted to achieve a plasma Ab concentration of about 1-1000 μg/ml and in some methods about 25-300 μg/ml.

Alternatively, the Ab can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the Ab in the patient. In general, human Abs show the longest half-life, followed by humanized Abs, chimeric Abs, and nonhuman Abs. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is typically administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient

can be administered a prophylactic regime.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being unduly toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts. A composition of the present invention can be administered via one or more routes of administration using one or more of a variety of methods well known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

Uses and Methods of the Invention

The Abs, Ab compositions, nucleic acids and methods of the present invention have numerous *in vitro* and *in vivo* utilities including, for example, methods to determine and quantify the expression of PD-1 or PD-L1 comprising binding of the Abs to the target polypeptides or measuring the amount of nucleic acid encoding these polypeptides, and a method for immunotherapy of a subject afflicted with a disease comprising administering to the subject a composition comprising a therapeutically effective amount of a therapeutic agent that inhibits signaling from an inhibitory immunoregulator. In preferred embodiments of the latter method, the inhibitory immunoregulator is a component of the PD-1/PD-L1 signaling pathway, and the therapeutic agent disrupts signaling of this pathway. More preferably, the therapeutic agent is an Ab that interferes with the interaction between PD-1 and PD-L1. In certain preferred embodiments of this method, the Ab binds specifically to PD-1 and blocks the interaction of PD-1 with PD-L1 and/or PD-L2. In other preferred embodiments, the therapeutic agent is an Ab that binds specifically to PD-L1 and blocks the interaction of PD-L1 with PD-1 and/or B7-1 (CD80). Thus, this disclosure provides methods for potentiating an immune response in a subject comprising administering an anti- PD-1 and/or an anti-PD-L1 Ab in order to disrupt the interaction between PD-1 and PD-L1, and methods of treating diseases

mediated by such a potentiation of the immune response. When Abs to PD-1 and PD-L1 are administered together, the two can be administered sequentially in either order or simultaneously. In certain aspects, this disclosure provides methods of modifying an immune response in a subject comprising administering to the subject an anti-PD-1 and/or an anti-PD-L1 Ab of the invention, or antigen-binding portion thereof, such that the immune response in the subject is modified. Preferably, the immune response is potentiated, enhanced, stimulated or up-regulated. In preferred embodiments, the Abs of the present invention are human Abs.

Preferred subjects include human patients in need of enhancement of an immune response. The immunotherapeutic methods disclosed herein are particularly suitable for treating human patients having a disorder that can be treated by potentiating a T-cell mediated immune response. In certain embodiments, the methods are employed for treatment of subjects afflicted with a disease caused by an infectious agent. In preferred embodiments, the methods are employed for treatment of subjects afflicted with, or at risk of being afflicted with, a cancer.

Cancer Immunotherapy

Blockade of PD-1/PD-L1 interaction has been shown to potentiate immune responses *in vitro* (U.S. Patent Nos. 8,008,449 and 7,943,743; Fife *et al,* 2009) and mediate preclinical antitumor activity (Dong *et al,* 2002; Iwai *et al,* 2002). However, the molecular interactions potentially blocked by these two Abs are not identical: anti-PD-1 Abs of the invention disrupt PD-1/PD-L1 and potentially PD-1/PD-L2 interactions; in contrast, whereas anti-PD-L1 Abs of the invention also disrupt PD-1/PD-L1 interactions, they do not block PD-1/PD-L2 interactions but instead may disrupt the PD-1-independent PD-L1/CD80 interaction, which has also been shown to down-modulate T-cell responses *in vitro* and *in vivo* (Park *et al,* 2010; Paterson *et al,* 2011; Yang *et al,* 2011; Butte *et al,* 2007; Butte *et al,* 2008). Thus, it is possible that among these varied ligand-receptor pairings, different interactions may dominate in different cancer types, contributing to dissimilar activity profiles for the two Abs.

Disruption of the PD-1/PD-L1 interaction by antagonistic Abs can enhance the immune response to cancerous cells in a patient. PD-L1 is not expressed in normal human cells, but is abundant in a variety of human cancers (Dong *et al,* 2002). The interaction between PD-1 and PD-L1 impairs T cell responses as manifested by a decrease in tumor-infiltrating lymphocytes (TILs) and a decrease in T-cell receptor-

mediated proliferation, resulting in T cell anergy, exhaustion or apoptosis, and immune evasion by the cancerous cells (Zou and Chen, 2008; Blank *et ah,* 2005; Konishi *et ah,* 2004; Dong *et ah,* 2003; Iwai *et ah,* 2002). Immune suppression can be reversed by inhibiting the local interaction between PD-L1 and PD-1 using an anti-PD-1 and/or an anti-PD-Ll Ab. These Abs may be used alone or in combination to inhibit the growth of cancerous tumors. In addition, either or both of these Abs may be used in conjunction with other immunogenic and/or anti-cancer agents including cytokines, standard cancer chemotherapies, vaccines, radiation, surgery, or other Abs.

Immunotherapy of Cancer Patients Using an Anti-PD-1 Antibody

This disclosure provides a method for immunotherapy of a subject afflicted with cancer, which method comprises administering to the subject a composition comprising a therapeutically effective amount of an Ab or an antigen-binding portion thereof that disrupts the interaction of PD-1 with PD-L1 and/or PD-L2. This disclosure also provides a method of inhibiting growth of tumor cells in a subject, comprising administering to the subject an Ab or an antigen-binding portion thereof that disrupts the interaction of PD-1 with PD-L1 and/or PD-L2 in an amount effective to inhibit growth of the tumor cells. In preferred embodiments, the subject is a human. In other preferred embodiments, the Ab or antigen-binding portion thereof is an anti-PD-1 Ab of the invention or an antigen-binding portion thereof. In certain embodiments, the Ab or antigen-binding portion thereof is of an IgGl or IgG4 isotype. In other embodiments, the Ab or antigen-binding portion thereof is a mAb or an antigen-binding portion thereof. In further embodiments, the Ab or antigen-binding portion thereof is a chimeric, humanized or human Ab or an antigen-binding portion thereof. In preferred embodiments for treating a human patient, the Ab or antigen-binding portion thereof is a human Ab or an antigen-binding portion thereof.

The clinical trials described in the Examples employed the anti-PD- 1 HuMAb, nivolumab (designated 5C4 in U.S. Patent No. 8,008,449), to treat cancer. While 5C4 was selected as the lead Ab for entering the clinic, it is notable that several anti-PD-1 Abs of the invention share with 5C4 functional properties that are important to the therapeutic activity of 5C4, including high affinity binding specifically to human PD-1, increasing T-cell proliferation, IL-2 secretion and interferon-γ production in an MLR assay, inhibiting the binding of PD-L1 and/or PD-L2 to PD-1, and inhibiting tumor cell growth *in vivo.* Moreover, certain of the anti-PD-1 Abs of the invention, 17D8, 2D3, 4H1 and 7D3 are

structurally related to 5C4 in comprising V_H and V_K regions that have sequences derived from V_H 3-33 and V_KL6 germline sequences, respectively. In addition, 5C4, 2D3, 7D3, 4H1 and 17D8 all cross-compete for binding to the same epitope region of hPD-1 (Example 1). Thus, the preclinical characterization of nivolumab and other anti-PD-1 HuMabs indicate that the methods of treating cancer provided herein may be performed using different Abs selected from the broad genus of anti-PD-1 Abs of the invention.

Accordingly, certain embodiments of the immunotherapy methods disclosed herein comprise administering to a patient an anti-PD- 1 Ab or antigen-binding portion thereof comprising: (a) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_H 3-33 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_KL6 germline sequence, or (b) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_H 4-39 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_KL15 germline sequence.

In certain other embodiments, the Ab or antigen-binding portion thereof that is administered to the patient cross-competes for binding to PD-1 with a reference Ab or a reference antigen-binding portion thereof comprising: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 1 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 8; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 2 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 9; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 3 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 10; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 4 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 11; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 5 and a human light chain variable region comprising consecutively linked amino

acids having the sequence set forth in SEQ ID NO: 12; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 6 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 13; or (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 7 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 14. In preferred embodiments, the Ab or antigen-binding portion thereof cross-competes for binding to PD-1 with nivolumab.

In certain embodiments of the immunotherapy methods disclosed herein, the anti-PD-1 Ab or antigen-binding portion thereof administered to the patient comprises: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 1 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 8; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 2 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 9; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 3 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 10; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 4 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 11; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 5 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 12; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 6 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 13; or (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 7 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 14. In preferred

embodiments, the anti-PD-1 Ab or antigen-binding portion comprises a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 4 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 11. In other preferred embodiments, the anti-PD-1 Ab is nivolumab.

In certain embodiments of the present methods, the anti-PD-1 Ab is formulated for intravenous administration. In preferred embodiments, the Ab is administered intravenously at a dose of 3 mg/kg over 60 minutes every 2 weeks. Typically, treatment is continued as long as clinical benefit is observed or until unmanageable toxicity or disease progression occurs.

In the clinical trials of anti-PD-1 immunotherapy described in the Examples below, intriguing ORs with durable clinical responses, even in heavily pretreated patients, were observed across multiple tumor types including a substantial proportion of NSCLC, MEL, and RCC patients and in various sites of metastasis including liver, lung, lymph nodes, and bone. MEL and RCC are considered to be immunogenic neoplasms, having previously been demonstrated to be responsive to cancer immunotherapy, *e.g.*, interferon-alfa and interleukin-2 in both MEL and RCC (Eton *et al.*., 2002; Coppin *et ah,* 2005; McDermott and Atkins, 2006) and anti-CTLA-4 Ab in MEL (Hodi *et al,* 2010). In MEL patients, significant ORRs were observed with nivolumab across different doses of 0.1, 0.3, 1, 3, or 10 mg/kg with an overall response rate of about 31%, and similarly comparable ORRs of about 30% were observed in RCC patients treated with nivolumab doses of 1 or 10 mg/kg *(see* Example 7). In contrast, immunotherapy has historically had minimal success in lung cancer. This lack of success has been attributed to NSCLC being "non-immunogenic" *(see, e.g.,* Holt and Disis, 2008; Holt *et al,* 2011). The ability of lung cancer to thwart the immune system results from multiple factors, including the secretion of immunosuppressive cytokines, loss of major histocompatibility complex antigen expression, and co-opting of T cell inhibitory pathways (Dasanu *et al.,* 2012; Marincola *et al.,* 2000; Brahmer *et al,* 2012).

Despite the limited efficacy of immunotherapy historically seen in lung cancer and the diverse mechanisms employed by lung cancer cells to evade immune system attack, various tumor cell vaccines *(e.g.,* belagenpumatucel-L, a whole-cell-based vaccine that blocks the action of TGF-p2) and antigen-based vaccines *(e.g.,* a humoral EGF vaccine and vaccines incorporating a MAGE-A3 fusion protein or portions of the tumor-

associated MUC1 antigen) that augment antigen-specific antitumor immunity are being evaluated in clinical trials (Holt *et ah,* 201 1; Shepherd *et ah,* 201 1; Dasanu *et ah,* 2012; Brahmer *et al,* 2012). However, while such antigen-specific vaccines have shown some promise for the treatment of NSCLC, Holt *et ah* (201 1) noted that trials involving nonspecific immunotherapeutic interventions had failed to improve outcomes in NSCLC, which indicated a need to combine them with antigen-specific vaccines. These authors expressed the view that truly efficacious immunotherapy of NSCLC would only result from implementation of strategies to both augment antitumor immunity and counteract tumor-mediated immunosuppression, and concluded that the nonexistent or weak nascent immune responses in NSCLC patients make it unlikely that nonspecific stimulation of the immune system or removal of immunosuppression would create changes in clinical outcomes.

Thus, the results reported herein with NSCLC are particularly striking, unexpected and surprising. In NSCLC patients, ORs were observed at nivolumab doses of 1, 3, or 10 mg/kg with response rates of 3%, 24%, and 20%, respectively *(see* Example 7). ORs were observed across NSCLC histologies: 9 responders of 54 squamous (17%), and 13 of 74 nonsquamous (18%). This level of activity seen with nivolumab in NSCLC patients with significant prior therapy (54% with 3 lines of previous therapy) and across histologies is unprecedented, especially in the squamous histology patients *(cf.* Gridelli *et ah,* 2008; Miller, 2006), and provides a very favorable benefit/risk dynamic regarding efficacy and safety compared to existing standard-of-care.

In certain embodiments of the present immunotherapy methods, the anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic squamous or non-squamous NSCLC after platinum based therapy (regardless of maintenance) and one other treatment. In other embodiments, the anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic squamous or non-squamous NSCLC after failure of platinum-based therapy and one other prior chemotherapy regimen. In further embodiments, the anti-PD- 1 Ab is indicated as monotherapy for locally advanced or metastatic squamous or non-squamous NSCLC after failure of two lines of therapy one of which must include platinum based regimen. In yet further embodiments, the anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic squamous or non-squamous NSCLC after at least one prior chemotherapy, after failure of at least one prior platinum based therapy, or after progression on at least one platinum doublet therapy. In

all of the immunotherapy methods disclosed herein, treatment may be continued as long as clinical benefit is observed or until unmanageable toxicity or disease progression occurs.

Durability of clinical responses to anti-PD-1 in heavily-pretreated cancer patients

The critical role of the PD-1 pathway in suppressing anti-tumor immunity, first revealed in laboratory models, has now been validated in clinical studies. As described herein, monotherapy with drugs blocking PD-1 (nivolumab) or its major ligand PD-L1 (BMS-936559) can mediate regression in patients with treatment-refractory advanced cancers. The objective response rate (ORR) in heavily pretreated NSCLC patients receiving anti-PD-1 Ab, including patients with squamous histology, is surprising and unexpected, as standard salvage therapies historically show modest benefit in these patients (Scagliotti *et al.*, 2011). As measured by standard RECIST in this study, ORs were long-lasting, with median response durations of about 18 months in 22 of 129 responders ((*see* Example 7). In addition, patterns of tumor regression consistent with immune-related patterns of response were observed.

These findings have established the PD-1 pathway as a new therapeutic focus in oncology (Pardoll, 2012; Topalian *et al.*, 2012c; Hamid and Carvajal, 2013). In the current study, in which 54% of patients had progressive disease following 3 or more prior systemic regimens, preliminary analysis has been conducted up to March 2013. This updated analysis has supported and reinforced the data obtained and conclusions reached from the earlier February 2012 analyses. Thus, conventional ORs or prolonged disease stabilization were documented in patients with NSCLC (17% and 10%, respectively), MEL (31%, 7%) and RCC (29%, 27%), across all doses tested (*see* Example 7). Additionally, 13 patients (4%) manifested unconventional, "immune-related" response patterns as previously described with anti-CTLA-4 therapy, several of which were sustained (Sharma *et al.*, 2011).

The durability of ORs across multiple cancer types in patients treated with the anti-PD-1 Ab is particularly notable. The updated analyses again underscored the durability of clinical activity in nivolumab-treated patients, which has not generally been observed with chemotherapy or small molecule inhibitors to date, but has been observed in some patients with advanced melanoma receiving immunotherapies, including ipilimumab and high-dose interleukin-2 (Topalian *et al.*, 2011; Hodi *et al.*, 2010). The persistence of partial tumor regressions following drug discontinuation suggests that PD-1

blockade has reset the immune equilibrium between tumor and host, and the OS benefit may ultimately prove to be significantly longer than has been measured to date. Further follow-up is needed to determine the ultimate durability of tumor regression and disease stabilization in patients on these clinical trials.

Significantly, the durable objective tumor regression and disease stabilization induced by nivolumab in heavily-pretreated patients with advanced NSCLC, MEL, and RCC translate to survival outcomes that surpass historical data for these patient populations treated with conventional chemotherapy and/or tyrosine-kinase inhibitor (TKI) treatments. In NSCLC, 1- and 2-year survival rates were 42% and 14%, respectively, with median OS of 9.2 and 10.1 months in patients with squamous and non-squamous cancers, respectively *(see* Example 7). This high level of efficacy is especially impressive since 54% of these patients had received 3 or more prior therapies. Moreover, because follow-up of many lung cancer patients was relatively limited, these figures may change as data mature. Historically, 2L chemotherapeutics for lung cancer *(i.e.,* docetaxel and pemetrexed) have achieved a median OS of 7.5-8.3 months, and one-year survival rates of approximately 30% (Shepherd *et al.,* 2000; Hanna *et al.,* 2004). In a 2L/3L population, erlotinib-treated patients had a median survival of 6.7 months, versus 4.7 months in placebo-treated patients (Shepherd *et al.,* 2005). No therapy is currently approved for use in lung cancer beyond the 3L setting, and minimal data exist to benchmark survival in this patient population, except in retrospective reviews with reported median survivals of 5.8-6.5 months and 1-year survivals of 25% (Girard *etal,* 2009; Scartozi *et al,* 2010).

In nivolumab-treated MEL patients, median OS of 16.8 months was achieved, with landmark survival rates of 62% (1-year) and 43% (2-year) *(see* Example 7). Survival outcomes in pretreated melanoma patients supported the recent FDA approvals of ipilimumab and vemurafenib. In a recent phase 3 trial enrolling melanoma patients with at least one prior treatment for metastatic disease, ipilimumab increased median OS from 6.4 to 10.1 months, compared to a gplOO peptide vaccine (Hodi *et al.,* 2010). In phase 2 trials of ipilimumab in previously treated patients, 2-year survival rates ranged from 24.2-32.8% (Lebbe *et al.,* 2012). Median OS in previously treated patients with BRAF-mutant melanoma enrolled on a large phase 2 of vemurafenib was 15.9 months, and 1-year survival was 58% (Sosman *et al.,* 2012).

Accordingly, in certain embodiments, immunotherapy with anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic MEL after therapy with dacarbazine (regardless of maintenance) and one other treatment. In other embodiments, the anti-PD- 1 Ab is indicated as monotherapy for locally advanced or metastatic MEL after failure of dacarbazine-based therapy. In further embodiments, the anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic MEL after failure of two lines of therapy one of which must include a dacarbazine-based regimen. In yet further embodiments, the anti-PD- 1 Ab is indicated as monotherapy for locally advanced or metastatic MEL after at least one prior chemotherapy, after failure of at least one prior dacarbazine-based therapy, or after progression on at least dacarbazine therapy. In all of the immunotherapy methods disclosed herein, treatment may be continued as long as clinical benefit is observed or until unmanageable toxicity or disease progression occurs.

In nivolumab-treated patients with RCC, among whom 45% received 3 or more prior therapies and 71% received prior anti-angiogenic therapy, median OS was reached at 22 months (as of the March 2013 date of analysis). Landmark survival rates of 70% (1-year) and 50% (2-year) were observed *(see* Example 7). In a recent Phase 3 trial enrolling kidney cancer patients whose disease progressed following anti-angiogenic therapy, everolimus was compared with placebo: median OS was 14.8 versus 14.4 months, respectively (Motzer *et ah,* 2008; Motzer *et ah,* 2010). A recent Phase 3 trial comparing sorafenib to temsirolimus in a sunitinib-refractory kidney cancer population yielded median OS of 16.6 andl2.3 months, respectively (Hutson *et ah,* 2012). Thus, as with NSCLC and MEL, treatment of a heavily -pretreated RCC patient population with nivolumab has yielded a considerably longer median OS (> 22 months) than treatment of a less refractory population with standard-of-care therapies. Controlled Phase 3 trials with prospective survival endpoints are underway in NSCLC, MEL and RCC (NCTO 1673 867, NCTO 172 1772, NCTO 1642004, NCTO 1668784, and NCTO 172 1746 *(see* Clinical Trials Website, http://wvvw.cjinicaltrials.gov). The results from these trials are expected to further demonstrate the high efficacy of, and durability of responses to, nivolumab in these cancers compared standard-of-care therapies.

In certain embodiments, immunotherapy with anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic RCC after therapy with an anti-angiogenic TKI or a mTOR inhibitor (regardless of maintenance) and one other treatment. In other embodiments, the anti-PD- 1 Ab is indicated as monotherapy for

locally advanced or metastatic RCC after failure of therapy with an anti-angiogenic TKI or a mTOR inhibitor. In further embodiments, the anti-PD-1 Ab is indicated as monotherapy for locally advanced or metastatic RCC after failure of two lines of therapy one of which must include an anti-angiogenic TKI or a mTOR inhibitor. In yet further embodiments, the anti-PD- 1 Ab is indicated as monotherapy for locally advanced or metastatic RCC after at least one prior chemotherapy, after failure of at least one prior anti-angiogenic TKI- or mTOR inhibitor-based therapy, or after progression on at least an anti-angiogenic TKI or a mTOR inhibitor therapy. Anti-PD- 1 immunotherapy may be continued as long as clinical benefit is observed or until unmanageable toxicity or disease progression occurs.

Notably, OS in lung cancer, melanoma and kidney cancer patients receiving nivolumab was considerably longer than PFS. These results mirror those reported for ipilimumab (Hodi *et ah,* 2010), and reflect the observation that early tumor enlargement or the appearance of new lesions in some patients receiving immune checkpoint blockade can evolve to disease stabilization or regression. These findings suggest that progression-free survival may not be an optimal endpoint for determining the efficacy of nivolumab and other agents in this class.

The data disclosed herein demonstrating the high efficacy, durability and broad applicability of anti-PD- 1 immunotherapy for treating cancer has led to nivolumab being tested for additional types of cancer. For example, on the basis that increased PD-L1 expression has been reported with various hematologic malignancies and may prevent the host immune response from exerting a beneficial impact on the malignant cells, a trial to confirm the ability of nivolumab to mediate antitumor activity in patients with hematologic malignancies (multiple myeloma, B-cell lymphoma, T-cell lymphoma, Hodgkin's lymphoma/primary mediastinal B-cell lymphoma, and chronic myelogenous leukemia) has been initiated (NCT0 15923 70). Nivolumab is also being tested as a monotherapy in advanced hepatocellular carcinoma (NCT01658878).

In summary, the results of anti-PD- 1 immunotherapy disclosed herein are remarkable in at least the following three respects. First, the efficacy of anti-PD-1 has been shown to surpass historical efficacy data for patients on standard-of-care treatments for cancer. Notably, this efficacy has been demonstrated in patient in heavily pretreated populations in which about half of the patients had progressive disease following 3 or more prior systemic regimens. Such patients, afflicted with advanced, metastatic and/or

refractory cancers, are notoriously difficult to treat. Accordingly, this disclosure provides methods for immunotherapy of a patient afflicted with an advanced, metastatic and/or refractory cancer, which method comprises administering to the patient a therapeutically effective amount of an Ab or an antigen-binding portion thereof that disrupts the interaction of PD-1 with PD-L1 and/or PD-L2. In certain embodiments of any of the therapeutic methods disclosed herein, the subject has been pre-treated for the cancer; for example, the subject had undergone at least one, two, or three prior lines of therapy for cancer.

Second, the present therapeutic methods have been shown to be applicable to a broad genus of different cancers. Based on the surprising discovery that even a "non-immunogenic" cancer such as NSCLC (Holt *et ah,* 201 1) and hard-to-treat cancers such as ovarian and gastric cancers (as well as other cancers tested, including MEL, RCC, and CRC) are amendable to treatment with anti-PD-1 and/or anti-PD-Ll *(see* Examples 7 and 14), this disclosure generally provides methods for immunotherapy of a patient afflicted with practically any of a very wide range of cancers.

Third, treatment with an anti-PD-1 or anti-PD-Ll Ab has been shown to produce strikingly durable clinical activity in cancer patients. Accordingly, this disclosure provides immunotherapeutic methods of inducing a durable clinical response in a cancer patient comprising administering to the patient a therapeutically effective amount of an Ab or an antigen-binding portion thereof that disrupts the interaction of PD-1 with PD-L1 and/or PD-L2. In preferred embodiments of any of the therapeutic methods described herein, the clinical response is a durable response.

As used herein, a "durable" response is a therapeutic or clinical response that exceeds the anticipated median OS rate in a patient population. The anticipated median OS rate varies with different cancers and different patient populations. In certain embodiments, a durable response exceeds the anticipated median OS rate in the relevant patient population by at least 10%, preferably by at least 20%, more preferably by at least 30%, and even more preferably by at least 50%. A major benefit of immunotherapeutic approaches based on PD- 1 pathway blockade may be the functional restoration of exhausted T cells with long-term generation of memory T cells that may maintain antitumor immune surveillance and inhibit tumor growth for prolonged periods extending to many years, even in the absence of continued therapy (Kim and Ahmed, 2010). Indeed, long-term follow-up studies on patients following cessation of nivolumab therapy have

confirmed that a patient with CRC experienced a complete response which was ongoing after 3 years; a patient with RCC experienced a partial response lasting 3 years off therapy, which converted to a complete response that was ongoing at 12 months; and a patient with melanoma achieved a partial response that was stable for 16 months off therapy, and recurrent disease was successfully treated with reinduction anti-PD-1 therapy (Lipson *et ah,* 2013).

Immune-related clinical responses

It has become evident that conventional response criteria may not adequately assess the activity of immunotherapeutic agents because progressive disease (by initial radiographic evaluation) does not necessarily reflect therapeutic failure. For example, treatment with the anti-CTLA-4 Ab, ipilimumab, has been shown to produce four distinct response patterns, all of which were associated with favorable survival: (a) shrinkage in baseline lesions, without new lesions; (b) durable stable disease (in some patients followed by a slow, steady decline in total tumor burden); (c) response after an increase in total tumor burden; and (d) response in the presence of new lesions. Accordingly, to properly evaluate immunotherapeutic agents, long-term effects on the target disease must also be captured. In this regard, systematic immune-related response criteria (irRC) that make allowances for an early increase in tumor burden and/or the appearance of new lesions, and which seek to enhance the characterization of immune-related response patterns, have been proposed (Wolchok *et ah,* 2009). While the full impact of these unconventional response patterns remains to be defined in randomized trials of nivolumab with survival endpoints, the present observations are reminiscent of findings with ipilimumab in which a significant extension of OS was observed in treated patients (Hodi *et ah,* 2010; Robert *et ah,* 201 1).

The overall risk/benefit profile of anti-PD-1 immunotherapy is also favorable, with a low incidence of more severe drug-related adverse events (AEs; > grade 3), the specific events observed to date being consistent with other immunotherapeutic agents. This suggests that anti-PD-1 immunotherapy can be delivered in an outpatient setting with minimal supportive care.

Broad spectrum of cancers treatable by anti-PD-1 immunotherapy

The clinical data presented herein demonstrate that immunotherapy based on PD-1 blockade is not limited to only "immunogenic" tumor types, such as MEL and RCC, but extends to tumor types not generally considered to be immune-responsive, including

NSCLC. The unexpected successes with treatment-refractory metastatic NSCLC underscore the possibility that any neoplasm can be "immunogenic" in the context of proper immune modulation, and suggest that PD-1 blockade as an immunotherapeutic approach is broadly applicable across a very diverse range of tumor types. Thus, cancers that may be treated using the anti-PD-1 Abs of the invention also include cancers typically responsive to immunotherapy as well as cancers that have traditionally been regarded as non-immunogenic. Non-limiting examples of preferred cancers for treatment include NSCLC, MEL, RCC, CRC, CRPC, HCC, squamous cell carcinoma of the head and neck, carcinomas of the esophagus, ovary, gastrointestinal tract and breast, and a hematologic malignancy. Although NSCLC is not generally considered responsive to immunotherapy, data disclosed herein unexpectedly demonstrate that both squamous and non-squamous NSCLC are responsive to treatment with an anti-PD-1 Ab. Additionally, the disclosure provides for the treatment of refractory or recurrent malignancies whose growth may be inhibited using an anti-PD-1 Ab of the invention.

Examples of cancers that may be treated using an anti-PD-1 Ab in the methods of the present invention, based on the indications of very broad applicability of anti-PD-1 immunotherapy provided herein, include liver cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, breast cancer, lung cancer, cutaneous or intraocular malignant melanoma, renal cancer, uterine cancer, ovarian cancer, colorectal cancer, colon cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, environmentally induced cancers including those induced by asbestos, hematologic malignancies including, for example, multiple myeloma, B-cell lymphoma, Hodgkin lymphoma/primary mediastinal B-cell lymphoma, non-Hodgkin's lymphomas, acute myeloid lymphoma, chronic myelogenous leukemia, chronic lymphoid leukemia,

follicular lymphoma, diffuse large B-cell lymphoma, Burkitt's lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, acute lymphoblastic leukemia, mycosis fungoides, anaplastic large cell lymphoma, T-cell lymphoma, and precursor T-lymphoblastic lymphoma, and any combinations of said cancers. The present invention is also applicable to treatment of metastatic cancers.

Medical uses of anti-PD-1 Abs

One aspect of this invention is the use of any anti-PD-1 Ab or antigen-binding portion thereof of the invention for the preparation of a medicament for inhibiting signaling from the PD-1/PD-L1 pathway so as to thereby potentiate an endogenous immune response in a subject afflicted with cancer. Another aspect is the use of any anti-PD-1 Ab or an antigen-binding portion thereof of the invention for the preparation of a medicament for immunotherapy of a subject afflicted with cancer comprising disrupting the interaction between PD-1 and PD-L1. These uses for the preparation of medicaments are broadly applicable to the full range of cancers disclosed herein. In preferred embodiments of these uses, the cancers include squamous NSCLC, non-squamous NSCLC, MEL, RCC, CRC, CRPC, HCC, squamous cell carcinoma of the head and neck, and carcinomas of the esophagus, ovary, gastrointestinal tract and breast, and a hematologic malignancy. This disclosure also provides medical uses of any anti-PD-1 Ab or antigen-binding portion thereof of the invention corresponding to all the embodiments of the methods of treatment employing an anti-PD-1 Ab described herein.

The disclosure also provides an anti-PD-1 Ab or an antigen-binding portion thereof of the invention for use in treating a subject afflicted with cancer comprising potentiating an endogenous immune response in the subject by inhibiting signaling from the PD-1/PD-L1 pathway. The disclosure further provides an anti-PD-1 Ab or an antigen-binding portion thereof of the invention for use in immunotherapy of a subject afflicted with cancer comprising disrupting the interaction between PD-1 and PD-L1. These Abs may be used in potentiating an endogenous immune response against, or in immunotherapy of, the full range of cancers disclosed herein. In preferred embodiments, the cancers include squamous NSCLC, non-squamous NSCLC, MEL *(e.g.,* metastatic malignant MEL), RCC, CRC, CRPC, HCC, squamous cell carcinoma of the head and neck, and carcinomas of the esophagus, ovary, gastrointestinal tract and breast, and a hematologic malignancy.

Combination Therapy Including Anti-PD-1 Antibodies

Whereas monotherapy with anti-PD-1 and anti-PD-Ll Abs has been shown herein to significantly increase the survival of patients with lung cancer, melanoma, kidney cancer, and potentially other malignancies, preclinical data indicate that synergistic treatment combinations based on PD-1 pathway blockade could have even more potent effects. Clinical evaluation of nivolumab combined with ipilimumab (anti-CTLA-4), whose mechanism of action is similar yet distinct from nivolumab's (Parry *et ah,* 2005; Mellman *et ah,* 201 1; Topalian *et ah,* 2012c), is ongoing and results from a Phase 1 trial are provided herein *(see, also,* NCT01024231; NCT01844505; NCT01783938; Wolchok *et ah,* 2013a; Wolchok *et ah,* 2013b; Hodi *et ah,* 2013). Clinical studies have also been initiated involving the administration of nivolumab in combination with melanoma vaccines (NCT01 176461, NCT01 176474; Weber *et ah,* 2013), with lirilumab (BMS-986015), a human IgG4 anti-KIR Ab, in patients with advanced solid tumors (NCT0 17 14739; Sanborn *et ah,* 2013), with cytokines, for example, IL-21, in patients with advanced or metastatic solid tumors (NCT01629758; Chow *et ah,* 2013), with chemotherapeutic drugs, for example, with platinum-based doublet chemotherapy in chemotherapy-naive NSCLC patients (NCT01454102; Rizvi *et ah,* 2013), and small-molecule targeted therapies in patients with metastatic RCC (NCT0 1472081; Amin *et ah,* 2013).

In certain aspects, this disclosure relates to the combination of an anti-PD-1 Ab with different cancer treatments, including chemotherapeutic regimes, radiation, surgery, hormone deprivation and angiogenesis inhibitors, for the treatment of various cancers. PD-1 blockade may also be effectively combined with an immunogenic agent, for example, a preparation of cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), antigen-presenting cells such as dendritic cells bearing tumor-associated antigens, cells transfected with genes encoding immune stimulating cytokines (He *et ah,* 2004), and/or another immunotherapeutic Ab *(e.g.,* an anti-CTLA-4, anti-PD-Ll and/or anti-LAG-3 Ab). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gplOO, MAGE antigens, Trp-2, MARTI and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF.

Combination of anti-PD-1 and anti-CTLA-4 Abs to treat advanced melanoma

Given that immunologic checkpoints are non-redundant and can inhibit T-cell activation, proliferation and effector function within lymph nodes and/or the tumor microenvironment, and based on preclinical data that the combination of anti-CTLA-4 and anti-PD-1 had a stronger anti-tumor effect in mouse tumor models than either Ab alone (*see* U.S. Patent No. 8,008,449), the hypothesis that combined blockade of CTLA-4 and PD-1 could produce greater anti-tumor activity than single agents was tested in a clinical trial in MEL patients (Example 15).

Although not formally compared in this study, the concurrent nivolumab/ipilimumab regimen, which comprised achieved ORRs that exceed the rates achieved with either nivolumab (Example 7) or ipilimumab alone (Hodi *et al.,* 2010). Most importantly, rapid and deep responses were achieved in a substantial portion of treated patients, a "deep" tumor response referring to a response in target lesions characterized by a reduction of 80% or more from baseline measurements by radiographic assessment. In the present study, the majority of responding patients, including some with extensive and bulky tumor burden, achieved > 80% tumor regression at the time of the initial tumor assessment. Particularly striking was the observation that 31% of response-evaluable patients treated with concurrent-regimens (which comprise (i) an induction dosing schedule with combined administration of the anti-PD- 1 and anti-CTLA-4 Abs followed by administration of the anti-PD-1 Ab alone, and (ii) a maintenance dosing schedule comprising less frequent combined administration of the anti-PD-1 and anti-CTLA-4 Abs) demonstrated > 80% tumor regression by week 12. At the MTD for the concurrent regimen, all 9 responding patients demonstrated > 80% tumor regression with 3 CRs. In contrast, in clinical experience to date, < 3% of MEL patients who received nivolumab or ipilimumab at 3 mg/kg achieved a CR (Example 7; Hodi *et al,* 2010). Thus, the overall activity of this immunotherapy combination in this preliminary phase 1 trial compares very favorably with that of other agents approved or being developed for advanced melanoma, including the targeted inhibitors of activated kinases (Chapman *et al.,* 201 1). An additional advantage of this combination is the durability of response as demonstrated in the present study as well as longer-term immunotherapy trials with nivolumab (as described herein) and ipilimumab (Wolchok *et al,* 2013d).

These initial data suggest that rapid responses of a greater magnitude may be achieved in patients treated with a nivolumab/ipilimumab combination compared with the

historical experience of either agent alone. Responses were generally durable, and were observed even in patients whose treatment was terminated early because of toxicity. Responding patients included those with elevated LDH, Mlc disease and bulky multi-focal tumor burden. Similar to prior reports regarding ipilimumab or nivolumab monotherapy, conventional ORRs may not fully capture the spectrum of clinical activity and potential benefit in patients treated with the concurrent nivolumab/ipilimumab regimen in that a number of patients experienced either long-term SD or unconventional immune-related patterns of response. Indeed, even among the 7 patients in the concurrent regimen with SD $\geq$ 24 weeks or irSD $\geq$ 24 weeks as best response, 6 demonstrated meaningful tumor regression of at least 19%, and the seventh patient has declining tumor burden after prolonged SD. Prior experience with checkpoint blockade monotherapy supports the observation that some patients may survive for extended periods of time with SD as the best OR, lending credence to the hypothesis that re-establishment of the equilibrium phase of immune surveillance is a desirable outcome (Screiber *et ah,* 201 1).

The observation that patients can achieve ORs when treated sequentially with nivolumab after prior ipilimumab indicates that lack of response to CTLA-4 blockade does not preclude clinical benefit from PD- 1 blockade and further supports the non-redundant nature of these co-inhibitory pathways. Notably, data disclosed herein (Example 8) suggest an association between the occurrence of response and tumor PD-Ll expression in patients receiving nivolumab, and prior data reveal a correlation between OS and increases in peripheral ALC in patients treated with ipilimumab (Berman *et ah,* 2009; Ku *et ah,* 2010; Postow *et ah,* 2012; Delyon *et ah,* 2013). In the present study of the nivolumab/ipilimumab combination, clinical responses were observed in patients irrespective of lymphocyte count or baseline tumor PD-Ll expression (Example 17), suggesting that the immune response generated by combination therapy has unique features compared to either monotherapy, While the data suggest that baseline tumor PD-L1 expression and lymphocyte count may be less relevant in the setting of active combination regimens capable of inducing rapid and pronounced tumor regression, it is also notable that a different anti-PD-Ll Ab (rabbit mAb 28-8 versus mouse 5H1 mAb) was used in a different IHC assay to measure PD-Ll expression in the combination therapy study compared to the nivolumab monotherapy study. In addition to changes in the IHC assay and Ab, the different results may also reflect differences in biopsy samples and tumor heterogeneity. The utility of PD-Ll expression as a biomarker for anti-PD-1

efficacy will be further evaluated prospectively in randomized phase 3 studies (*see, e.g.*, NCTO 172 1772, NCTO 1668784, and NCTO 172 1746).

The spectrum of adverse events observed among patients treated with the concurrent regimen was qualitatively similar to experience with nivolumab or ipilimumab monotherapy, although the rate of AEs was increased in patients treated with the combination. Grade 3-4 treatment-related AEs were observed in 53% of patients treated with the concurrent nivolumab/ipilimumab regimen, compared with historical rates of 20% in patients treated with ipilimumab monotherapy (Hodi *et al.*, 2010) and 17% in patients treated with nivolumab alone (Example 5) at a dose of 3 mg/kg. In the sequenced-regimen cohorts, 18% of patients experienced grade 3-4 treatment-related AEs. AEs experienced by patients treated with the concurrent and sequenced regimens were manageable and/or generally reversible using existing treatment algorithms.

Collectively, these results suggest that nivolumab and ipilimumab can be administered concurrently with a manageable safety profile and leading to durable clinical responses. More rapid and deeper clinical tumor responses were observed in patients treated with the combination compared with the responses obtained with either single agent.

As of the February 2013 clinical cut-off date for the study described in Example 15, of the 52 subjects on the concurrent regimen evaluable for response, 2 1 (40%) had an OR by modified World Health Organization (mWHO) criteria (Wolchok *et al,* 2009). In an additional 2 subjects (4%), there was an unconfirmed OR. In Cohort 1 (0.3 mg/kg nivolumab plus 3 mg/kg ipilimumab), 3 out of 14 evaluable subjects had an OR by mWHO (ORR: 21%, including 1 CR and 2 PRs). In Cohort 2 (1 mg/kg nivolumab plus 3 mg/kg ipilimumab), 9 out of 17 evaluable subjects had an OR by mWHO (ORR: 53%; including 3 CRs and 6 PRs). In Cohort 2a (3 mg/kg nivolumab plus 1 mg/kg ipilimumab), 6 out of 15 response evaluable subjects had an OR by mWHO (ORR: 40%; including 1 CR and 5 PRs). In Cohort 3 (3 mg/kg nivolumab plus 3 mg/kg ipilimumab), 3 out of 6 evaluable subjects had an objective response by mWHO (ORR: 50%, including 3 PRs). Based on these data, the invention disclosed herein includes a method for treating a subject afflicted with a cancer comprising administering to the subject: (a) an Ab or an antigen-binding portion thereof that specifically binds to and inhibits PD- 1; and (b) an Ab or an antigen-binding portion thereof that specifically binds to and inhibits CTLA-4; each Ab being administered at a dosage ranging from 0.1 to 20.0 mg/kg body weight in a

concurrent regimen comprising: (i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 10 doses, followed by administration of the anti-PD-1 Ab alone at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 12 doses; followed by (ii) a maintenance dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of at least once every 8, 12 or 16 weeks, or at least once a quarter, for at least 4, 6, 8, 10, 12 or 16 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs.

In certain embodiments of this method, the maintenance dosing schedule comprises combined administration of up to 4, 6, 8, 10, 12 or 16 doses of the anti-PD-1 and anti-CTLA-4 Abs. In other embodiments, the concurrent regimen comprises: (i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of once every 2, 3 or 4 weeks, or once a month, for 2, 4, 6 or 8 doses, followed by administration of the anti-PD-1 Ab alone at a dosing frequency of once every 2, 3 or 4 weeks, or once a month, for 2, 4, 6, 8 or 12 doses; followed by (ii) a maintenance dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of once every 8, 12 or 16 weeks, or once a quarter, for 4, 6, 8, 10, 12 or 16 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs. In certain other embodiments, each of the anti-PD-1 and anti-CTLA-4 Abs is individually administered at a dosage of 0.1, 0.3, 0.5, 1, 3, 5, 10 or 20 mg/kg. In further embodiments, the dosage of each of the anti-PD-1 and anti-CTLA-4 Abs is kept constant during the induction dosing schedule and the maintenance dosing schedule. In yet other embodiments, the anti-PD-1 and anti-CTLA-4 Abs are administered at the following dosages: (a) 0.1 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; (b) 0.3 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; (c) 1 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; (d) 3 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; (e) 5 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; (f) 10 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; (g) 0.1 mg/kg anti-PD-1 Ab and 1 mg/kg of anti-CTLA-4 Ab; (h) 0.3 mg/kg anti-PD-1 Ab and 1 mg/kg of anti-CTLA-4 Ab; (i) 1 mg/kg anti-PD-1 Ab and 1 mg/kg of anti-CTLA-4 Ab; (j) 3 mg/kg anti-PD-1 Ab and 1 mg/kg of anti-CTLA-4 Ab; (k) 5 mg/kg anti-PD-1 Ab and

1 mg/kg of anti-CTLA-4 Ab; or (1) 10 mg/kg anti-PD-1 Ab and 1 mg/kg of anti-CTLA-4 Ab.

In the protocol described in Example 15, the 3 mg/kg nivolumab plus 3 mg/kg ipilimumab dosing regimen exceeded the MTD (though combinations with ipilimumab and other anti-PD-1 Abs may have a higher or lower MTD), whereas both Cohort 2 (1 mg/kg nivolumab plus 3 mg/kg ipilimumab) and Cohort 2a (3 mg/kg nivolumab plus 1 mg/kg ipilimumab) had similar clinical activity. In addition, the majority of responses to the combination of nivolumab and ipilimumab occurred in the first 12 weeks. Given the uncertainty of whether the ipilimumab administered past week 12 contributes to the clinical benefit and the fact that the U.S. Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved schedule for ipilimumab is every 3 weeks for a total of four doses, in a preferred embodiment the anti-CTLA-4 Ab is administered during the induction dosing schedule once every 3 weeks for a total of 4 doses. Monotherapy treatment with nivolumab at 3 mg/kg every two weeks until progression has been shown to be associated with durable responses (Examples 4-7), and a maintenance dosing schedule comprising administration of nivolumab every 12 weeks has been shown to be efficacious (Example 15). Thus, starting at week 12, which is after the completion of the four doses of combined nivolumab and ipilimumab, nivolumab at 3 mg/kg may be administered every 2 to at least 12 weeks until progression. Accordingly, in preferred embodiments of the concurrent regimen method, the anti-PD-1 and anti-CTLA-4 Abs are administered at dosages of: (a) 1 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab; or (b) 3 mg/kg anti-PD-1 Ab and 1 mg/kg of anti-CTLA-4 Ab, and the concurrent regimen further comprises: (i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of once every 3 weeks for 4 doses, followed by administration of the anti-PD-1 alone at a dosing frequency of once every 3 weeks for 4 doses; followed by (ii) a maintenance dosing schedule comprising combined administration of the anti-PD- 1 and anti-CTLA-4 antibodies at a dosing frequency of once every 2 to 12 or more weeks for up to 8 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs.

Exposure-response analysis of nivolumab monotherapy across dose ranges of 1 mg/kg to 10 mg/kg reveals similar clinical activity (Example 7) while exposure-response analysis of 0.3 mg/kg, 3 mg/kg, and 10 mg/kg of ipilimumab monotherapy has

demonstrated increasing activity with increase in dose in a phase 2 trial (Wolchok *et ah,* 2010). Therefore, a dose of 3 mg/kg of ipilimumab (Cohort 2) may be more clinically impactful than selection of 3 mg/kg of nivolumab (Cohort 2a). Thus, in more preferred embodiments of the concurrent regimen method, the anti-PD-1 and anti-CTLA-4 Abs are administered at dosages of 1 mg/kg anti-PD-1 Ab and 3 mg/kg of anti-CTLA-4 Ab.

In certain embodiments of the present methods, the anti-PD-1 and anti-CTLA-4 Abs are formulated for intravenous administration. In certain other embodiments, when the anti-PD- 1 and anti-CTLA-4 Abs are administered in combination, they are administered within 30 minutes of each other. Either Ab may be administered first, that is, in certain embodiments, the anti-PD-1 Ab is administered before the anti-CTLA-4 Ab, whereas in other embodiments, the anti-CTLA-4 Ab is administered before the anti-PD- 1 Ab. Typically, each Ab is administered intravenously over a period of 60 minutes. In further embodiments, the anti-PD- 1 and anti-CTLA-4 Abs are administered concurrently, either admixed as a single composition in a pharmaceutically acceptable formulation for concurrent administration, or concurrently as separate compositions with each Ab in a pharmaceutically acceptable formulation.

Data disclosed in Example 7 demonstrate that immunotherapy with nivolumab produced significant clinical activity in MEL patients who were unresponsive to prior ipilimumab therapy. Accordingly, this disclosure provides a sequenced regimen method for treating a subject afflicted with a cancer, the subject having previously been treated with an anti-CTLA-4 Ab, which method comprises administering to the subject an Ab or an antigen-binding portion thereof that specifically binds to and inhibits PD-1 at a dosage ranging from 0.1 to 20.0 mg/kg body weight and at a dosing frequency of at least once every week, at least once every 2, 3 or 4 weeks, or at least once a month, for up to 6 to up to 72 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs. In certain embodiments of this method, administration of the anti-PD-1 Ab to the subject is initiated within 1-24 weeks after last treatment with the anti-CTLA-4 Ab. In other embodiments, administration of the anti-PD-1 Ab to the subject is initiated within 1, 2, 4, 8, 12, 16, 20 or 24 weeks after last treatment with the anti-CTLA-4 Ab. In preferred embodiments, administration of the anti-PD-1 Ab is initiated within 4, 8 or 12 weeks after last treatment of the subject with the anti-CTLA-4 Ab. Certain embodiments of the method comprise administering the anti-PD-1 Ab at a dosage of 0.1-20 mg/kg, *e.g.,* 0.1, 0.3, 0.5, 1, 3, 5, 10 or 20 mg/kg. In preferred embodiments, the

anti-PD-1 Ab is administered at a dosage of 1 or 3 mg/kg. In certain embodiments, the sequenced regimen comprises administering the anti-PD-1 Ab to the subject at a dosing frequency of once every week, once every 2, 3 or 4 weeks, or once a month for 6 to 72 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs. In preferred embodiments, the anti-PD-1 is administered at a dosage of 1 or 3 mg/kg at a dosing frequency of once every 2 weeks for up to 48 doses. In other preferred embodiments, the anti-PD-1 Ab is formulated for intravenous administration.

In certain aspects of any of the present concurrent or sequenced regimen methods, the treatment produces at least one therapeutic effect chosen from a reduction in size and/or growth of a tumor, elimination of the tumor, reduction in number of metastatic lesions over time, complete response, partial response, and stable disease. Based on the broad spectrum of cancers in which nivolumab has shown clinical response, the present combination therapy methods are also applicable to diverse cancers. Examples of cancers that may be treated by these methods include liver cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, breast cancer, lung cancer, cutaneous or intraocular malignant melanoma, renal cancer, uterine cancer, ovarian cancer, colorectal cancer, colon cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, solid tumors of childhood, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the CNS, primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, environmentally induced cancers including those induced by asbestos, hematologic malignancies including, for example, multiple myeloma, B-cell lymphoma, Hodgkin lymphoma/primary mediastinal B-cell lymphoma, non-Hodgkin's lymphomas, acute myeloid lymphoma, chronic myelogenous leukemia, chronic lymphoid leukemia, lymphocytic lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, Burkitt's lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, acute lymphoblastic leukemia, mycosis fungoides,

anaplastic large cell lymphoma, T-cell lymphoma, and precursor T-lymphoblastic lymphoma, and any combinations of said cancers. The present invention is also applicable to treatment of metastatic, refractory or recurring cancers. In preferred embodiments, the cancer to be treated is chosen from MEL, RCC, squamous NSCLC, non-squamous NSCLC, CRC, CRPC, OV, GC, HCC, PC, squamous cell carcinoma of the head and neck, carcinomas of the esophagus, gastrointestinal tract and breast, and a hematological malignancy. In more preferred embodiments, the cancer is MEL.

In certain embodiments, the subject has been pre-treated for the cancer. For example, the patient may have been treated with 1 or 2 or more prior systemic regimens of the type described herein as standard-of-care therapeutics. In certain other embodiments, the cancer is an advanced, recurring, metastatic and/or refractory cancer. In preferred embodiments, the concurrent or sequenced regimen treatment induces a durable clinical response in the subject. In preferred embodiments, the subject is a human, the anti-PD-1 Ab inhibits human PD-1, and the anti-CTLA-4 Ab inhibits human CTLA-4.

The anti-PD-1 Ab used in the present methods may be any therapeutic anti-PD-1 Ab of the invention. In preferred embodiments, the anti-PD-1 Ab is a mAb, which may be a chimeric, humanized or human Ab. In certain embodiments, the anti-PD-1 Ab comprises the CDR1, CDR2 and CDR3 domains in the heavy chain variable region and the CDR1, CDR2 and CDR3 domains in the light chain variable region of 17D8, 2D3, 4H1, 5C4 (nivolumab), 4A1 1, 7D3 or 5F4, respectively, as described and characterized in U.S. Patent No. 8,008,449. In further embodiments, the anti-PD-1 Ab comprises the heavy and light chain variable regions of 17D8, 2D3, 4H1, 5C4 (nivolumab), 4A1 1, 7D3 or 5F4, respectively. In additional embodiments, the anti-PD-1 Ab is 17D8, 2D3, 4H1, 5C4 (nivolumab), 4A1 1, 7D3 or 5F4. In preferred embodiments, the anti-PD-1 Ab is nivolumab.

Anti-CTLA-4 antibodies of the instant invention bind to human CTLA-4 so as to disrupt the interaction of CTLA-4 with a human B7 receptor. Because the interaction of CTLA-4 with B7 transduces a signal leading to inactivation of T-cells bearing the CTLA-4 receptor, disruption of the interaction effectively induces, enhances or prolongs the activation of such T cells, thereby inducing, enhancing or prolonging an immune response. Anti-CTLA-4 Abs are described in, for example, U.S. Patent Nos. 6,051,227, 7,034,121 in PCT Application Publication Nos. WO 00/37504 and WO 01/14424. An exemplary clinical anti-CTLA-4 Ab is the human mAb 10D1 (now known as ipilimumab

and marketed as YERVOY®) as disclosed in U.S. Patent No. 6,984,720. In certain aspects of any of the present methods, the anti-CTLA-4 Ab is a mAb. In certain other embodiments, the anti-CTLA-4 antibody is a chimeric, humanized or human antibody. In preferred embodiments, the anti-CTLA-4 antibody is ipilimumab.

This disclosure also provides the use of an anti-PD-1 Ab or an antigen-binding portion thereof in combination with an anti-CTLA-4 Ab or an antigen-binding portion thereof for the preparation of a co-administered medicament for treating a subject afflicted with a cancer in a concurrent regimen, wherein the anti-PD- 1 and anti-CTLA-4 Abs are each administered at a dosage ranging from 0.1 to 20.0 mg/kg body weight, and further wherein the concurrent regimen comprises: (i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 12 doses, followed by administration of the anti-PD-1 Ab alone at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 10 doses; followed by (ii) a maintenance dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 Abs at a dosing frequency of at least once every 8, 12 or 16 weeks, or at least once a quarter, for up to 4, 6, 8, 10, 12 or 16 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs. The disclosure provides uses of the combination of anti-PD-1 and anti-CTLA-4 Abs for the preparation of co-administered medicaments corresponding to all the embodiments of the methods of treatment employing these Abs described herein, and are broadly applicable to the full range of cancers disclosed herein.

The disclosure also provides an anti-PD- 1 Ab or an antigen-binding portion thereof for use in combination with an anti-CTLA-4 Ab or an antigen-binding portion thereof for treating a subject afflicted with a cancer in a concurrent regimen, wherein the anti-PD-1 and anti-CTLA-4 Abs are each administered at a dosage ranging from 0.1 to 20.0 mg/kg body weight, and further wherein the concurrent regimen comprises: (i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 antibodies at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 12 doses, followed by administration of the anti-PD-1 alone at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 10 doses; followed by (ii) a maintenance dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 antibodies at a

dosing frequency of at least once every 8, 12 or 16 weeks, or at least once a quarter, for up to 4, 6, 8, 10, 12 or 16 doses, or for as long as clinical benefit is observed, or until unmanageable toxicity or disease progression occurs.

Methods for screening a patient population and selecting a patient as suitable for immunotherapy with the combination of anti-PD-1 and anti-CTLA-4 using the concurrent or sequenced regimens, and methods of predicting efficacy of the Ab combination, based on a PD-L1 biomarker assay are performed as described for anti-PD-1 monotherapy, to the extent this biomarker is applicable to combination therapy with anti-PD-1 and anti-CTLA-4 Abs.

The disclosure additionally provides a kit for treating a subject afflicted with a cancer, the kit comprising: (a) a dosage ranging from 0.1 to 20.0 mg/kg body weight of an Ab or an antigen-binding portion thereof that specifically binds to and inhibits PD-1; (b) a dosage ranging from 0.1 to 20.0 mg/kg of an Ab or an antigen-binding portion thereof that specifically binds to and inhibits CTLA-4; and (c) instructions for using the combination of the anti-PD-1 and anti-CTLA-4 antibodies in any of the concurrent regimen methods. In certain embodiments, some of the dosages of the anti-PD-1 and anti-CTLA-4 Abs are admixed within a single pharmaceutical formulation for concurrent administration. In other embodiments, the dosages of the anti-PD- 1 and anti-CTLA-4 Abs are formulated as separate compositions with each Ab in a pharmaceutically acceptable formulation.

The disclosure further provides a kit for treating a subject afflicted with a cancer, the kit comprising: (a) a dosage ranging from 0.1 to 20.0 mg/kg body weight of an Ab or an antigen-binding portion thereof that specifically binds to and inhibits PD-1; and (b) instructions for using the anti-PD-1 Ab in any of the sequenced regimen methods.

A combined PD-1 and CTLA-4 blockade may also be further combined with standard cancer treatments. For example, a combined PD-1 and CTLA-4 blockade may be effectively combined with chemotherapeutic regimes, for example, further combination with dacarbazine or IL-2, for the treatment of MEL. In these instances, it may be possible to reduce the dose of the chemotherapeutic reagent. The scientific rationale behind the combined use of PD-1 and CTLA-4 blockade with chemotherapy is that cell death, which is a consequence of the cytotoxic action of most chemotherapeutic compounds, should result in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with a combined PD- 1 and CTLA-4

blockade through cell death include radiation, surgery, or hormone deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors may also be combined with a combined PD-1 and CTLA-4 blockade. Inhibition of angiogenesis leads to tumor cell death, which may also be a source of tumor antigen to be fed into host antigen presentation pathways.

Immunotherapy of Cancer Patients Using an Anti-PD-Ll Antibody

PD-L1 is the primary PD-1 ligand up-regulated within solid tumors, where it can inhibit cytokine production and the cytolytic activity of PD-1-positive, tumor-infiltrating $CD4^+$ and $CD8^+$ T-cells, respectively (Dong *et al,* 2002; Hino *et al,* 2010; Taube *et al,* 2012). These properties make PD-L1 a promising target for cancer immunotherapy. The clinical trials of anti-PD-Ll immunotherapy described in the Examples demonstrate for the first time that mAb blockade of the immune inhibitory ligand, PD-L1, produces both durable tumor regression and prolonged (> 24 weeks) disease stabilization in patients with metastatic NSCLC, MEL, RCC and OV, including those with extensive prior therapy. The human anti-PD-Ll HuMAb, BMS-936559, had a favorable safety profile overall at doses up to and including 10 mg/kg, as is evident from the low (9%) incidence of grade 3-4 drug-related AEs. These findings are consistent with the mild autoimmune phenotype seen in PD-L1 $^{-/-}$ mice (Dong *et al,* 2004) and the more severe hyperproliferation seen in CTLA-$4^{-/-}$ mice relative to PD-1 $^{-/-}$ mice (Phan *et al.,* 2003; Tivol *et al,* 1995; Nishimura *et al,* 1999). Most of the toxicities associated with anti-PD-L1 administration in patients were immune-related, suggesting on-target effects. The spectrum and frequency of adverse events of special interest (AEOSIs) is somewhat different between anti-PD-Ll and anti-CTLA-4, emphasizing the distinct biology of these pathways (Ribas *et al,* 2005). Infusion reactions were observed with BMS-936559, although they were mild in most patients. Severe colitis, a drug-related AE observed in ipilimumab-treated patients (Beck *et al.,* 2006), was infrequently noted with anti-PD-Ll.

As noted above for anti-PD- 1 immunotherapy, another important feature of anti-PD-Ll therapy is the durability of responses across multiple tumor types. This is particularly notable considering the advanced disease and prior treatment of patients on the current study. Although not compared directly, this durability appears greater than that observed with most chemotherapies and kinase inhibitors used to treat these cancers.

Because peripheral blood T-cells express PD-L1, it is possible to assess *in vivo* RO by BMS-963559 as a pharmacodynamic measure. Median RO was 65.8%, 66.2%,

and 72.4% for the doses tested. Whereas these studies provide a direct assessment and evidence of target engagement in patients treated with BMS-936559, relationships between RO in peripheral blood and the tumor microenvironment remain poorly understood.

Based on the clinical data disclosed herein, this disclosure provides a method for immunotherapy of a subject afflicted with cancer, which method comprises administering to the subject a composition comprising a therapeutically effective amount of an anti-PD-L1 Ab of the invention or an antigen-binding portion thereof. The disclosure also provides a method of inhibiting growth of tumor cells in a subject, comprising administering to the subject an anti-PD-L1 Ab of the invention or an antigen-binding portion thereof. In preferred embodiments, the subject is a human. In certain embodiments, the Ab or antigen-binding portion thereof is of an IgG1 or IgG4 isotype. In other embodiments, the Ab or antigen-binding portion thereof is a mAb or an antigen-binding portion thereof. In further embodiments, the Ab or antigen-binding portion thereof is a chimeric, humanized or human Ab or an antigen-binding portion thereof. In preferred embodiments for treating a human patient, the Ab or antigen-binding portion thereof is a human Ab or an antigen-binding portion thereof.

Clinical trials described in the Examples employed the anti-PD-L1 HuMAb BMS-936559 to treat cancer. While BMS-936559 (designated HuMAb 12A4 in U.S. Patent No. 7,943,743) was selected as the lead anti-PD-L1 Ab for entering the clinic, it is notable that several anti-PD-L1 Abs of the invention share with 12A4 functional properties that are important to the therapeutic activity of 12A4, including high affinity binding specifically to human PD-L1, increasing T-cell proliferation, IL-2 secretion and interferon-γ production in an MLR assay, inhibiting the binding of PD-L1 to PD-1, and reversing the suppressive effect of T regulatory cells on T cell effector cells and/or dendritic cells. Moreover, certain of the anti-PD-L1 Abs of the invention, namely 1B12, 7H1 and 12B7 are structurally related to 12A4 in comprising V_H and V_K regions that have sequences derived from V_H 1-69 and V_K L6 germline sequences, respectively. In addition, at least 12B7, 3G10, 1B12 and 13G4 cross-compete with 12A4 for binding to the same epitope region of hPD-L1, whereas 5F8 and 10A5 may bind to the same or an overlapping epitope region as 12A4 (Examples 2 and 3). Thus, the preclinical characterization of 12A4 and other anti-PD-L1 HuMabs indicate that the methods of treating cancer provided herein may be performed using any of the broad genus of anti-

PD-L1 Abs of the invention.

Accordingly, this disclosure provides immunotherapy methods comprising administering to a patient an anti-PD-L1 Ab or antigen-binding portion thereof comprising (a) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-18 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL6 germline sequence; (b) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_H 1-69 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL6 germline sequence; (c) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human $Y H$ 1-3 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human V_KL15 germline sequence; (d) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human $Y H$ 1-69 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_K*All* germline sequence; (e) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human $Y H$ 3-9 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL15germline sequence; or (f) a heavy chain variable region that comprises consecutively linked amino acids having a sequence derived from a human $Y H$ 3-9 germline sequence, and a light chain variable region that comprises consecutively linked amino acids having a sequence derived from a human Y_KL I8 germline sequence.

In certain embodiments, the anti-PD-L1 Ab or antigen-binding portion thereof administered to the patient cross-competes for binding to PD-L1 with a reference Ab or a reference antigen-binding portion thereof comprising: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 15 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 25; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 16 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 26; (c) a

human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 17 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 27; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 18 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 28; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 19 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 29; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 20 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 30; (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 21 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 31; (h) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 22 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 32; (i) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 23 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 33; or (j) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 24 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 34. In preferred embodiments, the Ab or antigen-binding portion thereof cross-competes for binding to PD-1 with a reference Ab or reference antigen-binding portion thereof comprising a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 16 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 26.

In certain preferred embodiments of the immunotherapy methods disclosed herein, the anti-PD-L1 Ab or antigen-binding portion thereof administered to the subject

comprises: (a) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 15 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 25; (b) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 16 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 26; (c) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 17 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 27; (d) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 18 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 28; (e) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 19 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 29; (f) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 20 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 30; (g) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 21 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 31; (h) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 22 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 32; (i) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 23 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEO ID NO: 33; or (j) a human heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 24 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 34. In more preferred embodiments, the anti-PD-L1 Ab or antigen-binding portion comprises a human heavy chain variable

region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 16 and a human light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 26.

Broad spectrum of cancers treatable by anti-PD-Ll immunotherapy

The clinical activity of anti-PD-Ll in patients with advanced NSCLC, similar to the activity of anti-PD-1 in these patients, was surprising and unexpected since NSCLC has been considered to be poorly responsive to immune-based therapies (Holt and Disis, 2008; Holt *et ah,* 201 1). The present clinical data obtained with BMS-936559, an anti-PD-Ll Ab of the invention, substantiate and extend the evidence obtained using the anti-PD-1 Ab that immunotherapy based on PD-1 blockade is not applicable only to "immunogenic" tumor types, such as MEL and RCC, but is also effective with a broad range of cancers, including treatment-refractory metastatic NSCLC, that are generally not considered to be immune-responsive. Preferred cancers that may be treated using the anti-PD-Ll Abs of the invention include MEL *(e.g.,* metastatic malignant melanoma), RCC, squamous NSCLC, non-squamous NSCLC, CRC, ovarian cancer (OV), gastric cancer (GC), breast cancer (BC), pancreatic carcinoma (PC) and carcinoma of the esophagus. Additionally, the invention includes refractory or recurrent malignancies whose growth may be inhibited using the anti-PD-Ll Abs of the invention.

Accordingly, examples of cancers that may be treated using an anti-PD-Ll Ab in the methods of the invention, based on the indications of very broad applicability of anti-PD-Ll immunotherapy provided herein, include bone cancer, skin cancer, cancer of the head or neck, breast cancer, lung cancer, cutaneous or intraocular malignant melanoma, renal cancer, uterine cancer, castration-resistant prostate cancer, colon cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, carcinomas of the ovary, gastrointestinal tract and breast, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the

central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, multiple myeloma, environmentally induced cancers including those induced by asbestos, metastatic cancers, and any combinations of said cancers. The present invention is also applicable to treatment of metastatic cancers.

Combination therapy with anti-PD-Ll Abs

Optionally, Abs to PD-L1 can be combined with an immunogenic agent, for example a preparation of cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), antigen-presenting cells such as dendritic cells bearing tumor-associated antigens, and cells transfected with genes encoding immune stimulating cytokines (He *et ah,* 2004). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gplOO, MAGE antigens, Trp-2, MARTI and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF. PD-L1 blockade may also be effectively combined with standard cancer treatments, including chemotherapeutic regimes, radiation, surgery, hormone deprivation and angiogenesis inhibitors, as well as another immunotherapeutic Ab *(e.g.,* an anti-PD-1, anti-CTLA-4 or anti-LAG-3 Ab).

Uses of anti-PD-Ll Abs

This disclosure provides the use of any anti-PD-Ll Ab or antigen-binding portion thereof of the invention for the preparation of a medicament for inhibiting signaling from the PD-1/PD-L1 pathway so as to thereby potentiate an endogenous immune response in a subject afflicted with cancer. This disclosure also provides the use of any anti-PD-Ll Ab or antigen-binding portion thereof of the invention for the preparation of a medicament for immunotherapy of a subject afflicted with cancer comprising disrupting the interaction between PD-1 and PD-L1. The disclosure provides medical uses of any anti-PD-Ll Ab or antigen-binding portion thereof of the invention corresponding to all the embodiments of the methods of treatment employing an anti-PD-Ll Ab described herein.

This disclosure also provides an anti-PD-Ll Ab or an antigen-binding portion thereof of the invention for use in treating a subject afflicted with cancer comprising potentiating an endogenous immune response in a subject afflicted with cancer by inhibiting signaling from the PD-1/PD-L1 pathway. The disclosure further provides an anti-PD-Ll Ab or an antigen-binding portion thereof of the invention for use in

immunotherapy of a subject afflicted with cancer comprising disrupting the interaction between PD-1 and PD-Ll. These Abs may be used in potentiating an endogenous immune response against, or in immunotherapy of, the full range of cancers disclosed herein. In preferred embodiments, the cancers include MEL *(e.g.,* metastatic malignant MEL), RCC, squamous NSCLC, non-squamous NSCLC, CRC, ovarian cancer (OV), gastric cancer (GC), breast cancer (BC), pancreatic carcinoma (PC) and carcinoma of the esophagus.

Validation of Cancer Immunotherapy by Immune Checkpoint Blockade

A major implication of the clinical activity of immune checkpoint blockade is that significant endogenous immune responses to tumor antigens are generated and these responses may be harnessed therapeutically to mediate clinical tumor regression upon checkpoint inhibition. In fact, there is evidence that inhibitory ligands such as PD-Ll are induced in response to immune attack, a mechanism termed adaptive resistance (Gajewski *et ah,* 2010; Taube *et ah,* 2012). This potential mechanism of immune resistance by tumors suggests that PD-1/PD-Ll -directed therapy might synergize with other treatments that enhance endogenous antitumor immunity. Follow-up studies have verified that patients continue to demonstrate tumor control after cessation of PD-l/PD-Ll pathway blockade (Lipson *et ah,* 2013). Such tumor control may reflect a persistent antitumor immune response and the generation of effective immunologic memory to enable sustained control of tumor growth.

The data disclosed herein on the clinical testing of Abs that block the immunoregulatory receptor, PD-1, and also of Abs that block one of its cognate ligands, PD-Ll, are unprecedented. These data constitute the largest clinical experience to date with PD- 1 pathway-directed cancer immunotherapy, and the first report specifically describing the safety, tolerability, and initial clinical activity of an anti-PD-Ll -directed agent. These findings show that both anti-PD-1 and anti-PD-Ll have favorable overall safety profiles and provide clear evidence of clinical activity across diverse cancers, including NSCLC, a tumor not historically considered responsive to immunotherapy, as well as tumors known to respond to immunotherapy, including MEL, RCC and OV. Thus, these data strongly validate the PD-l/PD-Ll pathway as an important target for therapeutic intervention in cancer.

The remarkable similarities observed between the patterns of clinical activity obtained with the anti-PD-1 and anti-PD-Ll mAbs, and among the tumor types analyzed

to date, validate the general importance of the PD-1/PD-L1 signaling pathway in tumor immune resistance and as a target for therapeutic intervention. Although the molecular interactions blocked by these two Abs are not identical, it has been clearly demonstrated herein that, irrespective of mechanistic details, both anti-PD-1 and anti-PD-Ll Abs of the invention are effective in treating patients afflicted with a wide variety of cancers.

Infectious Diseases

Other methods of the invention are used to treat patients that have been exposed to particular toxins or pathogens. For example, another aspect of this disclosure provides a method of treating an infectious disease in a subject comprising administering to the subject an anti-PDl or an anti-PD-Ll Ab, or antigen-binding portion thereof, of the invention such that the subject is treated for the infectious disease. Preferably, the Ab is a human anti-human PD-1 or PD-L1 Ab (such as any of the human Abs described herein). Alternatively, the Ab is a chimeric or humanized Ab.

Similar to its application to tumors as discussed above, Ab-mediated PD-1 or PD-LI blockade can be used alone, or as an adjuvant, in combination with vaccines, to potentiate an immune response to pathogens, toxins, and/or self-antigens. Examples of pathogens for which this therapeutic approach may be particularly useful include pathogens for which there is currently no effective vaccine, or pathogens for which conventional vaccines are less than completely effective. These include, but are not limited to HIV, Hepatitis (A, B, and C), Influenza, Herpes, Giardia, Malaria, Leishmania, Staphylococcus aureus, Pseudomonas aeruginosa. PD-1 and/or PD-L1 blockade is particularly useful against established infections by agents such as HIV that present altered antigens over the course of an infection. Novel epitopes on these antigens are recognized as foreign at the time of anti-human PD-1 or PD-L1 administration, thus provoking a strong T cell response that is not dampened by negative signals through the PD-1/PD-L1 pathway.

In the above methods, PD-1 or PD-L1 blockade can be combined with other forms of immunotherapy such as cytokine treatment *(e.g.,* administration of interferons, GM-CSF, G-CSF or IL-2).

Kits

Also within the scope of the present invention are kits, including pharmaceutical kits, comprising an anti-PD-1 and/or an anti-PD-Ll Ab of the invention, or a combination of an anti-PD-1 and an anti-CTLA-4 Ab, for therapeutic uses, and diagnostic kits

comprising an anti-PD-L1 Ab of the invention for assaying membranous PD-L1 expression as a biomarker for screening patients for immunotherapy or for predicting the efficacy of an immunotherapeutic agent. Kits typically include a label indicating the intended use of the contents of the kit and instructions for use. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit. In certain embodiments of a pharmaceutical kit, the anti-PD-1 and/or anti-PD-L1 Abs may be co-packaged with other therapeutic agents in unit dosage form. In certain embodiments of a diagnostic kit, the anti-PD-L1 Ab may be co-packaged with other reagents for performing an assay to detect and/or quantify PD-L1 expression.

In certain preferred embodiments, the pharmaceutical kit comprises the anti-human PD-1 HuMAb, nivolumab. In other preferred embodiments, the pharmaceutical kit comprises the anti-human PD-L1 HuMAb, BMS-936559. In yet other preferred embodiments, the pharmaceutical kit comprises the anti-human CTLA-4 HuMAb, ipilimumab. In certain preferred embodiments, the diagnostic kit comprises the rabbit anti-human PD-L1 mAb, 28-8, comprising the V*H* and V_K regions whose amino acid sequences are set forth in SEQ ID NOs. 35 and 36, respectively. In other preferred embodiments, the diagnostic kit comprises the murine anti-human PD-L1 mAb, 5H1 (Dong *et al.,* 2002).

PD-L1 Biomarker for Predicting anti-PD-1 Efficacy

A particular challenge in cancer immunotherapy has been the identification of mechanism-based predictive biomarkers to enable patient selection and guide on-treatment management. Data disclosed in the Examples below indicate that cell surface PD-L1 expression in tumors is a useful molecular marker for predicting the efficacy of, and selecting patients for, immunotherapy with anti-PD-1 and potentially other immune checkpoint inhibitors.

There are conflicting reports in the literature about the clinical implications of PD-L1 being expressed in tumors. Several studies have concluded that PD-L1 expression in tumors correlates with a poor prognosis for the patient. *See, e.g.,* Hino *et al.* (2010) (MEL); Hamanishi *et al.* (2007) (OV); Thompson *et al.* (2006) (RCC). These findings may be rationalized on the basis that the interaction of PD-L1 on tumor cells and PD-1 on T cells helps abrogate immune responses directed against the tumor, resulting in immune evasion from tumor-specific T cells (Blank *et al.,* 2005). However, in contrast to the foregoing studies, Gadiot *et al.* (2011) and Taube *et al.* (2012) have recently reported that

PD-L1 expression in melanoma tumors correlates with a trend toward better survival. These seemingly contradictory data may reflect the relatively small numbers of patients analyzed, different histologic subtypes studied, or different methodologies used, *e.g.*, the use of different Abs to stain PD-L1, the use of frozen versus paraffin-embedded material for IHC, and the detection of membranous and/or cytoplasmic staining of PD-L1. Taube *et al.* (2012) note that PD-L1 is a type I transmembrane molecule, and hypothesize that while the cytoplasmic presence of PD-L1 may represent intracellular stores of this polypeptide that may be deployed to the cell surface upon appropriate stimulation, it is cell surface PD-L1 expression of that is biologically relevant as a potential biomarker for predicting clinical response to PD-1 blockade. *See, also,* Brahmer *et al.* (2010), which describes preliminary evidence, obtained on a small sample size of only 9 patients, of a correlation between membranous PD-L1 expression and anti-PD-1 efficacy. The data described in the Examples below on the use of membranous PD-L1 expression as a biomarker for anti-PD-1 efficacy, which was obtained from analysis of a much larger sample, substantiate the hypothesis that PD-L1 expression may be used as a biomarker for predicting anti-PD-1 clinical response and for screening patients to identify suitable candidates for immunotherapy with an anti-PD-1 Ab. Though the utility of this biomarker was demonstrated for screening patients for, or predicting the clinical response to, anti-PD-1 immunotherapy, PD-L1 expression may also potentially be applicable more broadly as a companion biomarker for other types of inhibitors of inhibitory immunoregulators.

Specifically, membranous PD-L1 expression was assayed using an automated IHC protocol and a rabbit anti-hPD-L1 Ab. Strikingly, in the initial set of data analyzed *(see* Example 8), no patients with cell surface PD-L1 -negative tumors (MEL, NSCLC, CRC, RCC and CRPC) experienced an OR following treatment with the anti-PD-1 Ab, nivolumab. In contrast, cell surface expression of PD-L1 on tumor cells in pretreatment biopsies may be associated with an increased rate of OR among patients treated with nivolumab. While tumor cell expression of PD-L1 may be driven by constitutive oncogenic pathways, it may also reflect "adaptive immune resistance" in response to an endogenous antitumor immune response, part of a host inflammatory response, which may remain in check unless unleashed by blockade of the PD-1/PD-L1 pathway (Taube *et al.,* 2012). This emerging concept of adaptive immune resistance in cancer immunology suggests that inhibitory ligands such as PD-L1 are induced in response to immune attack (Gajewski *et al.,* 2010; Taube *et al.,* 2012). A major implication of the clinical activity of

immune checkpoint blockade as described herein is that significant endogenous immune responses to tumor antigens are generated and these responses may be harnessed therapeutically to mediate clinical tumor regression upon checkpoint inhibition. This potential mechanism of immune resistance by tumors suggests that PD-1/PD-L1 -directed therapy might synergize with other treatments that enhance endogenous antitumor immunity.

Assaying Cell-Surface PD-L1 Expression by Automated IHC

As described in the Examples, an automated IHC method was developed for assaying the expression of PD-L1 on the surface of cells in FFPE tissue specimens. This disclosure provides methods for detecting the presence of human PD-L1 antigen in a test tissue sample, or quantifying the level of human PD-L1 antigen or the proportion of cells in the sample that express the antigen, which methods comprise contacting the test sample, and a negative control sample, with a mAb that specifically binds to human PD-Ll, under conditions that allow for formation of a complex between the Ab or portion thereof and human PD-L1. Preferably, the test and control tissue samples are FFPE samples. The formation of a complex is then detected, wherein a difference in complex formation between the test sample and the negative control sample is indicative of the presence of human PD-L1 antigen in the sample. Various methods are used to quantify PD-L1 expression.

In a particular embodiment, the automated IHC method comprises: (a) deparaffinizing and rehydrating mounted tissue sections in an autostainer; (b) retrieving antigen using a decloaking chamber and pH 6 buffer, heated to 110°C for 10 min; (c) setting up reagents on an autostainer; and (d) running the autostainer to include steps of neutralizing endogenous peroxidase in the tissue specimen; blocking non-specific protein-binding sites on the slides; incubating the slides with primary Ab; incubating with a post-primary blocking agent; incubating with NovoLink Polymer; adding a chromogen substrate and developing; and counterstaining with hematoxylin.

For assessing PD-L1 expression in tumor tissue samples, a pathologist examines the number of membrane PD-L1 $^+$ tumor cells in each field under a microscope and mentally estimates the percentage of cells that are positive, then averages them to come to the final percentage. The different staining intensities are defined as 0/negative, 1+/weak, 2+/moderate, and 3+/strong. Typically, percentage values are first assigned to the 0 and 3+ buckets, and then the intermediate 1+ and 2+ intensities are considered. For highly

heterogeneous tissues, the specimen is divided into zones, and each zone is scored separately and then combined into a single set of percentage values. The percentages of negative and positive cells for the different staining intensities are determined from each area and a median value is given to each zone. A final percentage value is given to the tissue for each staining intensity category: negative, 1+, 2+, and 3+. The sum of all staining intensities needs to be 100%.

Staining is also assessed in tumor-infiltrating inflammatory cells such as macrophages and lymphocytes. In most cases macrophages serve as an internal positive control since staining is observed in a large proportion of macrophages. While not required to stain with 3+ intensity, an absence of staining of macrophages should be taken into account to rule out any technical failure. Macrophages and lymphocytes are assessed for plasma membrane staining and only recorded for all samples as being positive or negative for each cell category. Staining is also characterized according to an outside/inside tumor immune cell designation. "Inside" means the immune cell is within the tumor tissue and/or on the boundaries of the tumor region without being physically intercalated among the tumor cells. "Outside" means that there is no physical association with the tumor, the immune cells being found in the periphery associated with connective or any associated adjacent tissue.

In certain embodiments of these scoring methods, the samples are scored by two pathologists operating independently and the scores are subsequently consolidated. In certain other embodiments, the identification of positive and negative cells is scored using appropriate software.

A histoscore is used as a more quantitative measure of the IHC data. The histoscore is calculated as follows:

Histoscore = [(% tumor x 1 (low intensity)) + (% tumor x 2 (medium intensity)) + (% tumor x 3 (high intensity)]

To determine the histoscore, the pathologist estimates the percentage of stained cells in each intensity category within a specimen. Because expression of most biomarkers is heterogeneous the histoscore is a truer representation of the overall expression. The final histoscore range is 0 (no expression) to 300 (maximum expression).

An alternative means of quantifying PD-L1 expression in a test tissue sample IHC is to determine the adjusted inflammation score (AIS) score defined as the density of

inflammation multiplied by the percent PD-L1 expression by tumor-infiltrating inflammatory cells (Taube *et al.*, 2012).

Cancer Immunotherapy with Anti-PD-1 Comprising a Patient Selection Step

This disclosure also provides a method for immunotherapy of a subject afflicted with cancer, which method comprises: (a) selecting a subject that is a suitable candidate for immunotherapy, *e.g.*, administration of an anti-PD-1 Ab, the selecting comprising (i) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells, (ii) assessing the proportion of cells in the test tissue sample that express PD-L1 on the cell surface, and (iii) selecting the subject as a suitable candidate based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface exceeds a predetermined threshold level; and (b) administering to the selected subject a composition comprising a therapeutically effective amount of an agent that inhibits signaling from an inhibitory immunoregulator *e.g.*, an anti-PD-1 Ab.

There is evidence that membranous PD-L1 expression is a surrogate for an endogenous antitumor immune response that is part of a host inflammatory response (Gajewski *et al.*, 2010; Taube *et al.*, 2012). Accordingly, cell surface expression of PD-L1 in tumors and/or inflammatory cells in the tumor microenvironment may be a marker not just for selecting cancer patients who would benefit from treatment with an anti-PD-1 Ab, but also for treatment with an anti-PD-L1 Ab as well as treatments targeting inhibitory immunoregulatory pathways other than the PD-1/PD-L1 pathway. For example, cell surface expression of PD-L1 in tumors and/or tumor-infiltrating inflammatory cells may be used as a marker for identifying or selecting suitable cancer patients who would benefit from immunotherapy with agents, including Abs, that target, and disrupt or inhibit signaling from, immune checkpoints such as PD-L1, Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4), B and T Lymphocyte Attenuator (BTLA), T cell Immunoglobulin and Mucin domain-3 (TIM-3), Lymphocyte Activation Gene-3 (LAG-3), Killer Immunoglobulin-like Receptor (KIR), Killer cell Lectin-like Receptor G1 (KLRG-1), Natural Killer Cell Receptor 2B4 (CD244), and CD160 (Pardoll, 2012; Baitsch et al, 2012). In certain preferred embodiments, the inhibitory immunoregulator is a component of the PD-1/PD-L1 signaling pathway. In other preferred embodiments, the inhibitory immunoregulator is an anti-PD-1 Ab of the invention. In yet other preferred embodiments, the inhibitory immunoregulator is an anti-PD-L1 Ab of the invention.

Where any immunotherapy methods comprising assaying PD-L1 expression, *i.e.,* employing a PD-L1 expression biomarker, are described below as comprising the selection of a patient who is, or is not, suitable for anti-PD-1 immunotherapy, or as comprising the administration of an anti-PD-1 Ab for immunotherapeutic purposes, it should be understood that these methods apply more broadly to the selection of a patient who is, or is not, suitable for immunotherapy with, or to the administration of an inhibitor of, an inhibitory immunoregulator *(e.g.,* CTLA-4, BTLA, TIM3, LAG3 or KIR) or a component or ligand thereof. Further, in any the methods comprising the measurement of PD-L1 expression in a test tissue sample, it should be understood that the step comprising the provision of a test tissue sample obtained from a patient is an optional step. That is, in certain embodiments the method includes this step, and in other embodiments, this step is not included in the method. It should also be understood that in certain preferred embodiments the "assessing" step to identify, or determine the number or proportion of, cells in the test tissue sample that express PD-L1 on the cell surface is performed by a transformative method of assaying for PD-L1 expression, for example by performing a reverse transcriptase-polymerase chain reaction (RT-PCR) assay or an IHC assay. In certain other embodiments, no transformative step is involved and PD-L1 expression is assessed by, for example, reviewing a report of test results from a laboratory. In certain embodiments, the steps of the methods up to, and including, assessing PD-L1 expression provides an intermediate result that may be provided to a physician or other medical practitioner for use in selecting a suitable candidate for immunotherapy and/or administering an immunotherapeutic agent to the patient. In certain embodiments, the steps that provide the intermediate result may be performed by a medical practitioner or someone acting under the direction of a medical practitioner. In other embodiments, these steps are performed by an independent laboratory or by an independent person such as a laboratory technician.

The disclosure also provides a method for treatment of a subject afflicted with cancer, which method comprises: (a) selecting a subject that is not suitable for treatment with an agent that inhibits an inhibitory immunoregulator, *e.g.,* anti-PD-1 Ab immunotherapy, the selecting comprising (i) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (ii) assessing the proportion of cells in the test tissue sample that express PD-L1 on the surface of the cells; and (iii) selecting the

subject as not suitable for immunotherapy with an inhibitor of an inhibitory immunoregulator, *e.g.*, an anti-PD-1 Ab, based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface is less than a predetermined threshold level; and (b) administering a standard-of-care therapeutic other than an inhibitor of an inhibitory immunoregulator, *e.g.*, an anti-PD-1 Ab, to the selected subject.

Measurement of PD-L1 expression

In certain embodiments of any of the present methods, the proportion of cells that express PD-L1 is assessed by performing an assay to determine the presence of PD-L1 RNA. In further embodiments, the presence of PD-L1 RNA is determined by RT-PCR, *in situ* hybridization or RNase protection. In other embodiments, the proportion of cells that express PD-L1 is assessed by performing an assay to determine the presence of PD-L1 polypeptide. In further embodiments, the presence of PD-L1 polypeptide is determined by immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), *in vivo* imaging, or flow cytometry. In preferred embodiments, PD-L1 expression is assayed by IHC. Flow cytometry may be particularly suitable for assaying PD-L1 expression in cells of hematologic tumors. In preferred embodiments of all of these methods, cell surface expression of PD-L1 is assayed using, *e.g.*, IHC or *in vivo* imaging.

Imaging techniques have provided important tools in cancer research and treatment. Recent developments in molecular imaging systems, including positron emission tomography (PET), single-photon emission computed tomography (SPECT), fluorescence reflectance imaging (FRI), fluorescence-mediated tomography (FMT), bioluminescence imaging (BLI), laser-scanning confocal microscopy (LSCM) and multiphoton microscopy (MPM), will likely herald even greater use of these techniques in cancer research. Some of these molecular imaging systems allow clinicians to not only see where a tumor is located in the body, but also to visualize the expression and activity of specific molecules, cells, and biological processes that influence tumor behavior and/or responsiveness to therapeutic drugs (Condeelis and Weissleder, 2010). Ab specificity, coupled with the sensitivity and resolution of PET, makes immunoPET imaging particularly attractive for monitoring and assaying expression of antigens in tissue samples (McCabe and Wu, 2010; Olafsen *et ah,* 2010). In certain embodiments of any of the present methods, PD-L1 expression is assayed by immunoPET imaging.

In certain embodiments of any of the present methods, the proportion of cells in a test tissue sample that express PD-Ll is assessed by performing an assay to determine the presence of PD-Ll polypeptide on the surface of cells in the test tissue sample. In certain embodiments, the test tissue sample is a FFPE tissue sample. In certain preferred embodiments, the presence of PD-Ll polypeptide is determined by IHC assay. In further embodiments, the IHC assay is performed using an automated process. In further embodiments, the IHC assay is performed using an anti-PD-Ll mAb to bind to the PD-Ll polypeptide.

Abs that bind specifically to cell-surface-expressed PD-Ll in FFPE tissues

An Ab may bind to an antigen in fresh tissues but completely fail to recognize the antigen in an FFPE tissue sample. This phenomenon, well known in the art, is thought to be due primarily to intra- and inter-molecular cross-linking of polypeptides induced by formalin fixation, which alters the epitope recognized by the Ab (Sompuram *et al.,* 2006). In addition, several factors known to influence staining in FFPE tissue, including variable time to fixation, inadequate fixation period, differences in fixative used, tissue processing, Ab clone and dilution, antigen retrieval, detection system, and interpretation of results using different threshold points are important variables that can affect tissue antigenicity and IHC measurements (Bordeaux *et ah,* 2010). In particular, a lack of anti-human PD-Ll Abs that stain PD-Ll in FFPE specimens has been noted in the art (Hamanishi *et al.,* 2007). Taube *et ah* (2012) and Gadiot *et ah* (201 1) have also reported difficulties in identifying anti-PD-Ll Abs that bind specifically to PD-Ll in FFPE tissues, and inconsistent test results on the same Abs. Our own analysis of five commercially available anti-hPD-Ll Abs shows that these Abs failed to distinguish FFPE cells expressing PD-Ll from cells that did not express PD-Ll (see Example 9, Table 7). Thus, the contradictory results reported by different groups on the implications of PD-Ll expression for prognosis of a tumor may, in part, reflect the differential abilities of anti-PD-Ll Abs used to detect PD-Ll polypeptide in FFPE tissue samples. Accordingly, in order to detect hPD-Ll on the surface of cells using an IHC assay on FFPE tissues, there is a need for anti-hPD-Ll Abs that bind specifically to cell surface-expressed PD-Ll in FFPE tissue samples.

This disclosure provides a mAb or an antigen-binding portion thereof that binds specifically to a cell surface-expressed PD-Ll antigen in a FFPE tissue sample. In preferred embodiments, the mAb or antigen-binding portion thereof does not bind to a

cytoplasmic PD-L1 polypeptide in the FFPE tissue sample or exhibits a very low level of background binding. In certain other embodiments, the presence or absence of binding specifically to a cell surface-expressed or a cytoplasmic PD-L1 polypeptide is detected by immunohistochemical staining. In certain preferred aspects of the invention, the mAb or antigen-binding portion is a rabbit Ab or a portion thereof. In other preferred embodiments, the mAb is the rabbit mAb designated 28-8, 28-1, 28-12, 29-8 or 20-12. In more preferred embodiments, the mAb is the rabbit mAb designated 28-8 or an antigen-binding portion thereof. In further embodiments, the mAb is an Ab comprising a heavy chain variable region *(YH)* comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 35 and a light chain variable region (V_K) comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 36. In other embodiments, the mAb comprises the CDR1, CDR2 and CDR3 domains in a *Y H* having the sequence set forth in SEQ ID NO: 35, and the CDR1, CDR2 and CDR3 domains in a Y_K having the sequence set forth in SEQ ID NO: 36.

It is known in the art that rabbit Abs have certain advantages over murine Abs. For example, rabbit Abs generally exhibit more diverse epitope recognition, improved immune response to small-size epitopes, and higher specificity and affinity compared to murine Abs *(see, e.g.,* Fischer *et ah,* 2008; Cheang *et ah,* 2006; Rossi *et ah,* 2005). For example, the rabbit's lower immune dominance and larger B-cell repertoire results in greater epitope recognition compared to murine Abs. Further, the high specificity and novel epitope recognition of rabbit antibodies translates to success with recognition of post-translational modifications (Epitomics, 2013). In addition, many protein targets relevant to signal transduction and disease are highly conserved between mice, rats and humans, and can therefore be recognized as self-antigens by a mouse or rat host, making them less immunogenic. This problem is avoided by generating Abs in rabbits. Moreover, in applications in which two antigen-specific Abs are required, it is more convenient to have the Abs come from two different species. Thus, for example, it is easier to multiplex a rabbit Ab such as 28-8 with other Abs (likely to be murine Abs since the best immune marking Abs are murine Abs) that can mark immune cells that also express PD-L1 *(e.g.,* macrophages and lymphocytes). Accordingly, rabbit anti-hPD-L1 mAbs, *e.g.,* 28-8, are particularly suited to IHC assays for detecting surface-expressed PD-L1 in FFPE tissue samples and potentially have distinct advantages over murine Abs, such as 5H1.

As described in Example 9, a large number (185) of Ab multiclones from both

rabbit and mouse immunizations were screened, and only ten rabbit Ab, but no mouse Ab, multiclones specifically detected the membranous form of hPD-Ll. After further extensive screening by multiple rounds of IHC, 15 purified rabbit subclones were selected based on their specificity and intensity of staining *(see* Table 5). Following further characterization of the antibodies to determine their binding affinity and cross-competition by surface plasmon resonance, as well as screening by IHC on FFPE tissues, mAb 28-8 was selected as the Ab with the best combination of binding to membranous PDF-LI with high affinity and specificity, and low background staining.

In certain aspects of this invention, the mAb or antigen-binding portion cross-competes with mouse mAb 5H1 for binding to PD-L1, which indicates that these antibodies bind to the same epitope region of PD-L1. In certain other aspects, the mAb or antigen-binding portion thereof does not cross-compete with mouse mAb 5H1 for binding to PD-L1, indicating that they do not bind to the same epitope region of PD-L1.

The disclosure also provides nucleic acids encoding all of the rabbit anti-hPD-Ll Abs or portions thereof disclosed herein.

Immunotherapeutic methods comprising measurement of cell surface PD-L1 expression

The availability of rabbit Abs that bind with high affinity specifically to membranous PD-L1 in FFPE tissue specimens facilitates methods comprising a step of detecting PD-L1 polypeptide on the surface of cells in FFPE tissue samples. Accordingly, this disclosure also provides a method for immunotherapy of a subject afflicted with cancer, which method comprises: (a) selecting a subject that is a suitable candidate for immunotherapy, the selecting comprising: (i) optionally providing a FFPE test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (ii) assessing the proportion of cells in the test tissue sample that express PD-L1 on the cell surface by IHC using a rabbit anti-human PD-L1 Ab, *e.g.,* mAb 28-8, to bind to the PD-L1; and (iii) selecting the subject as a suitable candidate based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface exceeds a predetermined threshold level; and (b) administering a composition comprising a therapeutically effective amount of an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, to the selected subject.

In certain embodiments of methods employing IHC to assay PD-L1 expression in FFPE tissues, an automated IHC assay is used. The automated IHC process is performed on an autostainer and comprises: (a) de-paraffinizing the FFPE sample with xylene and rehydrating the sample; (b) retrieving the antigen using a decloaking chamber; (c) blocking nonspecifc protein binding sites by incubation with a Protein Block; (d) incubating the sample with a primary anti-PD-Ll Ab; (e) adding a polymeric horseradish peroxidase (HRP)-conjugated secondary Ab; (f) detecting the bound secondary Ab comprising staining with a 3,3'-diaminobenzidine (DAB) chromogen; and/or (g) counterstaining with hematoxylin. This automated IHC process has been optimized by minimization of the number of steps, optimization of incubation times, and selection of primary Abs, blocking and detection reagents that produce strong specific staining with a low level of background staining. In preferred embodiments of this automated IHC assay, the primary anti-PD-Ll Ab is rabbit mAb 28-8 or murine mAb 5H1. In certain embodiments of this invention, this IHC assay, and any other IHC assay described herein to measure PD-L1 expression, may be used as part of a method of immunotherapy. In other embodiments, any of the IHC methods described herein is used independently of any therapeutic process requiring the administration of a therapeutic, *i.e.*, solely as a diagnostic method to assay PD-L1 expression.

In certain embodiments any of the immunotherapy methods described herein, the Ab administered to the selected subject is any anti-PD-1 or anti-PD-Ll Ab or antigen-binding portion thereof of the invention. In certain preferred embodiments, the subject is a human. In other preferred embodiments, the Ab is a human Ab or antigen-binding portion thereof. In more preferred embodiments, the anti-PD-1 Ab is nivolumab and the anti-PD-Ll Ab is BMS-936559. In certain other embodiments, the anti-PD-1 Ab is an Ab or antigen-binding portion thereof that cross-competes with nivolumab for binding to PD-1, and the anti-PD-Ll Ab is an Ab or antigen-binding portion thereof that cross-competes with BMS-936559 for binding to PD-L1. In certain preferred embodiments, the cancer to be treated is selected from the group consisting MEL, RCC, squamous NSCLC, non-squamous NSCLC, CRC, castration-resistant prostate cancer CRPC, HCC, squamous cell carcinoma of the head and neck, carcinomas of the esophagus, ovary, gastrointestinal tract and breast, and a hematological malignancy.

In certain embodiments of the disclosed methods, the predetermined threshold is based on a proportion of (a) tumor cells, (b) tumor-infiltrating inflammatory cells, (c)

particular tumor-infiltrating inflammatory cells, *e.g.,* TILs or macrophages, or (d) a combination of tumor cells and tumor-infiltrating inflammatory cells, in a test tissue sample that expresses PD-L1 on the cell surface. In certain embodiments, the predetermined threshold is at least 0.001% of tumor cells expressing membranous PD-L1 as determined by IHC. In other embodiments, the predetermined threshold is at least 0.01%, preferably at least 0.1%, more preferably at least 1% of tumor cells expressing membranous PD-L1, as determined by IHC. In certain embodiments, the predetermined threshold is at least 5% of tumor cells expressing membranous PD-L1 as determined by IHC. In certain embodiments, the predetermined threshold is at least 0.01%, at least 0.1%, at least 1%, or at least 5% of tumor cells expressing membranous PD-L1 as determined by IHC, and/or a single tumor-infiltrating inflammatory cell expressing membranous PD-L1 as determined by IHC. In certain other embodiments, the predetermined threshold is at least 0.01%, at least 0.1%, at least 1%, or at least 5% of a tumor-infiltrating inflammatory cell expressing membranous PD-L1 as determined by IHC. In certain other embodiments, the predetermined threshold is at least 0.01%, at least 0.1%, at least 1%, or at least 5% of a tumor-infiltrating lymphocyte expressing membranous PD-L1 as determined by IHC. In certain other embodiments, the predetermined threshold is at least 0.01%, at least 0.1%, at least 1%, or at least 5% of a tumor-infiltrating macrophage expressing membranous PD-L1 as determined by IHC. In yet other embodiments, the predetermined threshold is at least a single tumor cell or a single tumor-infiltrating inflammatory cell expressing membranous PD-L1 as determined by IHC. Preferably, PD-L1 expression is assayed by automated IHC using mAb 28-8 or 5H1 as the primary Ab.

This disclosure also provides a method for treatment of a subject afflicted with cancer, which method comprises: (a) screening a plurality of subjects to identify a subject that is not a suitable candidate for immunotherapy comprising the administration of an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, to the subject, the screening comprising: (i) optionally providing test tissue samples from the plurality of subjects, the test tissue samples comprising tumor cells and tumor-infiltrating inflammatory cells; (ii) assessing the proportion of cells in the test tissue samples that express PD-L1 on the surface of the cells; and (iii) selecting the subject as a candidate that is not suitable for immunotherapy based on an assessment that the proportion of cells that express PD-L1 on the surface of cells in the subject's test tissue sample is below a predetermined threshold level; and (b) administering a standard-of-care therapeutic other

than an agent that inhibits an inhibitory immunoregulator, *e.g.*, an anti-PD-1 Ab, to the selected subject.

This disclosure further provides a method for immunotherapy of a subject afflicted with cancer, which method comprises: (a) screening a plurality of subjects to identify a subject that is a suitable candidate for immunotherapy, the screening comprising: (i) optionally providing test tissue samples from the plurality of subjects, the test tissue samples comprising tumor cells and tumor-infiltrating inflammatory cells; (ii) assessing the proportion of cells in the test tissue samples that express PD-L1 on the surface of the cells; and (iii) selecting the subject as a candidate that is suitable for immunotherapy with an agent that inhibits an inhibitory immunoregulator, *e.g.*, an anti-PD-1 Ab, based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface exceeds a predetermined threshold level; and (b) administering a composition comprising a therapeutically effective amount of said agent to the selected subject.

This disclosure additionally provides a method for treatment of a subject afflicted with cancer, which method comprises: (a) screening a plurality of subjects to identify a subject that is a suitable candidate for the treatment, the screening comprising: (i) optionally providing test tissue samples from the plurality of subjects, the test tissue samples comprising tumor cells and tumor-infiltrating inflammatory cells; (ii) assessing the proportion of cells in the test tissue samples that express PD-L1 on the surface of the cells, wherein the subject is identified as a suitable candidate for anti-PD-1 Ab immunotherapy if the proportion of cells in the tissue sample that express PD-L1 on the cell surface exceeds a predetermined threshold level, and the subject is identified as a candidate that is not a suitable candidate for anti-PD-1 Ab immunotherapy if the proportion of cells in the test tissue sample that express PD-L1 on the cell surface is below a predetermined threshold level; and (b) administering a composition comprising a therapeutically effective amount of an agent that inhibits an inhibitory immunoregulator, *e.g.*, an anti-PD-1 Ab, to the subject identified as a suitable candidate for anti-PD-1 Ab immunotherapy, or (c) administering a standard-of-care therapeutic other than said agent to the subject identified as not a suitable candidate for anti-PD-1 Ab immunotherapy.

One aspect of this invention is a method for immunotherapy of a subject afflicted with cancer, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor

cells and tumor-infiltrating inflammatory cells; (b) determining that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface is above a predetermined threshold level; and (c) based on that determination administering a composition comprising a therapeutically effective amount of an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, to the subject. Another aspect of the invention is a method for treatment of a subject afflicted with cancer, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) determining that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface is below a predetermined threshold level; and (c) based on that determination administering a standard-of-care therapeutic other than an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, to the subject.

Yet another aspect of the invention is a method for immunotherapy of a subject afflicted with cancer, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) determining the proportion of cells in the test tissue sample that express PD-L1 on the cell surface; (c) selecting an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, as a treatment for the subject based on the recognition that an anti-PD-1 Ab is effective in patients whose test tissue sample contains a proportion of cells above a predetermined threshold level that express PD-L1 on the cell surface; and (d) administering a composition comprising a therapeutically effective amount of said agent to the subject. In another embodiment, this disclosure provides a method for treatment of a subject afflicted with cancer, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) determining the proportion of cells in the test tissue sample that express PD-L1 on the cell surface; (c) selecting a standard-of-care therapeutic other than an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, as a treatment for the subject based on the recognition that said agent is ineffective in patients in whom the proportion of cells that express PD-L1 on the cell surface in the test tissue sample is below a predetermined threshold level; and (d) administering the standard-of-care therapeutic to the subject.

This disclosure also provides a method of selecting an immunotherapy for a subject afflicted with cancer, which method comprises: (a) assaying cells of a test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells to assess the proportion of cells in the test tissue sample that express PD-L1; and (b) based on an assessment that the proportion of cells that express membranous PD-L1 is above a predetermined threshold level, selecting an immunotherapy comprising a therapeutically effective amount of an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, for the subject. The disclosure further provides a method of selecting a treatment for a subject afflicted with cancer, which method comprises: (a) assaying cells of a test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells to assess the proportion of cells in the test tissue sample that express PD-L1; and (b) based on an assessment that the proportion of cells that express membranous PD-L1 is below a predetermined threshold level, selecting a standard-of-care treatment other than an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, for the subject.

In addition, this disclosure provides a method for treatment of a subject afflicted with cancer, which method comprises administering to the subject a composition comprising a therapeutically effective amount of an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, the subject having been selected on the basis that the proportion of cells in a test tissue sample from the subject that express PD-L1 is determined to exceed a predetermined threshold level, wherein the test tissue sample comprises tumor cells and tumor-infiltrating inflammatory cells. This disclosure also provides a method for treatment of a subject afflicted with cancer, which method comprises administering to the subject a standard-of-care treatment other than an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, the subject having been selected on the basis that the proportion of cells in a test tissue sample from the subject that express PD-L1 is determined to be below a predetermined threshold level, wherein the test tissue sample comprises tumor cells and tumor-infiltrating inflammatory cells.

This disclosure further provides a method for selecting a cancer patient for immunotherapy with an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) assaying the test tissue sample to determine the

proportion of cells therein that express PD-L1 on the cell surface; (c) comparing the proportion of cells that express PD-L1 on the cell surface with a predetermined threshold proportion; and (d) selecting the patient for immunotherapy based on an assessment that the proportion of cells in the test tissue sample that express surface PD-L1 is above the predetermined threshold level.

In any of the methods described herein comprising a step for assessing PD-L1 expression, the test tissue sample may be a FFPE tissue sample and PD-L1 polypeptide on the cell surface is detected by IHC using an anti-PD-L1 Ab, *e.g.,* mAb 28-8 or 5H1.

In addition, in any method where an immunotherapy is selected or administered based on an assessment that the proportion of cells in a test tissue sample from the subject expresses PD-L1 at a level above a predetermined threshold level, it follows that a complementary method of treatment may be performed wherein a standard-of-care treatment other than the immunotherapy is selected or administered based on an assessment that the proportion of cells in a test tissue sample from the subject expresses PD-L1 at a level below the predetermined threshold level.

This disclosure further provides a method for predicting the therapeutic effectiveness of an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, for treating a cancer patient, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) assaying the test tissue sample to determine the proportion of cells therein that express PD-L1 on the cell surface; (c) comparing the proportion of cells that express PD-L1 on the cell surface with a predetermined threshold value; and (d) predicting the therapeutic effectiveness of said agent, wherein if the proportion of cells that express PD-L1 on the cell surface exceeds the threshold proportion the agent is predicted to be effective in treating the patient, and wherein if the proportion of cells that express PD-L1 on the cell surface is below the threshold proportion the agent is predicted to not be effective in treating the patient.

This disclosure also provides a method for determining an immunotherapeutic regimen comprising an agent that inhibits an inhibitory immunoregulator, *e.g.,* an anti-PD-1 Ab, for treating a cancer patient, which method comprises: (a) optionally providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells; (b) assaying the test tissue sample to determine the proportion of cells therein that express PD-L1 on the

cell surface; (c) comparing the proportion of cells that express PD-L1 on the cell surface with a predetermined threshold proportion; and (d) determining an immunotherapeutic regimen comprising said agent based on the determination that the proportion of cells that express PD-L1 on the cell surface exceeds the predetermined threshold proportion.

Standard-of-Care Therapeutics

Several of the methods of treatment described herein comprise the administration of a standard-of-care therapeutic to a patient. As used herein, a "standard-of-care therapeutic" is a treatment process, including a drug or combination of drugs, radiation therapy (RT), surgery or other medical intervention that is recognized by medical practitioners as appropriate, accepted, and/or widely used for a certain type of patient, disease or clinical circumstance. Standard-of-care therapies for different types of cancer are well known by persons of skill in the art. For example, the National Comprehensive Cancer Network (NCCN), an alliance of 21 major cancer centers in the USA, publishes the NCCN Clinical Practice Guidelines in Oncology (NCCN GUIDELINES®) that provide detailed up-to-date information on the standard-of-care treatments for a wide variety of cancers (*see* NCCN GUIDELINES®, 2013). By way of example, standard-of-care treatments for MEL, RCC and NSCLC are summarized below.

Melanoma

MEL is a malignant tumor of melanocytes, the melanin-producing cells found predominantly in skin. Though less common than other skin cancers, it is the most dangerous of skin cancers if not diagnosed early and causes the majority (75%) of skin cancer deaths. The incidence of MEL is increasing worldwide in Caucasian populations, especially where peoples with low amounts of skin pigmentation receive excessive ultraviolet light exposure from the sun. In Europe, the incidence rate is < 10-20 per 100,000 population; in the USA 20-30 per 100,000; and in Australia, where the highest incidence is observed, 50-60 per 100,000 (Garbe *et al.*, 2012). MEL accounts for about 5% of all new cases of cancer in the United States (U.S.), and the incidence continues to rise by almost 3% per year. This translates to an estimated 76,690 new cases in the U.S. in 2013 with 9,480 associated deaths (Siegel *etal.* (2013).

For *in situ* (stage 0) or early-stage MEL (Stages I-II), surgical excision is the primary treatment. In general, the prognosis is excellent for patients with localized disease and tumors 1.0 mm or less in thickness, with 5-year survival rates of more than 90% (NCCN GUIDELINES®, 2013 - Melanoma). Where surgical excision is not

feasible for *in situ* melanoma due to comorbidity or cosmetically sensitive tumor location, topical imiquimod and radiotherapy are emerging as treatments, especially for lentigo maligna. Chemotherapeutic agents for treating MEL include dacarbazine, temozolomide and imatinib for melanoma with a c-KIT mutation, high-dose interleukin-2, and paclitaxel with or without carboplatin. However, these treatments have modest success, with response rates below 20% in first-line (1L) and second-line (2L) settings.

For patients with localized melanomas more than 1.0 mm in thickness, survival rates range from 50-90%. The likelihood of regional nodal involvement increases with increasing tumor thickness. With Stage III MEL (clinically positive nodes and/or in-transit disease), 5-year survival rates range from 20-70%. By far the most lethal is Stage IV MEL where long-term survival in patients with distant metastatic melanoma is less than 10% (NCCN GUIDELINES®, 2013 - Melanoma).

There is no consensus on the best treatments for metastatic MEL, though a variety of treatments including excision to clear margins, intralesional injections, laser ablation, radiation and biochemotherapy (combination of chemotherapy and biological agents such as interferon-alpha and IL-2) are being investigated. The therapeutic landscape for metastatic MEL has recently seen dramatic improvements with the development of novel drugs such as vemurafenib and ipilimumab. Vemurafenib specifically inhibits signaling by a mutated intracellular kinase, BRAF, that is present in about 50% of patients with metastatic MEL. In clinical trials, vemurafenib delivered an estimated progression-free survival (PFS) of 5.3 months in BRAF mutation-positive patients versus merely 1.6 months for dacarbazine, and a median OS of 15.9 months for vemurafenib versus 5.6-7.8 months for dacarbazine (Chapman *et al,* 201 1; Sosman *et al,* 2012). Ipilimumab is a HuMAb that inhibits the immune checkpoint receptor, CTLA-4, and thereby stimulates a T cell immune response. Ipilimumab, with or without a glycoprotein 100 (gplOO) peptide vaccine, improved median OS in patients with previously treated metastatic MEL to 10.0-10.1 months as compared with 6.4 months among patients receiving gplOO alone (Hodi *et al,* 2010). Besides these two agents, no other agent has demonstrated an OS benefit in a Phase 3 randomized study. Dacarbazine is approved by the FDA and the EMA for treatment of metastatic MEL with a reported objective response rate of 5-20% and a median OS of about 6.4 months, but these responses are short-lived. Other drugs such as temozolomide and fotemustine have not resulted in significant improvement in survival when compared to dacarbazine. IL-2 has also been approved by the FDA for the

treatment of metastatic MEL as it is associated with a 15-20% response rate including 4-6% complete responses which can be durable, but it is associated with significant toxicities including hypotension, cardiac arrhythmias, and pulmonary edema. Further details on standard-of-care treatments for melanoma are provided by Garbe *et al.* (2012) and the NCCN GUIDELINES®, 2013 - Melanoma. Despite the recent approval of ipilimumab and vemurafenib for advanced MEL, there is still a large unmet need for patients who have progressed on anti-CTLA-4 therapy and a BRAF inhibitor (depending on BRAF status) or patients with previously untreated, unresectable or metastatic BRAF wild-type MEL. The 5-year survival rate for late-stage MEL is currently only 15%.

Renal Cell Carcinoma

RCC is the most common type of kidney cancer in adults, responsible for approximately 90% of renal tumors, and 80-90% of these are clear cell tumors (NCCN GUIDELINES®, 2013 - Kidney Cancer). It is also the most lethal of all the genitourinary tumors. An estimated 65,150 patients will be diagnosed with renal cancer and 13,680 will die of the disease in the United States in 2013 (Siegel *et al.* (2013).

For clinically localized RCC (Stage IA and IB), surgical resection, including radical nephrectomy and nephron-sparing surgery, is an effective therapy. Partial nephrectomy is generally not suitable for patients with locally advanced tumors (Stage II and III), in which case radical nephrectomy is preferred. Where the tumor is confined to the renal parenchyma, the 5-year survival rate is 60-70%, but this is lowered considerably in Stage IV disease where metastases have spread. Stage IV RCC is relatively resistant to RT and chemotherapy, although patients may benefit from surgery, and cytoreductive nephrectomy before systemic therapy is recommended for patients with a potentially surgically resectable primary and multiple resectable metastases.

Until recently, the cytokines IL-2 and IFNa were the only active systemic treatments for advanced or metastatic RCC, both providing reported ORRs of 5-27%. However, due to each of these agent's limited clinical benefit and substantial toxicity profile, newer targeted agents have largely replaced cytokines in the treatment of advanced or metastatic renal cell carcinoma. The recognition of the importance of hypoxia inducible factor alpha (HIFa) signaling in the pathogenesis of clear-cell RCC has led to widespread study of two classes of targeted therapies, anti-angiogenic tyrosine kinase inhibitors (TKIs) and mammalian target of rapamycin (mTOR) inhibitors, in 1L and 2L treatments (Mulders, 2009). Targeting of angiogenesis is rational because

constitutive HIFa activation leads to the upregulation or activation of several proteins including vascular endothelial growth factor (VEGF), which can subsequently lead to tumor proliferation and neovasculature formation. Targeting of the mTOR pathway is important because activation of the upstream PI3K/Akt/mTOR signaling pathway is one method by which constitutive HIFa activation or upregulation occurs (Mulders, 2009). Agents that target angiogenesis include VEGF-receptor (VEGFr) TKIs *(e.g.,* sorafenib, sunitinib, pazopanib, axitinib, and tivozanib) and VEGF-binding mAbs *(e.g.,* bevacizumab), while agents that target the mTOR pathway include the mTOR inhibitors *(e.g.,* everolimus and temsirolimus) (Mulders, 2009; NCCN GUIDELINES®, 2013 - Kidney Cancer). However, most patients develop resistance, and OS improvement has only been shown in one phase 3 trial in poor-risk patients: temsirolimus showed a statistically significant benefit for OS in patients with advanced RCC compared to IFNa (10.9 months versus 7.3 months) (Hudes *etal,* 2007). Everolimus has also demonstrated a 2.1-month improvement in median PFS versus placebo, but with no OS improvement (Motzer et al, 2008). Among the five approved anti-angiogenic agents (sorafenib, sunitinib, bevacizumab, pazopanib, and axitinib) and two approved mTOR inhibitors (temsirolimus, everolimus), only everolimus is approved specifically for use after the failure of treatment with anti-angiogenic therapy. In the U.S., everolimus is indicated for the treatment of advanced RCC after failure of treatment with sunitinib or sorafenib, whereas in the EU, everolimus is more broadly indicated for patients with advanced RCC, whose disease has progressed on or after treatment with VEGF-targeted therapy.

Non-Small Cell Lung Cancer

NSCLC is the leading cause of cancer death in the U.S. and worldwide, exceeding breast, colon and prostate cancer combined. In the U.S., an estimated 228,190 new cases of lung and bronchial will be diagnosed in the U.S., and some 159,480 deaths will occur because of the disease (Siegel *et al.,* 2013). The majority of patients (approximately 78%) are diagnosed with advanced/recurrent or metastatic disease. Metastases to the adrenal gland from lung cancer are a common occurrence, with about 33% of patients having such metastases. NSCLC therapies have incrementally improved OS, but benefit has reached a plateau (median OS for late stage patients is just 1 year). Progression after 1L therapy occurred in nearly all of these subjects and the 5-year survival rate is only 3.6% in the refractory setting. From 2005 to 2009, the overall 5-year relative survival rate for

lung cancer in the U.S. was 15.9% (NCCN GUIDELINES®, 2013 - Non-Small Cell Lung Cancer).

Surgery, RT and chemotherapy are the three modalities commonly used to treat NSCLC patients. As a class, NSCLCs are relatively insensitive to chemotherapy and RT, compared to small cell carcinoma. In general, for patients with Stage I or II disease, surgical resection provides the best chance for cure, with chemotherapy increasingly being used both pre-operatively and post-operatively. RT can also be used as adjuvant therapy for patients with resectable NSCLC, the primary local treatment, or as palliative therapy for patients with incurable NSCLC.

Patients with Stage IV disease who have a good performance status (PS) benefit from chemotherapy. Many drugs, including platinum agents *(e.g.,* cisplatin, carboplatin), taxanes agents *(e.g.,* paclitaxel, albumin-bound paclitaxel, docetaxel), vinorelbine, vinblastine, etoposide, pemetrexed and gemcitabine are useful for Stage IV NSCLC. Combinations using many of these drugs produce 1-year survival rates of 30% to 40% and are superior to single agents. Specific targeted therapies have also been developed for the treatment of advanced lung cancer. For example, bevacizumab (AVASTIN®) is a mAb that blocks vascular endothelial growth factor A (VEGF-A). Erlotinib (TARCEVA®) is a small-molecule TKI of epidermal growth factor receptor (EGFR). Crizotinib (XALKORI®) is a small-molecule TKI that targets ALK and MET, and is used to treat NSCLC in patients carrying the mutated *ALK* fusion gene. Cetuximab (ERBITUX®) is a mAb that targets EGFR.

There is a particular unmet need among patients who have squamous cell NSCLC (representing up to 25% of all NSCLC) as there are few treatment options after 1L therapy. Single-agent chemotherapy is standard of care following progression with platinum-based doublet chemotherapy (Pt-doublet), resulting in median OS of approximately 7 months. Docetaxel remains the benchmark treatment in this line of therapy although erlotinib may also be used with less frequency. Pemetrexed has also been shown to produce clinically equivalent efficacy outcomes but with significantly fewer side effects compared with docetaxel in the 2L treatment of patients with advanced NSCLC (Hanna *et ah,* 2004). No therapy is currently approved for use in lung cancer beyond the 3L setting. Pemetrexed and bevacizumab are not approved in squamous NSCLC, and molecularly targeted therapies have limited application. The unmet need in advanced lung cancer has been compounded by the recent failure of Oncothyreon and

Merck KgaA's STIMUVAX® to improve OS in a phase 3 trial, inability of ArQule's and Daiichi Sankyo's c-Met kinase inhibitor, tivantinib, to meet survival endpoints, failure of Eli Lilly's ALIMTA® in combination with Roche's AVASTIN® to improve OS in a late-stage study, and Amgen's and Takeda Pharmaceutical's failure to meet clinical endpoints with the small-molecule VEGF-R antagonist, motesanib, in late-stage trials.

The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of all references cited throughout this application are expressly incorporated herein by reference.

EXAMPLE 1

Cross-Competition between Anti-PD-1 HuMAbs for Binding to CHO Cells Expressing Human PD-1

Chinese Hamster Ovary (CHO) cells transfected to express human PD-1 (CHO/PD-1 cells) were incubated with 10 μg/ml of Fab fragment of the anti-PD-1 HuMAb 5C4 or human IgG1 (hIgG1) isotype control Ab for 30 minutes at 4°C before addition of anti-PD-1 HuMAbs 2D3, 7D3 or 4H1 at a concentration of 0.2 μg/ml. Binding of 4H1, 2D3 or 7D3 to CHO/PD-1 cells were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-hIgG, Fc-gamma specific Ab. In the case of cross-competition assay with 5C4 and 17D8, CHO/PD-1 cells were incubated with the whole molecule of 5C4 before addition of FITC-labeled 17D8. Binding of 2D3, 7D3, 4H1 or 17D8 to the CHO/PD-1 cells was measured by flow cytometric analysis using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

The results are depicted in Figure 1. The data show that the 5C4 Fab fragment substantially blocked the binding of mAbs 5C4 itself, as well as the binding of 2D3, 7D3 (Figure 1A) and 4H1 (Figure IB), while the 5C4 whole mAb substantially blocked the binding of 17D8 (Figure 1C) to CHO/PD-1 cells as measured by mean fluorescence intensity (MFI) of staining.

EXAMPLE 2

Cross-Competition between Anti-PD-L1 HuMAbs for Binding to CHO Cells Expressing Human PD-L1

CHO cells transfected to express hPD-L1 (CHO/PD-L1 cells) were incubated with 10 μg/ml of each often unconjugated human anti-PD-L1 mAbs (5F8, 7H1, 10H10, 1B12, 3G10, 10A5, 11E6, 12A4, 12B7, and 13G4) or human IgG1 (hIgG1) isotype control Ab

for 20 min at 4°C. FITC-conjugated 10H10 (A), 3G10 (B), 10A5 (C), 11E6 (D), 12A4 (E) or 13G4 (F) was added to the cells to a final concentration of 0.09 μg/ml (B, D), 0.27 μg/ml (A, C), 0.91 μg/ml (F), or 2.73 μg/ml (E) for an additional 20 min at 4°C without prior washout of unbound, unconjugated Ab. Different quantities of the various FITC-conjugated HuMAbs were used due to differences in binding efficiency following labeling, and the optimal amounts of these FITC-conjugated HuMAbs were previously determined by dose-titration analysis of binding to CHO/PD-L1 cells. Binding of FITC-conjugated 10H10, 3G10, 10A5, 11E6, 12A4 or 13G4 to the CHO/PD-L1 cells was measured by flow cytometry.

The results are depicted in Figure 2. Binding of labeled 10H10 was partially blocked by 10A5, 11E6 and 13G4, but was substantially blocked only by itself (Figure 2A). Conversely, 10H10 substantially blocked the binding only of itself to CHO/PD-L1 cells. Each of anti-PD-L1 HuMAbs 5F8, 7H1, 1B12, 3G10, 10A5, 11E6, 12A4, 12B7 and 13G4 substantially blocked binding of labeled mAbs 3G10 (Figure 2B), 10A5 (Figure 2C), 11E6 (Figure 2D), 12A4 (Figure 2E) and 13G4 (Figure 2F) to CHO/PD-L1 cells as measured by MFI, though mAbs 5F8 and 13G4 generally blocked binding of the labeled mAbs to a slightly lesser extent.

EXAMPLE 3

Cross-Competition between Anti-PD-L1 mAbs for Binding to Ovarian Carcinoma Cells Expressing Human PD-L1

Anti-PD-L1 HuMAbs 5F8, 12B7, 3G10, 1B12, 13G4, 10H10, 10A5 and 12A4, and a human IgG1 (huIgG1) isotype control Ab were serially diluted from 10 μg/ml and incubated with hPD-L1 -expressing ES-2 ovarian carcinoma cells for 20 minutes at 4°C. Without washing, biotinylated-12A4 Ab was added to a final concentration of 0.4 μg/ml for an additional 20 minutes at 4°C. After washing, bound biotin-12A4 was detected using fluorescent streptavidin-PE secondary reagent and measured by flow cytometry. Figure 3 shows the fluorescence of bound biotin-12A4 plotted against the concentration of unlabeled hPD-L1 HuMAbs. Binding of biotin-12A4 to ES-2 cells was substantially blocked by 12A4 itself and by 1B12 and 12B7, and was moderately to significantly blocked by mAbs 5F8, 10A5, 13G4 and 3G10, but was not blocked by mAb 10H10.

EXAMPLE 4

Design of Phase 1 Clinical Study of Anti-PD-1 Ab

A Phase 1 study was conducted to assess the safety, antitumor activity, and pharmacokinetics of an anti-PD-1 in patients with selected advanced solid tumors. The human anti-PD-1 mAb, BMS-936558 (also referred to herein as nivolumab, and in U.S. Patent No. 8,008,449 as 5C4), was administered as an intravenous infusion every 2 weeks of each 8-week treatment cycle. Tumor status was re-assessed following each cycle. Patients continued treatment for up to 2 years (12 cycles), until they experienced complete remission, unacceptable toxicity, disease progression, or withdrew consent. In patients who were otherwise clinically stable, study treatment was continued beyond apparent initial disease progression until further progression was noted as recommended by proposed immune response criteria (Wolchok *et al,* 2009). Patients with stable disease (SD) or an ongoing objective response (OR: complete response [CR] or partial response [PR]) at the end of treatment were followed for 1 year and were offered retreatment for 1 additional year in the event of progression.

Dose escalation

Patients with advanced melanoma (MEL), non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), castration-resistant prostate cancer (CRPC) and colorectal cancer (CRC) were eligible to enroll. Cohorts of 3-6 patients per dose level were enrolled sequentially at 1.0, 3.0, and 10.0 mg/kg. Dose escalation proceeded when a minimum of 3 patients had completed the safety evaluation period (56 days) at the given dose level, with dose-limiting toxicity in less than one-third of patients. Intra-patient dose escalation was not permitted.

Cohort expansion

A maximum tolerated dose (MTD) was not reached. Initially, 5 expansion cohorts of approximately 16 patients each were enrolled at 10 mg/kg for MEL, NSCLC, RCC, CRPC and CRC. Based on initial signals of activity, and following a 6.5-month hiatus for protocol amendment, additional expansion cohorts of approximately 16 patients each were enrolled for MEL (at 1.0 and 3.0 mg/kg, followed by cohorts randomized to 0.1, 0.3, or 1.0 mg/kg), NSCLC (squamous or nonsquamous histology cohorts randomized to 1, 3, or 10 mg/kg), and RCC (at 1.0 mg/kg). Intra-patient dose escalation to 1 mg/kg was permitted for MEL patients with progressive disease after receiving 0.1 or 0.3 mg/kg.

Patients

Eligible patients had documented advanced solid tumors; age > 18 years; life expectancy >\2 weeks; Eastern Cooperative Oncology Group performance status of ≤ 2; measurable disease by Response Evaluation Criteria in Solid Tumors (RECIST), vl.O with modification *(see* Topalian *et al.,* 2012b); adequate hematologic, hepatic, and renal function; and received 1-5 prior systemic treatment regimens. Patients with stable treated brain metastases were enrolled. Exclusion criteria included a history of chronic autoimmune disease, prior therapy with T-cell modulating Abs *(e.g.,* anti-CTLA-4, anti-PD-1, anti-PD-Ll), conditions requiring immunosuppressive medications, and chronic infections *(e.g.,* HIV, hepatitis B or C).

A total of 296 patients with advanced solid tumors including MEL (n=104), NSCLC (n=122), RCC (n=34), CRPC (n=17), and CRC (n=19) were treated with BMS-936558 for 40 months up to February 2012. By March 2013, 306 patients including patients with non-small cell lung cancer (n=129), melanoma (n=107), RCC (n=34), CRPC (n=17), and CRC (n=19) had been treated with BMS-936558 from October 2008 through January 2012, all with a minimum of one year of observation. Two patients did not receive a full cycle of treatment and were not considered response-evaluable. Median age was 63 years (range, 29-85). ECOG performance score was 0 or 1 in 98% of patients. The majority of patients were heavily pretreated, with 47% having received at least 3 prior regimens. Notable prior therapies included immunotherapy (64%) and B-RAF inhibitor (8%) in MEL patients; platinum-based chemotherapy (94%) and tyrosine kinase inhibitors (TKIs, 34%) in NSCLC patients; and nephrectomy (94%), immunotherapy (59%), and anti-angiogenic therapy (74%) in RCC patients. Baseline characteristics of the total treated population (N=306) were similar to those of the efficacy population (response evaluable patients, N=270). Details on the patient pre-treatments are provided in Topalian *et al.* (2012b).

Statistical analysis

Baseline characteristics and adverse events for all treated patients (N=306), and efficacy results for 270 patients with lung cancer, melanoma, and kidney cancer as of March 2013 are reported. Pharmacokinetic and molecular-marker populations consisted of treated patients with available data as of the February 2012 date of preliminary analysis. The efficacy population consisted of response-evaluable patients commencing treatment at least 8 months before the date of analysis. Tumor measurements were

collected after each treatment cycle (4 doses) by investigators. Individual best objective responses based on the tumor measurements were assessed by the sponsor per modified RECIST vl.O. Objective response was confirmed by at least one sequential tumor assessment. Objective response and stable disease rates were estimated with confidence intervals using the Clopper-Pearson method. Time-to-event endpoints including PFS, OS, survival rates, and duration of response, were estimated using the Kaplan-Meier method. AEs were coded using Medical Dictionary for Regulatory Activities (MedDRA), version 15.1. Select adverse events with potential immunologic etiologies, also referred to as "immune-related adverse events" of "AEs of special interest" (AEOSIs), defined as adverse events that require more frequent monitoring and/or unique intervention, were identified using a pre-defined list of MeDRA terms. Individual best ORs were derived from investigator-reported data per modified RECIST vl.O. OR was confirmed by at least one sequential tumor assessment and OR rate (ORR={[CR + PR] ÷ n } × 100) was calculated.

EXAMPLE 5

Safety Evaluations on Patients Treated with Anti-PD-l Antibody

Safety evaluations, including clinical examination and laboratory assessments, were conducted in all treated patients at baseline and regular intervals up to 100 days following last administration of drug. The severity of AEs was graded based on the NCI Common Terminology Criteria for Adverse Events (NCI CTCAE), v3.0. Computed tomography (CT) or magnetic resonance imaging was performed for tumor assessment at baseline and following each treatment cycle.

A MTD was not defined across the doses of BMS-936558 tested on this study, up to the highest planned dose of 10 mg/kg. A relative BMS-936558 dose intensity of 90% or higher was achieved in 87% of patients *(see* Topalian *et ah,* 2012b; Topalian *et ah,* 2013, for details). AEs were coded using Medical Dictionary for Regulatory Activities (MedDRA), version 14.1. AEOSIs were identified using a pre-defined list of MeDRA terms. Fifteen of 296 (5%) patients discontinued treatment due to BMS-936558-related AEs. As of the February 2012 date of preliminary analysis, 62 (21%) patients had died, and by March 2013, 195 patients (64%) had died, with disease progression being the most common cause of death (Topalian *et ah,* 2012b; Topalian *et ah,* 2013).

The most common adverse events, regardless of causality included fatigue, decreased appetite, diarrhea, nausea, cough, dyspnea, constipation, vomiting, rash,

pyrexia and pruritus (Topalian *et ah,* 2012b; Topalian *et ah,* 2013). Common BMS-936558-related AEs included fatigue, rash, diarrhea, decreased appetite, and nausea. The majority of the events were low grade, with grade 3-4 drug-related AEs observed in 41 of 296 (14%) patients. Treatment-related AEs (any grade) were observed in 230 of 306 patients (75%), the most common being fatigue, rash, diarrhea and pruritus (Topalian *et ah,* 2013). The spectrum, frequency, and severity of BMS-936558-related AEs were generally similar across the dose levels tested, with no clear relationship to treatment duration. Fifty-two of 306 patients (17%) experienced Grade 3-4 treatment-related adverse events, with fatigue (2%), pneumonitis, lymphopenia, diarrhea, abdominal pain, and hypophosphatemia (1% each) being the most common. Treatment-related serious adverse events occurred in 42 of 306 patients (14%) (Topalian *et ah,* 2013).

Drug-related AEOSIs, with potential immune-related etiologies, were categorized by organ involvement to provide a more accurate estimate of frequency and included pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis among others. Treatment-related select adverse events of any grade were observed in 140 of 306 patients (46%), the most common being rash (15%), diarrhea (13%), and pruritus (11%) (Table 2). Grade 3-4 treatment-related select events were seen in 19 patients (6%).

Fifty-two of 230 patients (23%) with drug-related adverse events required management with systemic glucocorticoids and/or other immunosuppressive agents. Hepatic or gastrointestinal AEOSIs were managed with treatment interruption and, as necessary, with administration of corticosteroids. Among patients treated to-date, these AEs were reversible in all cases. Endocrine AEOSIs were managed with replacement therapy. Thirty-two of 306 patients (11%) discontinued therapy due to treatment-related adverse events. At the discretion of the treating physician, patients successfully reinitiated treatment with BMS-936558. Twenty-one (40%) were able to resume nivolumab therapy after toxicity resolved.

Drug-related pneumonitis (any grade) occurred in 12 of 306 (4%) patients (Topalian *et ah,* 2013). Clinical presentations ranged from radiographic abnormalities in asymptomatic patients, to progressive, diffuse pulmonary infiltrates associated with symptoms (cough, fever, dyspnea). Grade 3-4 pneumonitis developed in 4 patients (1%), of which 3 cases were fatal (2 NSCLC patients, 1 CRC). No clear relationship between the occurrence of pneumonitis and tumor type, dose level, or the number of doses received was noted. In 9 of 12 patients, pneumonitis was reversible with treatment

discontinuation and/or immunosuppression (glucocorticoids, infliximab, mycophenolate). Following the most recent pneumonitis-associated death in November 201 1, 79 patients continued to receive nivolumab (median 29 weeks, range 2-69 weeks) with no further mortalities from this or other treatment-related causes.

EXAMPLE 6

Pharmacokinetics/Pharmacodynamics Analyses on Anti-PD-l Antibody

For pharmacokinetic (PK) analyses, serial blood samples were collected and serum concentrations of BMS-936558 were quantified using by ELISA. For pharmacodynamic (PD) analysis, peripheral blood mononuclear cells were isolated from patients at baseline and following cycle 1 to estimate PD-1 receptor occupancy (RO) by BMS-936558 on circulating CD3+ T-cells via flow cytometry (Brahmer *et ah,* 2010).

The maximum concentration of BMS-936558 was observed at a median T_{max} of 1-4 hours after the start of infusion. The PK of BMS-936558 was linear with a dose proportional increase in C_{max} and $AUC_{(0\text{-}i4\ d)}$ in the dose range of 0.1-10 mg/kg (n=35). BMS-936558 PD was assessed by PD-1 RO on circulating T-cells. PBMCs from 65 MEL patients treated with one cycle of BMS-936558 at 0.1-10 mg/kg biweekly demonstrated median occupancy of PD-1 molecules on circulating $CD3^+$ T-cells by BMS-936558 ranging from 64%-70% *(see* Topalian *et ah,* 2012b, for details).

EXAMPLE 7

Antitumor Efficacy Exhibited by Anti-PD-l Antibody

Data analyzed as of February 2012

Clinical antitumor activity was observed at all BMS-936558 doses tested. ORs (confirmed CR or PR) were observed in a substantial portion of patients with NSCLC, MEL, and RCC (Tables 1 and 2; Figure 4), and in various sites of metastatic disease including liver, lung, lymph nodes, and bone (Figures 5-7 and not shown). Tumor regressions followed conventional as well as "immune-related" patterns of response, such as prolonged reduction in tumor burden in the presence of new lesions. Individual best overall responses were derived from investigator-reported data according to modified RECIST vl.0. OR was confirmed by at least one sequential tumor assessment. At the time of data analysis, 2 patients with NSCLC who were treated with 10 mg/kg had unconfirmed responses, and 8 additional patients (with MEL, NSCLC, or RCC) had a persistent reduction in baseline target lesions in the presence of new lesions, *(i.e.,* an

"immune-related" response pattern). None of these patients was categorized as a responder for the purpose of calculating OR rates. Antitumor responses and/or prolonged disease stabilization were observed in patients irrespective of prior therapies received *(see* summary of progression free interval for patients with OR and SD in Supplementary Appendix 4 of Topalian *et ah,* 2012b).

In NSCLC patients, 14 ORs were observed at BMS-936558 doses of 1, 3, or 10 mg/kg with response rates of 6%, 32%, and 18%, respectively. ORs were observed across NSCLC histologies: 6 responders of 18 squamous (33%), 7 responders of 56 nonsquamous (13%), and 1 of 2 unknown. All 14 patients with ORs started treatment ≥ 24 weeks before data analysis, and of these, 8 had response duration ≥ 24 weeks (Table 1). Stable disease (SD) lasting ≥ 24 weeks was observed in 5 (7%) NSCLC patients, all with nonsquamous histology. Among MEL patients, 26 ORs were observed at doses ranging from 0.1-10 mg/kg, with response rates ranging from 19%-41% per dose level. At the 3 mg/kg dose level, ORs were noted in 7 of 17 (41%) patients. Of 26 MEL patients who achieved an OR, 17 started treatment ≥ 1 year before data analysis, and of these, 13 patients had an OR duration ≥ 1 yr. The remaining 8 patients with OR were on study < 1 year and 6 had responses ranging from 1.9-5.6 months. SD lasting ≥ 24 weeks was observed in 6 (6%) patients. In RCC patients, ORs occurred in 4 of 17 (24%) patients treated with a BMS-936558 dose of 1 mg/kg and 5 of 16 (3 1%) patients treated with 10 mg/kg. Among 8 RCC patients with OR who started treatment ≥ 1 year prior to data analysis, 5 (63%) had OR duration ≥ 1 yr. SD lasting ≥ 24 weeks was observed in an additional 9 (27%) patients.

Table 1. Clinical Activity of BMS-936558 in the Efficacy Population* (N=236)† as Assessed to February 2012

Tumor Type	Dose (mg/kg)	n	ORR‡ No. Patients (%) [95% CI]†	SD ≥ 24 wk No. Patients (%) [95% CI]	PFSR§ at 24 wk (%) [95% CI]
MEL	0.1	14	4 (29) [8-58]	1 (7) [0.2-34]	40 [13-66]
	1.0	27	8 (30) [14-50]	3 (11) [2-29]	45 [26-65]
	3.0	17	7 (41) [18-67]	1 (6) [0.1-29]	55 [30-80]
	10.0	20	4 (20) [6-44]	0	30 [9-51]
ALL MEL		94	26 (28) [19-38]	6 (6) [2-13]	41 [30-51]

Tumor Type	Dose (mg/kg)	n	ORR‡ No. Patients (%) [95% CI]†	SD ≥ 24 wk No. Patients (%) [95% CI]	PFSR§ at 24 wk (%) [95% CI]
NSCLC**					
All	1	18	1 (6) [0.1-27]	1 (6) [0.1-27]	16 [0-34]
Squamous	1	5	0	0	0
Nonsquamous	1	12	0	1 (8) [0.2-39]	14 [0-37]
Unknown	1	1	1(100) [3-100]	0	1
All	3	19	6 (32) [13-57]	2 (11) [1-33]	41 [18-64]
Squamous	3	6	3 (50) [12-88]	0	50 [10-90]
Nonsquamous	3	13	3 (23) [5-54]	2 (15) [2-45]	37 [10-64]
All	10	39	7 (18) [8-34]	2 (5) [0.6-17]	24 [11-38]
Squamous	10	7	3 (43) [10-82]	0	43 [6-80]
Nonsquamous	10	31	4 (13) [4-30]	2 (7) [0.8-21]	21 [6-36]
Unknown	10	1	0	0	0
ALL NSCLC		76	14 (18) [11-29]	5 (7) [2-15]	26 [16-36]
All Squamous		18	6 (33) [13-59]	0	33 [12-55]
All Nonsquamous		56	7 (13) [5-24]	5 (9) [3-20]	22 [11-34]
RCC	1	17	4 (24) [7-50]	4 (24) [7-50]	47 [23-71]
	10	16	5 (31) [11-59]*	5 (31) [11-59]††	67 [43-91]
ALL RCC		33	9 (27) [13-46]	9 (27) [13-46]	56 [39-73

* The efficacy population consists of response-evaluable patients whose treatment was initiated at least 8 months before data analysis in February 2012, and had measurable disease at baseline and one of the following: at least 1 on-treatment scan or clinical evidence of disease progression or death.

† CR denotes complete response, MEL melanoma, NSCLC non-small cell lung cancer, ORR objective response rate, PFSR progression-free survival rate, PR partial response, RCC renal cell cancer, SD stable disease, n number of patients.

‡ Objective response rates ({[CR + PR] ÷ n } × 100) have been calculated based on confirmed responses with confidence intervals calculated using the Clopper-Pearson method. Individual patient responses were adjudicated per RECIST v1.O with

modification *(see* Topalian *et ah,* 2012b).

§ Progression-free survival rate was the proportion of patients who did not progress and were alive at 24 weeks calculated by the Kaplan-Meier methodology with confidence intervals using the Greenwood method.

[1] One CR.

**One NSCLC patient who was treated at the 3 mg/kg dose level had an initial evaluation of progressive disease, subsequently had a PR, and was classified as a responder.

Table 2. Duration of Objective Responses to BMS-936558* as Assessed to February 2012

Tumor Type	Dose (mg/kg)	No. of Patients with OR	Duration of Response (months)†
MEL	0.1	4	7.5+, 5.6+, 5.6, 5.6
	0.3	3	3.8+, 2.1+, 1.9+
	1	8	24.9+, 22.9, 20.3+, 19.3+, 18.4+, 7.6+, 5.6+, 5.3+
	3	7	22.4+, 18.3+, 15.2+, 12.9, 11.1, 9.3, 9.2+
	10	4	24.6+, 23.9+, 18.0+, 17.0
NSCLC§	1	1	9.2+
	3	6	30.8+, 7.6+, 5.5+, 3.7+, 1.9+, NA‡
	10	7	14.8+, 7.6+, 7.3+, 6.7, 4.2, 3.7+, 3.7
RCC	1	4	17.5+, 9.2+, 9.2, 5.6+
	10	5	22.3+. 21.7+, 12.9, 12.0, 8.4

* MEL denotes melanoma, NA not applicable, NSCLC non-small cell lung cancer, RCC renal cell cancer.

† Time from first response to time of documented progression, death, or for censored data, time to last tumor assessment.

‡ One patient was treated beyond an initial evaluation of progressive disease and subsequently had a PR; this patient was classified as a responder for the purposes of calculating response rates by RECIST vl.O but was not eligible for calculation of duration of response.

Data analyzed as of March 2013

Objective responses were observed in patients with NSCLC (17%), MEL (31%), and RCC (29%), but not with CRC or CRPC. Responses were observed across all nivolumab doses tested; response rates in NSCLC patients receiving 1 mg/kg, versus 3 or 10 mg/kg, appeared to be decreased (3%, versus 24% and 20%, respectively) (Tables 3 and 4). Thirteen of 270 patients (4.8%) with responding histologies had unconventional response patterns that did not meet RECIST criteria *(e.g.,* persistent reduction in target lesions in the presence of new lesions or regression following initial progression) (Wolchok *et al,* 2009). Additional patients manifested SD for 24 weeks or longer (10% NSCLC, 7% MEL, 27% RCC). Sustained survival, reflected by 1- and 2-year landmark OS rates, was noted in each of the responding populations as follows: NSCLC, 42% and 14%; MEL, 62% and 43%; and RCC, 70% and 50% (Table 3). Median OS of 9.6 months for lung cancer (9.2 and 10.1 months for squamous and non-squamous NSCLC histologies, respectively), 16.8 months for MEL, and more than 22 months for RCC, were observed; median PFS was 2.3 months in NSCLC, 3.7 months in MEL, and 7.3 months in RCC; and the median duration of response was 74, 104, and 56 weeks, respectively (Table 4). Among 16 responders who discontinued therapy for reasons other than disease progression and were followed for at least 24 weeks, 13 (81%) remained in response at the time of analysis (Figure 8).

Table 3. Clinical Activity of Nivolumab in the Efficacy Population (N = 306)* as Assessed to March 2013

Tumor Type	ORRs† No. of patients/total no. of patients (%) [95% CI]	Median Duration of Response‡, wk (range)	Stable Disease No. of patients/total no. of patients (%) [95% CI]		Median Progression-free Survival, mo (95% CI)	Median Overall Survival, mo (95% CI)	Overall Survival Rate, % (95% CI); patients at risk, n	
			≥ 24 wk	≥ 48 wk			1 yr	2 yr
Non-small cell lung cancer**	22/129 (17.1) [11.0, 24.7]	74.0 (6.1+, 133.9+)	13/129 (10.1) [5.5, 16.6]	6/129 (4.7) [1.7, 9.8]	2.3 (1.9, 3.7)	9.6 (7.8, 12.4)	42 (33, 51); 43	14 (4, 24); 5
Squamous	9/54 (16.7) [7.9, 29.3]	NR§ (16.1, 133.9+)	8/54 (14.8) [6.6, 27.1]	3/54 (5.6) [1.2, 15.4]	3.7 (1.8, 7.2)	9.2 (7.3, 12.5)	39 (25, 53):16	¶
Non-squamous	13/74 (17.6) [9.7, 28.2]	63.9 (6.1+, 74.0+)	5/74 (6.8) [2.2, 15.1]	3/74 (4.1) [0.8, 11.4]	2.0 (1.8, 3.6)	10.1 (7.2, 13.7)	43 (31, 54): 26	¶
Melanoma	33/107 (30.8) [22.3, 40.5]	104 (18.4, 117.0+)	7/107 (6.5) [2.7, 13.0]	4/107 (3.7) [1.0, 9.3]	3.7 (1.9, 9.1)	16.8 (12.5, 31.6)	62 (53, 72); 55	43 (32, 53); 26
Kidney cancer	10/34 (29.4) [15.1, 47.5]	56.1 (36.6, 126.7+)	9/34 (26.5) [12.9, 44.4]	2/34 (5.9) [0.7, 19.7]	7.3 (3.7, 12.9)	>22# (13.6, NE^)	70 (55, 86); 23	50 (31, 70); 8

* No objective responses were seen in 19 patients with colorectal cancer or 17 patients with castration-resistant prostate cancer.

† Objective response rates ({[CR + PR] ÷ n} × 100) have been calculated based on confirmed responses with confidence intervals calculated using the Clopper-Pearson method. Individual patient responses were adjudicated per RECIST vl.O with modification (see Methods SI and study protocol, NEJM.org).

‡ Time from first response to time of documented progression, death, or for censored data (denoted by "+"), time to last tumor assessment.

** Among 129 patients with non-small-cell lung cancer, 1 had an unknown histology and did not show an objective response. Others had squamous or non-squamous histologies, as indicated.

§ NR, not reached; the time point at which the probability that responders progress drops below 50% has not been reached due to insufficient number of events and/or follow-up.

¶ Insufficient period of follow-up.

‖ 1 CR was noted in melanoma, and 1 CR in kidney cancer.

The median overall survival was not reached at 22 months, the longest time to death so far in patients with kidney cancer in this trial.

^ NE, not estimable.

Table 4. Clinical Activity of Nivolumab by Dose Level as Assessed to March 2013

Tumor Type	Dose, mg/kg	ORRs* No. of patients/total no. of patients (%) [95% CI]	Median Duration of Response wk (range)†	Stable Disease No. of patients/total no. of patients (%) [95% CI]		Median Progression-free Survival, mo (95% CI)	Median Overall Survival, mo (95% CI)
				≥ 24 wk	≥ 48 wk		
Non-small cell lung cancer	All doses	22/129 (17.1) [11.0, 24.7]	74.0 (6.1+, 133.9+)	13/129 (10.1) [5.5, 16.6]	6/129 (4.7) [1.7, 9.8]	2.3 (1.9, 3.7)	9.6 (7.8, 12.4)
	1	1/33 (3.0) [0.1, 15.8]	63.9 (63.9, 63.9)	5/33 (15.2) [5.1, 31.9]	2/33 (6.1) [0.7, 20.2]	1.9 (1.8, 3.6)	9.2 (5.6, 11.1)
	3	9/37 (24.3) [11.8, 41.2]	NR‡ (16.1+, 133.9+)	3/37 (8.1) [1.7, 21.9]	2/37 (5.4) [0.7, 18.2]	1.9 (1.7, 7.3)	14.9 (9.5, NE^)
	10	12/59 (20.3) [11.0, 32.8]	83.1 (6.1+, 117.1+)	5/59 (8.5) [2.8, 18.7]	2/59 (3.4) [0.4, 11.7]	3.6 (1.9, 3.8)	9.2 (5.2, 12.4)
Squamous	All doses	9/54 (16.7) [7.9, 29.3]	NR (16.1, 133.9+)	8/54 (14.8) [6.6, 27.1]	3/54 (5.6) [1.2, 15.4]	3.7 (1.8, 7.2)	9.2 (7.3, 12.5)
	1	0	0	4/15 (26.7) [7.8, 55.1]	1/15 (6.7) [0.2, 31.9]	1.9 (1.8, 7.5)	8.0 (2.6, 13.3)
	3	4/18 (22.2) [6.4, 47.6]	NR (16.1, 133.9+)	1/18 (5.6) [0.1, 27.3]	1/18 (5.6) [0.1, 27.3]	3.8 (1.7, 14.9)	9.5, (6.7, NE)

Tumor Type	Dose, mg/kg	ORRs* No. of patients/total no. of patients (%) [95% CI]	Median Duration of Response wk (range) †	Stable Disease No. of patients/total no. of patients (%) [95% CI]		Median Progression-free Survival, mo (95% CI)	Median Overall Survival, mo (95% CI)
				≥ 24 wk	≥ 48 wk		
	10	5/21 (23.8) [8.2, 47.2]	83.1 (16.1, 117+)	3/21 (14.3) [3.0, 36.3]	1/21 (4.8) [0.1, 23.8]	4.1 (1.8, 7.6)	10.5 (7.8, 12.5)
Non-squamous	All doses	13/74 (17.6) [9.7, 28.2]	63.9 (6.1+, 74.0+)	5/74 (6.8) [2.2, 15.1]	3/74 (4.1) [0.8, 11.4]	2.0 (1.8, 3.6)	10.1 (7.2, 13.7)
	1	1/18 (5.6) [0.1, 27.3]	63.9 (63.9, 63.9)	1/18 (5.6) [0.1, 27.3]	1/18 (5.6) [0.1, 27.3]	1.8 (1.7, 3.6)	9.9 (5.6, 22.7)
	3	5/19 (26.3) [9.1, 51.2]	74.0 (24.3, 74.0+)	2/19 (10.5) [1.3, 33.1]	1/19 (5.3) [0.1, 26.0]	1.8 (1.7, 12.5)	18.2 (10.3, 18.2)
	10	7/37 (18.9) [8.0, 35.2]	NR (6.1+, 65.7+)	2/37 (5.4) [0.7, 18.2]	1/37 (2.7) [0.1, 14.2]	2.3 (1.9, 3.8)	7.4 (4.6, 12.4)
Melanoma §	All doses	33/107 (30.8) [22.3, 40.5]	104 (18.4, 117.0+)	7 (6.5) [2.7, 13.0]	4 (3.7) [1.0, 9.3]	3.7 (1.9, 9.1)	16.8 (12.5, 31.6)
	0.1¶	6/17 (35.3) [14.2, 61.7]	NR (24.1, 48.7+)	0	0	3.6 (1.7, 9.1)	16.2 (8.6, NE)
	0.3^	5/18 (27.8) [9.7, 53.5]	NR (18.4, 66.3+)	1 (5.6) [0.1, 27.3]	0	1.9 (1.8, 9.3)	12.5 (7.7, NE)

Tumor Type	Dose, mg/kg	ORRs* No. of patients/total no. of patients (%) [95% CI]	Median Duration of Response wk (range)†	Stable Disease No. of patients/total no. of patients (%) [95% CI]		Median Progression-free Survival, mo (95% CI)	Median Overall Survival, mo (95% CI)
				≥ 24 wk	≥ 48 wk		
	1	11/35 (31.4) [16.9, 49.3]	104 (32.4, 108.1+)	5/35 (14.3) [4.8, 30.3]	4/35 (11.4) [3.2, 26.7]	9.1 (1.8, 24.7)	25.3 (14.6, NE)
	3	7/17 (41.2) [16.9, 67.1]	75.9 (40.1+, 115.4+)	1/17 (5.9) [0.1, 28.7]	0	9.7 (1.9, 16.4)	20.3 (8.2, NE)
	10	4/20 (20.0) [5.7, 43.7]	112 (73.9, 117+)	0	0	3.7 (1.7, 20.50)	11.7 (7.2, 37.8)
Kidney cancer§	All doses	10/34 (29.4) [15.1, 47.5]	56.1 (36.6, 126.7+)	9/34 (26.5) [12.9, 44.4]	2/34 (5.9) [0.7, 19.7]	7.3 (3.7, 12.9)	>22# (13.6,NE)
	1	5/18 (27.8) [9.7, 53.5]	56.1 (40.1, 76.1+)	4/18 (22.2) [6.4, 47.6]	1/18 (5.6) [0.1, 27.3]	4.7 (1.9, 10.9)	NR (17.9, NE)
	10	5/16 (31.3) [11.0, 58.7]	56.1 (36.6, 126.7+)	5/16 (31.3) [11.0, 58.7]	1/16 (6.3) [0.2, 30.2]	8.0 (3.7, 14.0)	18.8 (11.6, NE)

* Objective response rates ({[CR + PR] ÷ n} × 100) have been calculated based on confirmed responses with confidence intervals calculated using the Clopper-Pearson method. Individual patient responses were adjudicated per RECIST vl.O with modification (see Methods SI and study protocol, NEJM.org).

† Time from first response to time of documented progression, death, or for censored data (denoted by "+"), time to last tumor assessment.

‡ NR, not reached; the time point at which the probability that responders progress drops below 50% has not been reached due to insufficient number of events and/or follow-up.

§ 1 CR was noted in melanoma, and 1 CR in kidney cancer.

¶ Five patients with tumor progression were dose-escalated from 0.1 to 1.0 mg/kg, and six from 0.3 to 1.0 mg/kg. None of these patients responded to therapy.

The median overall survival was not reached at 22 months, the longest time to death so far in patients with kidney cancer in this trial.

^ NE, not estimable.

EXAMPLE 8

Correlation between Membranous PD-L1 Expression and Anti-PD-l Response

IHC staining of PD-L1 was performed on pretreatment formalin-fixed paraffin-embedded (FFPE) tumor specimens using the murine anti-human PD-L1 mAb 5H1 (Dong *et ah,* 2002) in a standard IHC protocol (Taube *et ah,* 2012; Supp. Materials). Briefly, 5μιη-FFPE sections mounted on glass slides were deparaffinized in xylene and antigen retrieval was performed using a Tris-EDTA buffer, pH 9.0 at 120°C for 10 min in a Decloaking Chamber (Biocare Medical). Endogenous peroxidase, biotin and proteins were blocked (CAS system K1500, Dako; Avidin/biotin Blocking Kit, SP-2001, Vector Laboratories; Serotec Block ACE), and the primary 5H1 Ab was added at a concentration of 2 μg/ml and allowed to incubate at 4°C for 20 h. Secondary Ab (biotinylated anti-mouse IgGl, 553441 BD) was applied at a concentration of 1 μg/ml for 30 min at room temperature (RT). The signal was then developed with amplification according to the manufacturer's protocol (CAS system K1500, Dako). Sections were counterstained with hematoxylin, dehydrated in ethanol and cleared in xylene, and a coverslip was applied.

The percentage of tumor cells exhibiting cell surface staining for PD-L1 was scored by two independent pathologists who were blinded to treatment outcomes. PD-L1 positivity was defined per specimen by a 5% expression threshold (Taube *et ah,* 2012; Thompson *et ah,* 2006), and in cases with multiple specimens, if any specimen met this criterion. A Fisher's exact test was applied to assess the association between PD-L1 expression and OR, noting, however, that this analysis was based in part on optional biopsies from a non-random subset of the population and testing of a statistical hypothesis was not pre-specified.

Sixty-one pretreatment tumor specimens from 42 patients (18 MEL, 10 NSCLC, 7 CRC, 5 RCC, and 2 CRPC) were analyzed for tumor cell surface PD-L1 expression (Figure 8). Biopsy specimens from 25 of 42 patients were positive for PD-L1 expression by IHC. A Fisher's exact test was applied to assess the association between PD-L1 expression and OR in a post-hoc analysis. Among the 42 surface-PD-Ll $^{+}$ patients, 9 (36%) achieved an OR , whereas among 17 patients with PD-L1^{-} tumors, none achieved an OR (Figure 8A). Thus, in a subset of patients cell surface expression of PD-L1 on tumor cells in pretreatment biopsies is associated with an increased rate of OR among patients treated with BMS-936558, while no patients with documented PD-L1 -negative tumors experienced an OR. These data indicate that tumor PD-L1 expression is a

molecular marker that can enable patient selection for anti-PD-1 immunotherapy.

EXAMPLE 9

Isolation of Rabbit mAbs that Detect Membranous hPD-Ll Antigen in FFPE Tissues

Rabbit Abs against human PD-Ll polypeptide were prepared by Epitomics, Inc. (Burlingame, CA) by immunization of rabbits using a recombinant human PD-Ll fusion protein. Antiserum titers were evaluated using standard direct ELISA with the hPD-Ll antigen, and cell ELISA using transfected cells overexpressing hPD-Ll. These Abs were also screened for their ability to bind to PD-Ll by IHC assay of FFPE tissue sections. The rabbit with the highest Ab titer was selected for splenectomy. Lymphocytes isolated from the spleen were fused to myeloma cells in 40 x 96-well plates, and screened by ELISA against the immunizing PD-Ll antigen and by cell ELISA against cells overexpressing hPD-Ll. Positive clones were expanded into 24-well plates, and confirmatory screens were conducted by direct ELISA and cell ELISA. The supernatants (sups) of clones that were specific to the screening antigen were re-screened by IHC.

A set of mouse anti-hPD-Ll mAbs were also produced by immunization of mice using a protocol similar to that described above for the rabbit mAbs.

Out of a total of 185 multiclones from both rabbit and mouse immunizations screened, only ten rabbit multiclone Abs specifically detected the membranous form of hPD-Ll. None of the purified mouse subclones were found to specifically detect cell surface hPD-Ll. Sixty subclones from the top five rabbit multiclones (designated Nos. 13, 20, 28, 29 and 49, each comprising 12 subclones) were screened initially by IHC on FFPE low density tissue microarrays (TMAs), followed by confirmation and specificity verification in narrowed 25 subclones. Rabbit IgG was used as a negative isotype control, and mAb 5H1 (Dong *et al,* 2002) was used as the positive control. Specificity was further verified by antigen preabsorption assay. Through two rounds of IHC, the following 15 purified subclones were selected as the most promising Abs in terms of specificity and intensity of staining: 13-1, 13-3, 13-7, 13-8; 20-5, 20-7, 20-12, 20-6; 28-1, 28-8, 28-12; 29-8; 49-5, 49-7 and 49-9. Immunoreactivity data on these selected Abs are summarized in Table 5.

Table 5. Immunoreactivity of Rabbit Anti-hPD-L1 mAbs

	Specific Staining		Non-Specific staining
Antibody Name	Pos. vs Neg. Staining on PD-L1 Cells*	Intensity Range on Tissues† (Very High, High, Medium, Low)	Background Staining on Tissues (High, Medium, Low)
13-1	Pos	Low to Very High	None
13-3	Pos	Low to High	None
13-7	Pos	Low to High	None
13-8	Pos	Low to High	None
20-5	Pos	Low to Very High	High
20-6	Pos	Low to High	Medium
20-7	Pos	Low to High	Medium
20-12	Pos	Low to High	Medium
28-1	Pos	Low to Very High	None
28-8	Pos	Medium to Very High	None
28-10	Pos	Medium to Very High	Low
28-12	Pos	Low to Very High	None
49-5	Pos	Low to High	None
49-7	Pos	Low to High	Low
49-9	Pos	Low to High	None
5H1	Pos	High to Very High	None
Rb IgG	Neg	Neg	None

* PD-L1 stably transfected CHO cells vs. CHO-S control;

† PD-L1 positive tissues included placenta and one non-small cell lung cancer; Detection up to "very high" expression suggests better sensitivity at detecting membranous PD-L1.

Additional assays were performed to further characterize the purified Ab clones, including determining binding affinity and cross-competition among the Abs (to identify overlapping versus different epitope regions) by surface plasmon resonance. All the Abs exhibited high binding affinity (¾ $< 10^{-9}$ M). These 15 purified clones were also re-screened by IHC on FFPE low density TMA or regular sections against various cell and

tissue types known to be positive or negative for cell surface expression of PD-Ll. Rabbit IgG was used as the isotype control, and mAb 5H1 was used as the positive control. At high concentration (10 μg/ml), clones 28-x and 49-x displayed low to moderate levels of background staining in tissues, while clones 13-x exhibited no background staining, which suggests that the 13-x clones have a wider dynamic range. The 20-x clones displayed various degree of background staining which was primarily cytoplasmic and diffuse. The clone with most robust detection specifically of membranous PD-Ll, rabbit clone 28-8 (K_D = 100 p M, as determined by SPR), was selected as the lead Ab for subsequent IHC assays. MAbs 28-1, 28-12, 20-12 and 29-8 had K_D values of 130 pM, 94 pM, 160 pM and 1200 pM, respectively. The sequences of the heavy chain variable (V_H) and light (kappa) chain variable (V_K) regions of mAb 28-8 are set forth in SEQ ID NOs. 35 and 36, respectively. The 28-8 Ab was shown to recognize a different epitope from mouse mAb 5H1, based on SPR analysis. Clones 28-1, 28-12, 29-8 and 20-12 were the next best Abs in terms of robust detection of membranous PD-Ll in FFPE tissues. Although mAb 13-1 had the best specificity in terms of detection of membranous PD-Ll, the maximal detection level was lower than that of the other lead Abs. Western blotting was also performed with plus/minus antigen competition to verify the specificity of the top selected Abs for PD-Ll.

The binding of mAbs 5H1 and 28-8 to membranous PD-Ll in FFPE test tissue samples comprising tumor cells and tumor-infiltrating inflammatory cells from different types of tumors was compared. Membranous PD-Ll expression was evaluated using the histoscore method performed by 2 independent pathologists. Four NSCLC, 2 MEL, and 2 RCC tumors were stained with 28-8 at 2 mg/ml and 5H1 at 5 mg/ml. The data are tabulated in Table 6, and shown graphically in Figure 9. The rabbit mAb 28-8 showed better detection (higher histoscores) for 7 out of 10 samples using 2.5-fold less Ab, and in only one sample was the histoscore for 5H1 slightly higher than for mAb 28-8.

Table 6. Comparison of mAbs 28-8 and 5H1 by histoscore analysis

Tissue	Sample I.D.	Histoscore Average (5H1)	Histoscore Average (28-8)
NSCLC	1080754B	245	261
NSCLC	1080766B	103	130
NSCLC	1080790	40	37

Tissue	Sample ID.	Histoscore Average (5H1)	Histoscore Average (28-8)
NSCLC	1080993B	12	16
Mel	T030668	98	113
Mel	T980744	98	123
Mel	1168657B	0	0
Mel	T980747	1	9
RCC	1164619B	4	4
RCC	1167809B	108	125

Taube *et al.* (2012) demonstrated by flow cytometry on cultured cells that mAb 5H1 bound to the cell surface, and the specificity of binding to PD-L1 was confirmed using a PD-L1 fusion protein to competitively block binding of the 5H1 mAb to tissue sections. These authors also compared 5H1 with a rabbit polyclonal anti-hPD-L1 Ab, 4059, previously described by Gadiot *et al.* (2011), and found that whereas 5H1 showed a cell surface staining pattern on FFPE samples, pAb 4059 demonstrated diffuse cytoplasmic staining. Further, when 5H1 was compared to pAb 4059 by western blot analysis, Ab 4059 bound to multiple proteins in lysates of melanoma cells in addition to a 50 kDa protein corresponding to the expected mass of glycosylated PD-L1, in contrast to 5H1 which specifically detected the 50 kDa band of glycosylated PD-L1 (Taube *et al.*, 2012). Contrary to the results of Taube *et al.* (2012) and the results disclosed herein (summarized in Table 7), Gadiot *et al.* (2011) reported that mAb 5H1 produced a high level background staining in FFPE tissue samples, whereas they found that pAb 4059 produced satisfactory specific staining of PD-L1 in FFPE samples. Thirteen other Abs tested by Gadiot *et al.* (2011) either did not stain FFPE tissues, gave a high background staining, or were not blocked by PD-L1 fusion protein competition, highlighting the difficulties in obtaining anti-PD-L1 Abs that bind specifically to PD-L1 in FFPE tissues.

In the present study, an automated IHC assay (*see* Example 10) was used to evaluate the binding of several commercially available anti-PD-L1 Abs and 5H1 (Dong *et al.*, 2002) to FFPE tissue samples containing various cells expressing PD-L1. The results, summarized in Table 7, show that none of the commercially available Abs tested specifically recognized membranous PD-L1 expression in human tissues known to express PD-L1, or to clearly distinguish CHO cells expressing PD-L1 versus the

untransfected parent CHO cells that did not express PD-L1. The inability of the polyclonal Ab (pAb) 4059 to bind specifically recognized membranous PD-L1 is consistent with the findings of Taube *et al.* (2012). The binding of 28-8 was similar to that of 5H1 in this assay, though histoscore analysis suggests that 28-8 performs better than 5Hl.

Table 7. Binding of mAbs to FFPE Samples Containing PD-L1 -expressing cells

Source	Antibody Types	Clone No. (mAb) /Catalog No. (pAb)	Pos. vs. Neg. Staining on PD-L1 Cells*	Human Positive Tissues†
MBL	mAb	27A2	Failed	Failed
BioLegend	mAb	29E.2A3	Failed	Failed
eBiosciences	mAb	M1H1	No Staining	No staining
Collaborator	mAb	5H1	Passed	Passed
ProSci	pAb	4059	Failed	Failed
LifeSpan BioSciences	pAb	LS-B480/0604	Failed	Failed
Bristol-Myers Squibb	mAb	28-8	Passed	Passed

*PD-L1 stably transfected CHO cells vs. parent CHO-S negative control;

†PD-L1 positive tissues included tonsil and/or thymus;

mAb, mouse monoclonal Ab; pAb, rabbit polyclonal Ab.

EXAMPLE 10

Development of Automated IHC Protocolfor Assessing PD-L1 Expression

An automated IHC protocol was developed to assay PD-L1 expression in FFPE specimens. Tissue sections (4 μιη) were mounted on slides, deparaffinized in an autostainer (Leica) by soaking twice for 5 min in xylene, and re-hydrated by soaking twice for 2 min each time in 100% EtOH, twice in 95% (v/v) EtOH, once in 70% (v/v) EtOH, and once in de-ionized water (d¾0). Antigen retrieval was performed using a decloaking chamber (Biocare Medical Decloaking Chamber Plus) and Dako pH 6 buffer, heated to 110°C (PI) for 10 min, then moved to the next step (P2 FAN ON at 98°C; FAN OFF at 90°C). The slides were cooled at room temperature (RT) for 15 min and rinsed

with water for about 1 min.

Reagents were set up on an autostainer (BioGenex i6000), and the tissue area was defined using a pap pen. The IHC assay, run using the autostainer in research mode, comprised the following steps: neutralizing endogenous peroxidase using the Peroxidase Block (Leica) for 10 min, followed by rinsing 3 times with IHC wash buffer (Dako); applying Protein Block (Leica) to the slides, and incubating for 10 min at RT, followed by washing 3 times with wash buffer; applying the primary Ab to the slides (2 μg/ml) and incubating for 1 h at RT, followed by washing 6 times with wash buffer; adding Post Primary Block (NovoLink Kit) to the slides and incubating for 30 min, followed by washing 6 times with wash buffer; adding NovoLink Polymer (NovoLink Kit) to the slides and incubating for 30 min, followed by washing 6 times with wash buffer; adding the DAB chromogen substrates (NovoLink Kit) and developing for 3 min, followed by rinsing 5 times with d¾0 at RT; counterstaining with hematoxylin (NovoLink Kit) for 1 min at RT, followed by washing 3 times with d¾0 for 5 times at RT. The primary Ab was selected from the rabbit anti-PD-Ll Abs shown in Table 4; mAb 28-8 was the preferred Ab. As a negative control, rabbit IgG (Dako) was used. The tissue sections were dehydrated using a Leica autostainer by washing once for 2 min in 70% EtOH, twice for 2 min in 95% EtOH, and three times for 2 min in 70% EtOH, then cleared by washing three times for 5 min in xylene. The sections were permanently mounted with permount to the slide, covered with a coverslip, and transferred to a chemical hood to dry.

EXAMPLE 11

Design of Phase 1 Clinical Study on Anti-PD-Ll Antibody

Study design

A phase 1 study was conducted to assess the safety and tolerability of BMS-936559 (also referred to herein and in U.S. Patent No. 7,943,743 as 12A4) in patients with selected advanced solid tumors. Secondary objectives included initial assessment of the antitumor activity of BMS-936559 and pharmacokinetic evaluation. Pharmacodynamic measures were included under exploratory objectives. Patients were treated in 6-week cycles of BMS-936559 administered as a 60-minute intravenous infusion every 2 weeks on days 1, 15, and 29 of each cycle. Patients continued treatment for up to 16 cycles unless they experienced unacceptable toxicity, disease progression, or withdrew consent. In some patients who were clinically stable, treatment beyond initial disease progression was permitted until further progression was confirmed.

Dose escalation

Patients with advanced NSCLC, MEL, CRC, RCC, ovarian (OV), gastric (GC), breast (BC), and pancreatic (PC) carcinoma were eligible to enroll. Using an accelerated titration design, safety was assessed at doses of 0.3, 1, 3, and 10 mg/kg. One patient was enrolled in each successive cohort until there was a ≥ grade 2 drug-related AE during cycle 1. Two additional patients were then enrolled at that dose level and the study was transitioned to a standard 3+3 design. Intra-patient dose escalation or de-escalation was not permitted. The maximum tolerated dose (MTD) was defined as the highest dose where less than one-third of patients had a dose-limiting toxicity.

Cohort expansion

Initially, 5 expansion cohorts (n=16/cohort) were enrolled at 10 mg/kg for patients with NSCLC, MEL, RCC, OV, and CRC. Based on initial signals of activity, additional expansion cohorts (up to n=16/cohort) were enrolled for MEL (at 1.0 and 3.0 mg/kg), NSCLC (squamous or nonsquamous histology cohorts randomized to 1, 3, or 10 mg/kg), and at 10 mg/kg for PC, BC, and GC.

Patients

Patients were required to have documented advanced NSCLC, MEL, RCC, OV, CRC, PC, GC, or BC, and have failed at least one prior tumor-appropriate therapy for advanced/metastatic disease (except for PC or GC patients who could be treatment-naive). Other inclusion criteria included age ≥ 18 years, life expectancy ≥ 12 weeks, Eastern Cooperative Oncology Group performance status of ≤ 2, measurable disease as defined by RECIST vl.O, and adequate hematologic, hepatic, and renal function. Patients with treated brain metastases were allowed, if stable for at least 8 weeks. Major exclusion criteria included a history of autoimmune disease or other diseases requiring systemic steroids or immunosuppressive medication, prior therapy with T cell- modulating Abs (including anti-PD-1, anti-PD-Ll and anti-CTLA-4), history of HIV, or active hepatitis B or C.

In this ongoing study, 207 patients with NSCLC (n=75), MEL (n=55), CRC (n=18), RCC (n=17), OV (n=17), PC (n=14), GC (n=7), or BC (n=4) were treated with BMS-936559 during a 34-month period and are included in the safety data. Efficacy was characterized in 160 response-evaluable patients. The baseline demographic characteristics of the total and response-evaluable patient populations were very similar (Brahmer *et ah,* 2012). Among treated patients, 86% had received prior chemotherapy

and 28% immunologic or biological therapy. Prior therapies by tumor type included immunotherapy (56%) and B-RAF inhibitor (9%) in patients with MEL; platinum-based chemotherapy (95%) and tyrosine kinase inhibitors (TKIs; 41%) in patients with NSCLC; and nephrectomy (94%), anti-angiogenic therapy (82%), and immunotherapy (41%) in patients with RCC *(see* Brahmer *et al.,* 2012, for more details on patient pre-treatments).

Statistical analysis

All 207 patients commencing treatment as of the date of analysis were used for summaries of baseline characteristics and AEs. The efficacy population consisted of 160 response-evaluable patients who initiated treatment at least 7 months before the date of analysis. AEs were coded using MedDRA vl4. 1. Individual best overall responses were derived from radiographic scan measurements according to modified RECIST vl.O. ORs were confirmed by at least one sequential tumor assessment. Additional details regarding statistical methods are provided in Brahmer *et al.* (2012).

EXAMPLE 12

Safety Evaluations on Patients Treated with Anti-PD-Ll Antibody

Safety evaluations (clinical examination and laboratory assessments) were conducted on all treated patients at baseline and regular intervals (weekly during cycle 1 and biweekly thereafter). AE severity was graded based on the NCI CTCAE, v3.0. Disease assessment via computed tomography (CT) scans or magnetic resonance imaging was performed at baseline and prior to each treatment cycle.

A MTD was not reached up to the highest tested dose of 10 mg/kg of BMS-936559. The median duration of therapy was 12 weeks (range 2.0-1 11.1 weeks). A relative dose intensity of $\geq$ 90% was achieved in 86% of patients. Twelve of 207 patients (6%) discontinued treatment due to a BMS-936559-related AE *(see* Brahmer *et al,* 2012, for details).

AEs regardless of causality (any grade) were reported in 188 of 207 patients. Investigator-assessed BMS-936559-related AEs were noted in 126 of 207 (61%) patients. The most common drug-related AEs were fatigue, infusion reactions, diarrhea, arthralgia, rash, nausea, pruritus, and headache. Most events were low grade with BMS-936559-related grade 3-4 events noted in 19 of 207 (9%) patients (Brahmer *et al,* 2012). The spectrum, frequency, and severity of BMS-936559-related AEs were similar across the dose levels, with the exception of infusion reactions. Drug-related AEOSIs, with potential

immune-related etiologies, were observed in 81 of 207 (39%) of the patients and included rash, hypothyroidism, hepatitis, and single cases each of sarcoidosis, endophthalmitis, diabetes mellitus, and myasthenia gravis (Brahmer *et ah,* 2012). These AEs were predominantly grade 1-2 and generally reversible with treatment interruption or discontinuation. Notably, 9 patients were treated with corticosteroids for the management of AEs. AEs improved or resolved in all patients. Furthermore, 4 of these 9 patients maintained disease control despite treatment with corticosteroids. Endocrine AEs were managed with replacement therapy and patients reinitiated treatment with BMS-936559 at the discretion of the treating physician. Infusion reactions were observed in 21 of 207 (10%) patients, predominantly at 10 mg/kg. They were grade 1-2 with the exception of one grade 3 event at 10 mg/kg. Infusion reactions were generally rapidly reversible with antihistamines and antipyretics and, in some cases, corticosteroids. A prophylactic regimen with antihistamines and antipyretics was implemented during the study. Patients with grade 1-2 infusion reactions were able to continue treatment with BMS-936559 with prophylactic antihistamines and antipyretics and at a reduced infusion rate. BMS-936559-related serious AEs occurred in 11 of 207 (5%) patients. As of the data analysis date, 45 patients (22%) had died (Brahmer *et ah,* 2012); no drug-related deaths were observed.

EXAMPLE 13

Pharmacokinetics/Pharmacodynamics Analyses on Anti-PD-Ll Antibody

For PK analyses, serial blood samples were collected and serum concentrations of BMS-936559 were quantified by ELISA. Peripheral blood mononuclear cells were isolated from patients at baseline and following one treatment cycle to assay PD-L1 RO by BMS-936559 on circulating CD3-positive T-cells via flow cytometry (Brahmer *et ah,* 2010).

Serum concentrations of BMS-936559 increased in a dose-dependent manner from 1-10 mg/kg (n=13 1). Geometric mean area under the curve (0-14 days) for the 1, 3, and 10 mg/kg dose levels were 2210, 7750, and 36620 μg/mL·hr (coefficient of variation [CV] 34-59%), respectively; geometric mean peak concentrations at these dose levels were 27, 83, and 272 μg/mL (CV 30-34%), respectively, after the first dose. The half-life of BMS-936559 was estimated from population pharmacokinetic data as approximately 15 days. PD-L1 RO on CD3-positive peripheral blood lymphocytes was assessed in 29 MEL patients at the end of 1 cycle of treatment, at BMS-936559 doses from 1-10 mg/kg. Median RO exceeded 65% for all groups (Brahmer *et ah,* 2012).

EXAMPLE 14

Antitumor Efficacy Exhibited by Anti-PD-Ll Antibody

One-hundred and sixty patients out of the 207 treated were evaluable for response by February 2012 and included those with NSCLC, MEL, CRC, RCC, OV, and PC, but not patients with GC or BC. Clinical activity was observed at all doses ≥ 1 mg/kg (Brahmer *et ah,* 2012). ORs (confirmed complete [CR] or partial [PR] responses) were observed in patients with MEL, NSCLC, RCC, and OV (Table 8), as illustrated by representative spider plots and CT scans (Figures 10-13), and many ORs were also durable (Table 9). Four additional patients had a persistent reduction in target lesions in the presence of new lesions, consistent with an "immune-related" pattern of response; however, for the purpose of calculating response rates, they were not categorized as responders. Antitumor responses and/or prolonged stable disease (SD) were observed in patients with a variety of prior therapies received. ORs were observed even in patients with an extensive burden of metastatic disease.

In patients with MEL, there were 9 ORs across the 1, 3, and 10 mg/kg dose levels, with response rates of 6%, 29%, and 19%, respectively. Three MEL patients achieved a CR. All 9 MEL patients who experienced an OR started treatment ≥ 1 year prior to data analysis; of these 5 had a response duration ≥ 1 year. Additionally 14 MEL patients (27%) had SD lasting ≥ 24 weeks. In patients with NSCLC, there were 5 ORs amongst the 3 and 10 mg/kg dose levels, with response rates of 8% and 16%, respectively. There were ORs in patients with non-squamous (n=4) or squamous histology (n=l). All 5 NSCLC responders started treatment ≥ 24 weeks prior to data analysis; of these, 3 had responses lasting ≥ 24 weeks. Six additional NSCLC patients had SD lasting ≥ 24 weeks. There was 1 PR out of 17 patients with OV (6% response rate) and 3 patients (18%) with SD lasting ≥ 24 weeks, all at the 10 mg/kg dose. In patients with RCC, there were ORs in 2 of 17 (12%) patients treated at 10 mg/kg with responses lasting 4 and 18 months, respectively. Seven additional RCC patients had SD lasting ≥ 24 weeks.

Table 8. Clinical Activity of BMS-936559 in 160 Patients, Response Evaluable*

Tumor Type	Dose (mg/kg)	n	ORR[§] No. patients (%) [95% CI]	SD ≥ 24 wk No. patients (%) [95% CI]	PFSR** at 24 wk (%) [95% CI]
MEL	0.3	1	0 [0-98]	0 [0-98]	N/A
	1	18	1 (6) [0.1-27]	6 (33) [13-59]	39 [16-61]
	3	17	5 (29)[†] [10-56]	3 (18) [4-43]	47 [21-72]
	10	16	3 (19)[††] [4-46]	5 (31) [11-59]	44 [19-68]
ALL MEL		52	9 (17) (8-30)	14 (27) [16-41]	42 [28-56]
NSCLC[§]	1	11	0 [0-29]	0 [0-29]	N/A
Squamous	1	1	0 [0-98]	0 [0-98]	N/A
Non-Squamous	1	10	0 [0-31]	0 [0-31]	N/A
	3	13	1 (8) [0.2-36]	1 (8) [0.2-36]	34 [7-60]
Squamous	3	4	0 [0-60]	1 (25) [0.6-81]	50 [1-99]
Non-Squamous	3	9	1 (11) [0.3-48]	0 [0-34]	25 [0-55]
	10	25	4 (16) [5-36]	5 (20) [7-41]	46 [25-67]
Squamous	10	8	1 (13) [0.3-53]	2 (25) [3-65]	47 [10-83]
Non-Squamous	10	17	3 (18) [4-43]	3 (18) [4-43]	46 [20-72]
ALL NCSLC		49	5 (10) [3-22]	6 (12) [5-25]	31 [17-45]
ALL Squamous		13	1 (8) [0.2-36]	3 (23.1) [5-54]	43 [15-71]
ALL Non-Squamous		36	4 (11) [3-26]	3 (8) [2-23]	26 [10-42]
OV	3	1	0 [0-98]	0 [0-98]	N/A
	10	16	1 (6) [0.2-30]	3 (19) [4-46]	25 [4-46]
ALL OV		17	1 (6) [0.1-29]	3 (18) [4-43]	22 [2-43]
RCC	10	17	2 (12) [2-36]	7 (41) [18-67]	53 [29-77]

CI denotes Confidence intervals, MEL melanoma, RCC renal cell carcinoma, NSCLC non-small cell lung cancer, OV ovarian cancer, RCC renal cell carcinoma, N/A not applicable, ORR objective response rate (complete response + partial response), SD stable disease, and PFSR progression-free survival rate.

* Efficacy population consists of response-evaluable patients who initiated treatment at least 7 months prior to the date of analysis and had measurable disease at a baseline tumor assessment and at least one of the following: an on-study tumor assessment,

clinical progression, or death.

† Includes two CRs.

†† Includes one CR.

§ Objective response rates ({[CR + PR] ÷ n } × 100) are based on confirmed responses only, with confidence intervals calculated using the Clopper-Pearson method.

** Progression-free survival rate was the proportion of patients who did not progress, and were alive at 24 weeks, calculated by the Kaplan-Meier methodology, with confidence intervals using the Greenwood method.

Individual patient responses were adjudicated per RECIST vl.O with modification (see study protocol in Brahmer *et al.* (2012) *N Engl J Med* (submitted) for additional information).

Table 9. Duration of Objective Responses to BMS-936559 *

Tumor Type	Dose (mg/kg)	No. of Patients with OR	Duration of Response (months)†
MEL	1	1	6.9
	3	5	23.5+, 22.9+, 16.2+, 4.1+, 3.5
	10	4	24.6+, 23.9+, 18.0+, 17.0
NSCLC	1	0	9.2+
	3	1	2.3+
	10	4	16.6+, 12.6+, 9.8, 3.5
RCC	10	2	18, 4
OV	10	1	1.3+

* MEL denotes melanoma, NSCLC non-small cell lung cancer, RCC renal cell cancer, OV ovarian cancer.

† Time from first response to time of documented progression, death, or for censored data (denoted by +), time to last tumor assessment.

EXAMPLE 15

Design of Phase 1 Clinical Study on Anti-PD-l plus Anti-CTLA-4 in Advanced MEL

Study Design

In this phase 1 study, successive cohorts of patients were treated with escalating

doses of intravenous nivolumab and ipilimumab administered concurrently (concurrent regimen), and separately, two cohorts of patients treated previously with ipilimumab received nivolumab alone (sequenced regimen).

For the concurrent regimen, patients received during the induction period nivolumab and ipilimumab every 3 weeks for 4 doses, followed by nivolumab alone every 3 weeks for 4 doses. Combined treatment was subsequently continued during the maintenance period every 12 weeks for up to 8 doses. When both drugs were administered together, nivolumab was administered first. Within a cohort, nivolumab and ipilimumab doses were kept constant during the induction and maintenance periods. The dose-limiting-toxicity evaluation period was through week 9. Tumor assessments were at weeks 12, 18, 24, and 36, and then every 12 weeks thereafter.

In the sequenced regimen, patients previously treated with ipilimumab prior to study entry received nivolumab every 2 weeks for up to 48 doses. Nivolumab therapy was initiated within 4-12 weeks after ipilimumab monotherapy. Tumor assessments were at week 8 and then every 8 weeks thereafter. Tumor responses were adjudicated using mWHO and immune-related mWHO criteria for both regimens.

After completion of therapy, patients without confirmed disease progression were followed for up to 2.5 years. Patients with CR, PR, or SD $\geq$ 24 weeks and subsequent disease progression could be retreated with the original regimen. Safety evaluation was performed per protocol. The severity of AEs was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. Disease assessment, using CT and/or MRI as appropriate, was performed per protocol.

Dose escalation and cohort expansion

The study was initially planned to evaluate the concurrent regimen using a standard 3 + 3 design for the dose escalation phase, followed by cohort expansion to a total of up to 16 patients at the maximum tolerated dose or the maximum administered dose. The dose-limiting toxicity (DLT) evaluation period for dose escalation was 9 weeks. No intra-patient dose escalation was allowed and patients who experienced DLT were discontinued from therapy. Patients who withdrew from study during the DLT evaluation period for reasons other than drug-related toxicity could be replaced. The protocol was amended to permit expansion of any concurrent regimen cohort during dose escalation to N = up to 12 patients. Two sequenced-regimen cohorts (6 to 16 patients each) were later added; patients were treated with nivolumab (1 mg/kg or 3 mg/kg) after

having received prior ipilimumab.

Patients

Eligible patients were 18 years of age and had a diagnosis of measurable, unresectable stage III or IV melanoma; Eastern Cooperative Oncology Group performance status of 0-1, where 0 is asymptomatic and 1 is mildly symptomatic; adequate organ function; and life expectancy of $\geq$ 4 months. Patients with active, untreated central nervous system metastasis; history of autoimmune disease; prior therapy with T-cell modulating antibodies (excluding ipilimumab for sequenced-regimen cohorts); HIV; or hepatitis B or C were excluded.

In the sequenced-regimen cohorts, patients were required to have received 3 prior doses of ipilimumab, with the last dose administered within 4-12 weeks of initiation of nivolumab. Patients with CR, progression with evidence of clinical deterioration, or a history of high-grade AEs related to ipilimumab were excluded *(see* Wolchok *et al.* 2013a, for details of protocol).

Eighty-six patients were treated between December 2009 and February 2013, 53 with the concurrent regimen and 33 with the sequenced regimen. Baseline patient characteristics are detailed in Wolchok *et al.* (2013a). In the concurrent and sequenced regimens, 38% and 100% of patients, respectively, received prior systemic therapy. The majority of patients had Mlc disease and >30% had elevated serum lactate dehydrogenase (LDH). Most patients enrolled to the sequenced-regimen cohorts demonstrated radiographic progression (73%) with prior ipilimumab treatment.

PD-Ll immunohistochemistry

Pre-treatment PD-Ll expression was measured by IHC in FFPE tumor specimens using the rabbit anti-PD-Ll mAb, 28-8, and an automated assay developed by Dako (Carpinteria, CA). Ab specificity was assessed by western blotting against recombinant PD-Ll protein and lysates from PD-Ll expressing and non-expressing cell lines. An IHC assay with and without antigen competition and an assessment of staining patterns in normal human tissues was performed. Analytical sensitivity, specificity, repeatability, reproducibility, and robustness of the immunohistochemistry assay were tested and met all pre-specified acceptance criteria. Two pathologists, blinded to outcome, independently read and adjudicated scores for all clinical specimens. A sample was defined as PD-Ll - positive if 5% of tumor cells exhibited membrane PD-Ll staining of any intensity in a section with 100 evaluable cells.

Statistical analysis

All treated patients (N=86) as of February 2013 were used to describe baseline characteristics, safety, and absolute lymphocyte count (ALC), and analysis of PD-L1 staining. The efficacy population consisted of 82 response-evaluable patients who received at least one dose of study therapy, had measurable disease at baseline, and one of the following: > 1 on-treatment tumor evaluation, clinical progression, or death before the first on-treatment tumor evaluation. AEs were coded using MedDRA, version 15.1. Select AEs with potential immunologic etiologies were identified using a pre-defined list of MedDRA terms. Best overall responses were derived programmatically from tumor-measurements provided by the study-site radiologist and investigators per modified WHO (mWHO) or immune-related response criteria (Wolchok *et al.* 2009). Complete and partial responses were confirmed by at least one subsequent tumor assessment. An analysis also was performed to assess the magnitude of reduction in target lesions by radiographic assessment. A response was characterized as deep if a reduction of 80% from baseline measurements was noted. Unconfirmed responses as of the date of this analysis were also included in an estimate of aggregate clinical activity.

EXAMPLE 16

Safety Evaluations on MEL Patients Treated with Anti-PD-1 plus Anti-CTLA-4

For the concurrent regimen (n=53), AEs of any grade, irrespective of attribution, were observed in 98% of patients. Treatment-related AEs were observed in 93% of patients with the most common being rash (55%), pruritus (47%), fatigue (38%), and diarrhea (34%). Grade 3-4 AEs, irrespective of attribution, were observed in 72% of patients, while grade 3-4 treatment-related events were noted in 53%, with the most common being elevations of lipase (13%), aspartate aminotransferase (13%) and alanine aminotransferase (11%). Six of 28 (21%) patients had grade 3-4 dose- limiting treatment-related events. Treatment-related serious AEs were reported in 49% of patients. Common grade 3-4 treatment-related select AEs included hepatic (15%), gastrointestinal (9%), and renal (6%) events. Isolated cases of pneumonitis and uveitis were seen, consistent with historical monotherapy experiences. Eleven (21%) patients discontinued due to treatment-related AEs.

Cohort 3 (3 mg/kg nivolumab + 3 mg/kg ipilimumab) exceeded the MTD (3 of 6 patients experienced asymptomatic grade 3-4 elevated lipase that persisted for 3 weeks).

Cohort 2 (1 mg/kg nivolumab + 3 mg/kg ipilimumab) was identified as the MTD (grade 3 uveitis, grade 3 elevated AST/ALT in 1 patient each).

For the sequenced regimen (n=33), AEs of any grade, irrespective of attribution, were observed in 29 (88%) patients. Treatment-related AEs were observed in 24 (73%) patients, with the most common including pruritus (18%) and lipase elevation (12%). Grade 3-4 AEs, irrespective of attribution, were observed in 11 (33%) patients, while grade 3-4 treatment-related AEs were observed in 6 (18%) patients, with lipase elevation as the most common event (6%). Treatment-related serious AEs were reported in 7 (21%) patients. Grade 3-4 endocrine events were noted as treatment-related select AEs in 2 patients. One patient had grade-2 pneumonitis. Three (9%) patients discontinued due to treatment-related AEs.

For both the concurrent and sequenced regimens, treatment-related AEs were manageable and generally reversible with immunosuppressants and/or replacement therapy (for endocrinopathies) per previously algorithms previously established for ipilimumab (*see* YERVOY® package insert). Amongst the 86 patients treated on the study, 28 of 73 patients (38%) with drug-related adverse events required management with systemic glucocorticoids. Three patients required additional immunosuppressive therapy with infliximab (2 patients) or mycophenolate mofetil (1 patient). No treatment-related deaths were reported. Additional details of AEs and their management are provided in (Wolchok *et al* 2013a).

EXAMPLE 17

Efficacy Exhibited by Combination of Anti-PD-1 and Anti-CTLA-4 in MEL Patients

Clinical activity was observed with both the concurrent and sequenced regimens (Tables 10 and 11). In the concurrent-regimen cohorts, confirmed objective responses (OR) by mWHO criteria were observed in 21 of 52 (40%; 95% CI: 27-55) response-evaluable patients across all doses. After noting several patients who demonstrated major responses (approaching CR), the number of patients with at least 80% tumor reduction, an empirical threshold chosen because it represents a level of tumor regression that approaches complete response, was assessed. This depth of response was uncommon in published studies of checkpoint blockade (Hodi *et al,* 2010; Topalian *et al,* 2012b). Sixteen patients had ≥ 80% tumor reduction at 12 weeks, including 5 CRs (Table 10, Figures 14A and 15-17). In addition to the 21 patients with OR by mWHO criteria, 4 patients experienced an objective response by immune-related response criteria and 2 patients had

unconfirmed responses. These patients were not included in the calculation of ORRs. For the concurrent regimen, overall evidence of clinical activity (conventional, unconfirmed, or immune-related response or SD ≥ 24 weeks) was observed in 65% (95% CI: 51-78; Table 10) of patients. The profound impact of the concurrent combination can best be appreciated in the waterfall plot (Figure 14B). Responses were ongoing among 19 of 21 responders, with durations ranging from 6.1+ to 72.1+ weeks at the time of data analysis (Table 12). For patients treated at the MTD (cohort 2, 1 mg/kg nivolumab + 3 mg/kg ipilimumab), OR occurred in 9 of 17 (53%; 95% CI: 28-77) patients, including 3 CRs. All 9 responders achieved ≥ 80% tumor reduction at their first scheduled on-treatment assessment (Table 10 and Figure 14A).

For patients in the sequenced-regimen cohorts, 6 of 30 patients achieved OR (20%; 95% CI: 8-39) including 1 CR. Four (13%) patients achieved 80% tumor reduction at 8 weeks (Table 11 and Figure 18). Additional patients had immune-related (n=3) or unconfirmed (n=3) responses. When objective, immune-related or unconfirmed responses or SD ≥ 24 weeks are considered, evidence of clinical activity for the sequenced regimen was observed in 43% (95% CI: 26-63). The waterfall plot reveals that patients who did not respond to prior ipilimumab can respond to subsequent nivolumab (Figure 18C).

Table 10. Clinical Activity of Patients who Received the Concurrent Regimen of Nivolumab and Ipilimumab*

Cohort	Dose	Response-Evaluable Patients† n	CR n	PR n	uPR‡ n	irPR§ n	SD ≥24 wk n	irSD§ ≥24 wk n	Objective Response Rate¶ % [95% CI]	Aggregate Clinical Activity Rate‖ % [95% CI]	80% Tumor Reduction at 12 wk n (%)
1	0.3 mg/kg nivolumab + 3 mg/kg ipilimumab	14	1	2	0	2	2 (14)	0	21 [5-51]	50 [23-77]	4 (29)
2	1 mg/kg nivolumab + 3 mg/kg ipilimumab	17	3	6	0	0	0	2 (12)	53 [28-77]	65 [38-86]	7 (41)**
2a	3 mg/kg nivolumab + 1 mg/kg ipilimumab	15	1	5	2	1	2 (13)	0	40 [16-68]	73 [45-92]	5 (33)
3	3 mg/kg nivolumab + 3 mg/kg ipilimumab	6	0	3	0	1	0	1 (17)	50 [12-88]	83 [36-100]	0
All	Concurrent treatment	52	5	16	2	4	4 (8)	3 (6)	40 [27-55]	65 [51-78]	16 (31)

* CR denotes complete response, PR partial response, uPR unconfirmed partial response, irPR immune-related partial response, SD stable disease, irSD immune-related stable disease.

† Response-evaluable patients were those who received at least one dose of study therapy, had measurable disease at baseline, and one of the following: 1) at least one on-treatment tumor evaluation, 2) clinical progression, or 3) death prior to the first on-treatment tumor evaluation.

‡ Patients who had a PR after one tumor assessment but did not have sufficient follow-up time for confirmation of the initial PR.

¾ Patients who had target tumor-lesion reduction in the presence of new lesions, consistent with immune-related PR or SD.

¶ [(CR + PR) / no. response-evaluable patients] x 100. Confidence intervals were estimated by the Clopper-Pearson method.

‖ [(CR + PR + uCR + uPR + irPR + SD ≥24 wk + irSD ≥24 wk) / no. response-evaluable patients] x 100.

** Two additional patients in cohort 2 achieved >80% tumor reduction at their first scheduled assessment, which was conducted after week 12.

Table 11. Clinical Activity of Patients who Received the Sequenced Regimen of Nivolumab and Ipilimumab *

Cohort	Dose	Response-Evaluable Patients† n	CR n	PR n	uPR‡ n	irPR§ n	SD ≥ 24 wk n	irSD§ ≥ 24 wk n	Objective Response Rate¶ % [95% CI]	Aggregate Clinical Activity Rate‖ % [95% CI]	80% Tumor Reduction at 8 wk n (%)
6	1 mg/kg nivolumab + prior ipilimumab	16	1	5	2	2	1 (6)	0	38 [15-65]	69 [41-89]	4 (25)
7	3 mg/kg nivolumab + prior ipilimumab	14	0	0	1	1	0	0	0	14 [2-43]	0
All	Sequenced treatment	30	1	5	3	3	1 (3)	0	20 [8-39]	43 [26-63]	4 (13)

* CR denotes complete response, PR partial response, uPR unconfirmed partial response, irPR immune-related partial response, SD stable disease, irSD immune-related stable disease.

† Response-evaluable patients were those who received at least one dose of study therapy, had measurable disease at baseline, and one of the

following: 1) at least one on-treatment tumor evaluation, 2) clinical progression, or 3) death prior to the first on-treatment tumor evaluation.

‡ Patients who had a PR after one tumor assessment but did not have sufficient follow-up time for confirmation of the initial PR.

Table 12. Duration of Confirmed Objective Responses of Individual Patients

Cohort	Dose	Response	Duration of Response, weeks
1	0.3 mg/kg nivolumab + 3 mg/kg ipilimumab	CR	48.0+
		PR	72.1+, 65.4+
2	1 mg/kg nivolumab + 3 mg/kg ipilimumab	CR	36.1+, 28.9+, 24.7+
		PR	54.4+, 52.6, 23+, 18.4+, 14.0 +, 11.1+
2a	3 mg/kg nivolumab + 1 mg/kg ipilimumab	CR	7.7+
		PR	18.1+, 12.3, 6.6+, 6.4+, 6.1+
3	3 mg/kg nivolumab + 3 mg/kg ipilimumab	PR	38.7+, 31.3+, 12.1+
6	1 mg/kg nivolumab + prior ipilimumab	CR	24.1+
		PR	16.1+, 12.1+, 8.7+, 8.4+, 8.1+
7	3 mg/kg nivolumab + prior ipilimumab	CR	NA*
		PR	NA*

* Not available; as of the date of this analysis, no confirmed objective responses had been reported.

Assessment of tumor PD-L1 expression and absolute lymphocyte count

Tumor PD-L1 expression and alterations in the peripheral blood ALC have been explored as biomarkers for nivolumab and ipilimumab monotherapy, respectively (Topalian *et al,* 2012b; Berman *et al,* 2009; Ku *et al,* 2010; Postow *et al,* 2012; Delyon *et al,* 2013). Tumor PD-L1 expression was characterized via IHC staining and pharmacodynamic changes in peripheral blood ALC was analyzed. Using a $\geq$ 5% cut off to define PD-L1 positivity, tumor specimens from 21 of 56 (38%) patients were PD-L1 -positive. ORs were seen in patients with either PD-L1 -positive (6/13) or PD-L1 -negative (9/22) tumors amongst patients treated with the concurrent regimen (post-hoc P-value > 0.99; Fisher's exact test). In sequenced-regimen cohorts, a numerically higher number of overall responses were seen in patients with PD-L1 -positive tumor samples (4/8) compared with patients with PD-L1 -negative tumors (1/13), but the numbers are small.

In contrast to observations with ipilimumab monotherapy, a consistent rise in ALC from baseline was not detected in patients treated with the concurrent combination or in patients treated with nivolumab following ipilimumab therapy. In the concurrent-regimen cohorts, patients with a low ALC at weeks 5 to 7 (< 1000 cells^L) (Ku *et al,* 2010) had similar OR (43%) compared with patients with a normal ALC at weeks 5 to 7 (40%). Likewise, in the sequenced-regimen cohorts, 17% of patients with low ALC had OR and 23% of patients with normal or high ALC had OR.

Sequence Listing Summary

SEQ ID NO:	Description
1	VH amino acid sequence of 17D8
2	VH amino acid sequence of 2D3
3	VH amino acid sequence of 4H1
4	VH amino acid sequence of 5C4
5	VH amino acid sequence of 4A11
6	VH amino acid sequence of 7D3
7	VH amino acid sequence of 5F4
8	VL amino acid sequence of 17D8
9	VL amino acid sequence of 2D3
10	VL amino acid sequence of 4H1
11	VL amino acid sequence of 5C4

SEQ ID NO:	Description
12	VL amino acid sequence of 4A1 1
13	VL amino acid sequence of 7D3
14	VL amino acid sequence of 5F4
15	VH amino acid sequence of 3G10
16	VH amino acid sequence of 12A4
17	VH amino acid sequence of 10A5
18	VH amino acid sequence of 5F8
19	VH amino acid sequence of 10H10
20	VH amino acid sequence of IB 12
2 1	VH amino acid sequence of 7H1
22	VH amino acid sequence of 11E6
23	VH amino acid sequence of 12B7
24	VH amino acid sequence of 13G4
25	VL amino acid sequence of 3G 10
26	VL amino acid sequence of 12A4
27	VL amino acid sequence of 10A5
28	VL amino acid sequence of 5F8
29	VL amino acid sequence of lOH 10
30	VL amino acid sequence of IB 12
3 1	VL amino acid sequence of 7H1
32	VL amino acid sequence of 11E6
33	VL amino acid sequence of 12B7
34	VL amino acid sequence of 13G4
35	VH amino acid sequence of 28-8
36	VL amino acid sequence of 28-8
37	Heavy chain CDRl sequence of 28-8
38	Heavy chain CDR2 sequence of 28-8
39	Heavy chain CDR3 sequence of 28-8
40	Light chain CDRl sequence of 28-8
4 1	Light chain CDR2 sequence of 28-8
42	Light chain CDRl sequence of 28-8

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CLAIMS

What is claimed is:

1. A method of treating a subject afflicted with a cancer comprising administering to the subject a therapeutically effective amount of an antibody or an antigen-binding portion thereof that disrupts the interaction between Programmed Death- 1 (PD-1) and Programmed Death Ligand-1 (PD-L1, wherein the antibody or antigen-binding portion thereof binds specifically to PD-1 or to PD-L1.

2. The method of claim 1, wherein the cancer is selected from the group consisting of melanoma, renal cell carcinoma, squamous non-small cell lung cancer (NSCLC), non-squamous NSCLC, colorectal cancer, castration-resistant prostate cancer, ovarian cancer, gastric cancer, hepatocellular carcinoma, pancreatic carcinoma, squamous cell carcinoma of the head and neck, carcinomas of the esophagus, gastrointestinal tract and breast, and a hematological malignancy.

3. The method of claim 1, wherein the therapeutically effective amount of the antibody or antigen-binding portion thereof comprises a dose ranging from 0.1 to 10.0 mg/kg body weight which is administered at a dosing schedule of once per week, once every two weeks, or once a month.

4. A method for immunotherapy of a subject afflicted with cancer, which method comprises:

(a) selecting a subject that is a suitable candidate for immunotherapy, the selecting comprising:

(i) providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells;

(ii) assessing the proportion of cells in the test tissue sample that express PD-L1 on the cell surface; and

(iii) selecting the subject as a suitable candidate based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface exceeds a predetermined threshold level; and

(b) administering a composition comprising a therapeutically effective amount of an anti-PD-1 antibody to the selected subject.

5. The method of claim 4, wherein the proportion of cells that express PD-L1 is assessed by performing an assay to determine the presence of PD-L1 polypeptide on the surface of cells in the test tissue sample.

6. The method of claim 5, wherein the test tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

7. The method of claim 6, wherein the presence of PD-L1 polypeptide is determined using an automated IHC assay.

8. The method of claim 12, wherein the IHC assay is performed using an anti-PD-L1 monoclonal antibody to bind to the PD-L1 polypeptide, wherein the anti-PD-L1 monoclonal antibody is selected from 28-8, 28-1, 28-12, 29-8, and 5H1.

9. The method of claim 4, wherein the predetermined threshold level is 1% of tumor cells or a single tumor-infiltrating inflammatory cell expressing cell surface PD-L1 as determined by automated IHC using mAb 28-8.

10. A method for treatment of a subject afflicted with cancer, which method comprises:

(a) selecting a subject that is not suitable for anti-PD-1 antibody immunotherapy, the selecting comprising:

(i) providing a test tissue sample obtained from a patient with cancer of the tissue, the test tissue sample comprising tumor cells and tumor-infiltrating inflammatory cells;

(ii) assessing the proportion of cells in the test tissue sample that express PD-L1 on the cell surface; and

(iii) selecting the subject as not suitable for anti-PD-1 antibody immunotherapy based on an assessment that the proportion of cells in the test tissue sample that express PD-L1 on the cell surface is less than a predetermined threshold level; and

(b) administering a standard-of-care therapeutic other than an anti-PD-1 antibody to the selected subject.

11. A monoclonal antibody or an antigen-binding portion thereof that binds specifically to a cell surface-expressed PD-L1 polypeptide in a formalin-fixed, paraffin-embedded (FFPE) tissue sample.

12. The monoclonal antibody or antigen-binding portion thereof of claim 16, which antibody or portion thereof comprises the CDR1, CDR2 and CDR3 regions in a heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 35, and the CDR1, CDR2 and CDR3 regions in a light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 36.

13. The monoclonal antibody or antigen-binding portion thereof of claim 16, which antibody or portion thereof comprises a heavy chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 35, and a light chain variable region comprising consecutively linked amino acids having the sequence set forth in SEQ ID NO: 36.

14. A method of treating a subject afflicted with a cancer comprising administering to the subject:

(a) an antibody or an antigen-binding portion thereof that specifically binds to and inhibits Programmed Death- 1 (PD-1); and

(b) an antibody or an antigen-binding portion thereof that specifically binds to and inhibits Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4);

each antibody being administered at a dosage ranging from 0.1 to 20.0 mg/kg body weight in a concurrent regimen comprising:

(i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 antibodies at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 10 doses, followed by administration of the anti-PD-1 alone at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 12 doses; followed by

(ii) a maintenance dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 antibodies at a

dosing frequency of at least once every 8, 12 or 16 weeks, or at least once a quarter, for at least 4, 6, 8, 10, 12 or 16 doses.

15. The method of claim 14, wherein the anti-PD-1 and anti-CTLA-4 antibodies are administered at the following dosages:

(a) 0.1 mg/kg anti-PD-1 antibody and 3 mg/kg of anti-CTLA-4 antibody;
(b) 0.3 mg/kg anti-PD-1 antibody and 3 mg/kg of anti-CTLA-4 antibody;
(c) 1 mg/kg anti-PD-1 antibody and 3 mg/kg of anti-CTLA-4 antibody;
(d) 3 mg/kg anti-PD-1 antibody and 3 mg/kg of anti-CTLA-4 antibody;
(e) 5 mg/kg anti-PD-1 antibody and 3 mg/kg of anti-CTLA-4 antibody;
(f) 10 mg/kg anti-PD-1 antibody and 3 mg/kg of anti-CTLA-4 antibody;
(g) 0.1 mg/kg anti-PD-1 antibody and 1 mg/kg of anti-CTLA-4 antibody;
(h) 0.3 mg/kg anti-PD-1 antibody and 1 mg/kg of anti-CTLA-4 antibody;
(i) 1 mg/kg anti-PD-1 antibody and 1 mg/kg of anti-CTLA-4 antibody;
(j) 3 mg/kg anti-PD-1 antibody and 1 mg/kg of anti-CTLA-4 antibody;
(k) 5 mg/kg anti-PD-1 antibody and 1 mg/kg of anti-CTLA-4 antibody; or
(l) 10 mg/kg anti-PD-1 antibody and 1 mg/kg of anti-CTLA-4 antibody.

16. A method of treating a subject afflicted with a cancer, the subject having previously been treated with an anti-CTLA-4 antibody, which method comprises administering to the subject in a sequenced regimen an antibody or an antigen-binding portion thereof that specifically binds to and inhibits PD-1 at a dosage ranging from 0.1 to 20.0 mg/kg body weight and at a dosing frequency of at least once every week, at least once every 2, 3 or 4 weeks, or at least once a month, for up to 6 to up to 72 doses.

17. The method of claim 15 or 16, wherein the anti-PD-1 antibody is nivolumab.

18. The method of 15 or 16, wherein the anti-CTLA-4 antibody is ipilimumab.

19. An anti-PD-1 antibody or an antigen-binding portion thereof for use in treating a subject afflicted with a cancer in a concurrent regimen comprising combined administration with an anti-CTLA-4 antibody or an antigen-binding portion thereof, wherein the anti-PD-1 and anti-CTLA-4 antibodies are each administered

at a dosage ranging from 0.1 to 20.0 mg/kg body weight, and further wherein the concurrent regimen comprises:

(i) an induction dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 antibodies at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 12 doses, followed by administration of the anti-PD-1 alone at a dosing frequency of at least once every 2, 3 or 4 weeks, or at least once a month, for at least 2, 4, 6, 8 or 10 doses; followed by

(ii) a maintenance dosing schedule comprising combined administration of the anti-PD-1 and anti-CTLA-4 antibodies at a dosing frequency of at least once every 8, 12 or 16 weeks, or at least once a quarter, for up to 4, 6, 8, 10, 12 or 16 doses.

20. A kit for treating a subject afflicted with a cancer, the kit comprising:

(a) a dosage ranging from 0.1 to 20.0 mg/kg body weight of an antibody or an antigen-binding portion thereof that specifically binds to and inhibits PD-1;

(b) a dosage ranging from 0.1 to 20.0 mg/kg body weight of an antibody or an antigen-binding portion thereof that specifically binds to and inhibits CTLA-4; and

(c) instructions for using the anti-PD-1 and anti-CTLA-4 antibodies the concurrent regimen method of claim 14.

21. A kit for treating a subject afflicted with a cancer, the kit comprising:

(a) a dosage ranging from 0.1 to 20.0 mg/kg body weight of an antibody or an antigen-binding portion thereof that specifically binds to and inhibits PD-1; and

(b) instructions for using the anti-PD-1 antibody in the sequenced regimen method of claim 16.

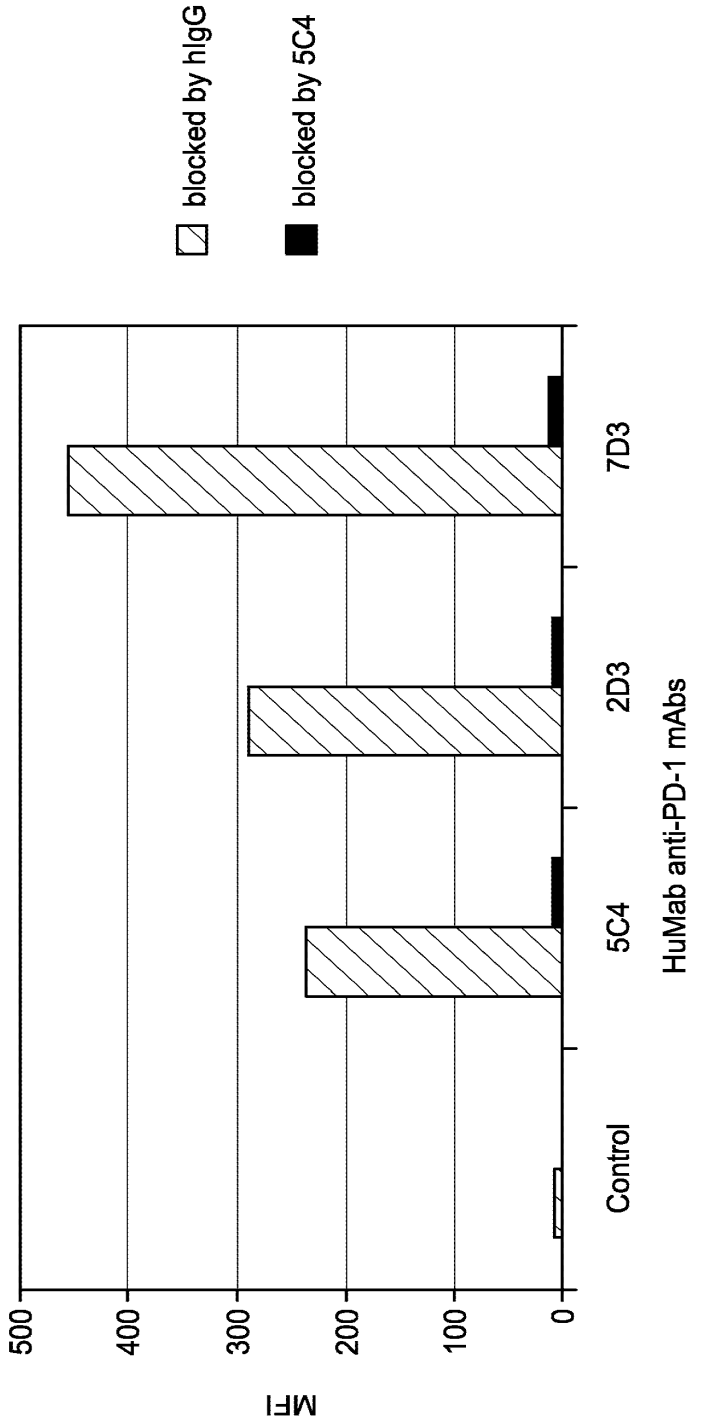

blocked by hIgG
blocked by 5C4
MFI
500
400
300
200
100
0
Control
5C4
2D3
7D3
HuMab anti-PD-1 mAbs

FIG. 1A

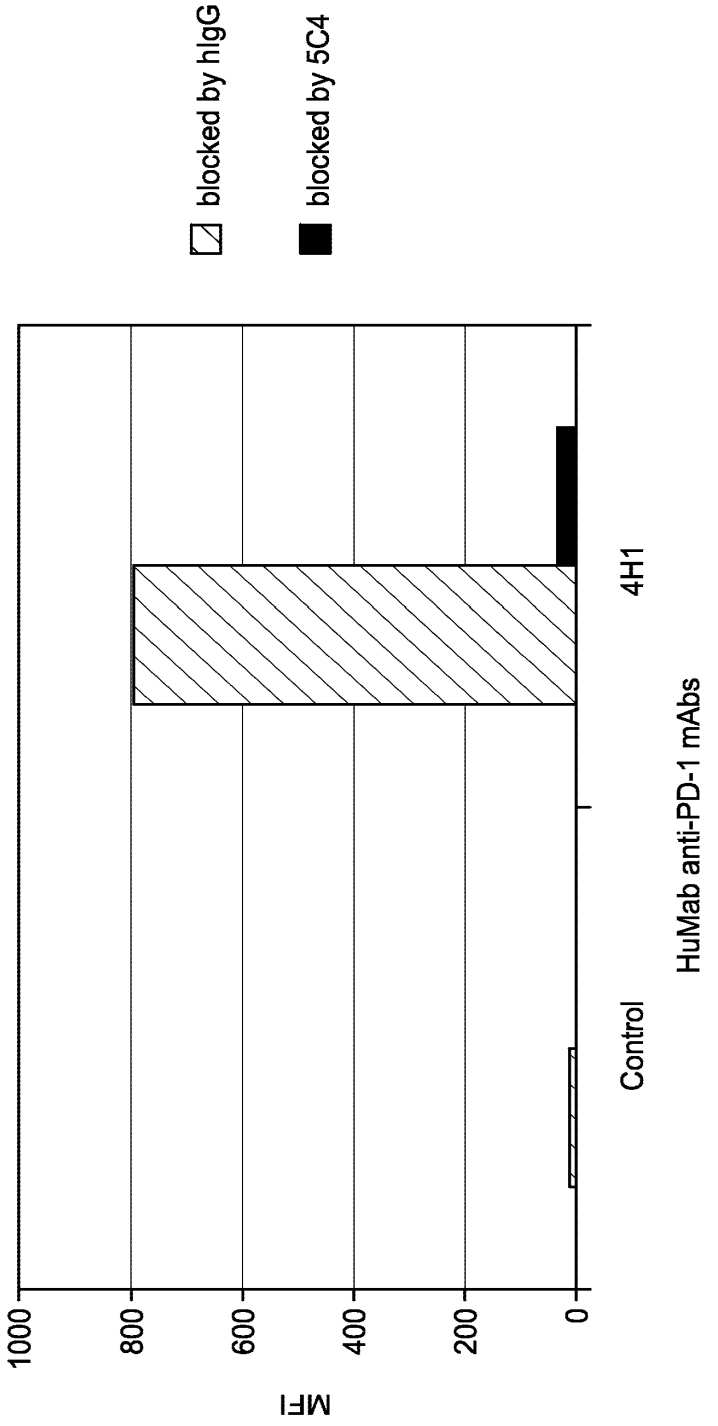

blocked by hIgG
blocked by 5C4
MFI
1000
800
600
400
200
0
Control
4H1
HuMab anti-PD-1 mAbs

FIG. 1B

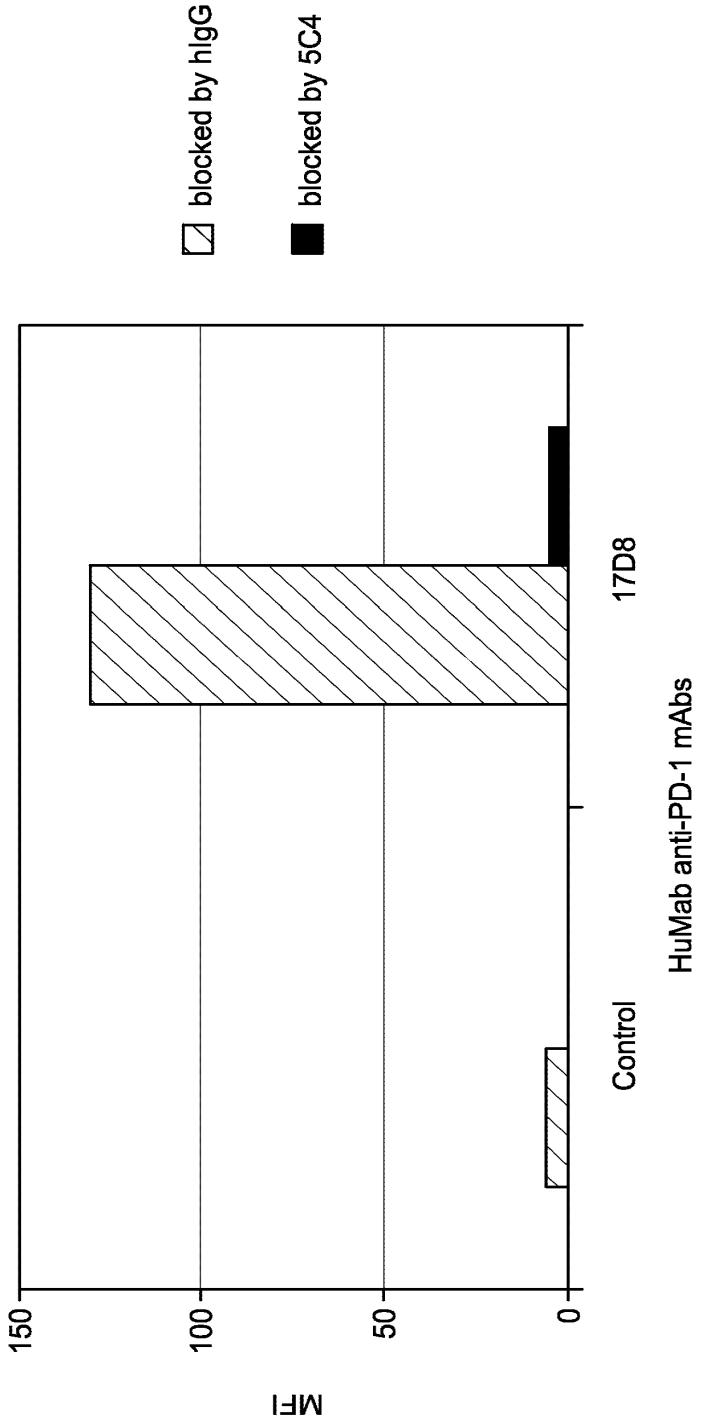

blocked by hIgG
blocked by 5C4
MFI
150
100
50
0
Control
17D8
HuMab anti-PD-1 mAbs

FIG. 1C

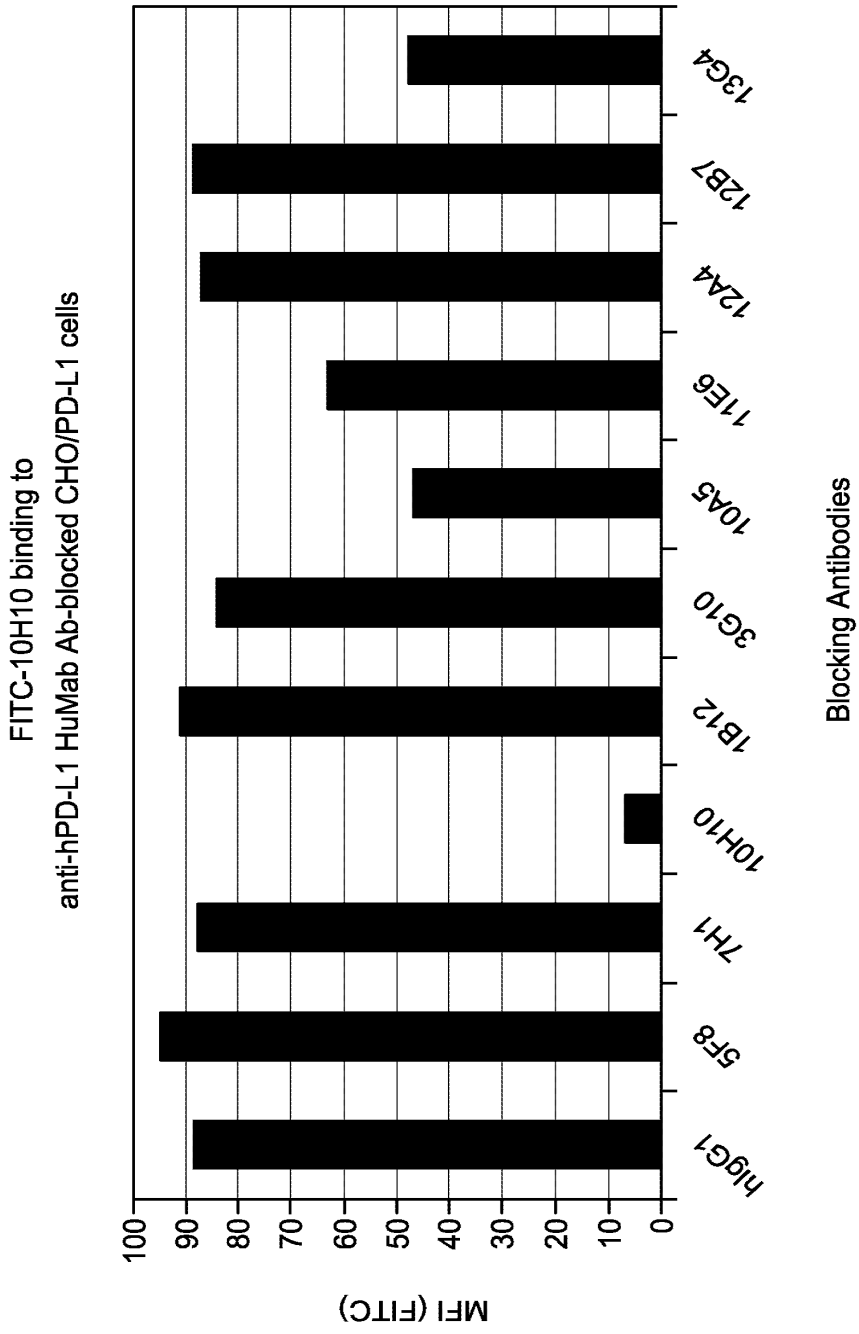

FITC-10H10 binding to
anti-hPD-L1 HuMab Ab-blocked CHO/PD-L1 cells
MFI (FITC)
0
10
20
30
40
50
60
70
80
90
100
hIgG1
5F8
7H1
10H10
1B12
3G10
10A5
11E6
12A4
12B7
13G4
Blocking Antibodies

FIG. 2A

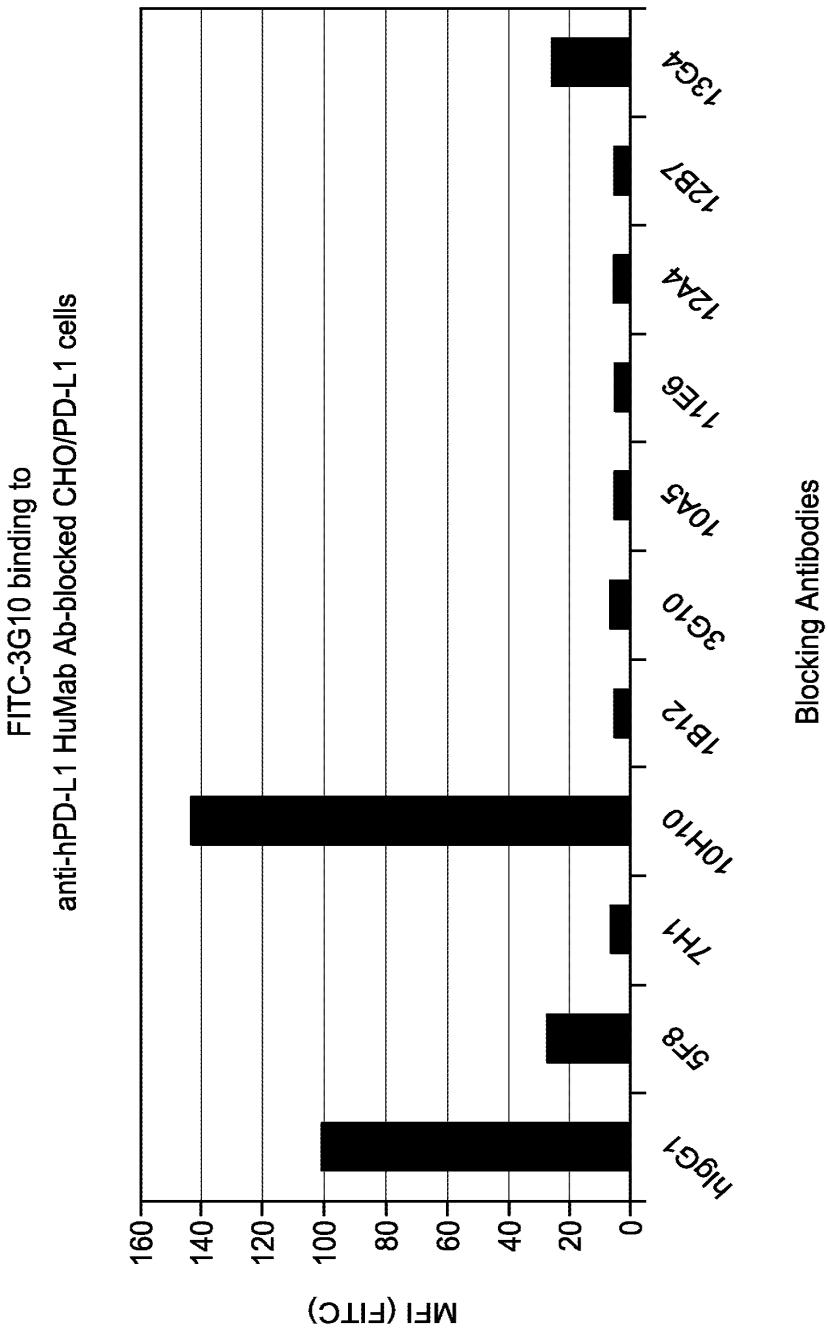

FITC-3G10 binding to
anti-hPD-L1 HuMab Ab-blocked CHO/PD-L1 cells
MFI (FITC)
160
140
120
100
80
60
40
20
0
hIgG1
5F8
7H1
10H10
1B12
3G10
10A5
11E6
12A4
12B7
13G4
Blocking Antibodies

FIG. 2B

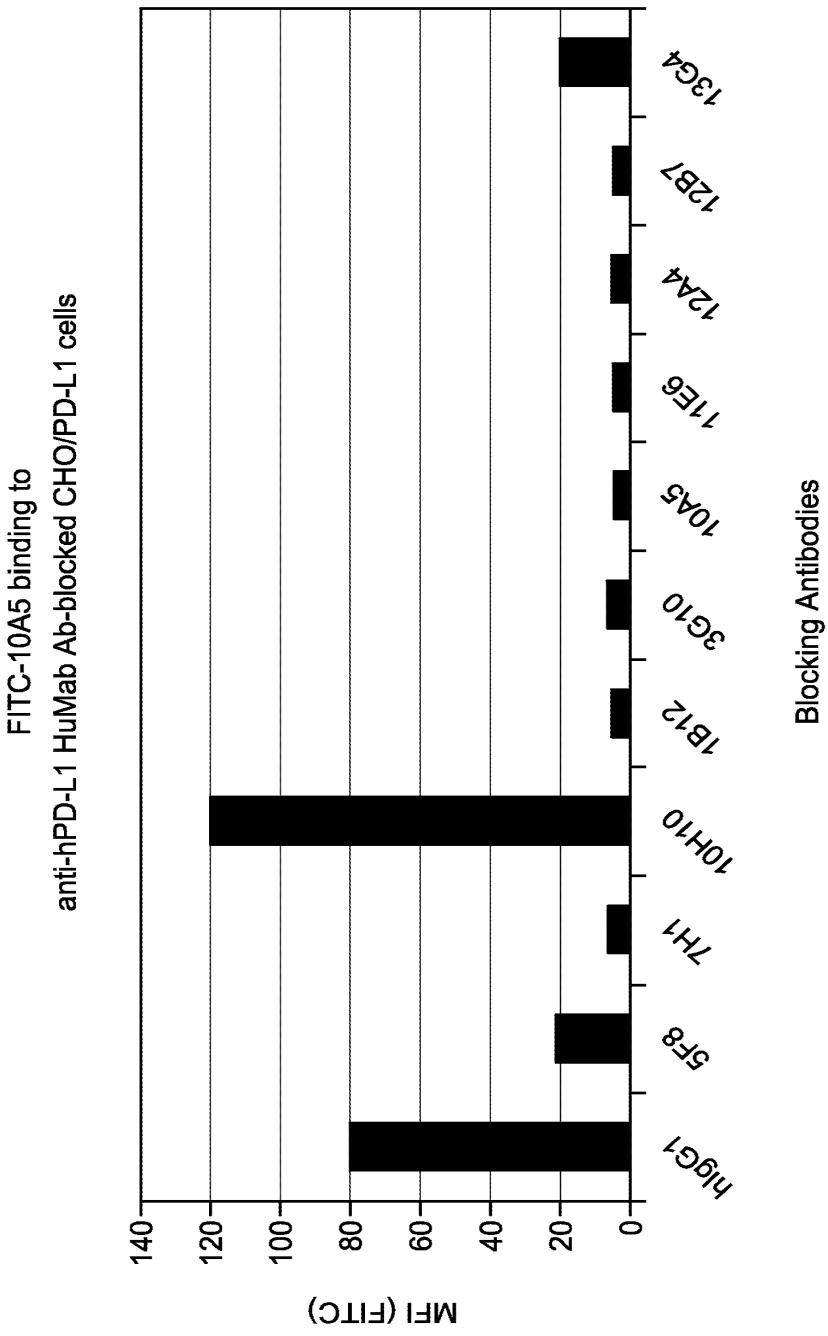

FITC-10A5 binding to
anti-hPD-L1 HuMab Ab-blocked CHO/PD-L1 cells
MFI (FITC)
0
20
40
60
80
100
120
140
hIgG1
5F8
7H1
10H10
1B12
3G10
10A5
11E6
12A4
12B7
13G4
Blocking Antibodies

FIG. 2C

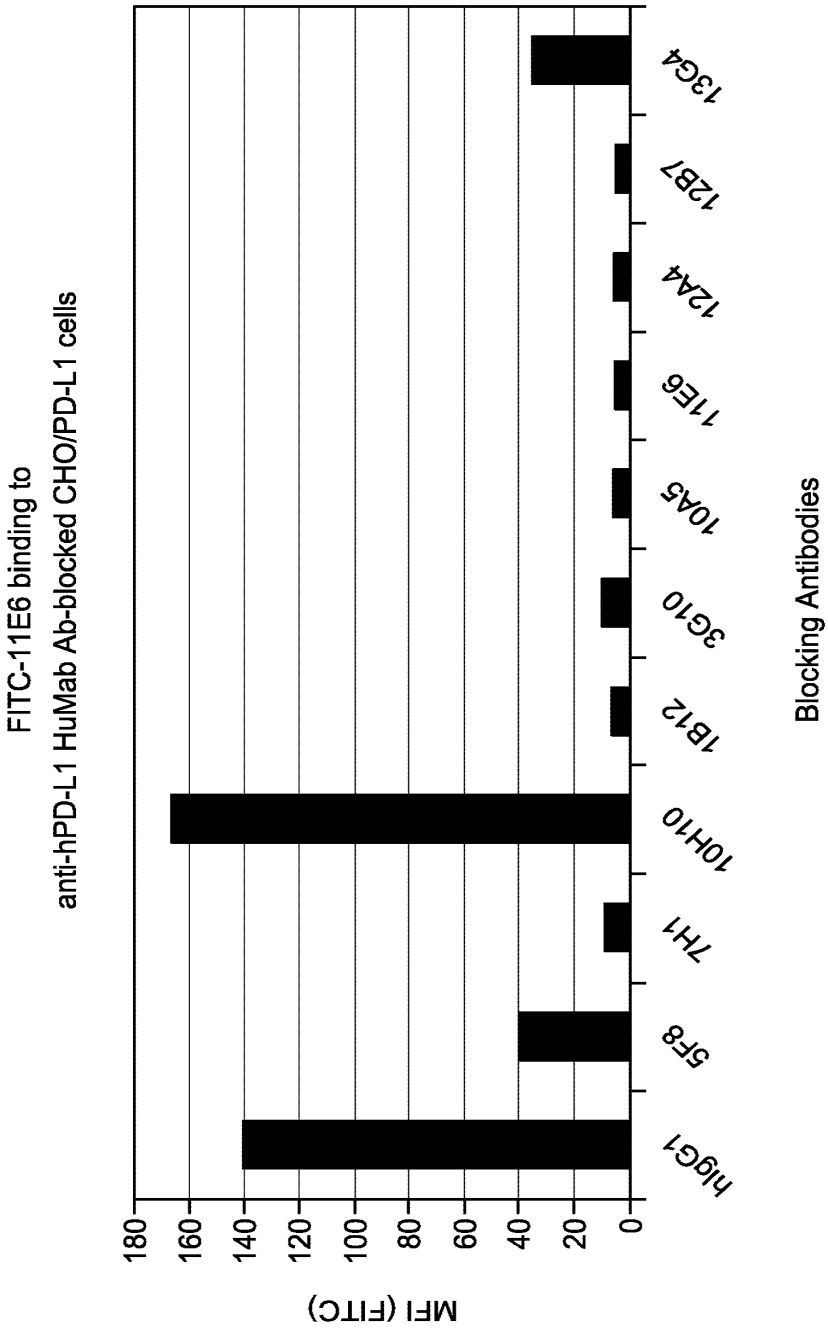

FITC-11E6 binding to
anti-hPD-L1 HuMab Ab-blocked CHO/PD-L1 cells
MFI (FITC)
0
20
40
60
80
100
120
140
160
180
hIgG1
5F8
7H1
10H10
1B12
3G10
10A5
11E6
12A4
12B7
13G4
Blocking Antibodies

FIG. 2D

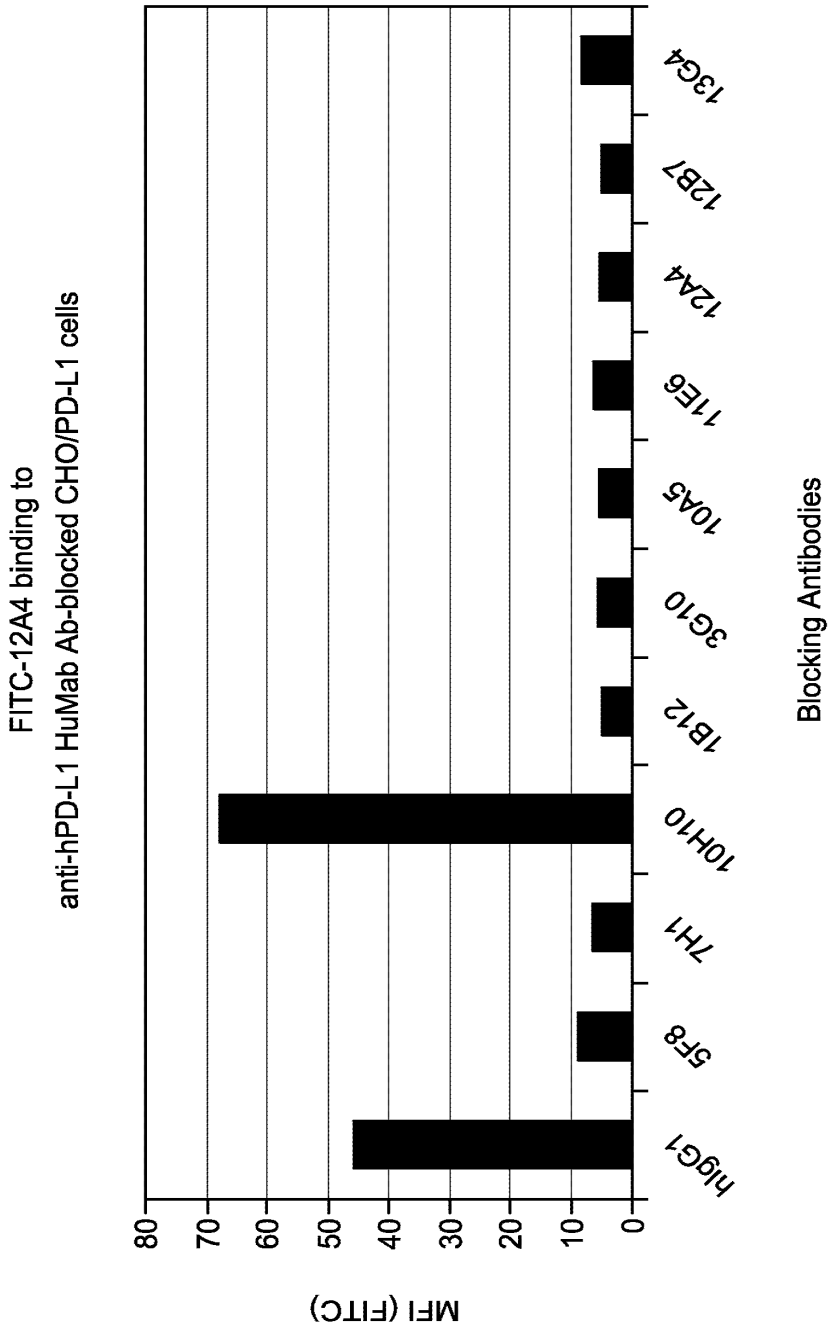

FITC-12A4 binding to
anti-hPD-L1 HuMab Ab-blocked CHO/PD-L1 cells
MFI (FITC)
0
10
20
30
40
50
60
70
80
hIgG1
5F8
7H1
10H10
1B12
3G10
10A5
11E6
12A4
12B7
13G4
Blocking Antibodies

FIG. 2E

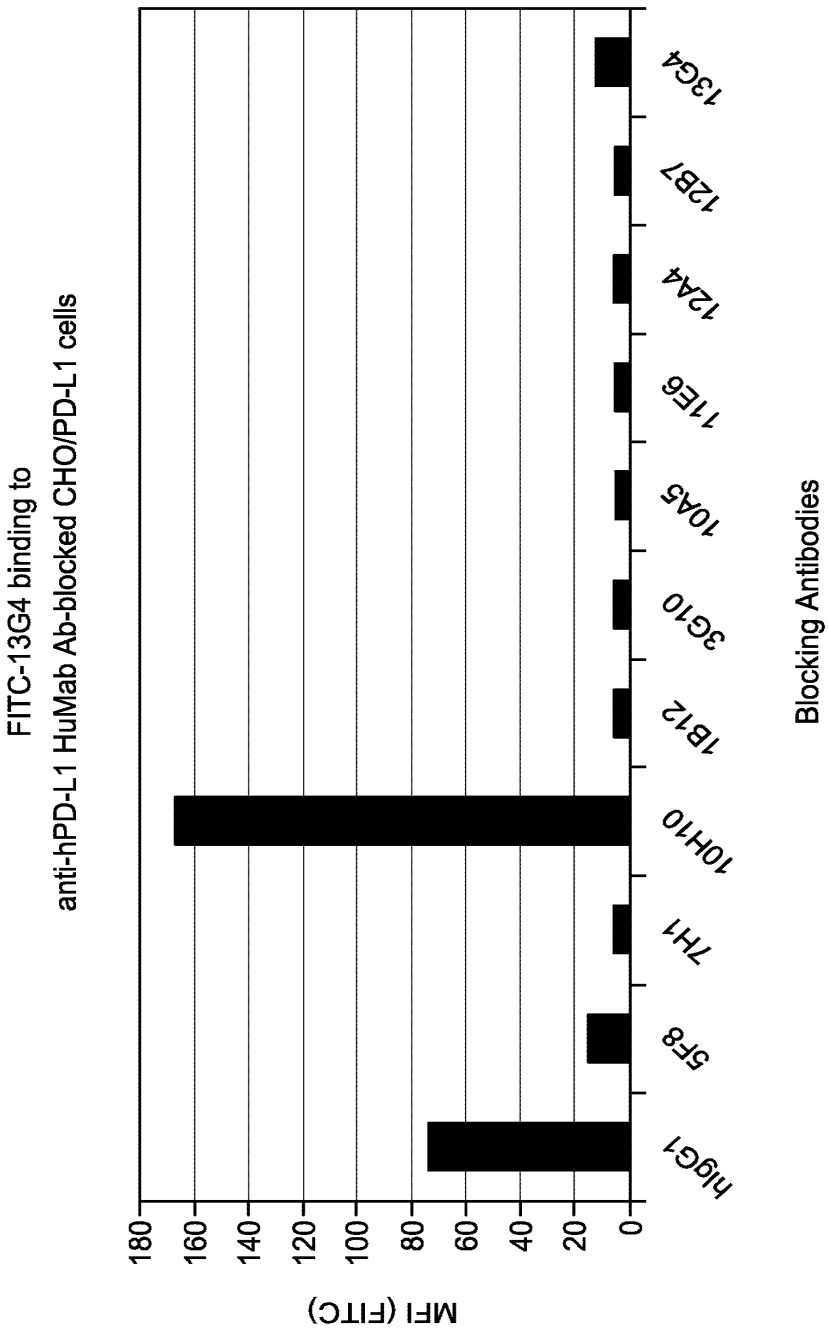

FITC-13G4 binding to
anti-hPD-L1 HuMab Ab-blocked CHO/PD-L1 cells
MFI (FITC)
0
20
40
60
80
100
120
140
160
180
hIgG1
5F8
7H1
10H10
1B12
3G10
10A5
11E6
12A4
12B7
13G4
Blocking Antibodies

FIG. 2F

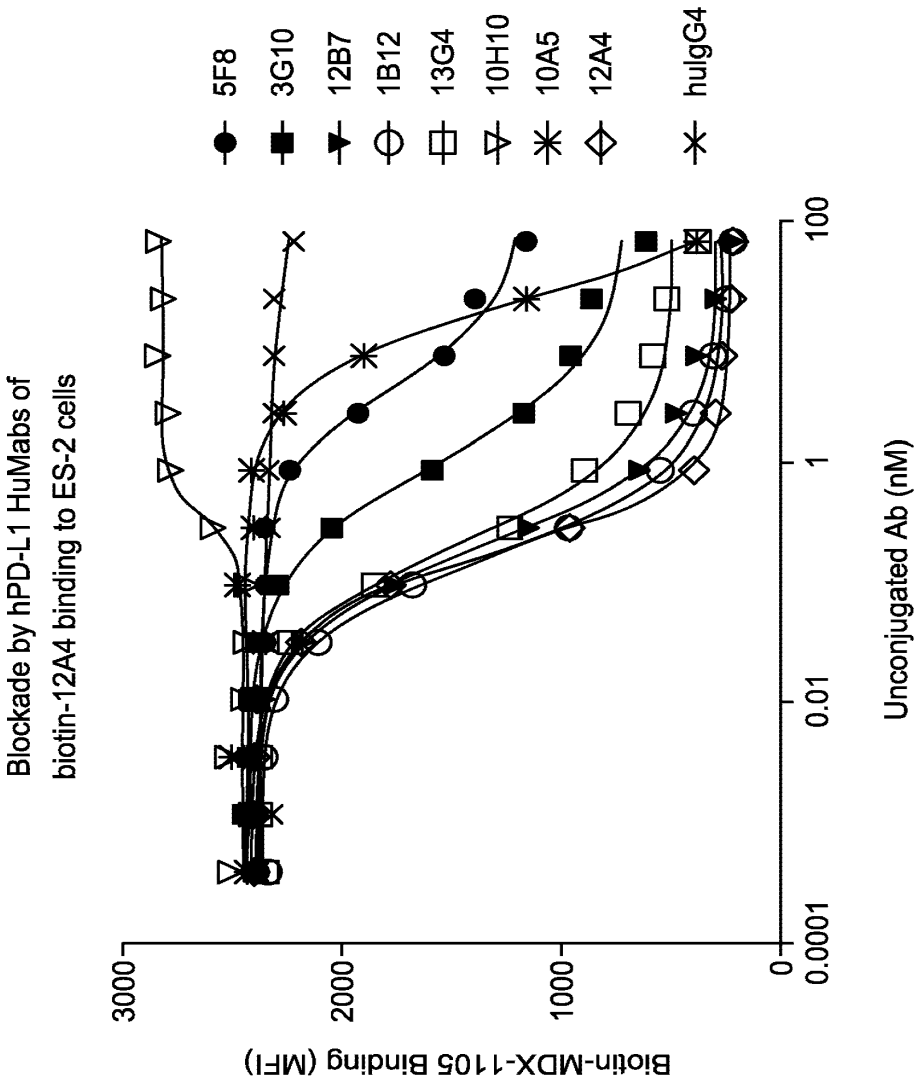

Blockade by hPD-L1 HuMabs of
biotin-12A4 binding to ES-2 cells
Biotin-MDX-1105 Binding (MFI)
3000
2000
1000
0
0.0001
0.01
1
100
Unconjugated Ab (nM)
5F8
3G10
12B7
1B12
13G4
10H10
10A5
12A4
hulgG4

FIG. 3

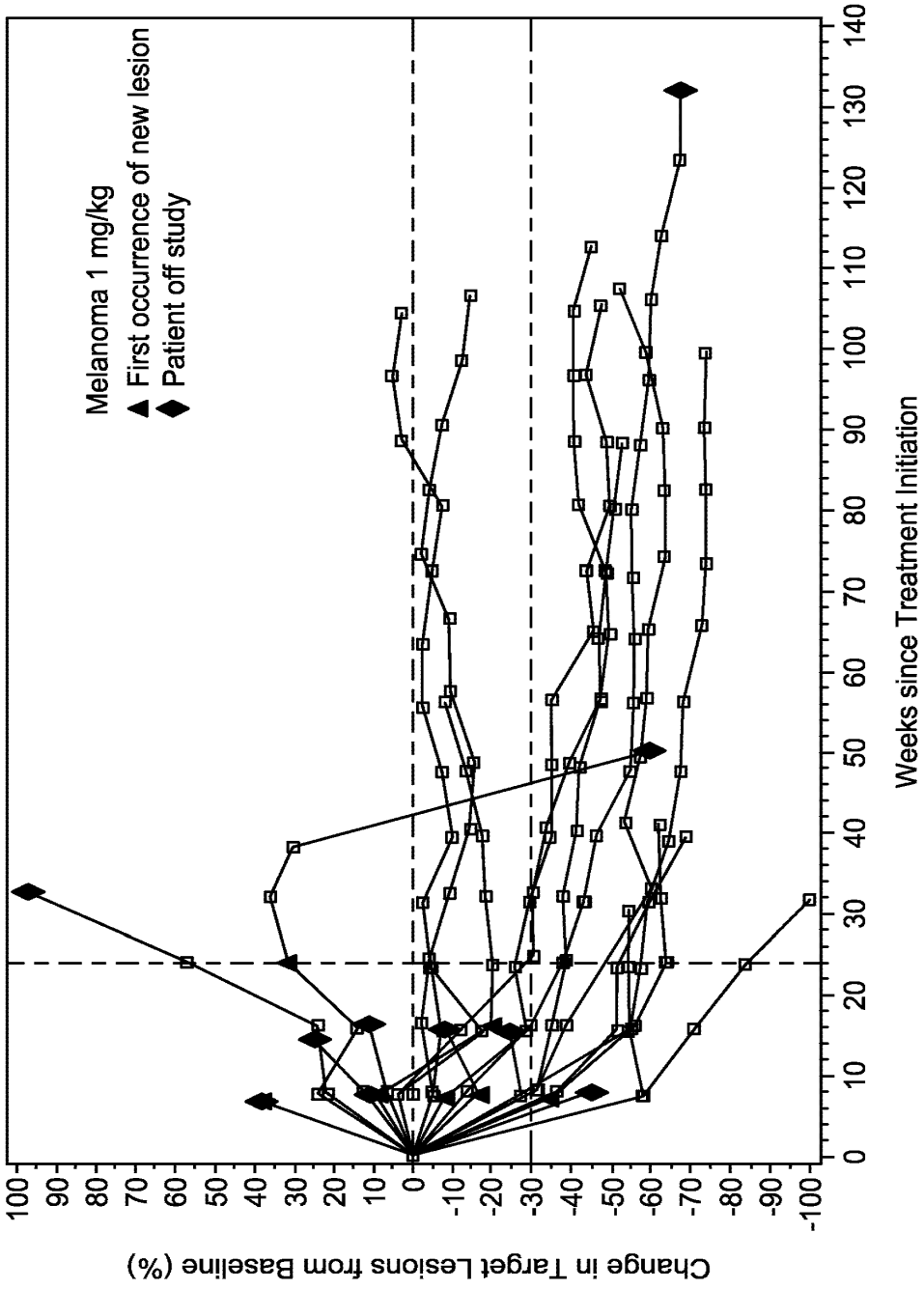

Melanoma 1 mg/kg
First occurrence of new lesion
Patient off study
Change in Target Lesions from Baseline (%)
Weeks since Treatment Initiation
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140

FIG. 4

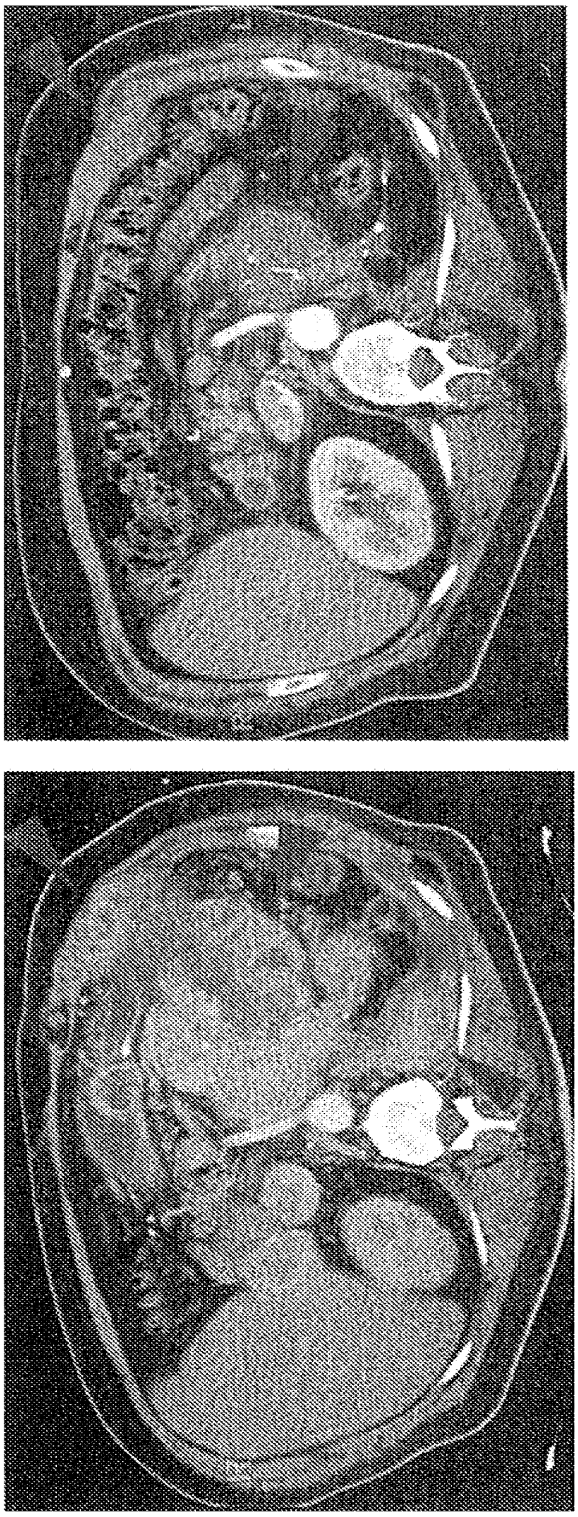


FIG. 5

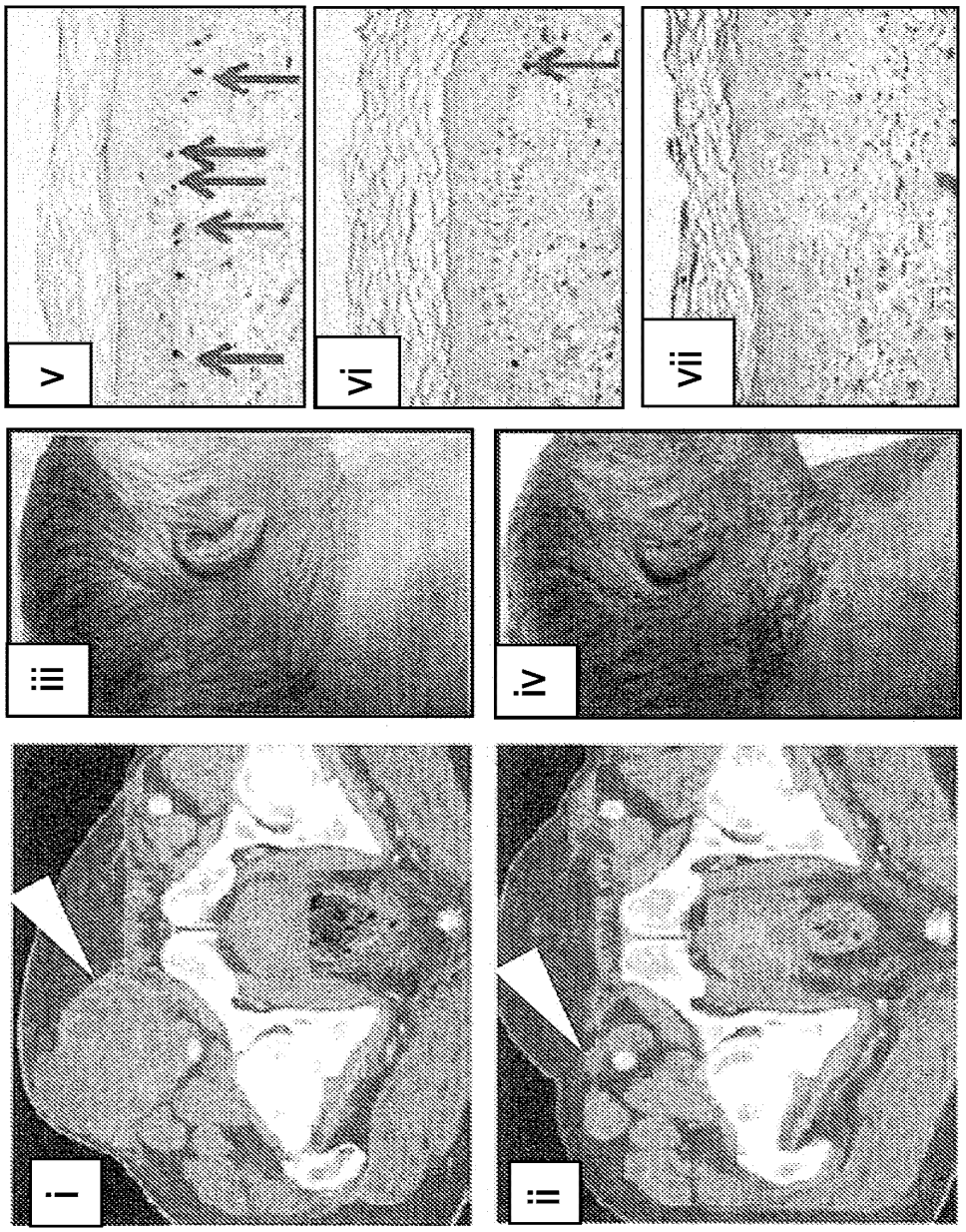


FIG. 6

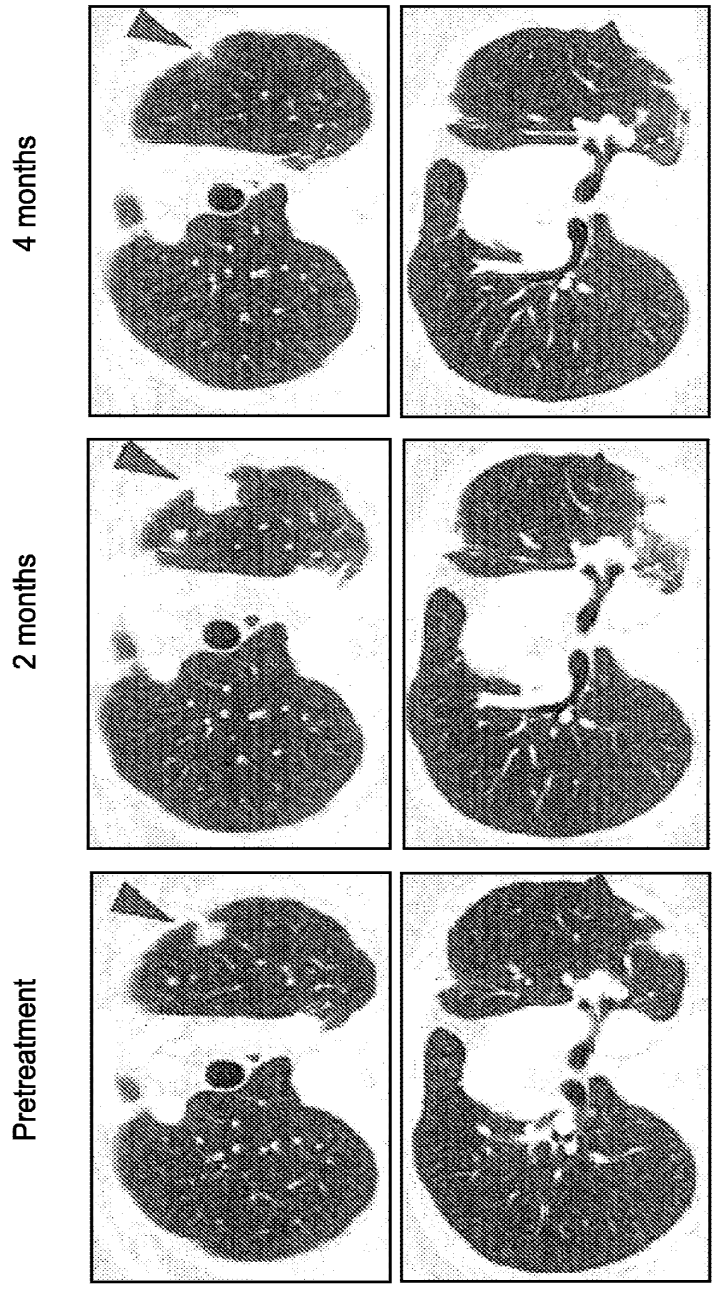
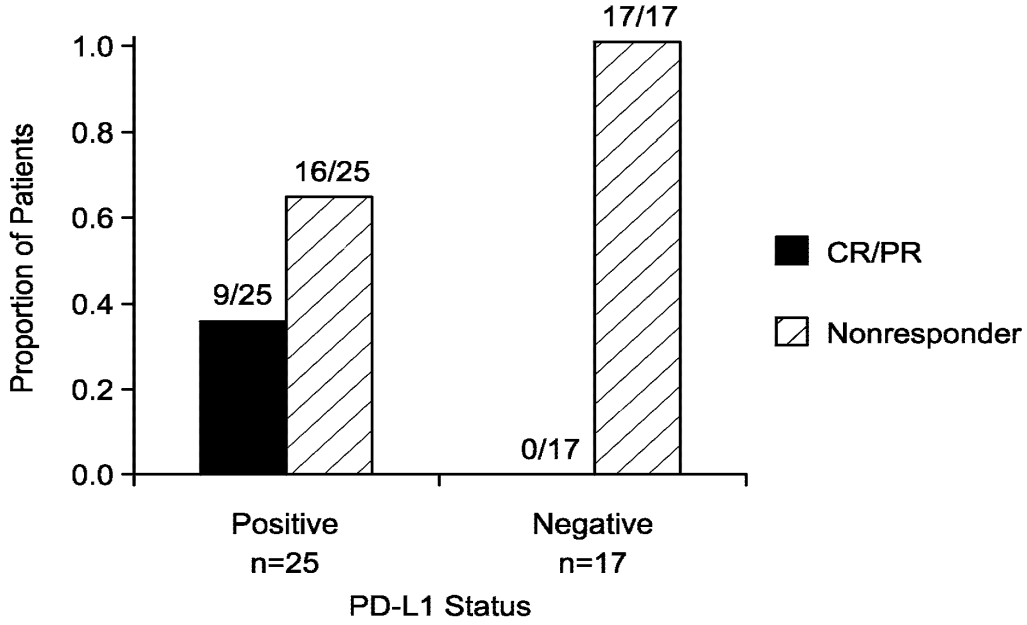


FIG. 7



Association Between Pretreatment Tumor PD-L1 Expression and Clinical Response			
Response Status	PD-L1-Positive no. (%)	PD-L1-Negative no. (%)	Total no. (%)
CR/PR	9(36)	0	9(21)
Nonresponder	16*(64)	17(100)	33(79)
All Patients	25	17	42

Fisher's exact test for association P=0.006.

*Two patients categorized as nonresponders at the time of data analysis are still under evaluation.

FIG. 8A

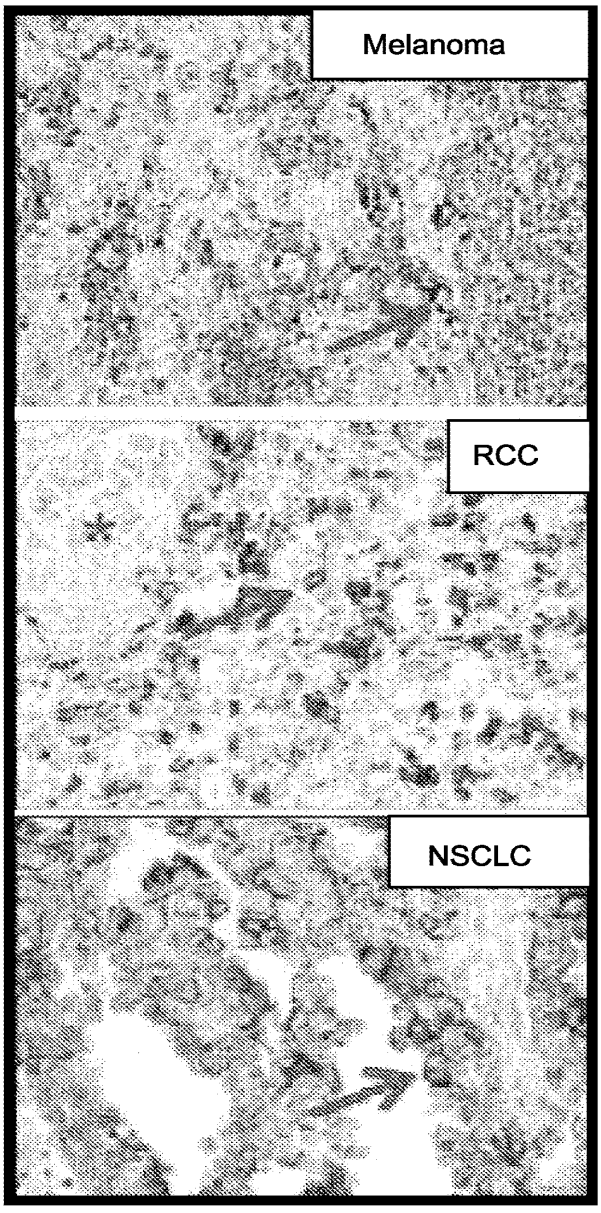


FIG. 8B

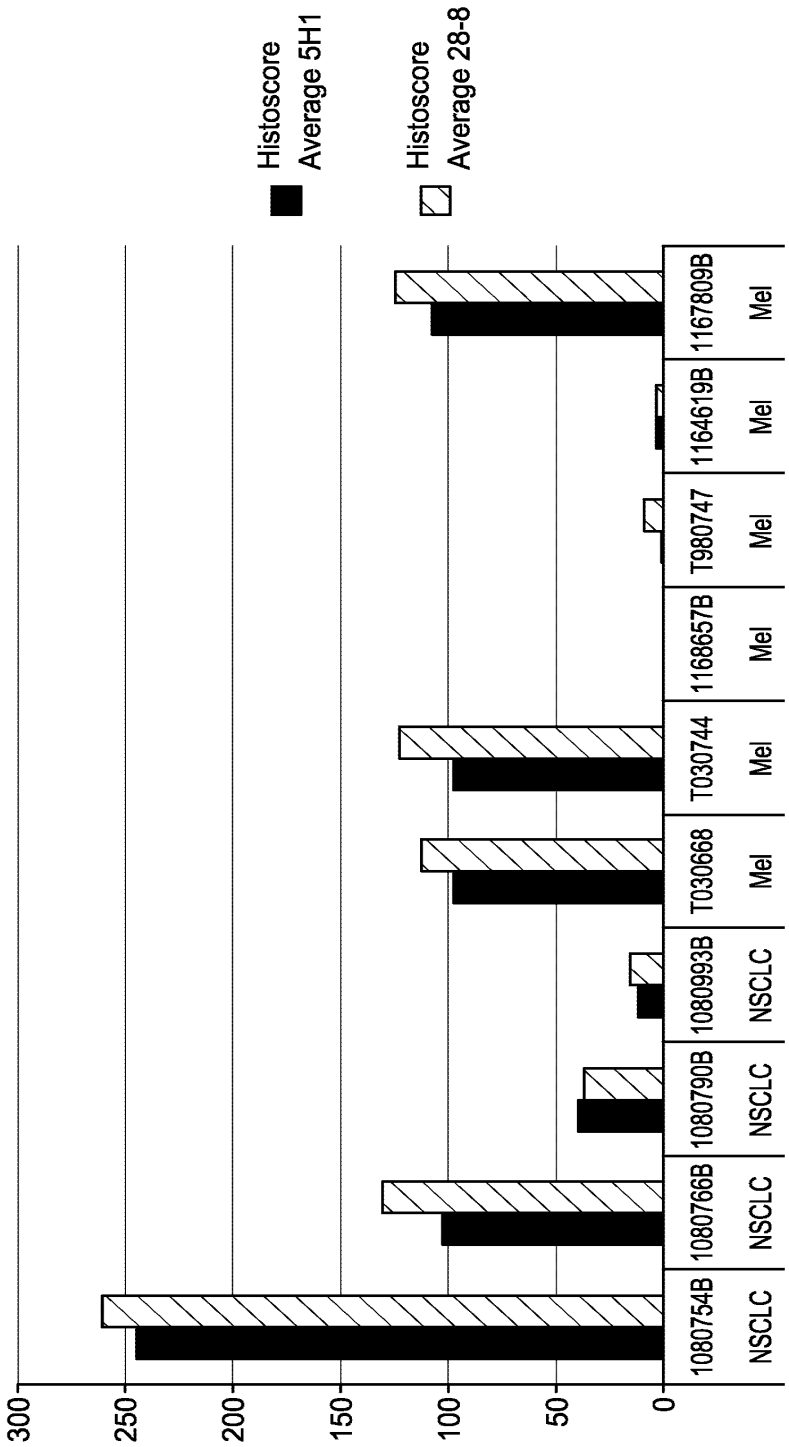


FIG. 9

EX1002 - Page 2140

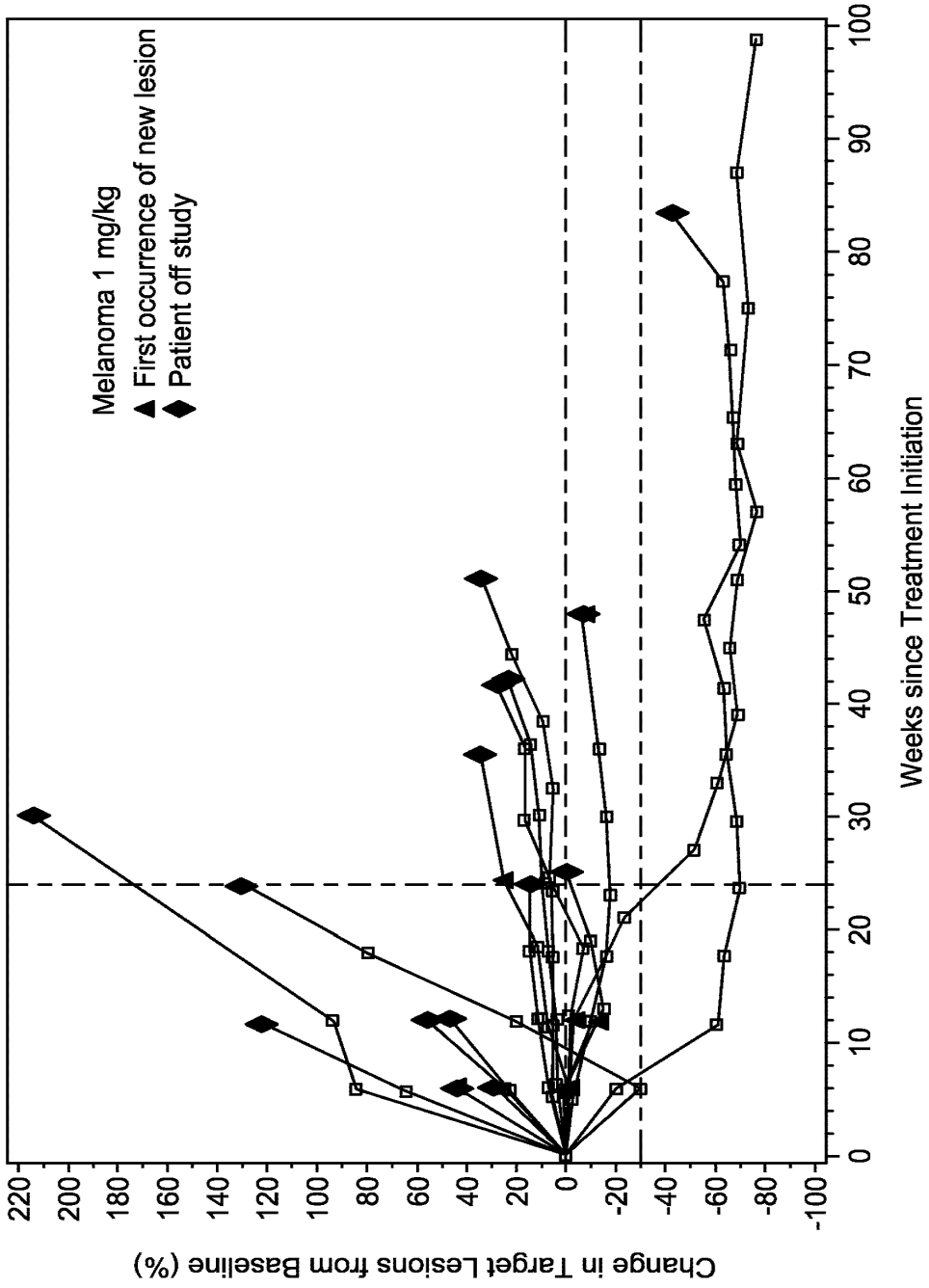

Melanoma 1 mg/kg
First occurrence of new lesion
Patient off study
Change in Target Lesions from Baseline (%)
Weeks since Treatment Initiation
220
200
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100

FIG. 10A

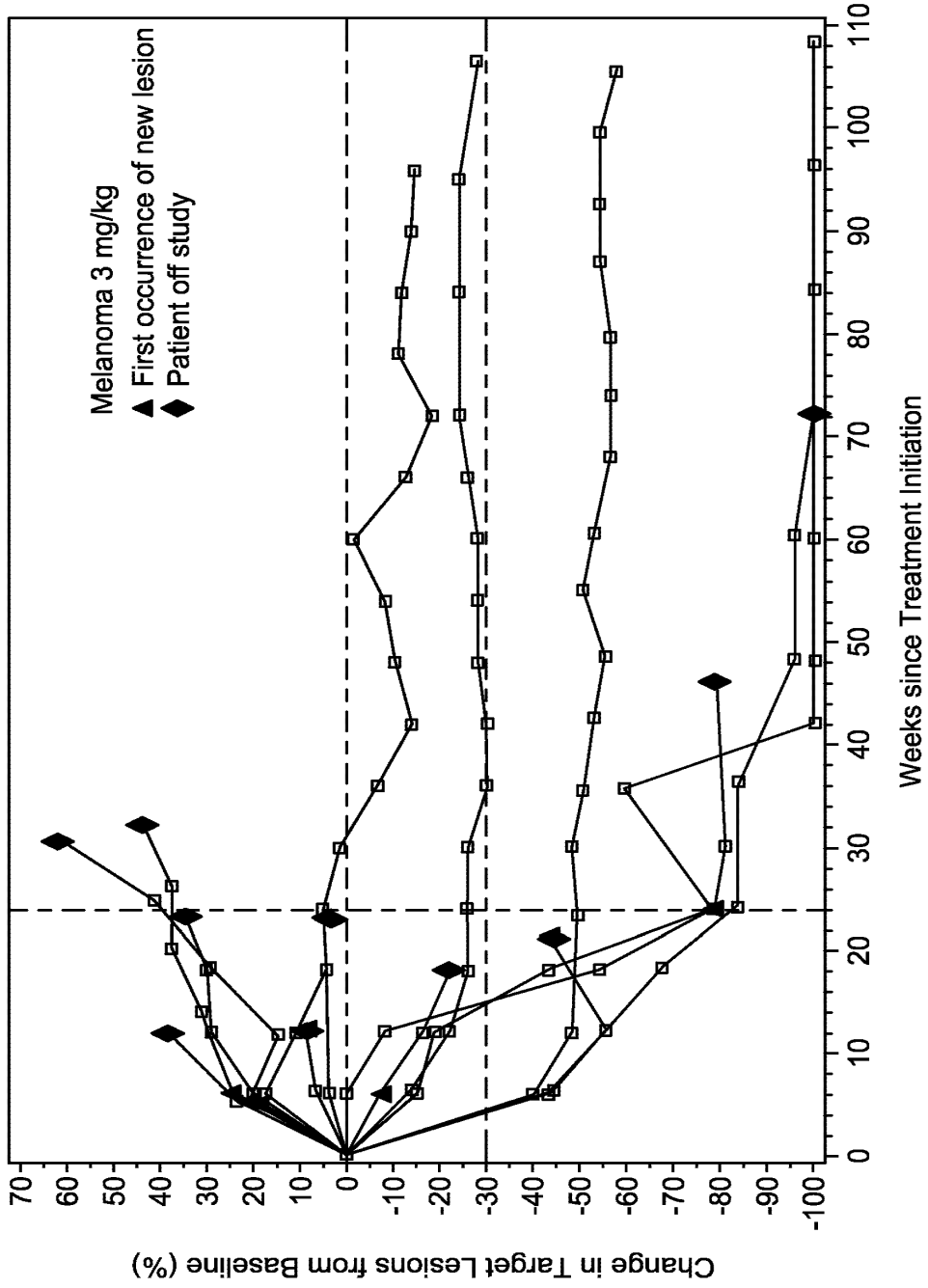

Melanoma 3 mg/kg
First occurrence of new lesion
Patient off study
Change in Target Lesions from Baseline (%)
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
0
10
20
30
40
50
60
70
80
90
100
110
Weeks since Treatment Initiation

FIG. 10B

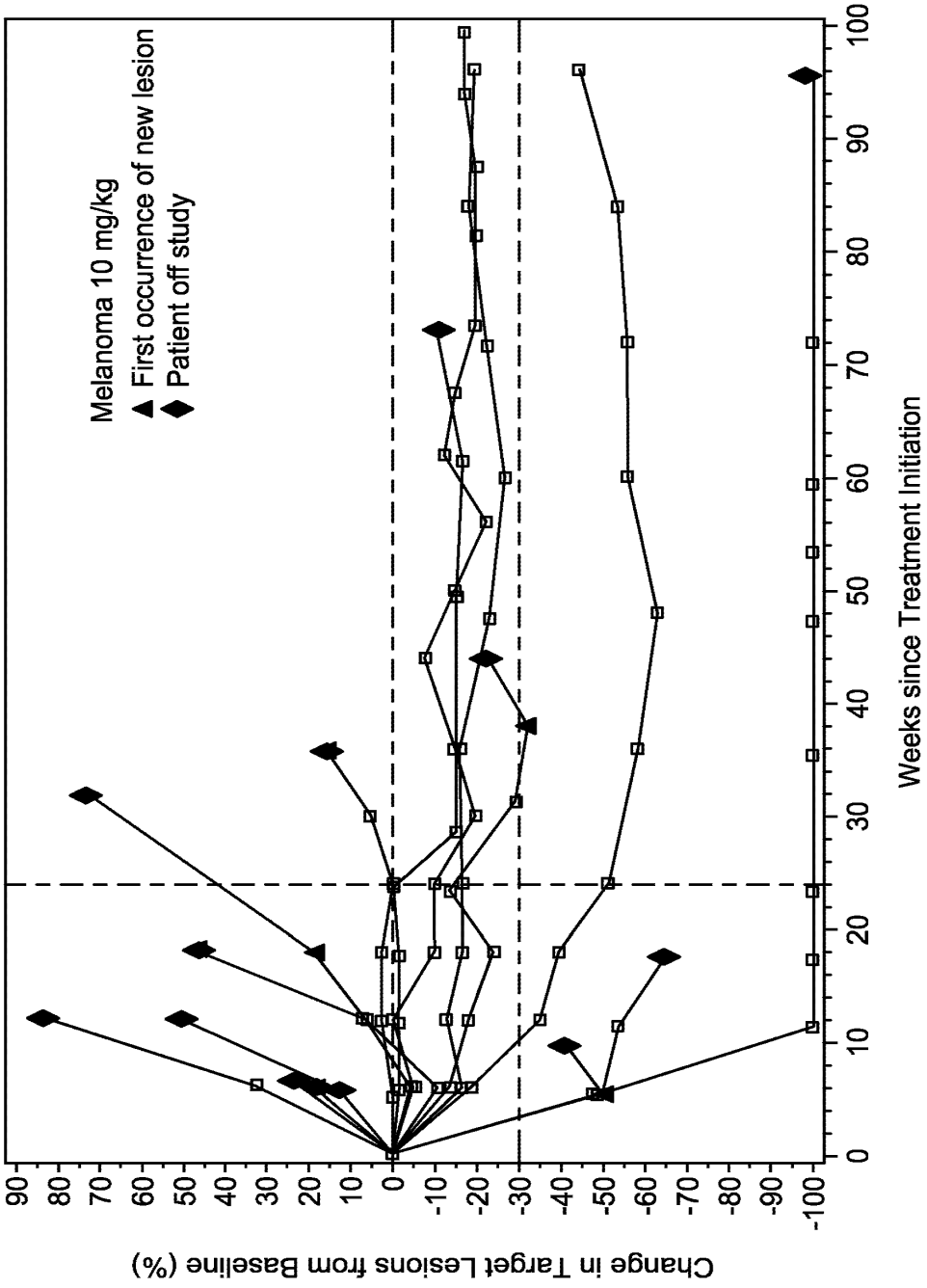

Melanoma 10 mg/kg
First occurrence of new lesion
Patient off study
Change in Target Lesions from Baseline (%)
Weeks since Treatment Initiation
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
0
10
20
30
40
50
60
70
80
90
100

FIG. 10C

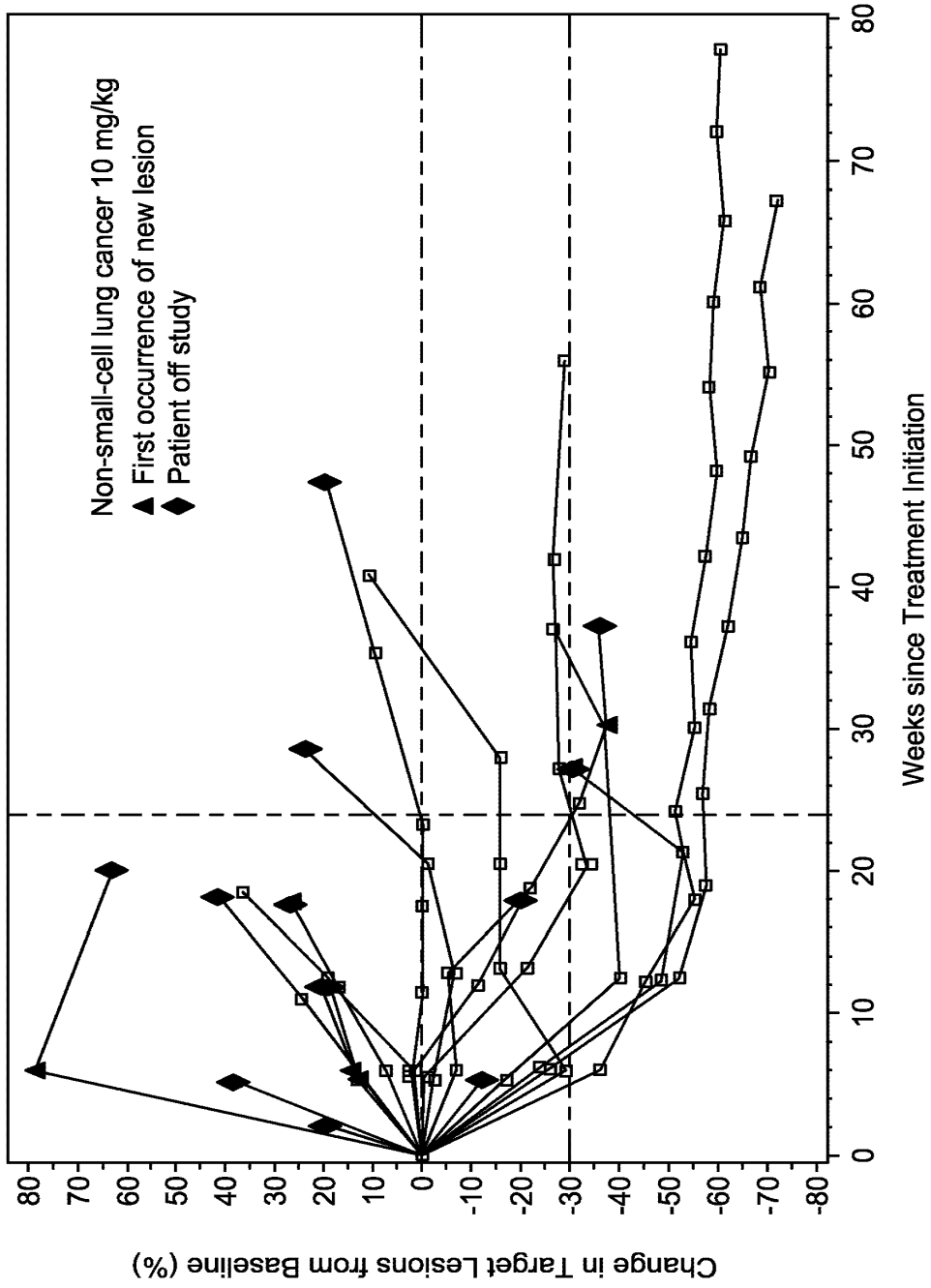

Non-small-cell lung cancer 10 mg/kg
First occurrence of new lesion
Patient off study
Change in Target Lesions from Baseline (%)
Weeks since Treatment Initiation
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
10
20
30
40
50
60
70
80

FIG. 10D

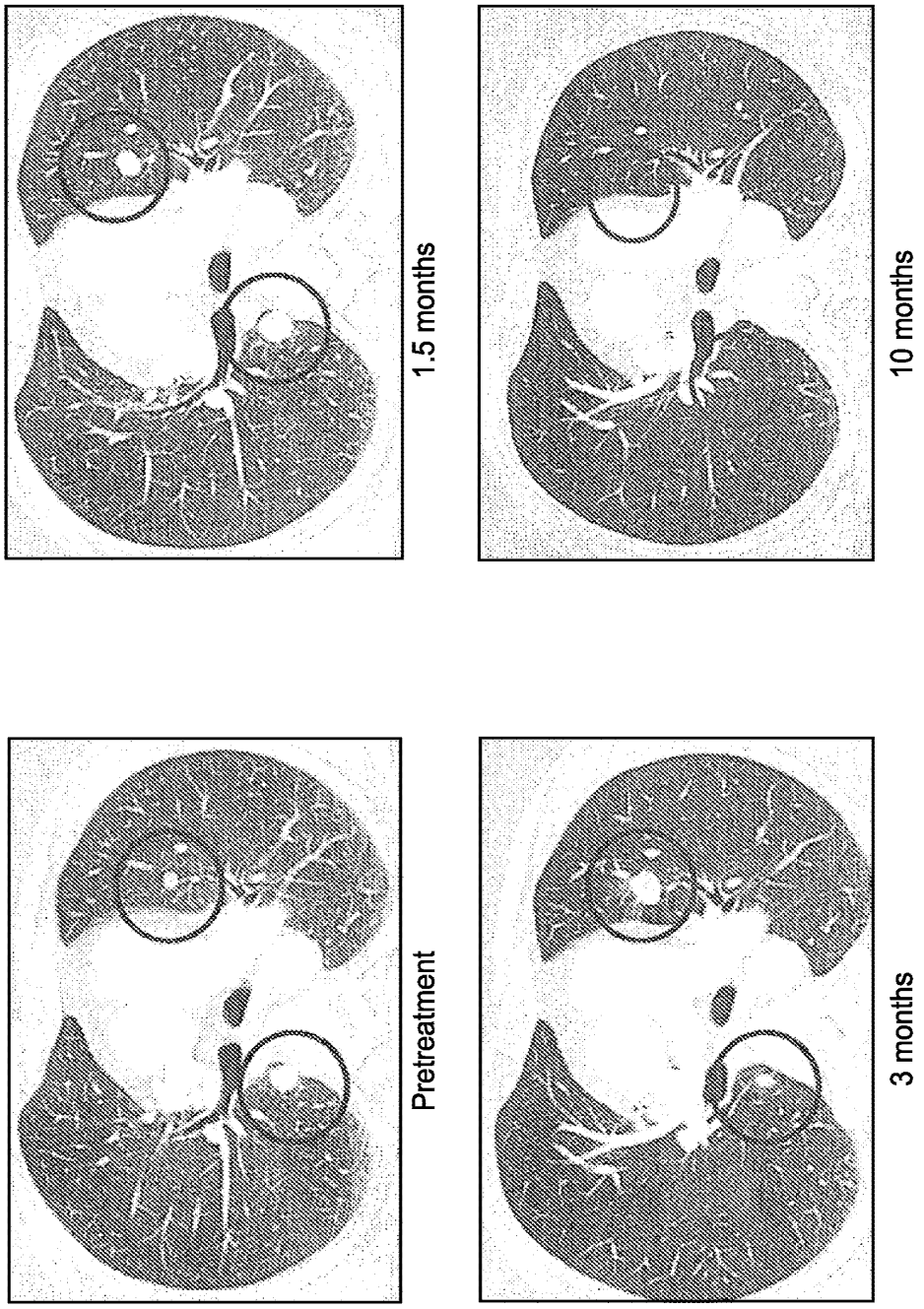


FIG. 11

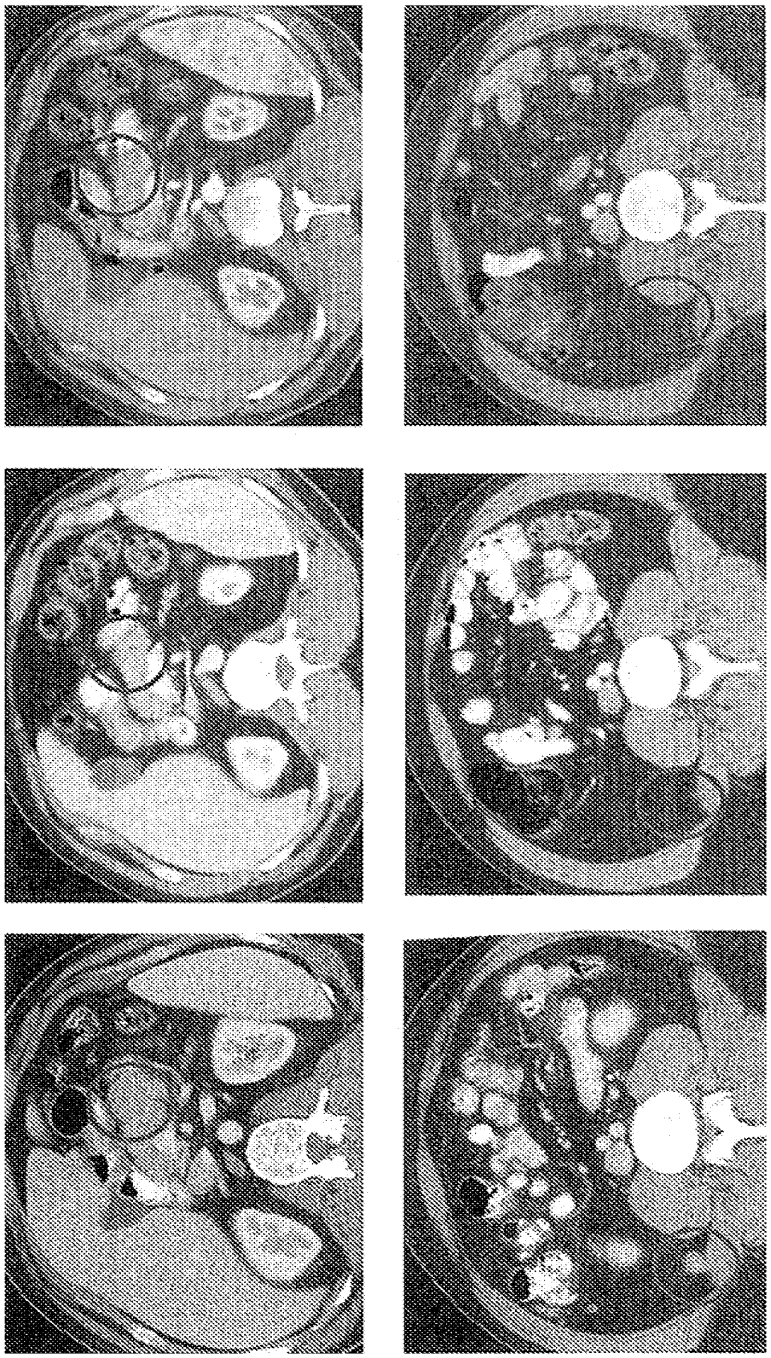


FIG. 12

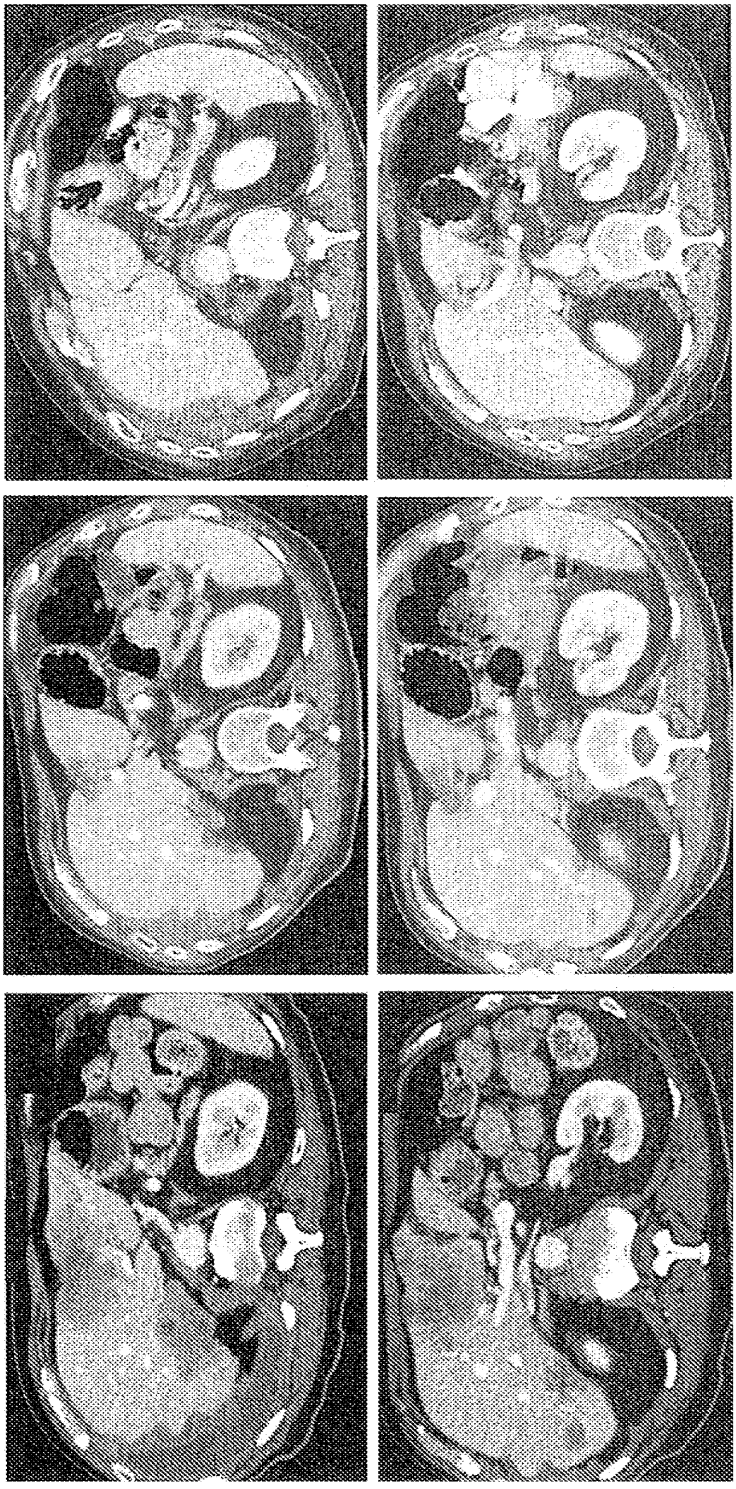


FIG. 13

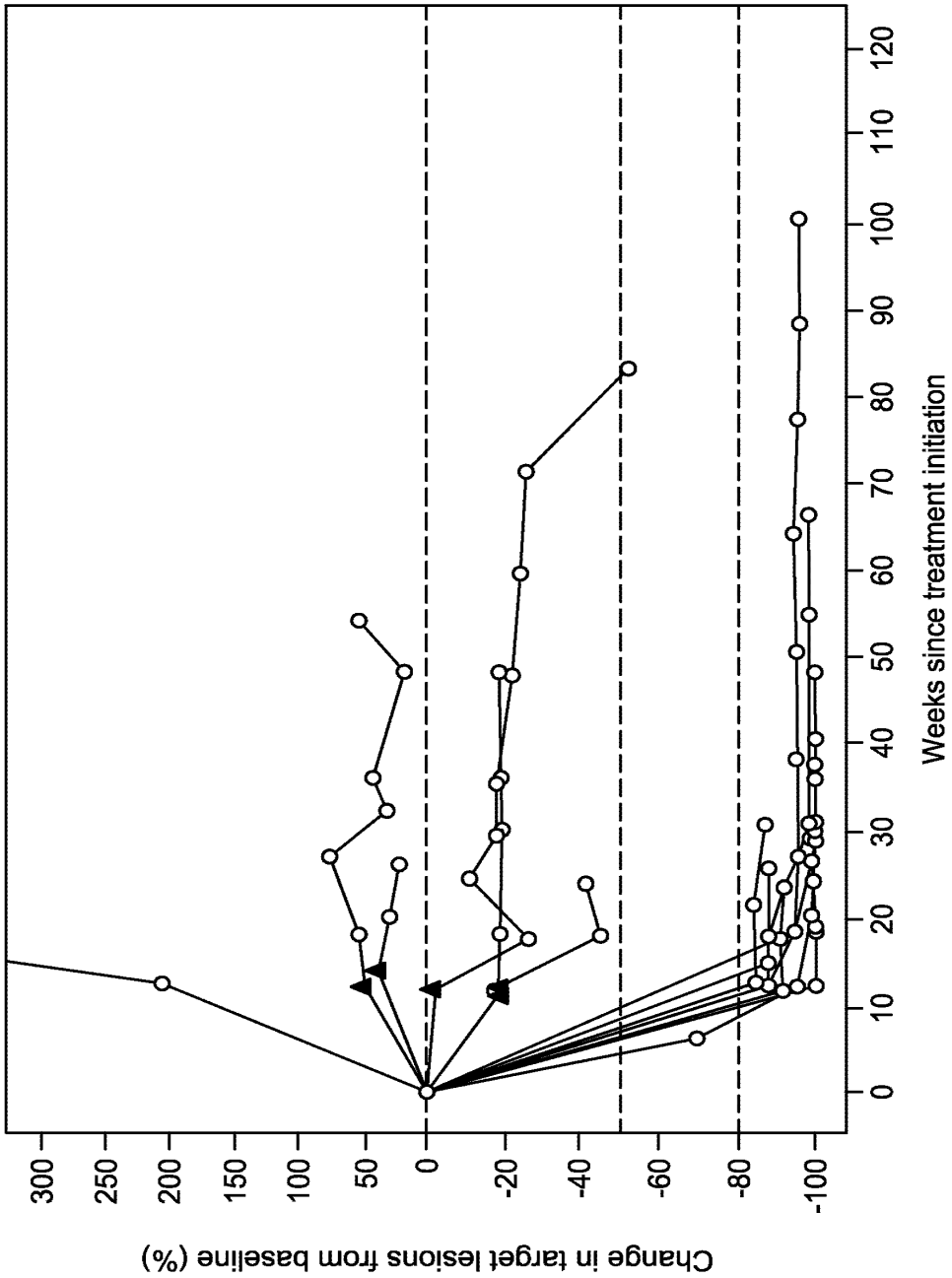

Change in target lesions from baseline (%)
300
250
200
150
100
50
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
Weeks since treatment initiation

FIG. 14A

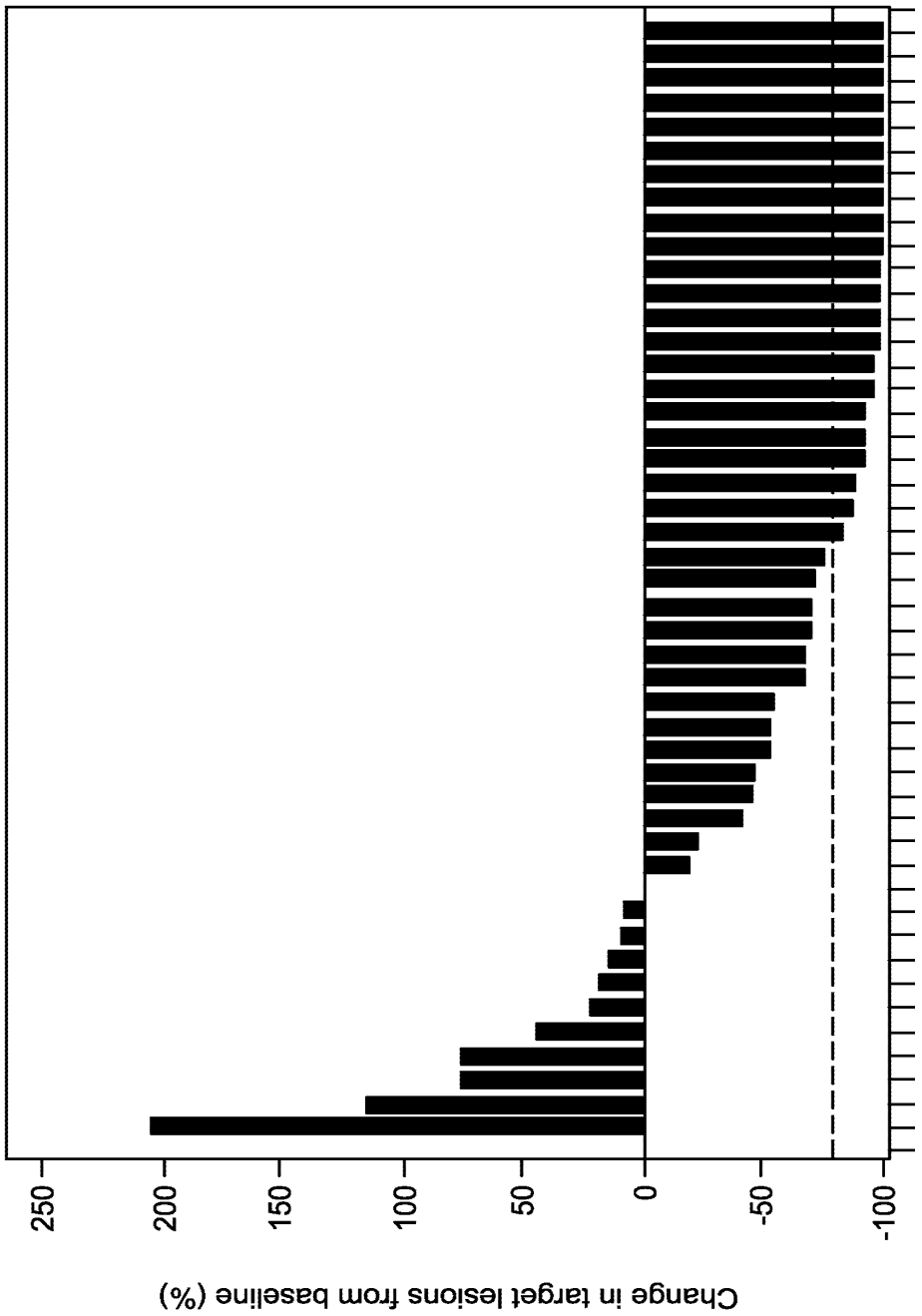

250
200
150
100
50
0
-50
-100
Change in target lesions from baseline (%)
Patients

FIG. 14B

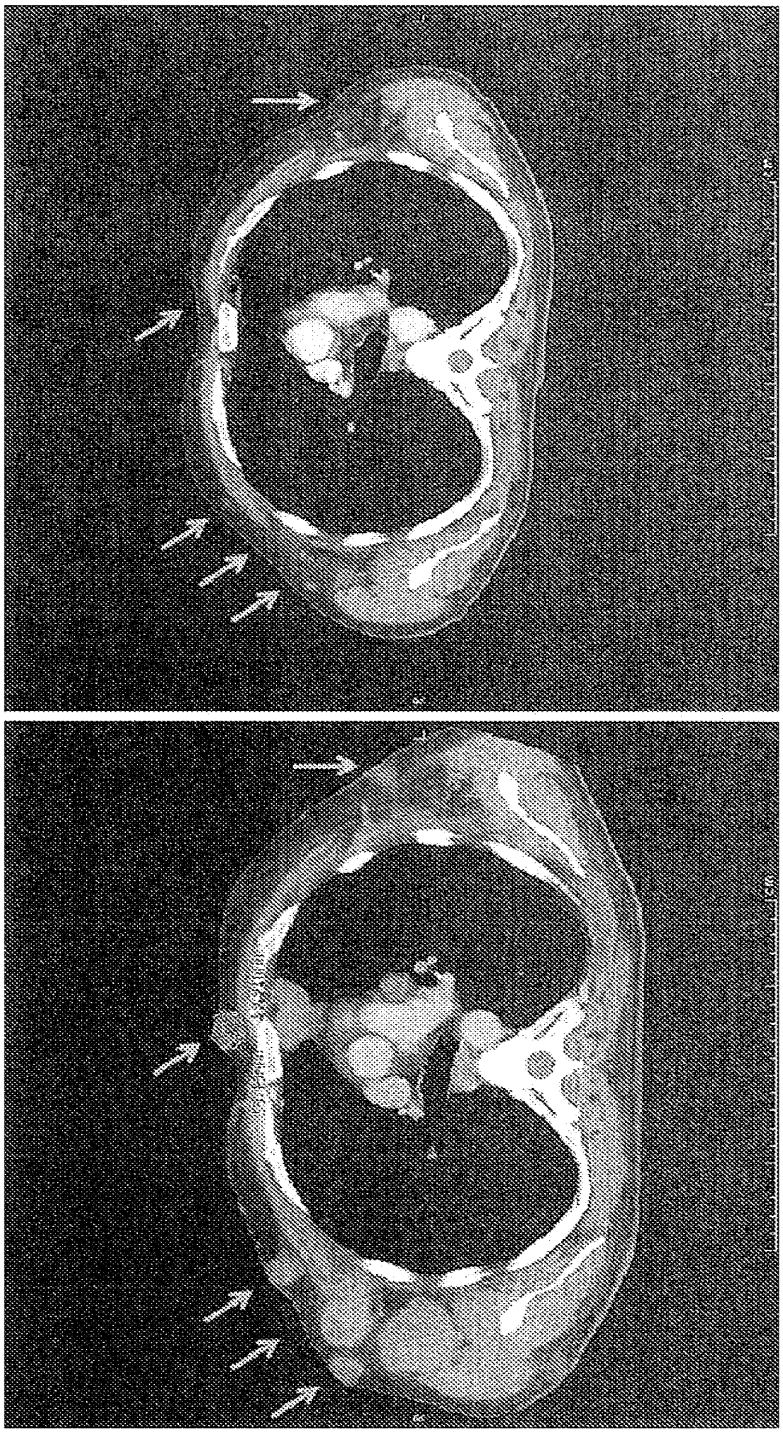


FIG. 15

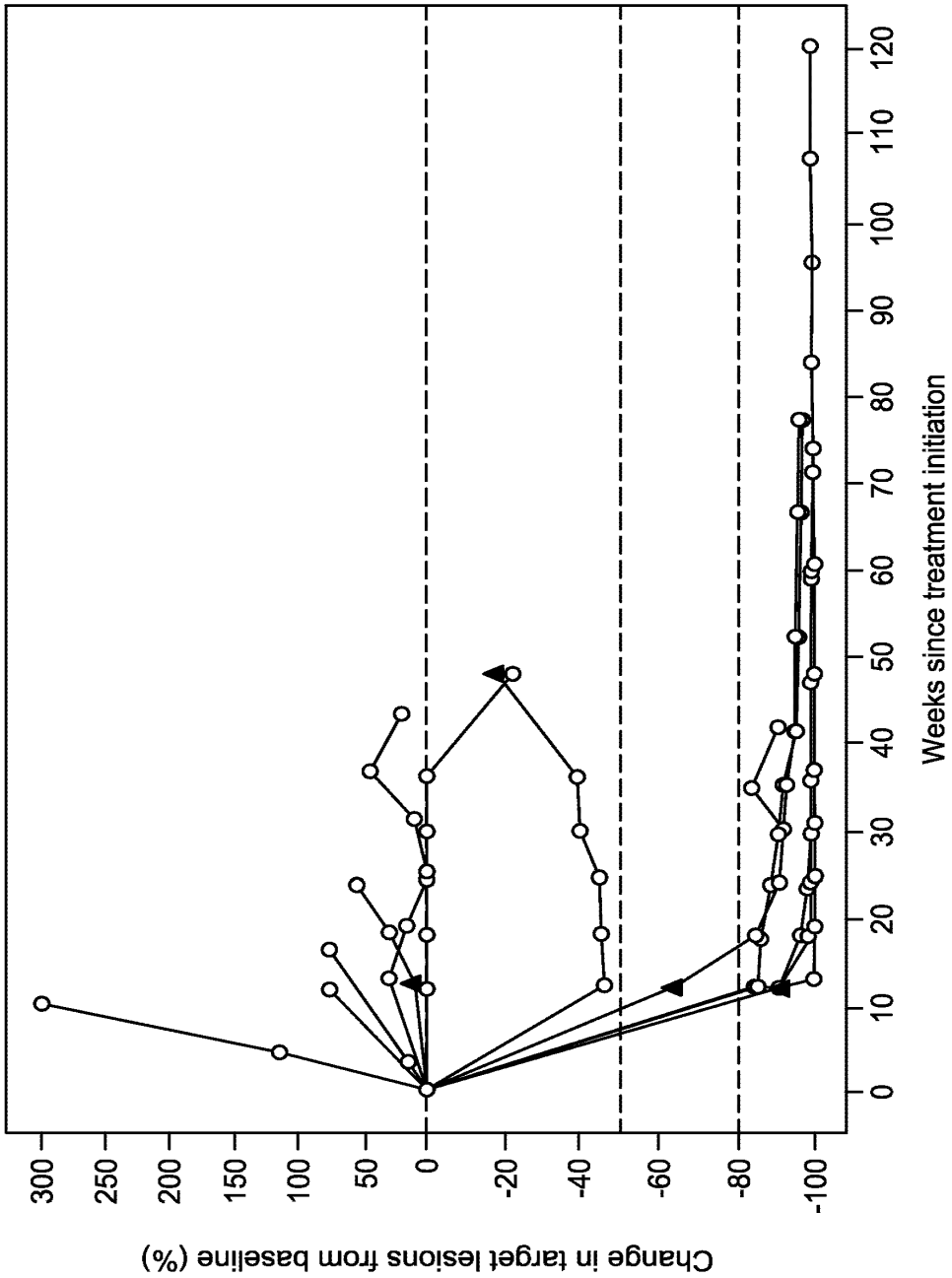

Change in target lesions from baseline (%)
300
250
200
150
100
50
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
Weeks since treatment initiation

FIG. 16A

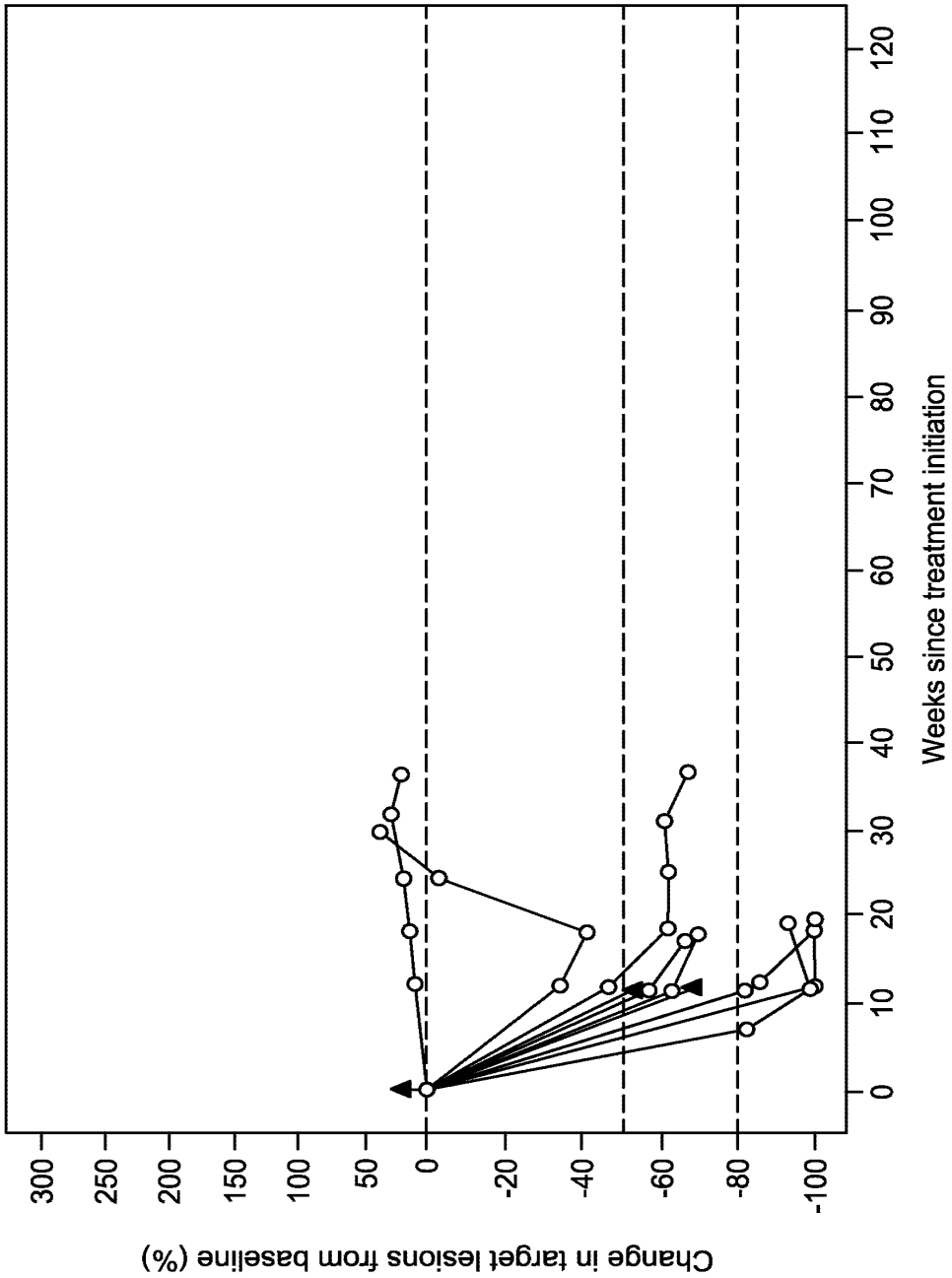

Change in target lesions from baseline (%)
300
250
200
150
100
50
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
Weeks since treatment initiation

FIG. 16B

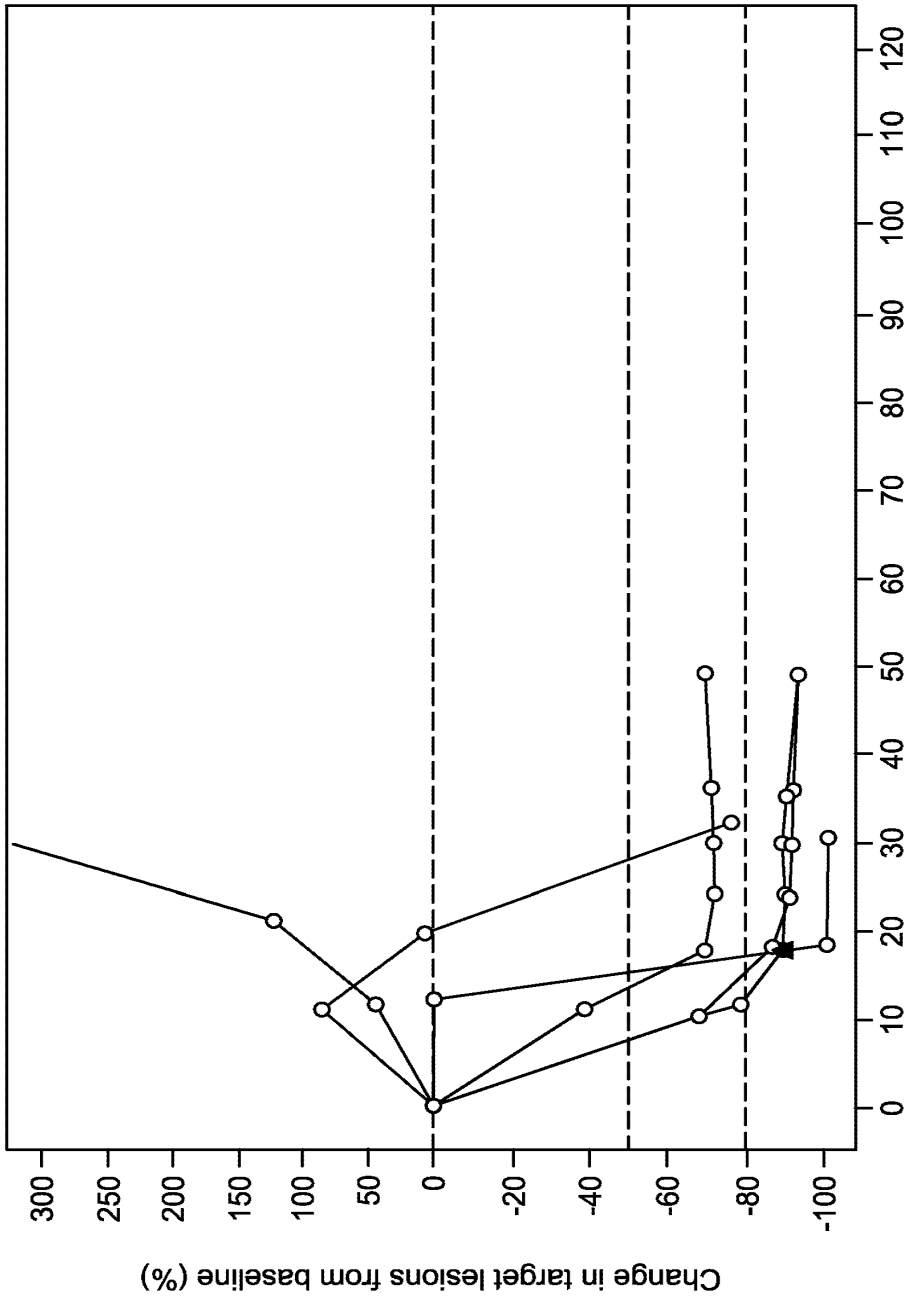

Change in target lesions from baseline (%)
300
250
200
150
100
50
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
Weeks since treatment initiation

FIG. 16C

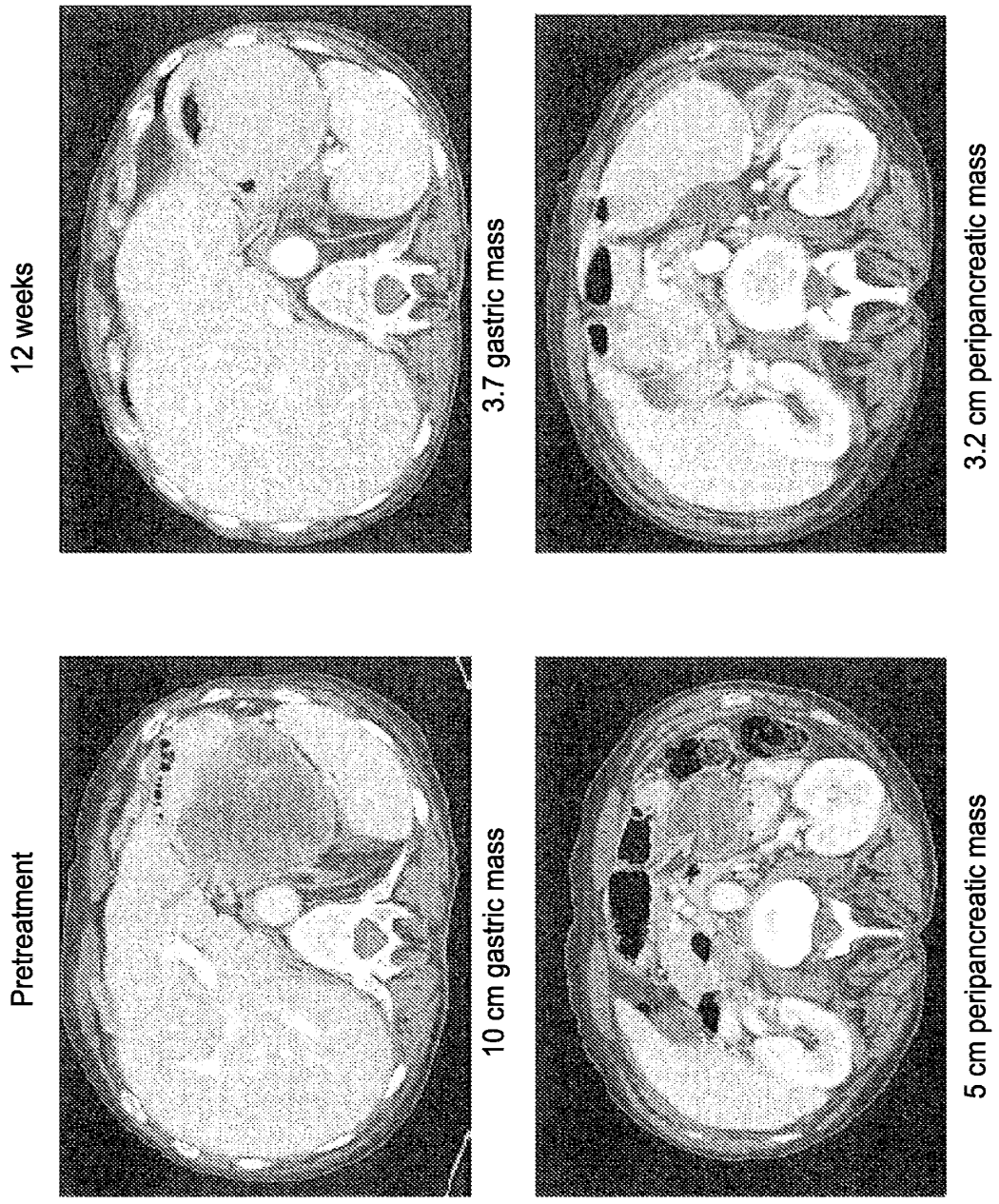

12 weeks
3.7 gastric mass
3.2 cm peripancreatic mass
Pretreatment
10 cm gastric mass
5 cm peripancreatic mass

FIG. 17

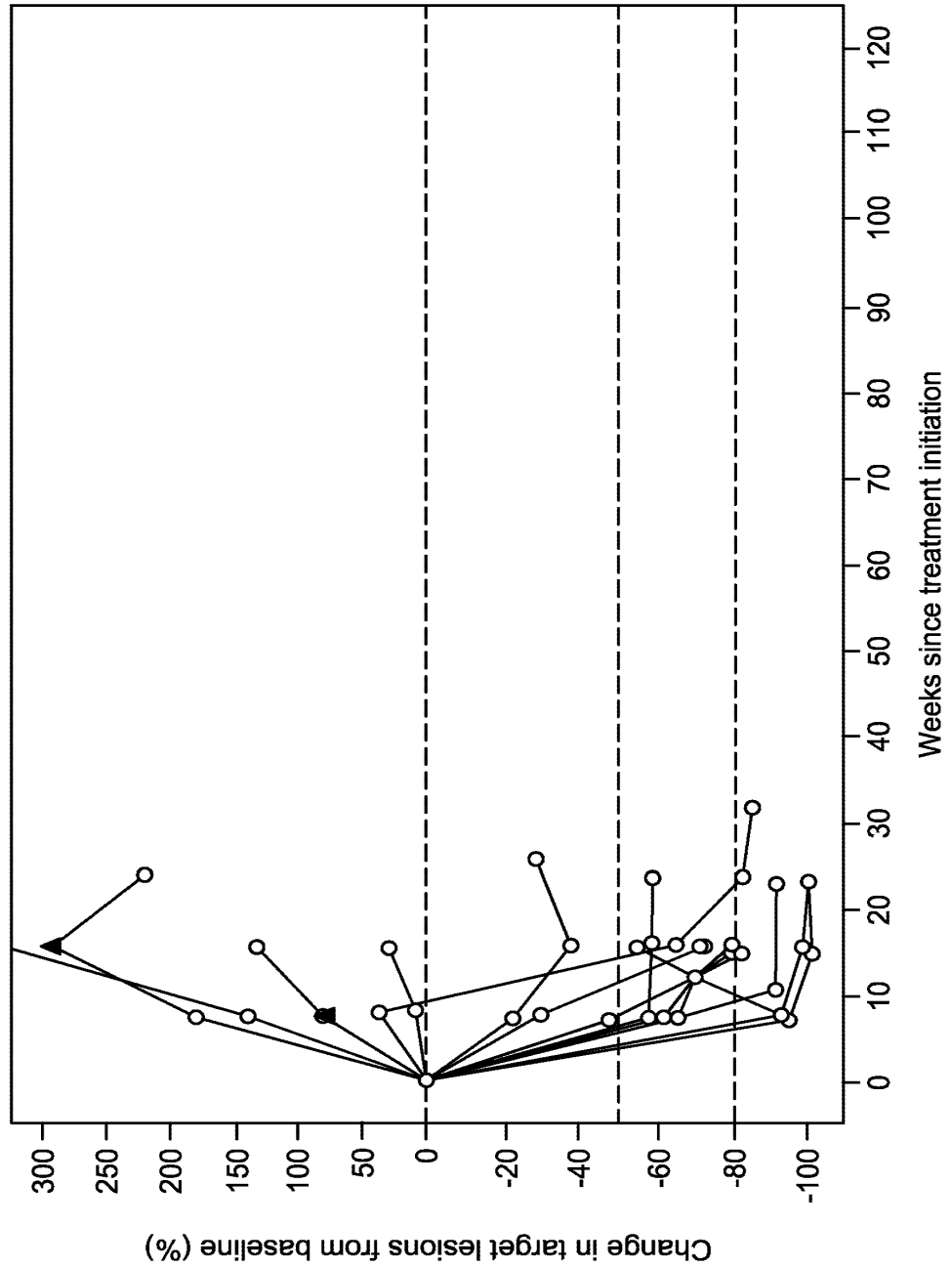

Change in target lesions from baseline (%)
300
250
200
150
100
50
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
Weeks since treatment initiation

FIG. 18A

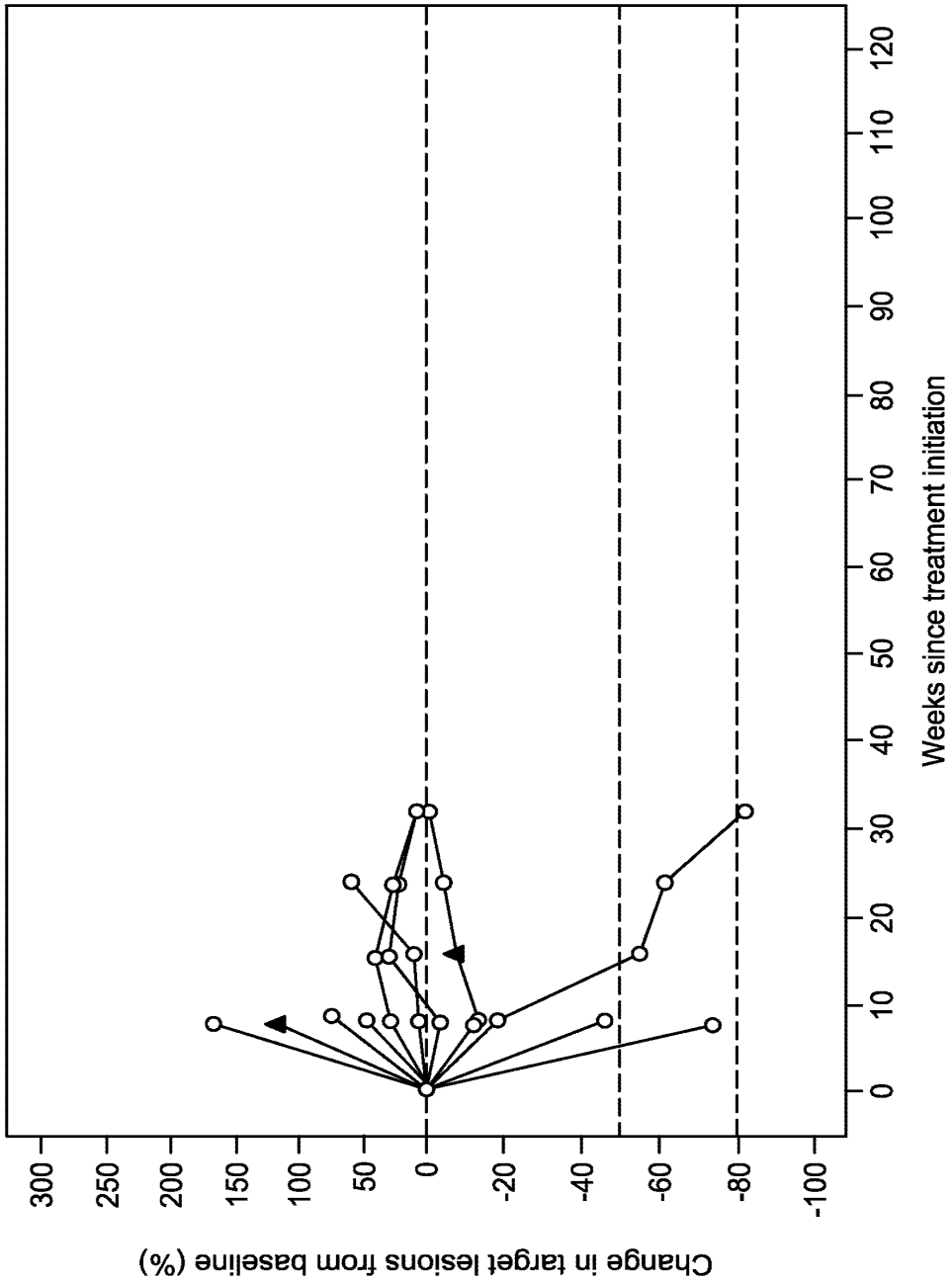

Change in target lesions from baseline (%)
300
250
200
150
100
50
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
80
90
100
110
120
Weeks since treatment initiation

FIG. 18B

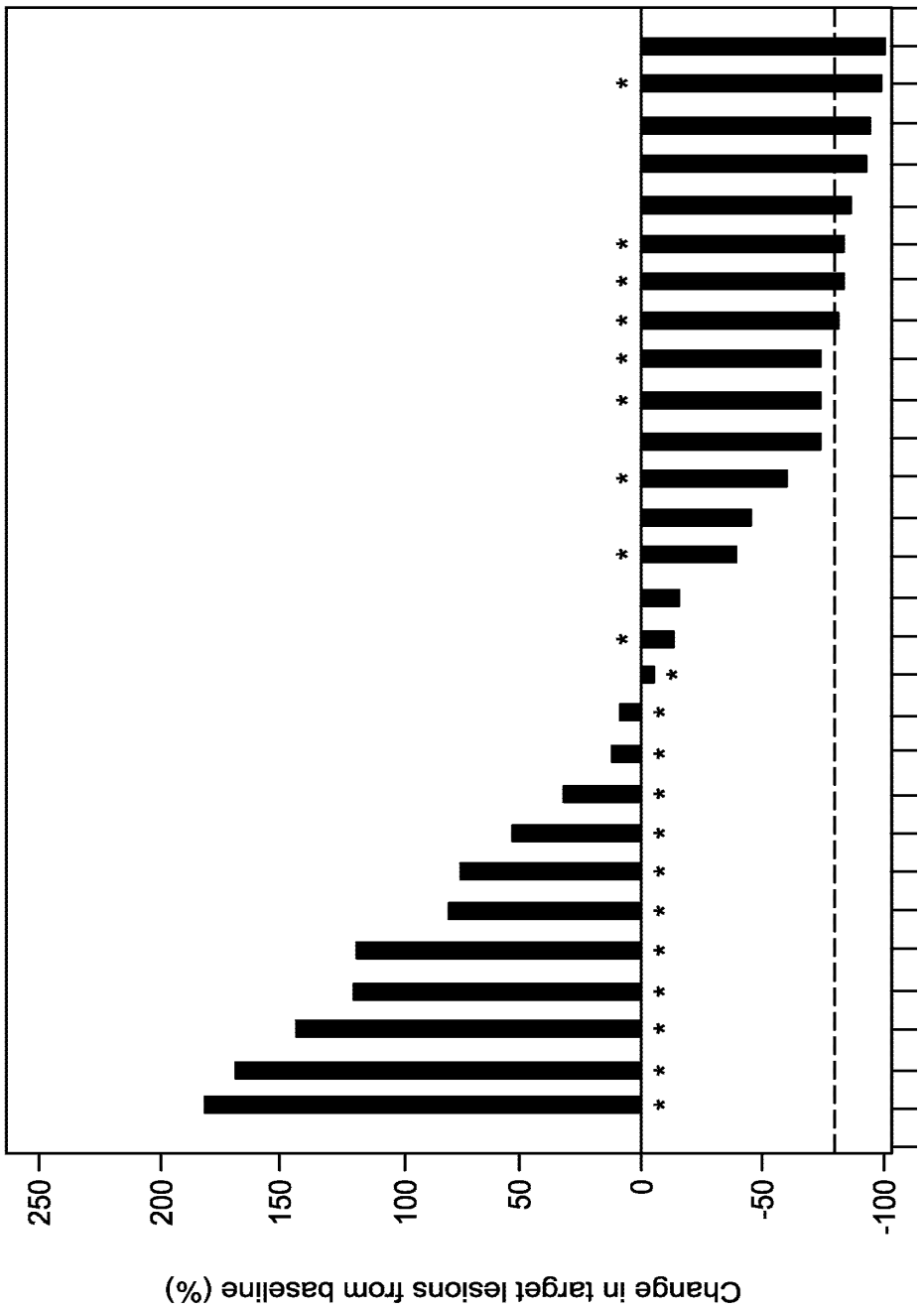

250
200
150
100
50
0
-50
-100
Change in target lesions from baseline (%)
Patients

FIG. 18C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/040764

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/005874 A2 (MEDAREX INC [US] ; KORMAN ALAN J [US] ; SELBY MARK J [US] ; WANG CHANGYU) 11 January 2007 (2007-01-11) pages 79,80,75 ; example all --- WO 2006/121168 A1 (ONO PHARMACEUTICAL CO [JP] ; MEDAREX INC [US] ; KORMAN ALAN J ; SRINIVASA) 16 November 2006 (2006-11-16) pages 58,59 ,55 ; example all --- WO 2004/056875 A1 (WYETH CORP [US] ; CAMBRIDGE ANTIBODY TECH [GB] ; COLLINS MARY [US] ; WOOD) 8 July 2004 (2004-07-08) pages 4,25 ,42 ; example all --- -/- .	1-3 1-3 1-3

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2013	31/10/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fellows , Edward

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/040764

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HIRANO FUMIYA ET AL: "Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity", CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 65, no. 3, 1 February 2005 (2005-02-01), pages 1089-1096, XP002419626, ISSN: 0008-5472 the whole document	1-3
A	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; April 2011 (2011-04), DODSON LINDZY F ET AL: "Potential targets for pancreatic cancer immunotherapeutics", XP002703660, Database accession no. PREV201100567322 the whole document & IMMUNOTHERAPY, vol. 3, no. 4, April 2011 (2011-04), pages 517-537, ISSN: 1750-743X, DOI: 10.2217/IMT.11.10	1-3
A	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; July 2011 (2011-07), ANDORSKY DAVID J ET AL: "Programmed Death Ligand 1 Is Expressed by Non-Hodgkin Lymphomas and Inhibits the Activity of Tumor-Associated T Cells", XP002703661, Database accession no. PREV201100493026 the whole document & CLINICAL CANCER RESEARCH, vol. 17, no. 13, July 2011 (2011-07), pages 4232-4244, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-10-2660	1-3

1

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2013/040764

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007005874 A2	11-01-2007	AU 2006265108 A1	11-01-2007
		BR PI0613361 A2	04-01-2011
		CA 2612241 A1	11-01-2007
		CN 101248089 A	20-08-2008
		EA 200800229 A1	30-06-2008
		EP 1907424 A2	09-04-2008
		HR P20080053 A2	31-08-2009
		JP 5252635 B2	31-07-2013
		JP 2008544755 A	11-12-2008
		JP 2013150606 A	08-08-2013
		KR 20080045674 A	23-05-2008
		NZ 564592 A	25-11-2011
		SG 163554 A1	30-08-2010
		US 2009055944 A1	26-02-2009
		US 2013122014 A1	16-05-2013
		WO 2007005874 A2	11-01-2007
		ZA 200710919 A	29-10-2008
WO 2006121168 A1	16-11-2006	AU 2006244885 A1	16-11-2006
		BR PI0610235 A2	08-06-2010
		CA 2607147 A1	16-11-2006
		CN 103059138 A	24-04-2013
		EP 1896582 A1	12-03-2008
		EP 2161336 A1	10-03-2010
		EP 2418278 A2	15-02-2012
		EP 2439272 A2	11-04-2012
		EP 2439273 A2	11-04-2012
		ES 2427646 T3	31-10-2013
		IL 187108 A	30-06-2011
		IL 208642 A	30-08-2012
		JP 4361545 B2	11-11-2009
		JP 5028700 B2	19-09-2012
		JP 2006340714 A	21-12-2006
		JP 2009155338 A	16-07-2009
		JP 2012158605 A	23-08-2012
		KR 20080011428 A	04-02-2008
		KR 20130032908 A	02-04-2013
		NZ 563193 A	28-05-2010
		RU 2010135087 A	27-02-2012
		TW 1379898 B	21-12-2012
		US 2009217401 A1	27-08-2009
		US 2013133091 A1	23-05-2013
		WO 2006121168 A1	16-11-2006
WO 2004056875 A1	08-07-2004	AT 514713 T	15-07-2011
		AU 2003288675 A1	14-07-2004
		AU 2010235966 A1	11-11-2010
		BR 0316880 A	25-10-2005
		CA 2508660 A1	08-07-2004
		CN 1753912 A	29-03-2006
		CN 101899114 A	01-12-2010
		EP 1576014 A1	21-09-2005
		ES 2367430 T3	03-11-2011
		HK 1083510 A1	16-12-2011
		IL 169152 A	30-11-2010
		JP 4511943 B2	28-07-2010
		JP 2006521783 A	28-09-2006
		JP 2010189395 A	02-09-2010

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/040764

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

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

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

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

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

1-3

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3

A method of treating a subject with cancer comprising administering to the subject a therapeutically effective amount of an antibody, or an antigen-binding portion thereof, that disrupts the interaction between PD-1 and PD-L1 wherein the antibody or antigen-binding portion thereof binds specifically to PD-1 or PD-L1 and related subject-matter.

---

2. claims: 4-9, 21

A method for immunotherapy of a subject with cancer comprising the testing of a tissue sample, assessing the portion of cells in the sample that express PD-L1 and administering a composition comprising a therapeutically effective amount of an anti-PD-1 antibody to a selected subject and related subject-matter.

---

3. claim: 10

A method for immunotherapy of a subject with cancer comprising selecting a subject who is not suitable of anti-PD-1 antibody immunotherapy and administering to the subject a standard-of-care therapeutic other than an anti-PD-1 antibody.

---

4. claims: 11-13

A monoclonal antibody, or an antigen-binding portion thereof, that binds specifically to a cell surface-expressed PD-L1 in a sample and related subject-matter.

---

5. claims: 14-20

A method of treating a subject with cancer comprising administering to the subject an antibody, or an antigen-binding portion thereof, that specifically binds and inhibits PD-1 and an antibody, or an antigen-binding portion thereof, that specifically binds and inhibits CTLA-4, each antibody be administered at a specific dosage range in a concurrent regimen and related subject-matter.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2013/040764

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		MX PA05006828 A	08-09-2005
		US 2004213795 A1	28-10-2004
		US 2006210567 A1	21-09-2006
		US 2008311117 A1	18-12-2008
		US 2010028330 A1	04-02-2010
		WO 2004056875 A1	08-07-2004

Form PCT/ISA/210 (patent family annex) (April 2005)

An Investigational Immuno-therapy Study of Nivolumab, and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread (CheckMate142)

The safety and scientific validity of this study is the responsibility of the study sponsor and investigators. Listing a study does not mean it has been evaluated by the U.S. Federal Government. Read our disclaimer for details.

ClinicalTrials.gov Identifier: NCT02060188

Recruitment Status : Active, not recruiting
First Posted : February 11, 2014
Last Update Posted : December 4, 2018

Sponsor:
Bristol-Myers Squibb

Information provided by (Responsible Party):
Bristol-Myers Squibb

Study Description

Go to

Brief Summary:
The purpose of this study is to examine if Nivolumab by itself, or Nivolumab in combination with other anti-cancer drugs, will result in meaningful tumor size reduction, in patients with colon cancer that has come back or has spread, and who have a specific biomarker in their tumors.

Condition or disease	Intervention/treatment	Phase
Microsatellite Unstable Colorectal Cancer Microsatellite Stable Colorectal Cancer	Drug: Ipilimumab Drug: Nivolumab	Phase 2

Mismatch Repair Proficient Colorectal Cancer Mismatch Repair Deficient Colorectal Cancer	Drug: Cobimetinib Drug: Daratumumab Drug: anti-LAG-3 antibody	

Detailed Description:

Allocation: The Microsatellite Instability High (MSI-High) and C4 and C6 Cohort Parts of the trial are Non-randomized, The Non-MSI high Dose Escalation Phase part of the trial contained a randomized portion

Study Design

Go to

Study Type :	Interventional (Clinical Trial)
Estimated Enrollment :	340 participants
Allocation:	Non-Randomized
Intervention Model:	Parallel Assignment
Masking:	None (Open Label)
Primary Purpose:	Treatment
Official Title:	A Phase 2 Clinical Trial of Nivolumab, or Nivolumab Combinations in Recurrent and Metastatic Microsatellite High (MSI-H) and Non-MSI-H Colon Cancer
Actual Study Start Date :	March 7, 2014
Estimated Primary Completion Date :	December 3, 2019
Estimated Study Completion Date :	December 30, 2020

Resource links provided by the National Library of Medicine NIH NLM

Drug Information available for: Nivolumab

U.S. FDA Resources

Arms and Interventions

Go to

Arm	Intervention/treatment
Experimental: Nivolumab Monotherapy Nivolumab administered as IV infusion at a dose of 3mg/kg every 2 weeks until disease progression	Drug: Nivolumab Other Names: • BMS-936558 • Opdivo

Experimental: Nivolumab (Nivo) + Ipilimumab (Ipi) • Nivo 3mg/Kg IV with Ipi 1 mg/Kg IV every 3 week (wk) for 4 doses followed by Nivo 3mg/Kg IV every 2wk until progression • Dose Escalation Phase: (Complete) • Dose Level (DL) 1: Nivo 0.3mg/Kg with Ipi 1 mg/Kg IV every 3wk for 4 doses followed by Nivo 3mg/Kg IV every 2wk until progression • DL 1: Nivo 1mg/Kg IV with Ipi 1 mg/Kg IV every 3 wk for 4 doses followed by Nivo 3mg/Kg IV every 2wk until progression • DL 2a: Nivo 1mg/Kg IV with Ipi 3 mg/Kg IV every 3wk for 4 doses followed by Nivo 3mg/Kg IV every 2 wk until progression • DL 2b: Nivo 3mg/Kg IV with Ipi 1 mg/Kg IV every 3wk for 4 doses followed by Nivo 3mg/Kg IV every 2 wk until progression	Drug: Ipilimumab Other Name: Yervoy Drug: Nivolumab Other Names: • BMS-936558 • Opdivo
Experimental: Nivolumab (Nivo) + Ipilimumab (Ipi) Cohort C3 Nivo IV dosed every 2wk with Ipi IV dosed every 6wk.	Drug: Ipilimumab Other Name: Yervoy Drug: Nivolumab Other Names: • BMS-936558 • Opdivo
Experimental: Nivolumab (Nivo) + Ipilimumab (Ipi) + Cobimetinib Cohort C4 Nivo IV dosed every 2wk, with Ipi IV dosed every 6wk, combined with Cobimetinib dosed orally once daily 21 days on/7 days off.	Drug: Ipilimumab Other Name: Yervoy Drug: Nivolumab Other Names: • BMS-936558 • Opdivo Drug: Cobimetinib Other Name: Cotellic

Experimental: Nivolumab (Nivo) + BMS-986016 Cohort C5 Nivo IV dosed every 2wk with BMS-986016 dosed every 2 wk	Drug: Nivolumab Other Names: • BMS-936558 • Opdivo Drug: anti-LAG-3 antibody Other Name: BMS-986016
Experimental: Nivolumab (Nivo) + Daratumumab Cohort C6 Daratumumab IV dosed weekly for week 1-8; then every 2 wks from Week 9-24; then every 4 wks on week 25; with Nivo dosed every 2 wks starting at week 3 and every 4 wks starting at week 25	Drug: Nivolumab Other Names: • BMS-936558 • Opdivo Drug: Daratumumab Other Name: Darzalex

Outcome Measures

Go to

Primary Outcome Measures :

1. Objective response rate (ORR) in all MSI-High and non-MSI-High subjects as determined by Investigators [Time Frame: The final analysis of the primary endpoint will occur at least 6 months after the last enrolled subject's first dose of study therapy (Approximately up to 34 months)]

   (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later)) (Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/-1 wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued (whichever occurs later))

Secondary Outcome Measures :

1. ORR in all MSI-H and non-MSI-H subjects based on IRRC determination [Time Frame: The final analysis of the secondary endpoint will occur the time of the primary endpoint analysis

(Approximately up to 34 months)]

Tumor imaging assessments will occur every 6 weeks from the date of first dose (+/- wk) for the first 24 weeks, then every 12 wks (+/- 1 wk) thereafter until disease progression or treatment is discontinued(whichever occurs later)

Eligibility Criteria

Go to

Information from the National Library of Medicine NIH NLM

Choosing to participate in a study is an important personal decision. Talk with your doctor and family members or friends about deciding to join a study. To learn more about this study, you or your doctor may contact the study research staff using the contacts provided below. For general information, Learn About Clinical Studies.

Ages Eligible for Study:	18 Years and older (Adult, Older Adult)
Sexes Eligible for Study:	All
Accepts Healthy Volunteers:	No

Criteria

For more information regarding BMS clinical trial participation, please visit www.BMSStudyConnect.com

Inclusion Criteria:

- Men and women ≥ 18 years of age
- Eastern Cooperative Oncology Group (ECOG) performance status 0 to 1
- Histologically confirmed recurrent or metastatic colorectal cancer
- Measurable disease by CT or MRI
- Testing for MSI Status (by an accredited lab)
    1. Subjects with microsatellite instability high (MSI-H) tumors will enroll in the MSI-H Cohort (mStage and cStage groups), the C3 Cohort, and the C5 Cohort.
    2. Subjects with phenotypes that are non-microsatellite instability high (non-MSI-H) will enroll in the non- MSI-H Safety Cohort and the C6, C4 Cohorts.
- Adequate organ function as defined by study-specific laboratory tests

- Must use acceptable form of birth control throughout the study. After the final dose of study drug, an acceptable form of birth control must be used for 23 weeks for women of childbearing potential (WOCBP) and 31 weeks for men who are sexually active with WOCBP
- Signed informed consent
- Willing and able to comply with study procedures
- Subjects enrolled into the C3 Cohort must have not had treatment for their metastatic disease

Exclusion Criteria:

- Active brain metastases or leptomeningeal metastases are not allowed.
- Prior treatment with an anti-Programmed Death Receptor (PD)-1, anti-PD-L1, anti-PD-L2, anti-Cytotoxic T-Cell Lymphoma-4 Antigen (CTLA-4) antibody, or any other antibody or drug specifically targeting T-cell co-stimulation or immune checkpoint pathways
- Prior malignancy active within the previous 3 years except for locally curable cancers
- Subjects with active, known or suspected autoimmune disease
- Subjects with a condition requiring systemic treatment with either corticosteroids or other immunosuppressive medications within 14 days of study drug administration

Other protocol defined inclusion/exclusion criteria could apply

Contacts and Locations

Go to

Information from the National Library of Medicine NIH NLM

To learn more about this study, you or your doctor may contact the study research staff using the contact information provided by the sponsor.

Please refer to this study by its ClinicalTrials.gov identifier (NCT number): ***NCT02060188***

Show 32 Study Locations

Sponsors and Collaborators

Bristol-Myers Squibb

Investigators

Study Director: Bristol-Myers Squibb Bristol-Myers Squibb

More Information

Go to

Additional Information:

BMS Clinical Trial Information | BMS Clinical Trial Patient Recruiting | Investigator Inquiry Form | FDA Safety Alerts and Recalls |

Publications automatically indexed to this study by ClinicalTrials.gov Identifier (NCT Number):

Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, Desai J, Hill A, Axelson M, Moss RA, Goldberg MV, Cao ZA, Ledeine JM, Maglinte GA, Kopetz S, André T. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. Lancet Oncol. 2017 Sep;18(9):1182-1191. doi: 10.1016/S1470-2045(17)30422-9. Epub 2017 Jul 19. Erratum in: Lancet Oncol. 2017 Sep;18(9):e510.

Responsible Party:	Bristol-Myers Squibb
ClinicalTrials.gov Identifier:	NCT02060188 History of Changes
Other Study ID Numbers:	CA209-142
	2013-003939-30 (EudraCT Number)
First Posted:	February 11, 2014 Key Record Dates
Last Update Posted:	December 4, 2018
Last Verified:	December 2018

Studies a U.S. FDA-regulated Drug Product:	Yes
Studies a U.S. FDA-regulated Device Product:	No

Additional relevant MeSH terms:

Colorectal Neoplasms	Rectal Diseases
Colonic Neoplasms	Antibodies
Intestinal Neoplasms	Antibodies, Monoclonal
Gastrointestinal Neoplasms	Nivolumab
Digestive System Neoplasms	Ipilimumab
Neoplasms by Site	Daratumumab
Neoplasms	Immunologic Factors
Digestive System Diseases	Physiological Effects of Drugs
Gastrointestinal Diseases	Antineoplastic Agents, Immunological
Colonic Diseases	Antineoplastic Agents
Intestinal Diseases	

Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors

The safety and scientific validity of this study is the responsibility of the study sponsor and investigators. Listing a study does not mean it has been evaluated by the U.S. Federal Government. Know the risks and potential benefits of clinical studies and talk to your health care provider before participating. Read our disclaimer for details.

ClinicalTrials.gov Identifier: NCT01876511

Recruitment Status : Recruiting
First Posted : June 12, 2013
Last Update Posted : March 6, 2019

See Contacts and Locations

Sponsor:
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Collaborator:
Merck Sharp & Dohme Corp.

Information provided by (Responsible Party):
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Study Description

Go to

Brief Summary:
This study will be looking at whether MK-3475 (an antibody that blocks negative signals to T cells) is effective (anti-tumor activity) and safe in three different patient populations. These include: 1. patients with MSI positive colon cancer, 2. patients with MSI negative colon cancer 3. patients with other MSI positive cancers, and 4. patients with MSI negative cancer with a mutator phenotype.

Condition or disease	Intervention/treatment	Phase
MSI Positive Colorectal Cancer MSI Negative Colorectal Cancer MSI Positive Non-Colorectal Cancers High Tumor Mutation Burden High TMB	Drug: MK-3475	Phase 2

Study Design

Go to

Study Type :	Interventional (Clinical Trial)
Estimated Enrollment :	171 participants
Allocation:	Non-Randomized
Intervention Model:	Parallel Assignment
Masking:	None (Open Label)
Primary Purpose:	Treatment
Official Title:	Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors
Study Start Date :	September 2013
Estimated Primary Completion Date :	June 2021
Estimated Study Completion Date :	June 2021

Resource links provided by the National Library of Medicine NIH NLM

Drug Information available for: Pembrolizumab

U.S. FDA Resources

Arms and Interventions

Go to

Arm	Intervention/treatment
Experimental: MSI Positive Colorectal Cancer	Drug: MK-3475 MK-3475 10 mg/kg every 14 days
Experimental: MSI Negative Colorectal Cancer	Drug: MK-3475 MK-3475 10 mg/kg every 14 days

Experimental: MSI Positive Non-Colorectal Cancer	Drug: MK-3475 MK-3475 10 mg/kg every 14 days
Experimental: MSI Negative with Mutator Phenotype	Drug: MK-3475 MK-3475 200mg flat dose every 21 days

Outcome Measures

Go to

Primary Outcome Measures :

1. Immune-related progression free survival (irPFS) rate at 20 weeks in patients with MSI positive and negative colorectal adenocarcinoma using immune related response criteria (irRC) [Time Frame: 4 years]
2. Objective response rate (irORR) at 20 weeks in patients with MSI positive and negative colorectal adenocarcinoma using immune related response criteria (irRC) [Time Frame: 4 years]
3. Immune-related progression free survival (irPFS) rate in patients with MSI positive non-colorectal adenocarcinoma using immune related response criteria (irRC) at 20 weeks [Time Frame: 4 years]
4. objective response rate in patients with MSI-negative cancer with a mutator phenotype using RECIST 1.1 criteria [Time Frame: 4 years]

Secondary Outcome Measures :

1. Overall survival [Time Frame: 4 years]
2. irPFS and PFS in patients with MSI positive and negative tumors at 28 weeks using irRC and RECIST 1.1 [Time Frame: 4 years]
3. Best overall response rate and disease control rate in patients with MSI positive and negative tumors [Time Frame: 4 years]
4. Number of participants experiencing immune-related toxicities (IRAEs) [Time Frame: 4 years]
5. Does MSI as a marker predict treatment response [Time Frame: 4 years]
6. Identify alternative markers of MSI status. This includes but is not limited to MLH 1, MSH 2, MSH 6, PMS2, BRAF pV600E, and TGFBR2. [Time Frame: 4 years]

Eligibility Criteria

Go to

Information from the National Library of Medicine

Choosing to participate in a study is an important personal decision. Talk with your doctor and family members or friends about deciding to join a study. To learn more about this study, you or your doctor may contact the study research staff using the contacts provided below. For general information, Learn About Clinical Studies.

Ages Eligible for Study:	18 Years and older (Adult, Older Adult)
Sexes Eligible for Study:	All
Accepts Healthy Volunteers:	No

Criteria

Inclusion Criteria:

- Arm 1 only: Patients with microsatellite instability (MSI) positive colorectal cancer
- Arm 2 only: Patients with MSI negative colorectal cancer
- Arm 3 only: Patients with MSI positive non-colorectal cancer
- Arm 4 only: patients with hypermutated MSI negative cancer
- Have measurable disease
- Eastern Cooperative Oncology Group (ECOG) Performance Status of 0 to 1
- Adequate organ function as defined by study-specified laboratory tests
- Must use acceptable form of birth control through the study and for 28 days after final dose of study drug
- Signed informed consent form
- Willing and able to comply with study procedures
- Agree to have a biopsy of their cancer
- Patients with colon cancer must have received at least two prior cancer therapy regimens.
- Patients with other cancer types must have received at least one prior cancer therapy
- Progressive disease

Exclusion Criteria:

- Patients with uncontrolled intercurrent illness, including but not limited to ongoing or active infection, systematic congestive heart failure, unstable angina pectoris, cardiac arrhythmia or psychiatric condition that would limit compliance with study requirements.
- Patients who have had chemotherapy or biological cancer therapy within 2 weeks prior to the first dose of study drug

- Patients who have had radiation within 2 weeks prior to the first dose of study drug
- Patients who have undergone major surgery within 4 weeks of dosing of investigational agent
- Patients who have received another investigational product or investigational device within 4 weeks prior to receiving study drug
- Patients who have received any of the following concomitant therapy: Interleukin-2 (IL-2), interferon, or other non-study immunotherapy regimens, immunosuppressive agents, other investigational therapies or chronic use of systemic corticosteroids within one week prior to first dose of study drug
- Patients who have received a live vaccine within 4 weeks prior to or after any dose of MK-3475 (exception: inactivated flu vaccines)
- Patients who have received growth factors, including but not limited to granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), erythropoietin, etc. within 2 weeks of study drug administration
- Patient who have had prior treatment with anti-PD-1 (anti-programmed cell death protein 1), anti-PD-L1, anti-PD-L2, anti-CD137, anti-OX-40, anti-CD40, or anti-CTLA-4 antibodies
- Patients with history of any autoimmune disease:inflammatory bowel disease, (including ulcerative colitis and Crohn's Disease), rheumatoid arthritis, systemic progressive sclerosis (scleroderma), systemic lupus erythematosus (SLE) autoimmune vasculitis, central nervous system (CNS) or motor neuropathy considered to be of autoimmune origin.
- Patients who have known history of infection with HIV, hepatitis B, or hepatitis C
- Patients with evidence of interstitial lung disease
- Systemically active steroid use
- Patients on home oxygen
- Patients with oxygen saturation of <92% on room air by pulse oximetry
- Pregnant or lactating
- Conditions, including alcohol or drug dependence, or intercurrent illness that would affect the patient's ability to comply with study visits and procedures
- Patient with known active central nervous system metastases and/or carcinomatous meningitis.
- Patients with primary brain tumors.
- Requires any other form of systemic or localized antineoplastic therapy while on study
- Has any tissue or organ allograft
- Patients with history of allogeneic hematopoeitic stem cell transplant

Contacts and Locations Go to

Information from the National Library of Medicine NIH NLM

To learn more about this study, you or your doctor may contact the study research staff using the contact information provided by the sponsor.

Please refer to this study by its ClinicalTrials.gov identifier (NCT number): ***NCT01876511***

Contacts

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University of Pittsburgh Cancer Institute

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Contact: James Lee, MD, PhD leejj@upmc.edu

Principal Investigator: James Lee, MD; PhD

Sponsors and Collaborators

Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Merck Sharp & Dohme Corp.

Investigators

Principal Investigator: Dung Le, MD Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

More Information

Go to

Publications:

Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Luber BS, Wong F, Azad NS, Rucki AA, Laheru D, Donehower R, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Greten TF, Duffy AG, Ciombor KK, Eyring AD, Lam BH, Joe A, Kang SP, Holdhoff M, Danilova L, Cope L, Meyer C, Zhou S, Goldberg RM, Armstrong DK, Bever KM, Fader AN, Taube J, Housseau F, Spetzler D, Xiao N, Pardoll DM, Papadopoulos N, Kinzler KW, Eshleman JR, Vogelstein B, Anders RA, Diaz LA Jr. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017 Jul 28;357(6349):409-413. doi: 10.1126/science.aan6733. Epub 2017 Jun 8.

Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015 Jun 25;372(26):2509-20. doi: 10.1056/NEJMoa1500596. Epub 2015 May 30.

Responsible Party:	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
ClinicalTrials.gov Identifier:	NCT01876511 History of Changes
Other Study ID Numbers:	J1365
	MK-3475-016 (Other Identifier: Merck)
	NA_00085756 (Other Identifier: JHMIRB)
First Posted:	June 12, 2013 Key Record Dates
Last Update Posted:	March 6, 2019
Last Verified:	March 2019

Keywords provided by Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins:

MSI	Mutation load
MSS (microsatellite unstable)	tumor mutation load
MLH 1	mutation burden
MSH 2	tumor mutation burden (TMB)
MSH 6	hypermutation
PMS2	POLE
BRAF pV600E	MSI Negative with Mutator Phenotype (High Tumor Mutation Burden)
TGFBR2	TMB

Additional relevant MeSH terms:

Colorectal Neoplasms	Gastrointestinal Diseases
Intestinal Neoplasms	Colonic Diseases
Gastrointestinal Neoplasms	Intestinal Diseases
Digestive System Neoplasms	Rectal Diseases
Neoplasms by Site	Pembrolizumab
Neoplasms	Antineoplastic Agents, Immunological
Digestive System Diseases	Antineoplastic Agents

blood

Nivolumab in Patients (Pts) with Relapsed or Refractory Classical Hodgkin Lymphoma (R/R cHL): Clinical Outcomes from Extended Follow-up of a Phase 1 Study (CA209-039)

Stephen Ansell, Philippe Armand, John M. Timmerman, Margaret A. Shipp, M Brigid Bradley Garelik, Lili Zhu, and Alexander M. Lesokhin

Blood 2015 126:583;

Abstract

Introduction: The programmed death-1 (PD-1) immune checkpoint pathway regulates T-cell-mediated antitumor immune responses in solid tumors and hematologic malignancies. Nivolumab (Bristol-Myers Squibb, Ono Pharmaceutical) is a fully human IgG4 PD-1-blocking monoclonal antibody with demonstrated efficacy in a range of tumors. Results from an independent cohort of 23 pts with R/R cHL in a phase 1 study (CA209-039) showed that nivolumab was well tolerated and yielded an overall response rate (ORR) of 87% (Ansell et al, *N Engl J Med*, 2015). This raises important questions including the necessary duration of treatment, the relevance of the depth of response (complete response [CR] vs partial response [PR]), the duration of response, and the feasibility of retreatment. Here, we present the clinical course and post-treatment outcomes from extended follow-up of these pts to shed some light on these questions.

Methods: Pts with R/R cHL received nivolumab 3 mg/kg at weeks (wks) 1 and 4, and then every 2 wks for up to 2 years (yrs). Therapy was stopped earlier in pts with intolerance to treatm progressive disease (PD) without evidence of clinical benefit. Pts who discontinued treatn to toxicity were followed for up to 120 days after discontinuation; other pts were followe after discontinuation. Responding pts discontinued after confirmed CR or 16 wks after

unconfirmed CR, or continued treatment for up to 2 yrs if they had PR or stable disease (SD). Pts who discontinued treatment with ongoing CR, PR, or SD could be retreated for confirmed PD occurring <1 yr after nivolumab discontinuation. Responses were evaluated using the Revised Response Criteria for Malignant Lymphoma (Cheson et al, *J Clin Oncol*, 2007). The primary endpoint was safety, and the key secondary endpoint was antitumor activity.

Results: A total of 23 pts with R/R cHL were treated. The median follow-up observation time is now 86 wks (range: 32-107 wks). Of 20 responders (14 PR, 6 CR), 10 have had durable responses per protocol assessment; their treatment durations and response characteristics are shown in Table 1. Responses were maintained in 2 pts (#5 and #6) after discontinuing nivolumab for >40 wks and in 1 pt (#7) after stopping due to toxicity. Eight pts with durable responses have received nivolumab for >1 yr, including 7 pts who have been in response for >1.5 yrs. One pt (#2) with an initial CR experienced a relapse 43 wks after treatment was discontinued, and achieved a second response (CR) after retreatment with nivolumab. Of the 10 remaining responders, 4 eventually progressed (time to progression [TTP] range: 21.4-92 wks), 1 discontinued treatment due to toxicity with no PD within the 120-day follow-up period, and 5 discontinued nivolumab to undergo stem cell transplant (SCT; 4 allogeneic, 1 autologous) after achieving remission. Time to CR for all responders ranged from 3-88 wks after starting nivolumab, including 2 pts with initial PRs that converted to CRs with continued treatment. All 5 pts who proceeded to SCT had responded to nivolumab within 16 wks of starting treatment (4 PR, 1 CR). Three pts had a best overall response of SD (1 discontinued due to toxicity without documented PD within the 120-day follow-up period; 2 subsequently discontinued for PD [TTP: 15 and 15.3 wks, respectively]). Overall, 3 pts discontinued nivolumab due to adverse events (AEs; Grade 2 peripheral neuropathy, Grade 3 myelodysplastic syndrome, Grade 3 pancreatitis). Grade 1 or 2 immune-related AEs (IR-AEs) occurred in 4 of 10 pts and resolved without treatment in 2 pts. The incidence of IR-AEs did not increase with time on treatment.

Conclusions: In pts with R/R cHL, nivolumab was well tolerated and produced a high ORR. Responses occurred within 16 wks of nivolumab initiation in 15 of 20 pts. Early responses to nivolumab allowed 5 pts to proceed to SCT and lasted ≥1 yr in 7 of 10 pts who did not pursue SCT. One pt achieved CR again after retreatment with nivolumab when relapse occurred within 1 yr of discontinuing treatment following an initial CR.

Pt #	Best Response	Duration of Response, wks	Time to First Response, wks	Time on Treatme
1	PR	90.7	3.6	96+
2	CR	82.1	7.1	91+
3	PR	73.1	7.6	82.4+

Pt #	Best Response	Duration of Response, wks	Time to First Response, wks	Time on Treatment, wks
4	PR	71.4	14.9	88+
5	CR	71.1	3.1	24.9
6	CR	65.1	7.1	22.9
7	PR	55.9	15.3	70.9
8	CR	48.3	39	87
9	CR	45.3	55	82.9
10	PR	41.7	38.7	82.1+

+Still on treatment

Table 1.

Treatment and Response Parameters for Pts with Durable Ongoing Responses

Disclosures Ansell: *Bristol-Myers Squibb:* Research Funding; *Celldex:* Research Funding. Armand: *BMS:* Research Funding; *Infinity:* Consultancy, Research Funding; *Sequenta, Inc.:* Research Funding; *Merck:* Consultancy, Research Funding. Timmerman: *Valor Biotherapeutics:* Research Funding; *Janssen:* Research Funding; *Bristol-Myers Squibb:* Honoraria, Research Funding. Shipp: *BMS:* Membership on an entity's Board of Directors or advisory committees, Research Funding; *Sanofi:* Research Funding; *Bayer:* Membership on an entity's Board of Directors or advisory committees, Research Funding; *Gilead:* Consultancy; *Merck:* Membership on an entity's Board of Directors or advisory committees. Bradley Garelik: *Bristol-Myers Squibb:* Employment. Zhu: *Bristol-Myers Squibb:* Employment. Lesokhin: *Efranat:* Consultancy; *Genentech:* Research Funding; *Aduro:* Consultancy; *Janssen:* Consultancy, Research Funding; *Bristol Myers Squibb:* Consultancy, Research Funding.

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* Asterisk with author names denotes non-ASH members.

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Potential Articles of Interest

Nivolumab for Relapsed or Refractory Classical Hodgkin Lymphoma (cHL) after Autologous

Nivolumab induced responses in melanoma refrac or naive to ipilimumab

Hematopoietic Cell Transplantation (auto-HCT): Extended Follow-up of the Phase 2 Single-Arm CheckMate 205 Study
Philippe Armand et al., Blood, 2018

Bendamustine in Combination with Gemcitabine and Vinorelbine (BEGEV) Is an Effective Regimen for Heavily Pretreated, Relapsed/Refractory Hodgkin Lymphoma Patients: A Multicenter, Retrospective Real-World Study
Antonio Russo et al., Blood, 2018

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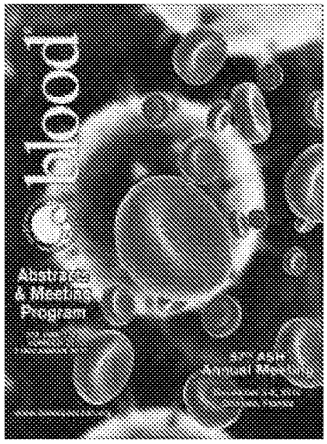


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Issue: 23

Pages: 583
DOI: https://doi.org/

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Comparative effectiveness of 2nd-line targeted therapies for metastatic renal cell carcinoma: analysis of two practice-based chart reviews

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Background: Second-line targeted therapies for metastatic renal cell carcinoma (mRCC) include mammalian target of rapamycin (mTOR) inhibitors and tyrosine kinase inhibitors (TKIs). This study aimed to compare practice-based effectiveness of these therapies in a recent chart review, and to compare findings with a previous chart review (Yang et al. 2012, ASCO).

Methods: Community-based medical oncologists/hematologists (N=36) reviewed charts for ≤15 patients each. Included patients were aged ≥18 years, received a 1st-line TKI and initiated 2nd-line targeted therapy in 2010 or later. The primary outcome was time from 2nd-line initiation to treatment failure (TTF; discontinuation, physician-assessed progression, or death, whichever occurred first). TTF was compared among patients receiving 2nd-line everolimus (EVE), temsirolimus (TEM), or TKI as a class (sunitinib, sorafenib, pazopanib or axitinib), using a multivariable Cox proportional hazards model adjusting for characteristics *including type of initial TKI and response, histological subtype, performance status, and sites of metastasis*. Hazard ratios (HRs) for TTF were pooled with previously-reported HRs for progression-free survival (PFS) from a previous chart review in a meta-analysis.

Results: A total of 138, 64 and 79 patients received 2nd-line therapy with EVE, TEM or a TKI, respectively. Mean age was 63 years, mean duration of mRCC 13.5 months, and median follow-up 6 months. After adjusting for baseline characteristics, EVE was associated with a 28% and 26% reduction in the hazard of TTF compared to TEM and TKI, respectively. Pooling both studies, EVE was associated with significantly reduced hazards of TTF compared to TEM and TKI (Table). TTF differences between TEM and TKI were not significant.

Conclusions: In two retrospective chart reviews EVE was associated with consistently reduced hazards of 2nd-line treatment failure in mRCC compared to TEM and TKIs.

Treatment/ Data	HR (95% Confidence Interval)
EVE vs. TEM	
Previous	0.73 (0.54, 0.97)
Current	0.72 (0.45, 1.16)
Pooled	0.73 (0.57, 0.93)
EVE vs. TKI	
Previous	0.76 (0.55, 1.04)
Current	0.74 (0.48, 1.15)
Pooled	0.75 (0.58, 0.97)

2

Survival following initiation of everolimus for 2nd-line treatment of metastatic renal cell carcinoma: prognostic factors in clinical practice and comparison to clinical trials

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Background: Current overall survival (OS) for metastatic renal cell carcinoma (mRCC) patients in clinical practice may differ from clinical trials. We aimed to identify and validate prognostic factors for OS in practice-based patients receiving 2nd-line everolimus (EVE), and to compare their OS vs. the RECORD-1 trial of EVE for mRCC.

Methods: Two separate samples of oncologists/hematologists reviewed charts for patients initiating 2nd-line EVE between 2009 and 2011 following a 1st-line tyrosine kinase inhibitor (TKI). OS was defined as time from EVE initiation to death (censored at last follow-up). In the 1st sample of charts (the study sample), prognostic factors were identified via multivariable Cox proportional hazards models with stepwise selection. Prognostic factors considered included age, duration of mRCC and 1st-line treatment, metastatic sites, diabetes, histological subtype, ECOG and KPS score, and progression during 1st-line treatment. Model performance was assessed in the 2nd sample (the validation sample). Kaplan-Meier (KM) estimates for OS were compared between chart data and RECORD-1.

Results: The study and validation samples included 220 and 97 patients, respectively. Significant prognostic factors were clear cell histology (hazard ratio (HR) = 2.9), KPS score <80% (HR = 2.9), duration of mRCC <1 year (HR = 2.7), progression on 1st-line TKI (HR = 2.2), and liver metastasis (HR = 1.9) (all $P < 0.05$). In the validation sample, KM estimates for 1-year OS were 90% for patients with 0–2 risk factors, 62% for patients with 3 risk factors, and 20% for patients with 4–5 risk factors (log-rank $P < 0.001$). OS estimates were consistent between both chart samples with 1-year OS probabilities of 67% and 68% and median OS of 19 and 23 months. In RECORD-1 1-year OS was 60% and median OS was 14.8 months (Motzer et al. 2010, Cancer).

Conclusions: Prognostic factors for OS following 2nd-line EVE for mRCC in clinical practice were consistent with those previously identified in trial data. However, OS with 2nd-line EVE in clinical practice was longer than observed in trial data, and was not associated with type of 1st-line TKI.

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Association between guideline-adherent imaging and overall survival following 2nd-line targeted therapy for metastatic renal cell carcinoma

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Background: NCCN guidelines recommend imaging at 2–6 months after initiation of targeted therapy for metastatic renal cell carcinoma (mRCC). This study assessed the association between guideline-adherent imaging and overall survival in mRCC.
Methods: Thirty-six community-based medical oncologists/hematologists reviewed charts for ≤15 mRCC patients each. Included patients were aged ≥18 years and initiated 2nd targeted therapy in 2010 or later. Patients alive up to month 6 were categorized into 3 groups based on time of first imaging test: early (0–60 days), guideline-adherent (61–180 days), and delayed (>180 days). Overall survival (OS) was compared using multivariable Cox proportional hazards models adjusted for age, gender, duration of mRCC, prior treatments, comorbidities, metastatic sites, 1st targeted therapy, ECOG and MSKCC status, 2nd targeted therapy, and progression status (based on symptoms or imaging) in the first 6 months on 2nd line therapy. Reasons for imaging were also assessed.
Results: Among the 192 patients included in the analysis, 25 received early imaging, 136 received guideline-adherent imaging, and 31 received delayed imaging. First imaging tests were more likely due to worsening symptoms or changes in a palpable mass, as opposed to routine monitoring, among patients with delayed vs. guideline adherent imaging (20.0% vs. 7.1%, P=0.005). After adjusting for baseline characteristics, guideline-adherent imaging was associated with a 64% lower hazard of death compared to delayed imaging (hazard ratio (HR)=0.36, 95% confidence interval (CI) 0.14 to 0.93, P=0.035). Adjusted 1-year survival was 82% in the guideline-adherent compared to 67% in the delayed imaging group. The hazard of death did not differ between those with early vs. guideline-adherent imaging (HR=1.05, 95% CI 0.35–3.12, P=0.927).
Conclusions: Among patients surviving 6 months after the initiation of 2nd targeted therapy for mRCC, timely first imaging within 2–6 months, consistent with guidelines, was associated with significantly prolonged overall survival compared to delayed imaging. Further investigation is warranted with larger samples.

4
Contrast enhancement on CT following renal cryoablation – does it represent treatment failure?

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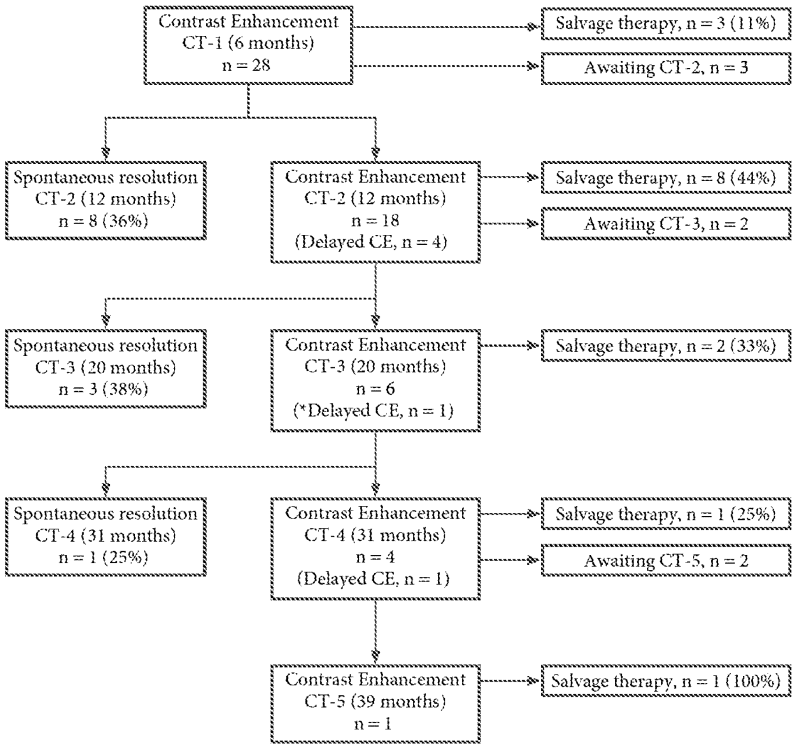
The project was kindly supported by The Danish Cancer Society.

Aim: Renal cryoablation is a valid treatment option for localized pT1a renal cancer. Treatment success is typically defined as absence of contrast enhancement (CE) on follow-up imaging. We investigate the development of lesions that demonstrate CE on follow-up CT after renal cryoablation.
Materials and Methods: A retrospective review of laparoscopic and percutaneous cryoablation procedures from 2005 to 2012 was conducted. A total of 113 patients with a localized pT1a biopsy verified malignant renal lesion was identified. Of these, 34 patients (12 females and 22 males) experienced postoperative CE. Mean patient age was 43 (59–66) yr. Mean tumor size was 25 (22–28) mm. RCC-subtypes: Clear cell (55%), Papillary (12%), Chromophobe (3%), Subtype not specified (30%). There was no statistically significant difference between the patient characteristics with or without CE lesions.
Results: A total of 28 patients (25%) were found to have CE on the initial follow-up CT and additional 6 patients had delayed CE (defined as no CE on prior CT). Spontaneous resolution of the CE was observed in 12 of the 34 patients (35%). Eleven percent of the patients with CE lesions had salvage therapy (including reablation) after the initial follow-up CT at 6 months compared with 44% and 33% after the second CT and third CT, respectively (p>0.05). Two of the 6 patients with delayed CE were reablated, one had oncological treatment due to performance status and three are still awaiting the next follow-up CT.
Conclusion: CE is evident in about ¼ of patients on the initial follow-up CT. At 12 months CE had spontaneously resolved in more than ⅔ of patients, but was continuously observed as late as 31 months. There was a tendency towards intervention if persistent CE was evident at 12 months, but later interventions were not uncommon. As there are no clear-cut criteria for monitoring treatment failure, careful evaluation by an experienced radiologist and urologist is recommend. The significance of delayed contrast enhancement is not clearly evident and needs further investigation.

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Fig. 1 Flow chart of patients with CE on follow-up imaging. Salvage therapy was defined as all secondary curative intended therapies, including reablation. *The patient with delayed CE in CT-3 had no CE on CT-2 but had CE on CT1.



5

The lifetime cost of everolimus vs. axitinib in metastatic renal cell carcinoma patients who have failed prior sunitinib therapy in the US

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Background: Everolimus and axitinib are approved in the US to treat metastatic renal cell carcinoma (mRCC) patients after failure on sunitinib or sorafenib, and one prior systemic therapy (e.g., sunitinib), respectively. Based on their respective clinical trial data, everolimus and axitinib appear to have similar progression-free survival (PFS) efficacies in sunitinib-refractory patients. Therefore, the purpose of this study is to compare the lifetime cost and resource utilization of these two therapies among mRCC patients following failure of sunitinib from a US payer perspective.

Methods: A Markov model was developed to simulate a cohort of sunitinib-refractory mRCC patients and estimate the cost of treating patients with of everolimus vs. axitinib. The following health states were included: stable disease without adverse events (AEs), stable disease with AEs, disease progression (PD), and death. Lacking an overall survival (OS) comparison between the two drugs, the model base case assumed OS equivalency between the arms based on the demonstrated association between PFS and OS (Delea et al. 2012). The model included the following resources: anti-tumor treatments, post-progression treatments, adverse events, physician and nurse visits, scans, blood tests, and palliative care. For drugs, the wholesale acquisition cost was used. Visit, procedure, and test costs were obtained from the Physician's Fee & Coding Guide. Adverse event management and end-of-life care costs were based on the Healthcare Cost & Utilization Project, secondary literature, and expert opinion. Resource utilization was obtained from clinical trial data and an analysis of the MarketScan® Research Database. A 3% annual discount rate was applied to costs and survival estimates. Scenario analyses were performed to determine the model's robustness.

Results: Base case results demonstrated that sunitinib-refractory patients treated with everolimus cost an average of $15,880 (15%) less over their lifetimes than sunitinib-refractory patients treated with axitinib. The primary difference in costs was related to anti-tumor treatment, which was largely driven by axitinib's higher dose intensity. Results remained consistent across sensitivity analyses for AE and PD treatment costs, as well as dose intensity.

Conclusions: Model results suggest that everolimus likely leads to lower lifetime costs than axitinib for sunitinib-refractory mRCC patients in the US.

6

FGF-mediated angiogenesis as independent mechanism of resistance to VEGF/VEGFR targeting

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Background: The growth of new blood vessels is regulated at multiple steps by interactions between several pro- and antiangiogenic factors. FGF/FGFR pathway plays important role in renal cell carcinoma (Tsimafeyeu et al. 2010 ASCO meeting). We believe that the angiogenesis induced by basic fibroblast growth factor (bFGF) is resistant to anti-VEGF/R (vascular endothelial growth factor/receptor) therapy.

Methods: In our first study, angiogenesis was measured with subcutaneously implanted Matrigel plugs containing: bFGF; VEGF; bFGF+bevacizumab; VEGF+bevacizumab; bFGF+anti-FGFR1 monoclonal antibody OM-RCA-01 (Tsimafeyeu et al. 2012 ASCO meeting); VEGF+OM-RCA-01. Control group was without stimulation or treatment. In

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second study, Corneal Micro Pocket Assay (CMPA) was performed. 70 Charles River female C57BL/6 mice were randomized to 7 arms: 1) bFGF; 2) VEGF; 3) bFGF negative, VEGF negative; 4) bFGF + sunitinib; 5) VEGF + sunitinib; 6) bFGF + bevacizumab; 7) VEGF + bevacizumab. Hydron pellets preparation, surgical procedure, and quantification of angiogenesis (angiogenic score) were performed as previously reported (Kenyon BM et al.).
Doses of bFGF, VEGF-A (R&D Systems), sunitinib (Pfizer), bevacizumab (Roche), OM-RCA-01 (OncoMax) were 100 ng, 200 ng, 50 mg/kg, 10 mg/kg, 10 mg/kg per animal, respectively.
Results. There was no neovascularization in bFGF negative, VEGF negative group (mean, 0). bFGF and VEGF strongly induced angiogenesis ($P < 0.001$) in both studies. There were no vessels and endothelial cells in anti-FGFR1 antibody FGF-stimulated group. In bFGF-induced angiogenesis, sunitinib (mean, 3.9; SEM, 0.1; $P = 0.2$) and bevacizumab (mean, 4.71; SEM, 0.33; $P = 0.85$ for CMPA and $P = 0.5$ for Matrigel assay) did not impact on neovascularization in comparison with bFGF positive control. The angiogenic effect of VEGF was significantly inhibited by both sunitinib (mean, 0.38; SEM, 0.06; $P = 0.001$) and bevacizumab (mean, 0.75; SEM, 0.05; $P = 0.001$ for CMPA and $P < 0.0001$ for Matrigel assay) in comparison with VEGF positive control. OM-RCA-01 not significantly inhibited growth of vessels in VEGF positive group ($P = 0.064$).
Conclusions. Targeting FGFR1 inhibited FGF-induced angiogenesis. Targeting VEGF(R) significantly impacted on VEGF-induced angiogenesis and not on bFGF-induced neovascularization.
The study was supported by Terry Fox Foundation.

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A phase 3 randomized study of cabozantinib (XL184) vs everolimus in subjects with clear cell renal cell carcinoma (METEOR)

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Background. Cabozantinib is an orally bioavailable tyrosine kinase inhibitor (TKI) with potent activity against MET and VEGFR2. Cabozantinib has demonstrated clinical activity in heavily pretreated subjects with clear cell carcinoma (ccRCC) in a single arm trial (Choueiri et al.; ASCO 2012, abstract 4504). VHL tumor suppressor gene dysfunction is commonly found in sporadic ccRCC and evident in all hereditary ccRCCs. This results in impaired HIF1α degradation and consequent activation of MET and VEGF pathways. Moreover, MET has been implicated in adaptive resistance to VEGF-pathway targeted agents. Based on clinical activity seen to date and scientific rationale, there is strong interest in the evaluation of cabozantinib in ccRCC.
Methods. We designed a Phase 3 randomized, open-label study to evaluate the effect of cabozantinib compared with everolimus in subjects with ccRCC (NCT01865747).The protocol-defined primary efficacy endpoint is progression-free survival (PFS) as determined by an independent radiology review committee (IRC). Secondary efficacy endpoints include overall survival (OS) and objective response rate (ORR). Exploratory analyses include safety and tolerability, tumor MET status, circulating tumor cells, bone markers, plasma biomarkers, skeletal-related events, and changes in health-related quality of life (HRQoL). Approximately 650 subjects will be randomized in 1:1 fashion to receive either cabozantinib or everolimus. Randomization will be stratified by number of prior VEGFR TKI therapy and MSKCC prognostic criteria (Motzer et al.; J Clin Oncol 22:454–463, 2004). Eligible subjects must have documented diagnosis of renal cell cancer with a clear-cell component and progressed after one or more prior VEGFR TKI therapy. Tumor assessments will include CT/MRI scans and technetium bone scans. Radiographic response will be evaluated by IRC using RECIST criteria (version 1.1). Skeletal related events are defined as radiation therapy to bone, pathological fracture, spinal cord compression, and surgery to bone. HRQoL will be assessed using the NCCN-Functional Assessment of Cancer Therapy (FACT) Kidney Symptom Index (FKSI-19) questionnaire and the EuroQol Health questionnaire (EQ-5D-5L). Survival of subjects will be followed at regular intervals.

8
Sunitinib expanded-access trial in metastatic renal cell carcinoma (mRCC) – final US results

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Background. Sunitinib is established as a reference standard of care and approved worldwide for mRCC patients. Prior to its regulatory approval, >4,500 patients were enrolled from 2005–2007 in a global mRCC expanded-access trial (ClinicalTrials.gov, NCT00130897; Pfizer) with sunitinib given to patients ineligible for other sunitinib trials. Sunitinib was shown to have a manageable safety profile and encouraging efficacy in this broad population of patients (Gore et al. 2009). Here we report final efficacy and safety results for the 320 patients in the US who enrolled in this trial.
Methods. Patients aged ≥18 years with treatment-naïve or previously treated

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mRCC received oral sunitinib at 50 mg/day on a 4-week-on/2-week-off schedule. Tumor measurements were scheduled per local practice and measured using RECIST. Safety was assessed regularly. Objective response rate (ORR), progression-free survival (PFS), overall survival (OS), and treatment-related adverse events (AEs) are reported. Analyses included patients receiving ≥1 sunitinib dose.
Results: The treated patients included 30% aged ≥65 years, 7% with non-clear cell RCC, 8% with ECOG performance status (PS) ≥2, and 12% with baseline brain metastases. Median treatment duration and follow-up were 5.7 and 12.7 months. 305 patients (95%) discontinued treatment; reasons included lack of efficacy (32%), death (19%), or AEs (15%). Overall ORR was 14%, with ORR in subgroups of interest as follows: aged ≥65 years, 9%; non-clear cell RCC, 9%; ECOG PS ≥2, 0%; and baseline brain metastases, 5%. Median PFS was 9.4 months (95% CI: 7.9–12.0) and median OS was 16.6 months (95% CI: 13.7–20.7). The most common any-grade treatment-related AEs were fatigue (64%), diarrhea (54%), nausea (41%), dysgeusia (35%), decreased appetite (31%), vomiting and mucosal inflammation (both 29%), hand-foot syndrome (26%), stomatitis (23%), hypertension (22%), thrombocytopenia (21%), and rash (20%). The most common grade 3/4 treatment-related AEs were fatigue (11%), thrombocytopenia and neutropenia (both 8%), hypertension (7%), and diarrhea (5%).
Conclusions: Among patients from the US treated in an mRCC global expanded-access trial, efficacy with sunitinib was favorable and the safety experience was similar for a broader patient population than was eligible for clinical trials, comparable to results previously reported in the overall population.

9
Multiplexed tissue protein assay as a predictor of response to targeted therapy in advanced renal cell carcinoma

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Background: Although a number of prognostic biomarkers have been explored in advanced renal cancer, to date none have been useful in therapeutic outcome prediction.
Methods: Following regulatory approval, formalin-fixed paraffin embedded pre-therapy (sunitinib and/or mTOR inhibitors) tissue samples and clinical data on kidney cancer patients (pts) were obtained. The samples were analyzed using layered immunohistochemistry which allows analysis of multiple biomarkers using a single tissue section. Multiplexed panels of protein biomarkers were used to probe tissue sections for proteins along the PI3K/AKT/mTOR and/or the VEGFR/PDGFR signaling pathways. Expression of biomarkers in the cancer was scored, and predictive scores which correlated with the patient's clinical outcome status generated.
Results: Tissue samples of 51 pts treated with sunitinib were analyzed. A predictive score was generated by combining the scores assigned to VEGFR1 and VEGFR2 and multiplying the sum with the score of VEGFA. If the predictive score was equal to or above 24, pt was predicted to be a responder; to have an objective response (OR)/stable disease (SD). If score <24 the pt was predicted to have no benefit from sunitinib. 27 of 33 OR/SD pts and 15 of 18 with progressive disease (PD) were accurately predicted. The accuracy of the test was noted to be 83.3% and sensitivity and specificity were 81.8% and 83.3% respectively. Median times to treatment failure were 12 months (95%CI 8–24 months) and 3 months (95%CI 3–12 months), respectively on the patient groups with scores >24 or <24.
Tissue samples of 33 pts treated with mTOR inhibitor were analyzed. 3 biomarkers in the mTOR pathway (pmTOR (Ser 2448), p4EBP1 (Ser 65), p4EBP1 (Thr 37-46)) were used to create a predictive score. 8 of 12 OR/SD pts (sensitivity 67%) and 17 of 21 PD pts (specificity 76%) were accurately predicted using a score cutoff of 6.
Conclusions: These results indicate that an assay based on multiplexed protein analysis of tumor tissue is capable of providing clinically applicable information to help guide therapy. Prospective testing with a larger sample size is required. Funding source: Supported in part by NCI BRIDGE Grant 5R44CA123994-06 and by NCI SBIR Contract No. HHSN261201000135C.

10
Role of cytoreductive nephrectomy (CN) in patients with metastatic non-clear cell renal cell carcinoma (nccRCC): results from a surveillance, epidemiology, and end results (SEER) database analysis of over 4,900 patients

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Background: The role of CN in patients with metastatic nccRCC is largely unknown. Additionally, the impact of CN in these patients in the era of molecularly targeted therapy (MTT) is not defined. Herein, we determine the impact of CN on mortality in patients with metastatic nccRCC.
Methods: We used the SEER database to identify 4,914 patients diagnosed with metastatic RCC between 2000–2009. Of these patients, 2,994 (61%) underwent CN and 591 (12%) had NCC histology. The primary outcome was RCC-specific mortality (RSM) and secondary outcome was overall survival (OS).
Results: Patients with NCC histology undergoing CN had a lower likelihood of RSM ($p<0.001$). This association was retained after adjustment for age at diagnosis, gender, race, marital status, year of diagnosis, geographic location, and different NCC histology, (Hazard Ratio (HR) 0.62, 95% Confidence Interval (CI) 0.48–0.80, $p<0.001$) (Table 1). Additionally, patients having undergone CN had improved OS compared to those who did not (14 versus 6 months, $p<0.001$). In multivariable analysis, CN

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was associated with a significant improvement in OS (HR 0.45, 95% CI 0.37–0.55, p<0.001). The association between CN and improved RSM and OS in patients treated in the era of MTT (2006–2009) remained significant (HR 0.50 and HR 0.43, respectively).
Conclusions: CN in patients with metastatic nccRCC appears to be associated with a survival benefit in patients treated in the era of MTT and regardless of the underlying nccRCC histology. CN remains a treatment option for patients with metastatic RCC who are appropriate surgical candidates. Limitations of this data include the absence of information regarding comorbidities, performance status, and disease burden.

standardized system has been developed in adults. The RENAL Nephrometry system is a valid instrument across institutions and between users which reliably predicts tumor complexity and clinical outcomes such as the ability to complete partial nephrectomy (PN) and the incidence of peri-operative complications. We propose that such a standardized system would be a useful in children, adolescents, and young adults with renal masses. Our goal was to describe RENAL Nephrometry in such a population and correlate this score with surgical and pathologic outcomes.
Methods: We reviewed all patients at our institution who underwent resection of a renal tumor between 2002–13, and who had pre-operative, contrast-enhanced, cross-sectional imaging available. We assessed the RENAL Nephrometry score for each affected kidney. RENAL Nephrometry is based on 5 features characterizing renal tumor anatomy as they relate to resectability. The mass is assigned low (4–6), moderate (7–9) or high (10–12) complexity based on the sum score. We then compared tumor characteristics and outcomes between the groups of low, moderate, and high complexity masses.
Results: We identified 65 patients and 67 kidneys meeting study criteria. Median age at diagnosis was 3.5 yr (0.1–27.6). We identified 5 low-complexity, 10 moderate-complexity, and 52 high-complexity lesions (Table). In comparing the clinical and pathologic features between groups, we observed that less complex masses were observed in older patients, more commonly managed with PN, and more often represented Renal Cell Carcinoma (RCC), and non-Wilms Tumor (WT) pathology. We did not observe a statistically significant correlation between tumor complexity and EBL, Operative time, Intraoperative transfusion, Positive margins, or Tumor rupture.
Conclusion: Typically, renal lesions in this population are highly complex. RENAL Nephrometry appears most useful in evaluating and discriminating RCC and masses in older children and adolescents. However, it requires further refinement to capture nuances in distinguishing the complexity of other pediatric renal malignancies such as WT.

Table 1 RSM and OS, based on CN status, in metastatic RCC.

Histology	RSM				OS			
	N (No CN/CN)	1-Year RSM (No CN/CN) (%)	HR (95% CI)	P-Value	N (No CN/CN)	Median OS (No CN/CN) (months)	HR (95% CI)	P-Value
Clear-Cell	1437/2322	61.0/80.1	0.48 (0.44–0.53)	<0.001	1775/2669	6/22	0.40 (0.37–0.43)	<0.001
Papillary	128/183	54.6/36.5	0.71 (0.53–0.96)	0.02	162/236	7/15	0.53 (0.42–0.67)	<0.001
Chromophobe	23/61	77.9/22.3	0.32 (0.16–0.65)	0.002	29/71	5/36	0.17 (0.10–0.29)	<0.001
Collecting Duct/ Medullary	26/66	79.3/68.4	0.52 (0.27–1.01)	0.05	32/81	4/7	0.44 (0.28–0.68)	<0.001

11
The initial experience with RENAL nephrometry in children, adolescents, and young adults with renal tumors

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Background: Currently, no standard manner exits to compare renal tumor complexity in children and adolescents, making it impossible to speak a "common language" when comparing lesions and the impact of tumor complexity on surgery and outcomes. Recently, such a

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Table Comparison of Renal Masses with Low, Moderate and High Complexity RENAL Nephrometry Scores.

	Low Complexity N=5	Moderate Complexity N=10	High Complexity N=52	Overall p-value	Low vs Mod p-value	Mod vs High p-value	Low vs High p-value
Median Age at Dx (Years)	15.9 (3.1–27.6)	6.7 (0.1–17.7)	3.3 (0.1–23.3)	**0.009**	0.075	0.11	**0.006**
Sex	1M:4F	4M:6F	28M:24F	0.29	0.6	0.52	0.194
R – Largest tumor diameter				**<0.001**	0.5	**<0.001**	**<0.001**
1: ≤4 cm	5 (100%)	7 (70%)	3 (5.8%)				
2: 4 to 7 cm	0 (0%)	3 (30%)	4 (7.7%)				
3: ≥7 cm	0 (0%)	0 (0%)	45 (86.5%)				
E – % Exophytic				**<0.001**	**0.001**	0.36	**<0.001**
1: ≥50%	4 (80%)	3 (30%)	0 (0%)				
2: <50%	1 (20%)	7 (70%)	8 (15.4%)				
3: Entirely Endophytic	0 (0%)	0 (0%)	44 (84.6%)				

	Low Complexity N=5	Moderate Complexity N=10	High Complexity N=52	Overall p-value	Low vs Mod p-value	Mod vs High p-value	Low vs High p-value
N – Nearness to CS/Sinus				**<0.001**	**0.004**	1	**<0.001**
1: ≥7 mm	4 (80%)	0 (0%)	0 (0%)				
2: 4 to 7 mm	1 (20%)	0 (0%)	0 (0%)				
3: ≤4 mm	0 (0%)	10 (100%)	52 (100%)				
A – Ant vs. Post Location				**0.012**	0.74	**0.003**	0.22
a: Completely Anterior	2 (40%)	3 (30%)	10 (19.2%)				
p: Completely Posterior	1 (20%)	4 (40%)	3 (5.8%)				
x: Both	2 (40%)	3 (30%)	39 (75.0%)				
L – Location to PLs				**<0.001**	**0.007**	**<0.001**	**<0.001**
1: Entirely above or below	5 (100%)	2 (20%)	0 (0%)				
2: Crosses the PL	0 (0%)	8 (80%)	3 (5.8%)				
3: >50% across or between	0 (0%)	0 (0%)	49 (94.2%)				
Sum Score	4: 2 (40%)	7: 0 (0%)	10: 6 (11.5%)				
	5: 2 (40%)	8: 2 (20%)	11: 11 (21.2%)				
	6: 1 (20%)	9: 8 (80%)	12: 35 (67.3%)				
Surgery							
Radical Nephrectomy (RN)	2 (40%)	3 (30%)	51 (98.1%)	**<0.001**	1	**<0.001**	**0.001**
Partial Nephrectomy (PN)	3 (60%)	7 (70%)	1 (1.9%)				
Completed Case as PN	3 of 3 (100%)	7 of 7 (100%)	1 of 1 (100%)				
Positive Margin	1 (20%)	1 (10%)	5 (9.6%)	0.77	1	1	0.44
Tumor Spill	0 (0%)	0 (0%)	3 (5.8%)	0.64	1	1	1
EBL (mL)	75 (25–700)	150 (65–550)	50 (5–1860)	0.31	0.76	0.19	0.35
Intraoperative Transfusion	1 (20%)	2 (20%)	12 (23.1%)	0.97	1	1	1
Operative Time (min)	199 (159–240)	261.5 (215–508)	283.5 (77–711)	0.22	0.34	0.98	0.08
Pathology				**0.014**	0.45	**0.039**	**0.006**
Wilms Tumor (WT)	1 (20%)	4 (40%)	37 (71.2%)				
Renal Cell Carcinoma	4 (80%)	3 (30%)	4 (7.7%)				
Clear Cell Sarcoma (CCSK)	0 (0%)	0 (0%)	4 (7.7%)				
Mesoblastic Nephroma	0 (0%)	1 (10%)	2 (3.8%)				
Rhabdomyosarcoma	0 (0%)	0 (0%)	2 (3.8%)				
Inf. Myofibroblastic Tumor	0 (0%)	1 (10%)	0 (0%)				
Fibrosarcoma	0 (0%)	0 (0%)	1 (1.9%)				
Juxtaglomerular Tumor	0 (0%)	1 (10%)	0 (0%)				
Cystic Nephroma	0 (0%)	0 (0%)	1 (1.9%)				
Metanephric Adenoma	0 (0%)	0 (0%)	1 (1.9%)				
RCC vs.	4 (80%)	3 (30%)	4 (7.7%)	**<0.001**	0.12	0.076	0.194
All other pathologies	1 (20%)	7 (70%)	48 (92.3%)				
Wilms Tumor/CCSK vs.	1 (20%)	4 (40%)	41 (78.8%)	**0.003**	0.6	**0.02**	**0.014**
All other pathologies	4 (80%)	6 (60%)	11 (21.2%)				

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Quadrant biopsy technique improves diagnostic ability for large renal masses

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Objectives: Standard percutaneous biopsy is generally obtained from one location within a renal mass and thus prone to sampling error, particularly in large heterogeneous tumors. Indeterminate results or failure to detect poor prognostic features is common even when several samples are obtained from a single area. The purpose of this study was to evaluate the accuracy of percutaneous biopsy for large renal masses using a modified technique that includes core biopsies from multiple locations within each tumor compared with standard biopsy.
Patients and Methods: Clinical and pathologic data for all patients with ≥cT2 renal masses who underwent percutaneous biopsy at a single institution from 5/2009–6/2013 was reviewed. The quadrant biopsy technique was developed in 2011 and includes core biopsies from four separate solid enhancing areas within the tumor using an 18-gauge needle. An expert genitourinary pathologist performed a blinded central review of each core and nephrectomy specimen. Results from quadrant biopsy technique were compared with those from standard biopsy.
Results: Of 72 biopsies performed in 68 patients, 30 used standard and 42 utilized a quadrant technique. Median number of cores obtained in standard technique and quadrant technique was 2 and 5 respectively. Biopsy was diagnostic in 26/30 (86.6%) standard biopsies and 42/42 (100%) quadrant biopsies. When evaluating individual cores, fibrotic/necrotic material or benign kidney was present in 18.3% of quadrant biopsy specimens. Nephrectomy was performed in 37/68 (54.4%) of patients after biopsy. When comparing nephrectomy to standard or quadrant biopsy technique, 2/8 (25%) and 0/29 (0%) patients had an inaccurate diagnosis of the presence of malignancy made from each technique respectively. Sarcomatoid de-differentiation was identified in 1/30 (3.3%) standard and 7/42 (16.6%) quadrant biopsies. Using the quadrant technique, sensitivity for detecting sarcomatoid features was 85% and 81% when compared to nephrectomy specimens or among all biopsy cores, respectively. Post-procedure complications were reported in 1/72 (1.4%) patients. One patient was admitted to the hospital after standard technique biopsy for hematoma and required no further intervention.
Conclusions: Sampling multiple locations in large renal tumors may increase diagnostic capability to guide entrance into pre-surgical clinical trials for metastatic RCC, facilitate surgical planning or improve informed consent. Sensitivity for detecting poor prognostic features is increased using quadrant technique when compared with historical series.

13
Proposal of an algorithm for utilization of percutaneous renal mass biopsy based on the results of 1000 case series

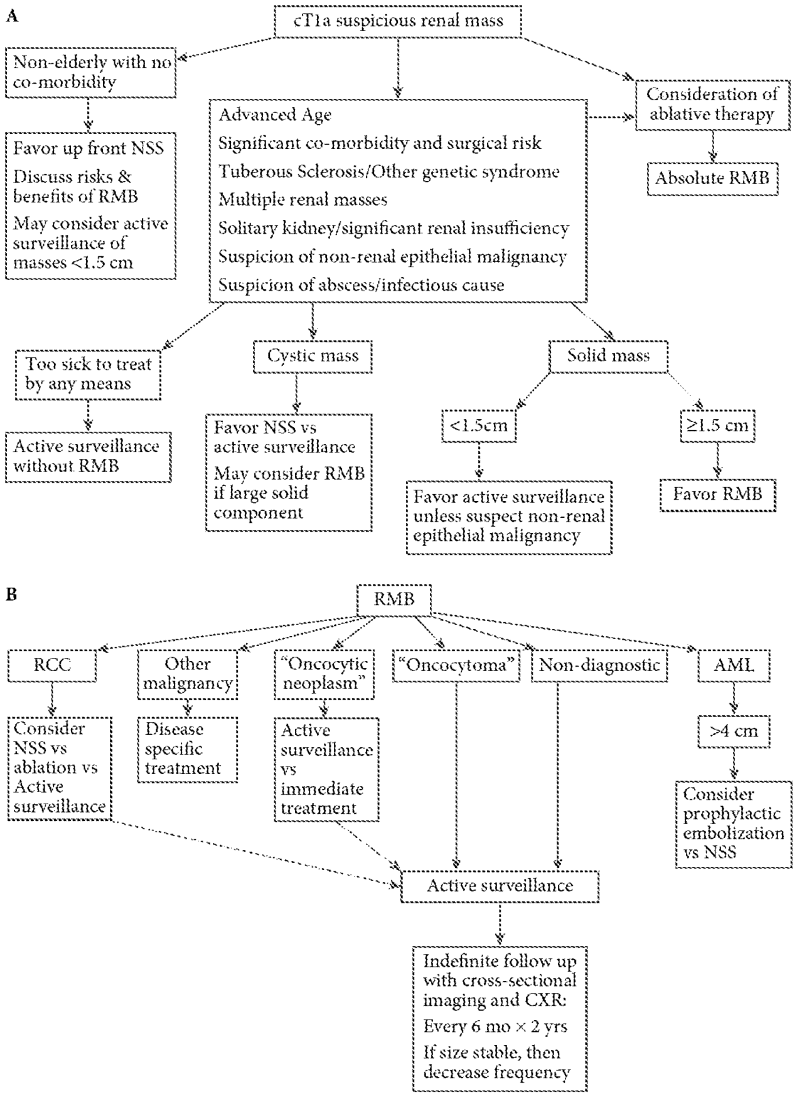
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Introduction: Renal mass biopsy (RMB) is a diagnostic option for evaluation of suspicious renal masses. Current guidelines offer little guidance about selection of appropriate masses for diagnostic biopsy and management after RMB results have been obtained. We propose an algorithm for utilization of RMB based on our database of 1000 RMBs.
Methods: Under IRB approval, we performed a retrospective review of our database of 1000 adult patients who underwent a RMB at Massachusetts General Hospital (1997–2010). The decision for RMB was at the discretion of the urologist and patient, although all masses considered for ablative therapy were biopsied routinely. Core biopsies were performed using 15–18 gauge coaxial needles and fine needle aspirations (FNA) were performed with 20–23 gauge Chiba needles.
Results: 80% of tumors were 0–4 cm, 16% were >4–7 cm, 4% were >7 cm. 82% were solid and 18% were cystic. 94% underwent core biopsies + FNA, 5% had core biopsies only and 1% had FNA only. 216/1000 (22%) biopsies were non-diagnostic. Solid lesions had a non-diagnostic rate of 13% (104/821) compared with 63% (112/179) in cystic lesions ($p < 0.0001$). The non-diagnostic rate increased with a decrease in tumor size (<2 cm: 21%, 2–4 cm: 9.5%, >4–7 cm: 11%, >7 cm: 6.3%; $p < 0.0001$) and the non-diagnostic rate for tumors <1.5 cm was 42%. Of diagnostic biopsies, the identification of RCC increased with tumor size (<2 cm: 65%, 2–4 cm: 73%, >4–7 cm: 79%; >7 cm: 87%; $p < 0.0001$). Of the 216 tumors with an initial non-diagnostic RMB, 55 underwent eventual repeat biopsy, revealing 26 (47%) RCC or other malignancy, 32 (58%) non-diagnostic, 3 (5.5%) oncocytoma and 2 (3.6%) angiomyolipoma. 33/216 non-diagnostic RMB eventually had surgery, with 30 being RCC or other malignancy on pathology. Based on our results we propose an algorithm (see Figure opposite) for selection of small renal masses for biopsy and management after RMB.
Conclusions: RMB can be an effective method for diagnostic evaluation of a suspicious renal mass. However, utilization of RMB and management afterwards should be based on both the properties of the mass as well as the clinical history of the patient. We present an effective algorithm for approaching the suspicious cT1a renal mass.

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Figure



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Safety, durable clinical benefit, and remission resulting from nivolumab (Anti-PD-1; BMS-936558; ONO-4538) in a phase 1 trial in patients with previously treated metastatic renal cell carcinoma (mRCC): long-term patient follow-up

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Background: Programmed death-1 (PD-1) is an immune checkpoint receptor that negatively regulates T-cell activation. PD-L1, a PD-1 ligand, is associated with poor prognosis in mRCC. In a phase 1 study, the PD-1 receptor-blocking antibody nivolumab was generally well tolerated and showed encouraging antitumor activity in patients with previously treated mRCC and other solid tumors. We report overall survival (OS), long-term safety, and updated response data after a minimum 1-year follow-up for the mRCC cohort.

Methods: Patients received nivolumab 0.1–10 mg/kg every 2 weeks intravenously in 8-week treatment cycles for 24 months or until discontinuation criteria were met. A maximum tolerated dose was not reached up to 10 mg/kg; cohorts were expanded at the 1- and 10-mg/kg dose levels.

Results: 34 patients received nivolumab at 1 mg/kg (n = 18) or 10 mg/kg (n = 16). Of these, 15 patients (44%) had received ≥3 prior therapies. Responses were noted in 10/34 patients (Table). An additional 3 patients showed unconventional "immune-related" responses. Among responding patients, 40% had responses at first tumor assessment (8 weeks). As of March 2013, 4/10 patients continued to respond. Median duration of response was 56.1 weeks for both doses (range: 36.6–126.7+). Among 7 patients who discontinued treatment while responding for reasons other than progressive disease, responses persisted in 4 patients for ≥19 weeks off

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treatment (19, 45+, 51+, and 59+ weeks). OS rates at 1 and 2 years were 70% and 50%, respectively, with 19 patients alive after 24 months median follow-up. The incidence of all-grade and grade 3–4 treatment-related adverse events (AEs) was 85% and 18%, respectively. The most common AEs included fatigue, rash, diarrhea, and pruritus. Treatment-related pneumonitis occurred in 1 patient (grade 1). Select, potentially immune-related AEs requiring more frequent monitoring and/or specific intervention included skin (35%), endocrinopathies (18%), gastrointestinal (18%), and hepatic (12%).
Conclusions: In a cohort of heavily pretreated patients with mRCC, long-term continuous dosing of nivolumab was generally well tolerated and produced durable clinical benefit, which was maintained off therapy in a subset of patients. These findings provide the basis for an ongoing randomized phase 3 trial of nivolumab in mRCC (NCT01668784).

Dose, mg/kg	Objective Response Rate, % (n/N)	Stable Disease Rate, % (n/N)		Median Progression-Free Survival, Months	Median OS, Months
		≥24 Weeks	≥48 Weeks		
All doses	29 (10/34)	26 (9/34)	6 (2/34)	7.3	>22[a]
1	28 (5/18)	22 (4/18)	6 (1/18)	4.7	Not reached
10	31[b] (5/16)	31 (5/16)	6 (1/16)	8.0	18.8

[a]Median OS not reached at 22 months (the longest time to death so far)
[b]1 complete response

Clinical Trial Registration Number: NCT00730639.

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A phase 3 comparative study of nivolumab (anti-PD-1; BMS-936558; ONO-4538) versus everolimus in patients with advanced or metastatic renal cell carcinoma (mRCC) previously treated with anti-angiogenic therapy

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Background: Standard approved treatments for patients with mRCC include interleukin-2, VEGF TKIs (e.g. sunitinib, pazopanib, axitinib, sorafenib) or mTOR inhibitors (temsirolimus, everolimus). However, many patients develop resistance, and overall survival (OS) improvement has only been shown in one phase 3 trial in poor-risk, untreated patients. Although sequential treatment with targeted therapies may improve OS for some patients with mRCC, more data are needed to confirm this finding. Everolimus demonstrated a 3-month improvement in median progression-free survival (PFS) but showed no improvement in OS versus placebo in patients who progressed following VEGF-targeted therapy (Motzer RJ, et al. Lancet. 2008;372:449–56). Programmed death-1 (PD-1) is an immune checkpoint receptor that negatively regulates T-cell activation and PD-1 overexpression by tumor infiltrating lymphocytes has been associated with poor prognosis in multiple tumor types including mRCC. A phase 1 study of the fully human PD-1 receptor blocking monoclonal antibody nivolumab showed encouraging antitumor activity in previously treated mRCC (objective response rate [ORR], 29% (10/34); stable disease at ≥24 weeks, 27% [9/34]; poster presentation at ASCO 2013; JCO 2013;31(15 suppl):abstract 4514). Therefore, an ongoing, randomized, open-label, global phase 3 trial was developed to evaluate the clinical benefit of nivolumab in patients with mRCC previously treated with anti-angiogenic therapy.
Methods: 822 patients with advanced or metastatic clear-cell mRCC who have received ≤2 prior anti-angiogenic therapies and ≤3 total prior systemic regimens will be randomized 1:1 to receive nivolumab 3 mg/kg intravenously every 2 weeks (Arm A) or everolimus 10 mg orally daily (Arm B). Patients will be stratified by region, MSKCC risk group, and number of prior anti-angiogenic therapy regimens. Tumor response will be assessed using RECIST 1.1 every 8 weeks following randomization for the first year and then every 12 weeks until disease progression or treatment discontinuation, whichever occurs later. The primary endpoint is OS. Secondary endpoints include PFS, ORR, OR duration, adverse events, OS in PD ligand 1 (PD-L1) positive or negative subgroups, and patient-reported outcomes. The trial is open and enrolling patients.

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Impact of bone metastases (BM) and bisphosphonate use in patients with metastatic renal cell carcinoma (mRCC) treated with targeted therapy: Results from a pooled clinical trials database

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Background: BM are frequently present in patients with mRCC and are associated with high rates of skeletal-related events (SREs), defined as pathologic fractures, need for bone radiotherapy or surgery, and spinal cord compression. The objective of this study was to investigate the prognostic significance of BM and to analyze the role of bisphosphonate therapy in patients with mRCC treated with targeted therapy.
Methods: We conducted a pooled retrospective analysis of patients with mRCC treated from 2003–2011 on phase III (NCT00083889, NCT00065468, NCT00678392) and phase II clinical trials (NCT00054886, NCT00077974, NCT00267748, NCT00338884, NCT00137423). Statistical analyses were performed using Cox regression and the Kaplan-Meier method.
Results: We identified 2,749 patients treated with sunitinib (n=1,059), sorafenib (n=355), axitinib (n=359), temsirolimus (n=208), temsirolimus+interferon-alfa (IFN-α) (n=208), or IFN-α (n=560), of whom 28% had BM. Most patients were less than 65 years of age (66.5%), with clear-cell histology (89.1%), and prior nephrectomy (83.6%). 285 patients (10.4%) received treatment with bisphosphonate therapy. No patient received denosumab.

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The presence of BM was associated with a shorter progression-free survival (PFS) and overall survival (OS) when compared to those without BM (Table 1). The use of bisphosphonate therapy was not independently associated with improved survival. The rate of SREs in patients with BM receiving treatment with a bisphosphonate was 1.8% (n=14) compared to 4.6% (n=36) in patients not receiving a bisphosphonate (p=0.191). Adverse events for patients treated with bisphosphonate therapy compared to patients not receiving bisphosphonate therapy included: osteonecrosis of the jaw (2.1% versus <0.1%), hypocalcemia (7.4% versus 3.0%), and renal insufficiency (4.9% versus 2.5%).

Conclusions: In this analysis, we confirm that the presence of bone metastases is associated with shorter survival in patients with mRCC. Bisphosphonate therapy did not have an impact on survival.

Table 1 PFS and OS based on presence of BM and treatment with bisphosphonate therapy

	Median PFS (months)	Median OS (months)
Presence of BM (n=781)	5.05	13.17
Absence of BM (n=1968)	6.73	20.20
	p=0.0008	p=0.0001
	HR=0.837	HR=0.774
Treated with bisphosphonate therapy (n=162)*	5.12	13.34
Not treated with bisphosphonate therapy (n=619)*	4.93	13.11
	p=0.1785	p=0.3891
	HR=1.154	HR=1.106

HR=Hazard Ratio.
*Patients with BM.

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Combined *low activity GSTA1* and *active GSTT1* genotypes modify risk for renal cell carcinoma

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Objective: Obesity, hypertension, smoking and professional exposure to carcinogens are the recognized risk factors for clear renal cell carcinoma (cRCC). Cytosolic glutathione transferases (GST) catalyze conjugation reaction of electrophylic compounds to glutathione. Although polymorphic expression of GST enzymes confers increased risk for various cancers, the role of GST polymorphisms in susceptibility to cRCC is still controversial. We aimed to assess whether common GST polymorphisms are associated with higher risk for cRCC, independently or in conjunction with recognized risk factors, as well as, to identify their value in tumor progression.

Methods: A hospital-based case-control study recruited 98 patients with cRCC and 240 healthy controls. *GSTA1*, *GSTT1*, *GSTP1* and *GSTO1* genotypes were determined by PCR. The associations between the genotypes and cRCC risk were examined by using logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs).

Results: Regression analysis showed that *low activity GSTA1 (CT/TT)* and *active GSTT1* genotypes were associated with higher, but statistically non-significant effect on risk for cRCC (OR=1.4 CI:0.7–2.4 and OR=1.8 CI:0.9–3.5, respectively) after adjustment for age, gender and obesity. However, when the effect of these two genotypes was analyzed in combination, patients with combined *low activity GSTA1/active GSTT1* genotype exhibited 5-fold higher risk (CI:1.1–22.9, p=0.034) than those with *GSTA1 CC/GSTT1 null* genotype. Patients homozygous for *GSTO1 A* and *GSTP1 Ile* alleles exhibited higher, but non-significant risk compared to the patients homozygous for *GSTO1 C* and *GSTP1 Val* alleles (OR=1.6, CI:0.7–3.8, OR=1.5 CI:0.6–3.6, respectively). Concerning the association of GST genotypes with cRCC progression, patients with *active GSTT1* genotype, or carrying *GSTO1 A* and *GSTP1 Ile* alleles had tumors of higher grade than those with *GSTT1 null* genotype or homozygous for *GSTO1 C* or *GSTP1 Val* alleles.

Conclusions: Patients with combined *low activity GSTA1* and *active GSTT1* genotypes are at higher risk of developing cRCC.

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ADAPT: An Ongoing international phase 3 randomized trial of autologous dendritic cell immunotherapy (AGS 003) plus standard treatment in advanced renal cell carcinoma (RCC)

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Background: AGS-003 is an autologous immunotherapy designed to induce a T-cell response specific to a patient's tumor antigens. It is prepared from matured monocyte-derived dendritic cells co-electroporated with patients' amplified tumor RNA plus synthetic CD40L RNA. Sunitinib is a tyrosine kinase inhibitor (TKI) that has been well established as a standard first-line treatment for advanced RCC. The antitumor activity of sunitinib combined with its ability to reverse the immune suppression observed in RCC patients provides strong rationale to combine sunitinib with AGS-003. In a single arm phase 2 study, AGS-003 plus sunitinib was safe and yielded a median overall survival (OS) of over 30 months in newly diagnosed, synchronous, unfavorable risk mRCC patients.

Methods: The ADAPT study is a randomized (2:1), international phase 3 study comparing standard targeted therapy ± AGS-003. While standard targeted therapy initiates with sunitinib for all subjects, changes to other TKIs or mTOR inhibitors is permitted for early progression or toxicity. Patients randomized to the combination arm will receive AGS-003 once every 3 weeks for 5 doses, followed by quarterly booster doses. At week 48, patients with stable disease or

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better will continue booster dosing every 12 weeks until progression.
The primary objective is to compare the median OS between treatment arms. Additional endpoints include PFS, safety, immunologic and tumor response.
The study is enrolling adults with synchronous, metastatic clear cell RCC who are good candidates for surgery and targeted therapy initiating with sunitinib, 1–4 Heng risk factors (intermediate or poor risk), KPS ≥ 70%, life expectancy of at least 6 months and adequate end organ function. Patients will be excluded for prior systemic RCC treatment, brain metastases or if they require chronic immunosuppressive therapy.
More than 120 sites in North America, Europe and Israel will participate to recruit approximately 900 subjects for tumor collection. Currently, 78 global sites have been activated with 70 tumors collected and 16 subjects randomized. Further details regarding enrollment and study progress will be presented.
Clinical Trials registry number: NCT 1582672

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Hypoxia response regulates clear cell renal cell carcinoma tumor initiating cells

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Scientific Background: Clear cell renal cancer (ccRCC) is associated with *VHL* gene mutations that lead to stabilization of hypoxia inducible factors (*HIF-1α* and *HIF-2α*). In turn, this activates the transcription of a palette of genes involved in glucose uptake and metabolism, angiogenesis and cell cycle. Tumor initiating cells (TICs), often referred as to cancer stem cells, are neoplastic cells characterized by self-renewal, multilineage differentiation and initiation of tumor growth *in vivo* abilities. Previous reports have indicated ccRCC $CD105^+$ cells population to harbor stem cell properties, including clonogenic ability, expression of nestin, Nanog, Oct4, lack of differentiative epithelial markers expression, ability to grow in nonadhesive spheroids, *in vitro* differentiation into epithelial and endothelial cell types, and finally generation of transplantable carcinomas *in vivo*. At the same time ccRCC $CD133^+$ cells are to significantly enhance tumor development and growth as they differentiate into endothelial and epithelial cells and contribute to tumor vascularization. Moreover hypoxia was pointed as a critical microenvironmental factor regulating cancer stem cells' self-renewal potential, enhancing the activity of stem cell factors and maintaining stem cell populations but its roles in ccRCC stem cells biology is largely unknown.
Research design: The present study was to evaluate the influence of hypoxia on propagation of $CD105^+$ and $CD133^+$ ccRCC cells. In the first step viability and proliferation of ccRCC cells carrying wild type and mutant VHL gene was analyzed in 21% and 2% oxygene tension. In the second step the presence of $CD105^+$ and $CD133^+$ ccRCC cells was verified in these conditions. Clonogenicity – colony formation potential – (anchorage-independent growth) and aggregation (metastatic potential) property of cancer cells was measured. The anti-proliferative effects of selected TKI on ccRCC and ccRCC TICs in 2D and 3D cultures under 21% and 2% oxygene tension was verified. In 3D approach ccRCC and TICs were grown in aggregates and in hanging drop culture. ccRCC spheroids were also treated with TKI under normoxic or hypoxic conditions.
Current research: Tumor micro-environmental hypoxia and VHL gene status concordantly influence propagation of $CD105^+$ and $CD133^+$ ccRCC cells. TICs are targets of TKI and hypoxia modulates its' effect.
Grant registry number: This project was supported by the National Science Centre research grant NCN – UMO-2011/01/B/NZ5/02822, Foundation for Polish Science research grant TEAM 6/2011 and Military Institute of Medicine statutory funding.

20
Chronic myeloid leukaemia development in the course of ccRCC treatment with tyrosine kinase inhibitors

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Scientific Background: Targeted agents are a novel therapeutic approach developed to disrupt different cellular signalling pathways. Tyrosine kinase inhibitors (TKIs), including sunitinib and sorafenib, block multiple tyrosine kinase receptors that are involved in the progression of many tumours, including clear cell renal cell cancer (ccRCC). Although TKIs are primarily designed to target defined receptor tyrosine kinases (RTKs), it is increasingly apparent that many TKIs may also unexpectedly inhibit other targeted kinases and trigger the appearance of undesirable side effects by influencing kinases on non-cancerous cells. Patients should be examined for the presence and development of comorbidities before starting TKI treatment as well as during the therapy.
Treatment and eligibility criteria: 259 patients with clear cell renal cancer (ccRCC) were treated with sunitinib or sorafenib as I line ccRCC treatment in standard dose and schedule in the Military Institute of Medicine in Warsaw. Patients were eligible for treatment under standard MSKCC criteria. Treatment was initialized between March 2004 and July 2009 and monitoring ended in May 2013. Analyzed cohort represent typical patients in oncology ward hospitalized care in large oncology center.
Main Outcome: Development of 3 cases of chronic myeloid leukemia (CML) during tyrosine kinase inhibitor treatment was observed and defined as unexpected medical coincidence.
Current research: Hypothetical model picturing inhibition of multiple molecular pathways in bone marrow stem cells by TKIs has been proposed to explain co-incidence of TKI treatment and CML development.
Grant registry number: This study was supported by Military Institute of Medicine statutory founding, treatment was covered

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according to National Health Fund regulations.

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Survival, safety and treatment response duration in "real world" patients with metastatic clear cell renal cancer – an update from clinical practice

A.M. CZARNECKA, P. SOBCZUK*†, K. BOGUSZ*†, M. SPYCHALSKA*† and C. SZCZYLIK**

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Scientific Background: Clinical trials are considered the gold standard to assess the efficacy and safety of treatment. However, because trials are conducted in standardized conditions, discrepancies in patient selection or treatment conditions may alter both the effectiveness and risks of treatment. Progression-free survival (PFS) durations reported from randomized trials of targeted therapies may also vary considerably from the real world of prescription and use. Level 1 evidence supports sequential use of tyrosine kinase inhibitors, as well as these agents followed by everolimus and sequential therapy with targeted agents provides significant benefit. Classical scheme of treatment, approved in Poland, combines nephrectomy and sequential treatment of metastatic disease with Sunitinib (TKI) and Everolimus (mTOR inhibitor).

Treatment and eligibility criteria: 153 (117M, 36F) patients with metastatic ccRCC were treated in the Department of Oncology of Military Institute of Medicine in Warsaw. Patients stared therapy between January 2007 and December 2011. Overall Survival (OS) since nephrectomy was evaluated and duration of first-line therapy (Sunitinib) and second-line therapy (Everolimus) was estimated. Analyzed cohort represent typical patients in oncology ward hospitalized care in large oncology center. Common side effects were monitored and treated accordingly. Cardiovascular risk associated with sunitinib treatment was investigated.

Current research: On-treatment patients are monitored. PFS on Sunitinib vary favorably form reported in clinical trials. Negative and positive predictive factors of TKI treatment are recognized.

Grant registry number: This study was supported by Military Institute of Medicine statutory founding, treatment was covered according to National Health Fund regulations.

22

Brain metastasis from renal cell carcinoma, are we missing our chance to treat?

M. HANZLY, A. ABOUMOHAMED, K. AITWOOD, D. MEHEDINT, E. KAUFFMAN and T. SCHWAAB

Roswell Park Cancer Institute

Introduction: Brain metastasis occurs in approximately 8% of patients with primary renal cell carcinoma [1]. Current guidelines from the NCCN, European Association of Urology, and American Urologic Association recommend cerebral imaging only in patients who have symptomatology [2–4]. The purpose of our study was to examine characteristics of RCC patients with brain metastasis, including symptomatology at diagnosis, treatment and overall survival.

Methods: An IRB-approved prospectively-maintained kidney cancer data base at Roswell Park Cancer Institute was reviewed for cases of primary RCC from 1995 to 2012. Brain metastases from a primary RCC was identified in 52 patients. Clinical and perioperative variables examined include family and social history, primary tumor histology and treatment modality, time between primary tumor diagnosis and brain metastasis, symptomatology, metastasis treatment modality and overall survival from the time of brain metastasis diagnosis.

Results: Primary RCC was diagnosed in 898 patients. Among the 52 patients with brain metastasis, 79% were male and 100% were Caucasian, 87% had no family history of renal malignancy and 51% had a history of tobacco abuse, with a 39 pack-year average. Primary treatment for RCC included 75% radical nephrectomy, 10% observation, 7.6% cytoreductive nephrectomy, 5.4% sunitinib, 2% interleukin-2 treatment. Histological examination of the primary tumors identified 88% pure clear cell, 6% clear cell with sarcomatoid features, 4% clear cell with granular features and 2% were poorly differentiated. Central nervous system (CNS) symptoms were absent in 37% of patients diagnosed with brain metastases, while 73% presented with symptoms. The average number of metastases was 1.78 per patient in the asymptomatic group, and 2.7 metastases in patients with CNS symptoms ($p=0.18$). Elapsed time from primary RCC treatment to diagnosis of brain metastases was an average of 730 days in the asymptomatic group and 1170 days for the symptomatic group ($p=0.02$). The symptomatic group overall survival (OS) at one and three years was 81% and 69%, respectively, while the asymptomatic group was 81% and 31%, respectively. Pulmonary metastasis was present in 75% of both cohorts ($p=0.002$). Two patients had only brain metastases and no other sites of metastasis. Clinical trial enrolment was cited as the number one reason for brain imaging and diagnosed 75% of asymptomatic patients.

Conclusion: Patients without CNS symptoms with brain metastases may have a worse overall survival. Patients with pulmonary metastasis are more likely to have brain metastases. Urologic guidelines recommending CNS imaging only in patients with symptoms may be missing a subset of the metastatic RCC population who could potentially benefit from early therapeutic intervention.

References

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23

Utilization of renal mass biopsy in patients with renal cell carcinoma

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Acknowledgements: The Urologic Diseases in America Project is sponsored by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH). Dr. Leppert is supported by K23 DK089086. Dr. Chertow is supported by K24 DK085446. The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH/NIDDK.

Purpose: Renal mass biopsy (RMB) may play an important role in personalized treatment for patients with renal cell carcinoma (RCC). We examined the patient, tumor, and temporal factors associated with receipt of RMB in a contemporary nationally representative sample.

Materials and Methods: We queried the Surveillance, Epidemiology, and End Results-Medicare dataset for incident cases of RCC diagnosed between 1992 and 2007. We tested for associations among receipt of RMB and patient and tumor characteristics, type of therapy, and procedure type. Temporal trends in receipt of RMB were characterized over the study period.

Results: Approximately one in five (20.7%) patients diagnosed with RCC (n=24,702) underwent RMB before instituting therapy. There was a steady and modest increase in RMB utilization, with the highest utilization (30%) occurring in the final study year. Among patients who underwent radical (n=15,666) or partial (n=2,211) nephrectomy, 17% and 20%, respectively, underwent RMB in advance of surgery. Sixty-five percent of patients who underwent ablation (n=314) underwent RMB before or in conjunction with the procedure. Roughly half of patients (50.4%) treated with systemic therapy alone underwent RMB. Factors independently associated with use of RMB included younger age, black race, Hispanic ethnicity, tumor size >7 cm, and metastatic disease at presentation.

Conclusions: At present, most patients who eventually undergo radical or partial nephrectomy do not undergo RMB, whereas most patients who eventually undergo ablation or systemic therapy do. The optimal use of RMB in the evaluation of kidney tumors has yet to be determined.

24

Nano-scale proteomic profiling to define diagnostic signatures and biomarkers of therapeutic activity in patients with RCC

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Acknowledgements: This work is supported by the NCI/NIH (R21 CA169964), by a Stanford Cancer Institute 2012 Developmental Cancer Research Award Grant, and by a Translational Research and Applied Medicine Pilot Grant Award. Dr. Leppert is supported by K23 DK089086 from the NIDDK/NIH.

Background: Novel agents that inhibit kinases in the hypoxia and MTOR pathways can achieve response rates of 30–60% in renal cell carcinoma (RCC); however, protein biomarkers to diagnose RCC and guide treatment have not been identified.

Methods: We developed the use of two high-throughput, nanoscale immunoassays to profile hypoxia and downstream signaling in clinical specimens from patients with RCC. These automated technologies minimize errors due to manual variability, and make 96 independent measurements per overnight experiment. We used size-based protein separation (Sally Instrument, Protein Simple) to quantify proteins using antibody-specific detection, normalized to loading control. Next, we used a nano-immunoassay with charge-based separation (NIA, Nanopro1000 Instrument, Protein Simple) to distinguish multiple charged modifications of individual proteins, and measure relative ratios of individual unphosphorylated and phosphorylated isoforms.

Results: We first optimized assays for proteins in MAPK, PI3K and STAT pathways, and loading controls, to analyze frozen surgical specimens and ex-vivo fine needle aspirates (FNA's) performed immediately following nephrectomy. We analyzed more than 200 FNA's from solid tumors, comparing RCC with paired adjacent non-tumor tissue, and other epithelial malignancies. Basal MAPK signaling of tumors varied across 3 logs of expression. Unique to NIA, we analyzed percent phosphorylation and resolved differences in single phosphorylations. Subtle differences in basal MAPK signaling between tumor cells and adjacent non-tumor tissue can distinguish RCC from other malignancies. Lastly, we prospectively collected and analyzed blood peripheral mononuclear cells from 20 RCC patients before and during treatment with standard targeted therapies. Kinase inhibitors can preferentially inhibit or activate specific protein phospho-isoforms.

Conclusions: Our studies demonstrate that rapid and quantitative nanoproteomic profiling in very small amounts of clinical specimen may accelerate translational studies for novel diagnostic and predictive biomarkers.

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Utilization of cytoreductive nephrectomy and patient survival in the targeted therapy era

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Acknowledgements: Dr. Leppert is supported by Award Number DK089086 from the National Institutes of Health

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(NIH), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Dr. Chertow is supported by K24 DK085446. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH/NIDDK.

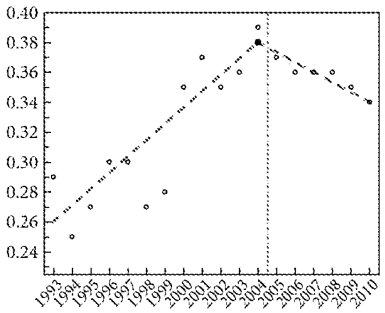
Background: We sought to analyze utilization and survival outcomes of cytoreductive nephrectomy in patients with metastatic renal cell carcinoma (RCC) before and after introduction of targeted therapy.

Methods: We identified patients with metastatic RCC between 1993 and 2010 in the SEER registry and examined temporal trends in utilization, as well as patient and tumor characteristics associated with receipt of cytoreductive nephrectomy. We performed a joinpoint regression to determine when changes in utilization of cytoreductive nephrectomy occurred. We fitted multivariable proportional hazard models to determine the association of cytoreductive nephrectomy with mortality in full and propensity score-matched cohorts. We performed a difference-in-difference analysis to compare survival outcomes before and after introduction of targeted therapy.

Results: The proportion of patients undergoing cytoreductive nephrectomy increased from 1993 to 2004, from 29% to 39%. We identified a primary joinpoint of 2004, just prior to the introduction of targeted therapy. Beginning in 2005, there was a modest decrease in utilization of CN. Cytoreductive nephrectomy was associated with a lower adjusted relative hazard (0·41, 95% confidence interval 0·34 to 0·43). Median survival among patients receiving cytoreductive nephrectomy increased in the targeted therapy era (19 *versus* 13 months), while median survival among patients not receiving cytoreductive nephrectomy increased only slightly (4 *versus* 3 months). Difference in difference analysis showed that there was a significant decrease in hazard of death among patients who received cytoreductive nephrectomy in the targeted therapy era.

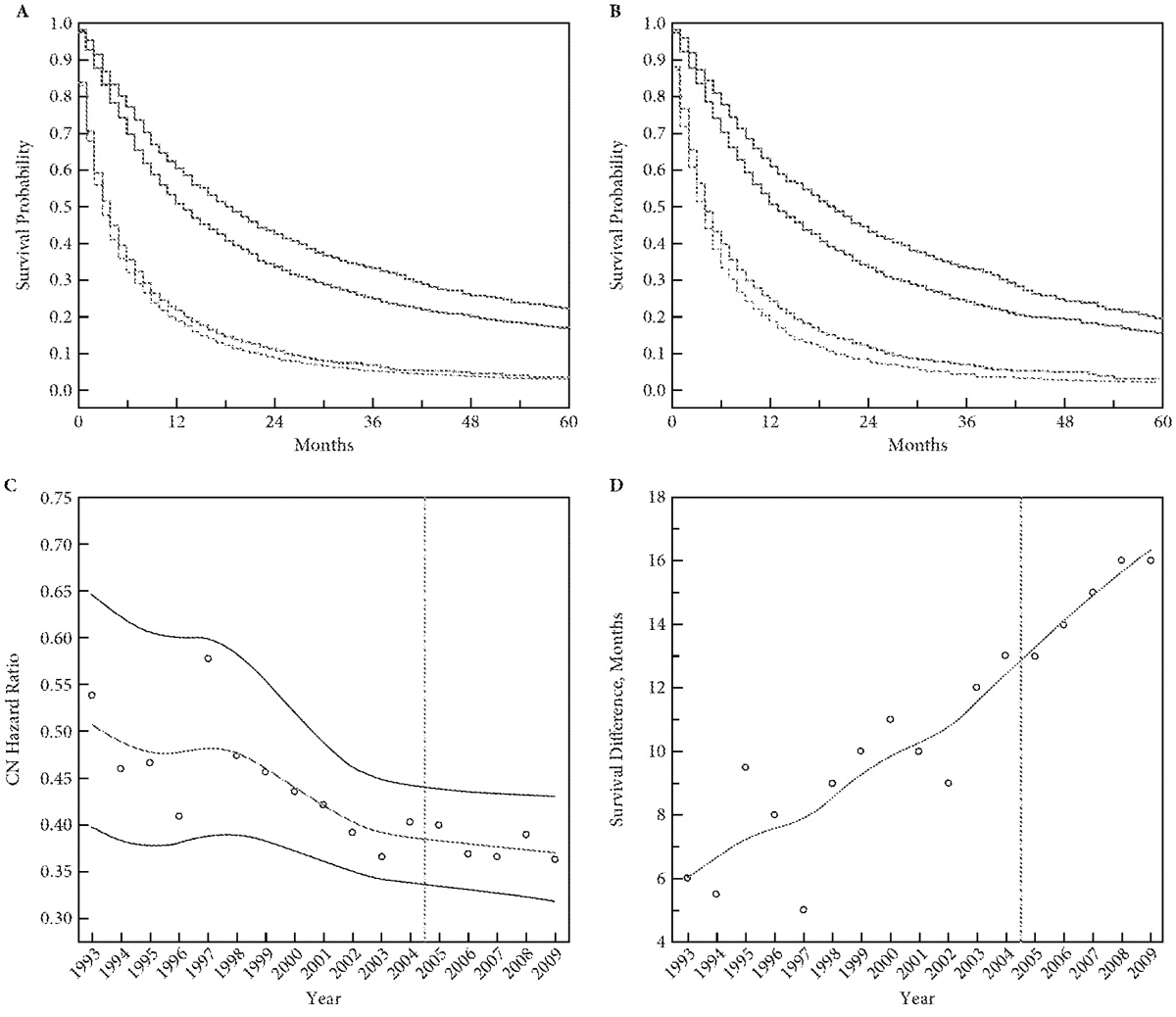
Conclusions: Despite decreased utilization in the targeted therapy era, cytoreductive nephrectomy remains associated with improved survival. Prospective randomized trials are needed to confirm the benefit of cytoreductive nephrectomy among patients with metastatic RCC treated with novel targeted therapies.

Fig. 1 Proportion of patients with metastatic RCC receiving cytoreductive nephrectomy by year of diagnosis. We identified 2004 as the year when utilization trends changed (joinpoint). This coincides with the introduction of targeted therapies in 2005, represented by the vertical dotted reference line.



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Fig. 2 Product-limit survival estimates stratified by treatment era in the full (**A**) and propensity score-matched (**B**) cohorts. Patients treated in the targeted therapy era (≥2005) are shown in red. Patients treated before 2005 are shown in blue. Survival curves of patients treated with cytoreductive nephrectomy are represented as a solid line, while those not receiving cytoreductive nephrectomy are represented as dotted lines. Median overall survival of patients treated with CN increased to 19 months from 13 months in the targeted therapy era. Median overall survival of patients not receiving CN, increased to 4 from 3 months. (**C**) The temporal trend of the adjusted hazard of mortality associated with cytoreductive nephrectomy by diagnosis year with 95% CI. (**D**) The increasing difference in median survival for patients receiving cytoreductive nephrectomy. The beginning of the targeted therapy era is represented by the vertical dotted reference line.



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Poorly functioning kidneys recover from ischemia during partial nephrectomy as well as strongly functioning kidneys

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Objective: Some authors have proposed that poorly functioning kidneys may not recover from ischemia as well as strongly functioning kidneys, which would have impact on the surgical approaches to partial nephrectomy (PN).
Materials and Methods: A total of 122 consecutive patients undergoing PN at our institution with appropriate studies to facilitate analysis of function and parenchymal mass volume in the operated kidney (CT and SCr within 2 months prior and 4–12 months postoperative) were analyzed. Patients with a contralateral kidney also required renal scans in the same time frame to provide split renal function. Volumetric CT was utilized to measure the volume of functional parenchyma pre and post PN in the operated kidney and the contralateral kidney. GFR was determined by the MDRD2 equation. Recovery of nephron function (GP/VS=% GFR preserved/% volume saved) would be 100% if all nephrons recovered from the ischemic insult.
Results: Median age was 61 years with approximately 50% of patients having open procedures. Median RENAL score was 7. Cold ischemia was used in 50 patients (median 26 min), while 72 had limited warm ischemia (median 20 min). Median %GFR preserved in the operated kidney was 79.5%. Median parenchymal volume saved was 84%. Function in the contralateral kidney remained stable without significant increase. Overall recovery of nephron function (GP/VS) was 95.5%, and was 100.7% for the cold ischemia subgroup and 93% in the limited warm ischemia subgroup. Recovery of nephron function (GP/VS) was similar for all strata of preoperative eGFR in the operated kidney (<30, 21–45, 46–60, and >60) as detailed in the table ($p > 0.18$ for all comparisons), even in the limited warm ischemia subgroup.
Conclusions: Preoperative GFR at PN does not correlate with how well the kidney will recover from the ischemia insult. Our results suggest that the amount vascularized parenchyma preserved by the PN is the main determinant of the postoperative GFR. Even poorly functioning kidneys recover well from the ischemic insult as long as limited warm ischemia or hypothermia are utilized.

Recovery Nephron Function (GP/VS).

Preop eGFR	Overall (n=122)	Cold Ischemia (n=50)	Warm Ischemia (n=72)
<30 (20)	101.0 (90–116)	109.0 (105–117)	96.2 (80–113)
31–45 (39)	93.2 (83–109)	97.0 (88–105)	90.6 (77–109)
46–60 (43)	95.5 (87–101)	100.6 (94–107)	92.2 (86–99)
>60 (20)	98.7 (91–102)	100.5 (94–104)	96.6 (91–98)
	p=0.206	p=0.181	p=0.679

All analyses refer specifically to the operated kidney.

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cytotoxic T-lymphocyte-associated protein 4 [Homo sapiens]

GenBank: AAB59385.1

Identical Proteins FASTA Graphics

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  TITLE     CTLA-4 and CD28 activated lymphocyte molecules are closely related
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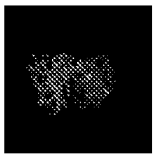
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Protein 3D Structure



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Source: Homo sapiens
Method: X-Ray Diffraction
Resolution: 2

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A novel approach to investigation of the pathogenesis of pterygium [Gene. 2016]

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RefSeq genomic sequence
See the genomic reference sequence for the CTLA4 gene (NG_011502.1).

RefSeq protein isoforms
See 2 reference sequence protein isoforms for the CTLA4 gene.

More about the CTLA4 gene

This gene is a member of the immunoglobulin superfamily and

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encodes a protein which transmits an inhibitory signal to T cells. The protein c...

Also Known As: ALPS5, CD, CD152, CELIA...

Homologs of the CTLA4 gene

The CTLA4 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, and frog.

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FASTA Graphics

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            Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 45 to 965)
  AUTHORS   Shinohara,T., Taniwaki,M., Ishida,Y., Kawaichi,M. and Honjo,T.
  TITLE     Structure and chromosomal localization of the human PD-1 gene
            (PDCD1)
  JOURNAL   Genomics 23 (3), 704-706 (1994)
   PUBMED   7851902
REFERENCE   2  (bases 1 to 2106)
  AUTHORS   Finger,L.R., Pu,J., Wasserman,R., Vibhakar,R., Louie,E.,
            Hardy,R.R., Burrows,P.D. and Billips,L.G.
  TITLE     The human PD-1 gene: complete cDNA, genomic organization, and
            developmentally regulated expression in B cell progenitors
  JOURNAL   Gene 197 (1-2), 177-187 (1997)
   PUBMED   9332365
REFERENCE   3  (bases 1 to 2106)
  AUTHORS   Finger,L.R.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUL-1996) The Cancer Research Institute, New York
            Medical College, 100 Grasslands Road, Elmsford, New York 10523, USA
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```

Analyze this sequence

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Highlight Sequence Features

Find in this Sequence

Articles about the PDCD1 gene

Aberrant PD-L1 expression through 3'-UTR disruption in multipk [Nature. 2016]

Non-Small Cell Lung Cancer, PD-L1, and the Pa [Arch Pathol Lab Med. 2016]

Prospective identification of neoantigen-specific lymphocytes in [Nat Med. 2016]

See all...

Pathways for the PDCD1 gene

PD-1 signaling

Costimulation by the CD28 family

Adaptive Immune System

See all...

Reference sequence information

RefSeq alternative splicing

See 3 reference mRNA sequence splice variants for the PDCD1 gene.

More about the PDCD1 gene

This gene encodes a cell surface membrane protein of the immunoglobulin superfamily. This protein is expressed in pro-B-cells and is thought...

Also Known As: CD279, PD-1, PD1, SLEB2...

Homologs of the PDCD1 gene

The PDCD1 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and chicken.

Related information

BioSystems

```
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```

Component Of
Full text in PMC
Gene
Map Viewer
OMIM
Probe
Protein
PubMed
PubMed (Weighted)
Taxonomy
UniGene
GEO Profiles

LinkOut to external resources

Order full-length cDNA clone
[GeneCopoeia Inc.]

Order full-length cDNA clone
[PlasmID Repository at Harvard...]

Recent activity

Turn Off Clear

- Human hPD-1 (hPD-1) mRNA, complete cds — Nucleotide
- RecName: Full=Programmed cell death 1 ligand 1; Short=PD-L — Protein
- Homo sapiens killer cell immunoglobulin like recept — Nucleotide
- Human p58 natural killer cell receptor precursor mRNA, — Nucleotide
- KIR2DL1 (158) — Protein

See more...

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//
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Sign in to NCBI

Protein

Protein

Go

Advanced

Help

GenPept

Send to:

Change region shown

Customize view

RecName: Full=Programmed cell death 1 ligand 1; Short=PD-L1; Short=PDCD1 ligand 1; Short=Programmed death ligand 1; AltName: Full=B7 homolog 1; Short=B7-H1; AltName: CD_antigen=CD274; Flags: Precursor

UniProtKB/Swiss-Prot: Q9NZQ7.1

Identical Proteins FASTA Graphics

Go to:

```
LOCUS       PDIL1_HUMAN               290 aa            linear   PRI 02-NOV-2016
DEFINITION  RecName: Full=Programmed cell death 1 ligand 1; Short=PD-L1;
            Short=PDCD1 ligand 1; Short=Programmed death ligand 1; AltName:
            Full=B7 homolog 1; Short=B7-H1; AltName: CD_antigen=CD274; Flags:
            Precursor.
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            annotation updated: Nov 2, 2016.
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Analyze this sequence

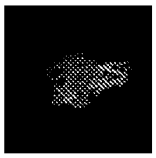
Run BLAST

Identify Conserved Domains

Highlight Sequence Features

Find in this Sequence

Protein 3D Structure



Pd-l1 In Complex With Bms-936559 Fab
PDB: 5GGT
Source: Homo sapiens
Method: X-Ray Diffraction
Resolution: 2.8

See all 11 structures...

Articles about the CD274 gene

[IFN-γ up-regulates PD-L1 expression in h [Xi Bao Yu Fen Zi Mian Yi Xue Z...]

PD-L1-expressing neutrophils as a novel indicator [Arthritis Res Ther. 2016]

Predictive relevance of PD-L1 expression combin [Eur J Cancer. 2016]

See all...

Pathways for the CD274 gene

Cell adhesion molecules (CAMs)

PD-1 signaling

Costimulation by the CD28 family

See all...

Reference sequence information

RefSeq protein isoforms
See 3 reference sequence protein isoforms for the CD274 gene.

More about the CD274 gene

This gene encodes an immune inhibitory receptor ligand that is expressed by hematopoietic and non-hematopoietic cells, such as T cells and B...

KEYWORDS 3D-structure; Alternative splicing; Cell membrane; Complete proteome; Disulfide bond; Glycoprotein; Immunoglobulin domain; Membrane; Receptor; Reference proteome; Repeat; Signal; Transmembrane; Transmembrane helix.
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.
REFERENCE 1 (residues 1 to 290)
AUTHORS Dong,H., Zhu,G., Tamada,K. and Chen,L.
TITLE B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion
JOURNAL Nat. Med. 5 (12), 1365-1369 (1999)
PUBMED 10581077
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1), FUNCTION, TISSUE SPECIFICITY, AND INDUCTION.
REFERENCE 2 (residues 1 to 290)
AUTHORS Freeman,G.J., Long,A.J., Iwai,Y., Bourque,K., Chernova,T., Nishimura,H., Fitz,L.J., Malenkovich,N., Okazaki,T., Byrne,M.C., Horton,H.F., Fouser,L., Carter,L., Ling,V., Bowman,M.R., Carreno,B.M., Collins,M., Wood,C.R. and Honjo,T.
TITLE Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation
JOURNAL J. Exp. Med. 192 (7), 1027-1034 (2000)
PUBMED 11015443
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1), FUNCTION, INTERACTION WITH PDCD1, TISSUE SPECIFICITY, AND INDUCTION.; TISSUE=Placenta
REFERENCE 3 (residues 1 to 290)
AUTHORS He,X.H., Xu,L.H. and Liu,Y.
TITLE Identification of a novel splice variant of human PD-L1 mRNA encoding an isoform-lacking Igv-like domain
JOURNAL Acta Pharmacol. Sin. 26 (4), 462-468 (2005)
PUBMED 15780196
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORMS 1; 2 AND 3), AND SUBCELLULAR LOCATION (ISOFORMS 1 AND 2).
REFERENCE 4 (residues 1 to 290)
AUTHORS Chi,X.-Y., Zha,Q.-B., Xu,L.-H., Jia,Q.-T., Li,F.-Y. and He,K.-H.
TITLE Direct Submission
JOURNAL Submitted (??-NOV-2005)
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 2).; TISSUE=Gastric carcinoma
REFERENCE 5 (residues 1 to 290)
AUTHORS Ota,T., Suzuki,Y., Nishikawa,T., Otsuki,T., Sugiyama,T., Irie,R., Wakamatsu,A., Hayashi,K., Sato,H., Nagai,K., Kimura,K., Makita,H., Sekine,M., Obayashi,M., Nishi,T., Shibahara,T., Tanaka,T., Ishii,S., Yamamoto,J., Saito,K., Kawai,Y., Isono,Y., Nakamura,Y., Nagahari,K., Murakami,K., Yasuda,T., Iwayanagi,T., Wagatsuma,M., Shiratori,A., Sudo,H., Hosoiri,T., Kaku,Y., Kodaira,H., Kondo,H., Sugawara,M., Takahashi,M., Kanda,K., Yokoi,T., Furuya,T., Kikkawa,E., Omura,Y., Abe,K., Kamihara,K., Katsuta,N., Sato,K., Tanikawa,M., Yamazaki,M., Ninomiya,K., Ishibashi,T., Yamashita,H., Murakawa,K., Fujimori,K., Tanai,H., Kimata,M., Watanabe,M., Hiraoka,S., Chiba,Y., Ishida,S., Ono,Y., Takiguchi,S., Watanabe,S., Yosida,M., Hotuta,T., Kusano,J., Kanehori,K., Takahashi-Fujii,A., Hara,H., Tanase,T.O., Nomura,Y., Togiya,S., Komai,F., Hara,R., Takeuchi,K., Arita,M., Imose,N., Musashino,K., Yuuki,H., Oshima,A., Sasaki,N., Aotsuka,S., Yoshikawa,Y., Matsunawa,H., Ichihara,T., Shiohata,N., Sano,S., Moriya,S., Momiyama,H., Satoh,N., Takami,S., Terashima,Y., Suzuki,O., Nakagawa,S., Senoh,A., Mizoguchi,H., Goto,Y., Shimizu,F., Wakebe,H., Hishigaki,H., Watanabe,T., Sugiyama,A., Takemoto,M., Kawakami,B., Yamazaki,M., Watanabe,K., Kumagai,A., Itakura,S., Fukuzumi,Y., Fujimori,Y., Komiyama,M., Tashiro,H., Tanigami,A., Fujiwara,T., Ono,T., Yamada,K., Fujii,Y., Ozaki,K., Hirao,M., Ohmori,Y., Kawabata,A., Hikiji,T., Kobatake,N., Inagaki,H., Ikema,Y., Okamoto,S., Okitani,R., Kawakami,T., Noguchi,S., Itoh,T., Shigeta,K., Senba,T., Matsumura,K., Nakajima,Y., Mizuno,T., Morinaga,M., Sasaki,M., Togashi,T., Oyama,M., Hata,H., Watanabe,M., Komatsu,T., Mizushima-Sugano,J., Satoh,T., Shirai,Y., Takahashi,Y., Nakagawa,K., Okumura,K., Nagase,T., Nomura,N., Kikuchi,H., Masuho,Y., Yamashita,R., Nakai,K., Yada,T., Nakamura,Y., Ohara,O., Isogai,T. and Sugano,S.
TITLE Complete sequencing and characterization of 21,243 full-length human cDNAs

Also Known As: B7-H; B7H1; PD-L1; PDCD...

Homologs of the CD274 gene

The CD274 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, and frog.

Related information

BLink
Related Sequences
BioSystems
CDD Search Results
Conserved Domains (Concise)
Conserved Domains (Full)
Domain Relatives
Full text in PMC
Gene
GeneView in dbSNP
Identical Structures
Map Viewer
OMIM
Protein (RefSeq)
Proteins with Similar Sequences
PubMed
PubMed (Weighted)
Related Structures (List)
Related Structures (Summary)
Taxonomy

LinkOut to external resources

Q9NZQ7 [Domain Mapping of Disease Mut...]
A selection of literature about the proteins [GoPubMed Proteins]
Transcript/Protein Information [PANTHER Classification System]
Pathway Commons [Pathway Commons]
Protein Ontology Consortium [Protein Ontology Consortium]
Reactome [Reactome]
SeekQuence Research Antibodies [SeekQuence Research Antibodies]
Evolutionary Trace of Functional Site [Evolutionary Trace of Functio...]

Recent activity

Turn Off Clear

RecName: Full=Programmed cell death 1 ligand 1; Short=PD-L Protein

Homo sapiens killer cell immunoglobulin like recept Nucleotide
Human p58 natural killer cell receptor precursor mRNA, Nucleotide
KIR2DL1 (158) Protein
lymphocyte activation gene 3 protein precursor [Mus muscu Protein
See more

```
    JOURNAL   Nat. Genet. 36 (1), 40-45 (2004)
     PUBMED   14702039
    REMARK    NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA] (ISOFORMS 1 AND 3).;
              TISSUE=Placenta
  REFERENCE   6  (residues 1 to 290)
    AUTHORS   Humphray,S.J., Oliver,K., Hunt,A.R., Plumb,R.W., Loveland,J.E.,
              Howe,K.L., Andrews,T.D., Searle,S., Hunt,S.E., Scott,C.E.,
              Jones,M.C., Ainscough,R., Almeida,J.P., Ambrose,K.D., Ashwell,R.I.,
              Babbage,A.K., Babbage,S., Bagguley,C.L., Bailey,J., Banerjee,R.,
              Barker,D.J., Barlow,K.F., Bates,K., Beasley,H., Beasley,O.,
              Bird,C.P., Bray-Allen,S., Brown,A.J., Brown,J.Y., Burford,D.,
              Burrill,W., Burton,J., Carder,C., Carter,N.P., Chapman,J.C.,
              Chen,Y., Clarke,G., Clark,S.Y., Clee,C.M., Clegg,S., Collier,R.E.,
              Corby,N., Crosier,M., Cummings,A.T., Davies,J., Dhami,P., Dunn,M.,
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              Frankish,A., Frankland,J.A., French,L., Fricker,D.G., Garner,P.,
              Garnett,J., Ghori,J., Gilbert,J.G., Glison,C., Grafham,D.V.,
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              Heath,P.D., Henderson,C.D., Hopkins,B.L., Howard,P.J., Howden,P.J.,
              Huckle,E., Johnson,C., Johnson,D., Joy,A.A., Kay,M., Keenan,S.,
              Kershaw,J.K., Kimberley,A.M., King,A., Knights,A., Laird,G.K.,
              Langford,C., Lawlor,S., Leongamornlert,D.A., Leversha,M., Lloyd,C.,
              Lloyd,D.M., Lovell,J., Martin,S., Mashreghi-Mohammadi,M.,
              Matthews,L., McLaren,S., McLay,K.E., McMurray,A., Milne,S.,
              Nickerson,T., Nisbett,J., Nordsiek,G., Pearce,A.V., Peck,A.I.,
              Porter,K.M., Pandian,R., Pelan,S., Phillimore,B., Povey,S.,
              Ramsey,Y., Rand,V., Scharfe,M., Sehra,H.K., Shownkeen,R.,
              Sims,S.K., Skuce,C.D., Smith,M., Steward,C.A., Swarbreck,D.,
              Sycamore,N., Tester,J., Thorpe,A., Tracey,A., Tromans,A.,
              Thomas,D.W., Wall,M., Wallis,J.M., West,A.P., Whitehead,S.L.,
              Willey,D.L., Williams,S.A., Wilming,L., Wray,P.W., Young,L.,
              Ashurst,J.L., Coulson,A., Blocker,H., Durbin,R., Sulston,J.E.,
              Hubbard,T., Jackson,M.J., Bentley,D.R., Beck,S., Rogers,J. and
              Dunham,I.
    TITLE     DNA sequence and analysis of human chromosome 9
    JOURNAL   Nature 429 (6990), 369-374 (2004)
     PUBMED   15164053
    REMARK    NUCLEOTIDE SEQUENCE [LARGE SCALE GENOMIC DNA].
  REFERENCE   7  (residues 1 to 290)
    AUTHORS   Mural,R.J., Istrail,S., Sutton,G.G., Florea,L., Halpern,A.L.,
              Mobarry,C.M., Lippert,R., Walenz,B., Shatkay,H., Dew,I.,
              Miller,J.R., Flanigan,M.J., Edwards,N.J., Bolanos,R., Fasulo,D.,
              Halldorsson,B.V., Hannenhalli,S., Turner,R., Yooseph,S., Lu,F.,
              Nusskern,D.R., Shue,B.C., Zheng,X.H., Zhong,F., Delcher,A.L.,
              Huson,D.H., Kravitz,S.A., Mouchard,L., Reinert,K., Remington,K.A.,
              Clark,A.G., Waterman,M.S., Eichler,E.E., Adams,M.D.,
              Hunkapiller,M.W., Myers,E.W. and Venter,J.C.
    TITLE     Direct Submission
    JOURNAL   Submitted (??-SEP-2005)
    REMARK    NUCLEOTIDE SEQUENCE [LARGE SCALE GENOMIC DNA].
  REFERENCE   8  (residues 1 to 290)
    AUTHORS   Gerhard,D.S., Wagner,L., Feingold,E.A., Shenmen,C.M., Grouse,L.H.,
              Schuler,G., Klein,S.L., Old,S., Rasooly,R., Good,P., Guyer,M.,
              Peck,A.M., Derge,J.G., Lipman,D., Collins,F.S., Jang,W., Sherry,S.,
              Feolo,M., Misquitta,L., Lee,E., Rotmistrovsky,K., Greenhut,S.F.,
              Schaefer,C.F., Buetow,K., Bonner,T.I., Haussler,D., Kent,J.,
              Kiekhaus,M., Furey,T., Brent,M., Prange,C., Schreiber,K.,
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              Soares,M.B., Casavant,T.L., Scheetz,T.E., Brown-stein,M.J.,
              Usdin,T.B., Toshiyuki,S., Carninci,P., Piao,Y., Dudekula,D.B.,
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              Smith,M.R., Simmons,B., Moore,T., Waterman,R., Johnson,S.L.,
              Ruan,Y., Wei,C.L., Mathavan,S., Gunaratne,P.H., Wu,J., Garcia,A.M.,
              Hulyk,S.W., Fuh,E., Yuan,Y., Sneed,A., Kowis,C., Hodgson,A.,
              Muzny,D.M., McPherson,J., Gibbs,R.A., Fahey,J., Helton,E.,
              Ketteman,M., Madan,A., Rodrigues,S., Sanchez,A., Whiting,M.,
              Madari,A., Young,A.C., Wetherby,K.D., Granite,S.J., Kwong,P.N.,
              Brinkley,C.P., Pearson,R.L., Bouffard,G.G., Blakesly,R.W.,
              Green,E.D., Dickson,M.C., Rodriguez,A.C., Grimwood,J., Schmutz,J.,
              Myers,R.M., Butterfield,Y.S., Griffith,M., Griffith,O.L.,
              Krzywinski,M.I., Liao,N., Morin,R., Palmquist,D., Petrescu,A.S.,
              Skalska,U., Smailus,D.E., Stott,J.M., Schnerch,A., Schein,J.E.,
              Jones,S.J., Holt,R.A., Baross,A., Marra,M.A., Clifton,S.,
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              Makowski,K.A., Bosak,S. and Malek,J.
    CONSRTM   MGC Project Team
    TITLE     The status, quality, and expansion of the NIH full-length cDNA
              project: the Mammalian Gene Collection (MGC)
    JOURNAL   Genome Res. 14 (10B), 2121-2127 (2004)
     PUBMED   15489334
    REMARK    NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA] (ISOFORM 1).;
              TISSUE=Lung
              Erratum:[Genome Res. 2006 Jun;16(6):804. Morrin, Ryan [corrected to
              Morin, Ryan]]
  REFERENCE   9  (residues 1 to 290)
    AUTHORS   Chen,R., Jiang,X., Sun,D., Han,G., Wang,F., Ye,M., Wang,L. and
              Zou,H.
    TITLE     Glycoproteomics analysis of human liver tissue by combination of
              multiple enzyme digestion and hydrazide chemistry
    JOURNAL   J. Proteome Res. 8 (2), 651-661 (2009)
     PUBMED   19159218
    REMARK    GLYCOSYLATION [LARGE SCALE ANALYSIS] AT ASN-192.;
              TISSUE=Liver
  REFERENCE   10 (residues 1 to 290)
    AUTHORS   Lin,D.Y., Tanaka,Y., Iwasaki,M., Gittis,A.G., Su,H.P., Mikami,B.,
              Okazaki,T., Honjo,T., Minato,N. and Garboczi,D.N.
    TITLE     The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of
              antibodies and T cell receptors
    JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 105 (8), 3011-3016 (2008)
     PUBMED   18287011
    REMARK    X-RAY CRYSTALLOGRAPHY (2.64 ANGSTROMS) OF 18-239 IN COMPLEX WITH
              MOUSE PDCD1, AND DISULFIDE BONDS.
  COMMENT     On or before Apr 22, 2014 this sequence version replaced
              gi:121948354, gi:121948736.
              [FUNCTION] Involved in the costimulatory signal, essential for
              T-cell proliferation and production of IL10 and IFNG, in an
              IL2-dependent and a PDCD1-independent manner. Interaction with
              PDCD1 inhibits T-cell proliferation and cytokine production.
              {ECO:0000269|PubMed:10581077, ECO:0000269|PubMed:11015443}.
              [SUBUNIT] Interacts with PDCD1. {ECO:0000269|PubMed:11015443,
              ECO:0000269|PubMed:18287011}.
              [INTERACTION] P33681:CD80; NbExp=2; IntAct=EBI-4314282,
              EBI-1031024; Q15116:PDCD1; NbExp=2; IntAct=EBI-4314282,
              EBI-4314328.
              [SUBCELLULAR LOCATION] Isoform 1: Cell membrane
              {ECO:0000269|PubMed:15780196}; Single-pass type I membrane protein
              {ECO:0000255}.
              [SUBCELLULAR LOCATION] Isoform 2: Endomembrane system
              {ECO:0000269|PubMed:15780196}; Single-pass type I membrane protein
              {ECO:0000255}.
              [ALTERNATIVE PRODUCTS] Event=Alternative splicing; Named
              isoforms=3; Name=1; Synonyms=PD-L1I; IsoId=Q9NZQ7-1;
              Sequence=Displayed; Name=2; Synonyms=PD-L1II; IsoId=Q9NZQ7-2;
              Sequence=VSP_013735; Name=3; IsoId=Q9NZQ7-3; Sequence=VSP_013736,
              VSP_013737; Note=No experimental confirmation available. May be
              produced at very low levels due to a premature stop codon in the
              mRNA, leading to nonsense-mediated mRNA decay.
              [TISSUE SPECIFICITY] Highly expressed in the heart, skeletal
              muscle, placenta and lung. Weakly expressed in the thymus, spleen,
              kidney and liver. Expressed on activated T- and B-cells, dendritic
              cells, keratinocytes and monocytes. {ECO:0000269|PubMed:10581077,
              ECO:0000269|PubMed:11015443}.
              [INDUCTION] Up-regulated on T- and B-cells, dendritic cells,
              keratinocytes and monocytes after LPS and IFNG activation.
              Up-regulated in B-cells activated by surface Ig cross-linking.
              {ECO:0000269|PubMed:10581077, ECO:0000269|PubMed:11015443}.
              [SIMILARITY] Belongs to the immunoglobulin superfamily. BTN/MOG
              family. {ECO:0000305}.
              [SIMILARITY] Contains 1 Ig-like C2-type (immunoglobulin-like)
              domain. {ECO:0000305}.
              [SIMILARITY] Contains 1 Ig-like V-type (immunoglobulin-like)
              domain. {ECO:0000305}.
              [SEQUENCE CAUTION] Sequence=BAA91966.1; Type=Erroneous initiation;
              Note=Translation N-terminally extended.; Evidence={ECO:0000305}.
  FEATURES             Location/Qualifiers
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                       /db_xref="taxon:9606"
       gene            1..290
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                     /note="PD-L1; PDCD1 ligand 1; Programmed death ligand 1;
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                     /gene_synonym="B7H1"
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                     /experiment="experimental evidence, no additional details
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                     /note="Extracellular. {ECO:0000255}."
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                     /gene="CD274"
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                     /FTId=VSP_013735."
     Region          19..127
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                     /gene_synonym="B7H1"
                     /gene_synonym="PDCD1L1"
                     /gene_synonym="PDCD1LG1"
                     /gene_synonym="PDL1"
                     /region_name="Domain"
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                     recorded"
                     /note="Ig-like V-type."
     Region          24..130
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                     /gene_synonym="B7H1"
                     /gene_synonym="PDCD1L1"
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                     /experiment="experimental evidence, no additional details
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                     /gene_synonym="PDCD1L1"
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                     /gene_synonym="PDL1"
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                     /experiment="experimental evidence, no additional details
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                     /experiment="experimental evidence, no additional details
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                     ECO:0000269|PubMed:18287011}."
     Region          45..47
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                     /gene_synonym="PDCD1L1"
                     /gene_synonym="PDCD1LG1"
                     /gene_synonym="PDL1"
                     /region_name="Beta-strand region"
                     /experiment="experimental evidence, no additional details
                     recorded"
                     /note="{ECO:0000244|PDB:5C3T}."
     Region          50..52
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                     /gene_synonym="B7H1"
                     /gene_synonym="PDCD1L1"
                     /gene_synonym="PDCD1LG1"
                     /gene_synonym="PDL1"
                     /region_name="Helical region"
                     /experiment="experimental evidence, no additional details
                     recorded"
                     /note="{ECO:0000244|PDB:5C3T}."
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                     /gene_synonym="B7H1"
                     /gene_synonym="PDCD1L1"
                     /gene_synonym="PDCD1LG1"
                     /gene_synonym="PDL1"
                     /region_name="Beta-strand region"
                     /experiment="experimental evidence, no additional details
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                     /note="{ECO:0000244|PDB:5C3T}."
     Region          62..68
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                     /gene_synonym="B7H1"
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                     /region_name="Beta-strand region"
                     /experiment="experimental evidence, no additional details
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                     /note="{ECO:0000244|PDB:5C3T}."
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                     /db_xref="CDD:298048"
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                     /gene_synonym="PDCD1LG1"
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                     /experiment="experimental evidence, no additional details
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                     /experiment="experimental evidence, no additional details
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                     /gene_synonym="PDCD1L1"
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                     /gene_synonym="PDL1"
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                     /experiment="experimental evidence, no additional details
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     Region          135..220
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                     /region_name="Ig"
                     /note="Immunoglobulin domain; cl11960"
                     /db_xref="CDD:238845"
     Region          138..145
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                     /experiment="experimental evidence, no additional details
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                     ECO:0000303|PubMed:15780196). /FTId=VSP_013737."
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                     /gene_synonym="PDCD1LG1"
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                     /experiment="experimental evidence, no additional details
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                     /gene_synonym="PDL1"
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                     /experiment="experimental evidence, no additional details
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                     /note="{ECO:0000244|PDB:4Z18)."
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                     /note="N-linked (GlcNAc...).
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     Region          206..213
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EXPERT OPINION

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Anti-programmed death-1 and anti-programmed death-ligand 1 antibodies in cancer therapy

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Introduction: Multiple agents targeting the immune "checkpoint" programmed death-1 (PD-1) pathway have demonstrated early evidence of durable clinical activity and an encouraging safety profile in patients with various tumor types, including some cancers, such as non-small-cell lung cancer, historically perceived as non-immunogenic and thus nonresponsive to immunotherapy.

Areas covered: Functions of the PD-1 pathway in normal immune responses are reviewed, along with the significance of expression of PD-1 and its ligands in malignant settings. Rationale for the development of PD-1 pathway-targeted therapies and associated clinical data are presented. Finally, efforts to date to identify and develop partner predictive or prognostic biomarkers for these new PD-1 pathway-targeted immunotherapies are discussed.

Expert opinion: Rather than targeting the tumor directly, immunotherapies inhibiting PD-1 pathway signaling modulate the antitumor immune response. Indeed, these agents have already demonstrated promising antitumor activity and manageable toxicity in various cancers. If future data continue to support encouraging clinical profiles of anti-PD-1 and anti-programmed death-ligand 1 antibodies, the current paradigm of cancer therapy may shift. In select patient populations (ideally identified by a predictive biomarker), PD-1 pathway-targeted immunotherapy has the potential to serve as the backbone of cancer treatment, and trials evaluating combinations with chemotherapy and/or molecularly targeted therapies will determine whether additive or even synergistic responses can be achieved.

Keywords: cancer, immunotherapy, nivolumab, programmed-death 1

Expert Opin. Biol. Ther. (2013) 13(6):847-861

1. Introduction

The concept of manipulating the immune system to fight cancer was first tested in the 1890s by William Coley, in his application of bacterial products to treat patients with solid tumors. However, difficulty in recapitulating Coley's (infrequent) successes, along with Medawar's experiments demonstrating acquired immunological tolerance and decades of generally contrary results from murine research, led to the pervasive opinion that the immune system had not, in fact, evolved to recognize and destroy cancer cells [1,2]. Burnet's theory of immunosurveillance briefly rekindled interest in this possibility, but it was not until the mid-1980s, with the identification of multiple novel tumor-associated antigens, that the prospect of cancer immunotherapy was truly revisited. Notable early experiences with immunotherapy included the landmark trials of interferon-alpha [3,4] and then high-dose interleukin-2 (IL-2) and so-called lymphokine-activated killer cells in patients with multiple tumor types [5,6]. These agents were capable of inducing durable responses, albeit with significant toxicity, in a small proportion of patients.

10.1517/14712598.2013.770836 © 2013 Informa UK, Ltd. ISSN 1471-2598, e-ISSN 1744-7682

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Article highlights.

- PD-1 ligation abrogates the anticancer T-cell response.
- Signaling via the PD-1 pathway is different from signaling via the CTLA-4 pathway – it induces distinct effects upon T-cell activation and function and acts at a different timepoint during the immune response.
- The PD-1/PD-1 ligand axis has been implicated in patient prognosis in several solid tumors, providing rationale for therapeutic inhibition.
- Early-stage clinical trials with multiple PD-1 pathway-targeted agents have demonstrated encouraging activity and safety profiles.
- Tumor PD-L1 expression is under examination as a possible predictive biomarker for patient benefit from PD-1 pathway-targeted therapy.
- Combination of other immunotherapeutic agents or anticancer therapies with anti-PD-1 treatment is forthcoming in an effort to improve outcomes.

This box summarizes key points contained in the article.

Modern tumor immunotherapy has advanced with small, but definitive victories; the last several years (termed a "renaissance" of sorts) have included the clinical targeting of immunoregulatory molecules, which appears to result in a more defined scope of antitumor effects and toxicities than does the systemic administration of cytokines. The first immunoregulatory "checkpoint" inhibitor approved by the Food and Drug Administration (FDA) was ipilimumab, a fully human anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibody, in early 2011. This in turn has led to initiation of the development of immunotherapeutic agents targeting other related molecules that govern T-cell activity (**Table 1**). Targeting another checkpoint pathway, the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway has resulted in encouraging antitumor activity in a number of tumor histologies [7,8]. This review will focus on blocking PD-1 and its ligands for cancer immunotherapy.

2. PD-1 and its ligands: physiological function

T-cell activation and function are exquisitely regulated processes that depend upon the signaling and cosignaling of several key molecules. One of these notable "checkpoint" molecules is PD-1, first reported in 1992 [9]. PD-1 pathway signaling functions to maintain central and peripheral tolerance [10-12], abrogate the development of autoimmunity [13,14], and minimize damage to healthy tissue during infection [12,15].

2.1 PD-1

PD-1 is expressed on T cells after activation [16] and is also present on exhausted T cells, activated B cells, natural killer (NK) cells, dendritic cells (DCs), and activated monocytes [12]. PD-1 has two principal ligands, known as PD-L1 (B7-H1) [17,18] and PD-L2 (B7-DC) [19,20], which are discussed in further detail below. The PD-1 molecule has immunoreceptor tyrosine-based inhibitory and switch motifs located in the cytoplasmic tail; phosphatase binding to these motifs is responsible for PD-1-mediated immunosuppression [12]. PD-1 ligation via either PD-L1 or PD-L2 inhibits signaling of the T-cell receptor (TCR), downregulates the secretion of immunostimulatory cytokines and expression of survival proteins [12], and increases T-cell production of the immunosuppressive cytokine IL-10 [13,17]. As multiple tumors express PD-L1 [21], anti-PD-1 antibodies prevent tumor PD-L1 from binding to and signaling through T-cell PD-1. A related ligand, PD-L2 (B7-H3), has been observed on multiple normal tissues and several tumor cell lines. Originally presumed to be a ligand for PD-1, PD-L2 appears to signal through an as-yet unidentified receptor distinct from PD-1 [22]. Recent data suggest that PD-L2 and another related ligand, PD-L4 (B7-H4), participate in the modulation of the immune response to a variety of tumors, suggesting a broader role of this family in cancer etiology. Further investigation of these ligands in the malignant setting is necessary.

2.2 Difference from CTLA-4

CTLA-4 binds the same antigen-presenting cell (APC) ligands (CD80 and CD86) as the costimulatory molecule responsible for delivering the second signal for T-cell activation, CD28, but with much higher affinity (**Figure 1**) [23,24]. CTLA-4 expression on T cells is induced upon TCR binding, and its ligation results in decreased T-cell activation, proliferation, and function [24,25]. However, unlike PD-1, the primary role of CTLA-4 is to regulate the amplitude of signals governing early activation of naïve and memory T cells; CTLA-4 achieves this inhibition via mechanisms distinct from those mediated by PD-1 [26,27]. CTLA-4 is also expressed on regulatory T cells (Tregs), where it likely supports their immunosuppressive function [28,29]. It is not surprising, then, that CTLA-4 also plays a key role in T-cell tolerance of self antigens and in the prevention of autoimmunity [11,14,30].

CTLA-4 signaling directly affects the interaction of naïve and memory T cells with APCs, preventing the T cells from achieving full activation. Accordingly, anti-CTLA-4 antibodies prevent CTLA-4 from downmodulating the initiation of immune responses. In contrast, PD-1 signaling works at the level of T-cell–tumor cell interaction [31], where PD-L1-expressing tumor cells can signal through PD-1 on tumor-infiltrating lymphocytes (TILs) to deactivate the antitumor T-cell response. Therapies targeting the PD-1 pathway allow the immune system to avoid this tumor-induced deactivation and thus retain the potential to evolve as the tumor mutates. Illustrative of the differences between CTLA-4 and PD-1 is the phenotype of the mice engineered to lack these proteins: CTLA-4 knockout mice die at ages < 4 weeks from autoimmunity and uncontrolled lymphoproliferative disease [32,33], whereas PD-1 knockout mice lacking

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Table 1. Key immunotherapies currently in clinical development [110-113].

Agent class	Mechanism of action
Anti-PD-1 antibody (antagonist)	Inhibits signaling of PD-L1 and PD-L2 through PD-1
Anti-PD-L1 antibody (antagonist)	Inhibits signaling of PD-L1 through PD-1 or CD80
Anti-LAG3 antibody (antagonist)	Blocks signaling of coinhibitory molecule LAG3
Anti-CD25 antibody (antagonist or toxin-linked)	Binds to and depletes $CD25^{hi}$ cells (including Tregs)
Anti-CD137 antibody (agonist)	Activates signaling through costimulatory molecule CD137
Anti-OX40 antibody (agonist)	Activates signaling though costimulatory molecule OX40
Indoleamine-2,3-dioxygenase (IDO) inhibitors	Inhibits activity of immunosuppressive enzyme IDO

Please see clinicaltrials.gov for additional information.

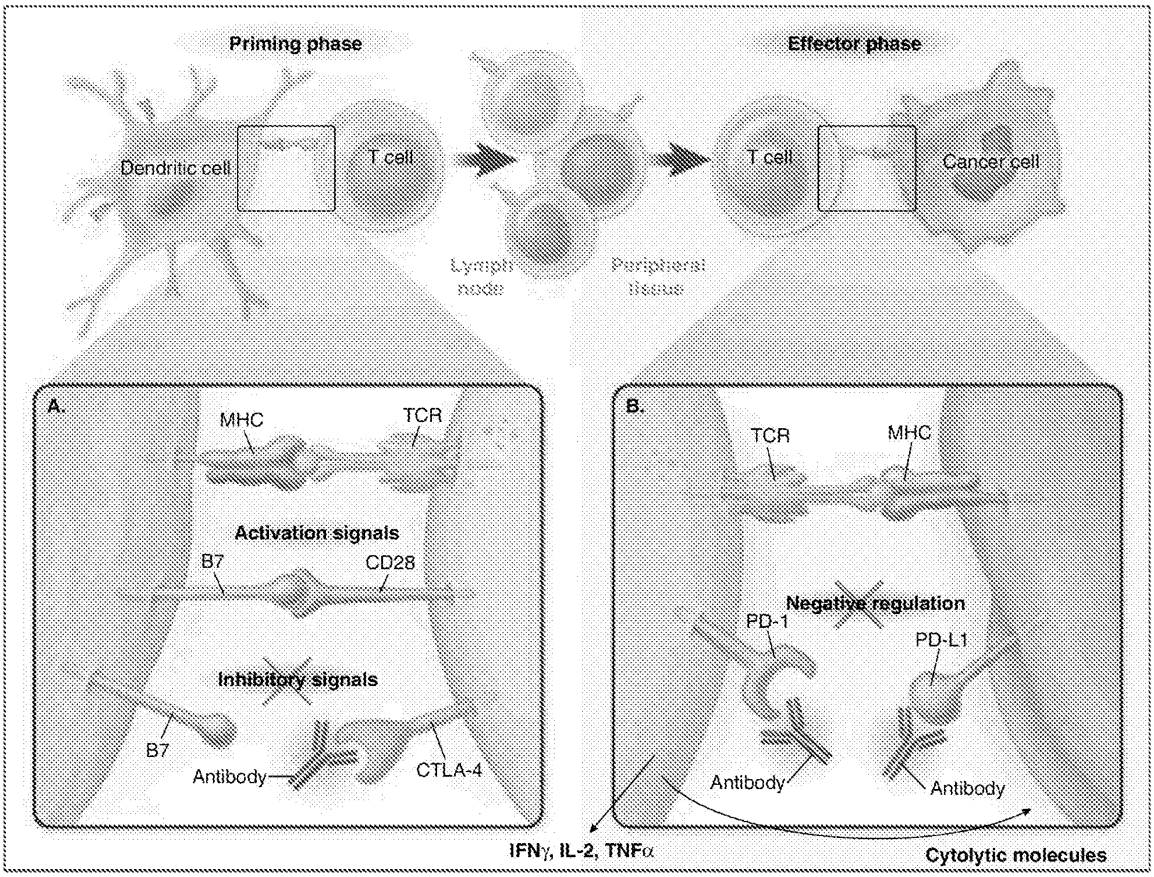


Figure 1. Blocking checkpoint inhibitors in cancer therapy. A. Blocking CTLA-4. Inhibition of CTLA-4 allows full activation of T cells during the priming phase of the immune response. **B.** Blocking PD-1. The PD-1 inhibitory receptor is expressed by T cells during long-term antigen exposure. Upon ligation with PD-L1 or PD-L2 (which can be expressed on tumor tissue), PD-1 signaling acts to turn off the effector T cell on which it is expressed. Blockade of the PD-1 signaling pathway results in the restoration of tumor-specific T-cell effector functions, including the killing of tumor cells and secretion of the immunostimulatory cytokines IFNγ, IL-2, and TNFα.

Adapted with permission from Ribas [120].

CTLA-4: Cytotoxic T lymphocyte antigen-4; IFNγ: Interferon gamma; IL-2: Interleukin-2; MHC: Major histocompatibility complex; TCR: T-cell receptor; TNFα: Tumor necrosis factor alpha.

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PD-1 develop late-onset organ-specific autoimmunity, which varies in intensity according to strain [34,35].

2.3 PD-L1

Although PD-L1 mRNA has been found at low levels in nearly all human tissues studied [21], constitutive expression of PD-L1 protein appears limited to certain populations of macrophages in various organs [36]. PD-L1 protein can, however, be induced in many cell types upon stimulation with inflammatory mediators [36-41], especially interferon gamma (IFNγ); IFNγ-induced PD-L1 expression is significant in the tumor microenvironment [36]. Data suggest that transcriptional control of PD-L1 is regulated by cooperation between signal transducer and activator of transcription 3 (STAT3) and c-Jun [42]. A recent publication investigating PD-L1 expression in melanoma samples [43] proposed the idea that tumors might express PD-L1 as a mechanism of "adaptive immune resistance" in response to TILs; this phenomenon, if proven correct, may indeed be a modification of the physiological expression – as a means of self-preservation – of PD-L1 in healthy tissue exposed to an aggravated inflammatory response. In addition to PD-1, PD-L1 can also interact with B7-1 (CD80) to reduce T-cell activation and elaboration of cytokines [44]. Thus, an anti-PD-L1 antibody can abrogate both the interaction of PD-L1 with PD-1 and the interaction of PD-L1 with CD80.

2.4 PD-L2

PD-L2 is expressed at low levels on human monocytes, myeloid DCs, and some nonhematopoetic tissues, including lung [19,45-49]. Expression of PD-L2 on myeloid cells can be stimulated by IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ, and certain toll-like receptor agonists [16,39,50]. PD-L2 is also expressed on activated T cells [51]. It should be noted that the cytokine milieu within the tumor microenvironment is often skewed toward a Th2 profile, which likely influences PD-1 ligand expression [50,52]. In addition to dampening TCR signaling via ligation of PD-1, PD-L2 can also "reverse" signal into the APC upon which it is expressed, decreasing their production of pro-inflammatory cytokines [53]. Interestingly, even though PD-L1 is thought to be the ligand most responsible for mediating PD-1-associated immunoregulation [54], the binding affinity of PD-1 for PD-L2 is two to six times greater than that of PD-1 for PD-L1 [55]. There is thus competition between PD-L1 and PD-L2 for access to PD-1.

PD-L2 is expressed on the tumors of certain subgroups of patients with various solid cancers and lymphoid malignancies [50,56-60]; it is a negative prognostic factor in patients with esophageal cancer [56] but has not been shown to correlate with prognosis in patients with pancreatic cancer [57]. PD-L2 has also been observed on cancer-associated fibroblasts [61], suggesting that the presence of these cells may affect anti-PD-1 antibody efficacy. Ongoing research aims to more precisely define the role(s) of PD-L2 in tumor development and interaction with the host immune response.

3. Solid tumor rationale: experience with anti-CTLA-4 antibodies

Part of the rationale for targeting the PD-1 pathway in cancer patients comes from experience with anti-CTLA-4 antibodies. Administration of anti-CTLA-4 antibody to mice demonstrated that treatment could mediate the regression of multiple implantable and spontaneous tumor types [62-67]. In light of these preclinical findings, a number of agents were developed for use in humans, the most notable being the fully human antibody ipilimumab [66]. Ipilimumab demonstrated activity against various tumor types in Phase I pharmacokinetic (PK) and safety studies [68-73]. Notably, an analysis of five early ipilimumab trials in patients with metastatic melanoma showed that the majority of patients who went on to experience an objective radiographic response had a delayed onset (typically after 12 weeks of treatment) of such responses [74].

In a Phase III trial of previously treated melanoma patients, ipilimumab alone or in combination with a glycoprotein 100 (gp100) vaccine demonstrated an improvement in overall survival compared with gp100 alone (10.1 or 10 versus 6.4 months) [75]. The most common adverse events (AEs) were treatment-related, and although most were low-grade, 10 – 15% of patients experienced severe (grade 3/4) drug-related AEs. Based on these findings, ipilimumab was approved in early 2011 for patients with advanced melanoma. Trials of ipilimumab are ongoing in patients with breast, prostate, pancreatic, urothelial, and small-cell and non-small-cell lung cancer (NSCLC) (www.clinicaltrials.gov).

4. Solid tumor rationale: prognostic roles of PD-1 and PD-L1 expression

Expression of PD-1 by TILs, which often indicates T-cell dysfunction or exhaustion [12,76-79], has been shown to correlate with poor prognosis in patients with renal cell cancer (RCC) [80] and hepatocellular carcinoma (HCC) [81], and with more advanced disease in patients with melanoma [82]. In parallel, tumor PD-L1 expression predicts poor outcome in patients with multiple tumor types [21], including NSCLC [83], HCC [84], and melanoma (Table 2) [82]. Interestingly, two recent reports have correlated tumor PD-L1 expression with improved prognosis and likelihood to benefit from immunotherapy (and more specifically, PD-1-targeted therapy) [7,43].

The location of PD-L1 on tumor cells is also relevant to its prognostic role. Cytosolic PD-L1 is not reported to correlate with patient response to immunotherapy [54], probably because agents targeting PD-L1 only block this molecule on the cell surface. However, there are few studies that have examined the relationship exclusively between cell-surface

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Table 2. Prognostic significance of PD-L1 expression in various tumors.

Tumor type	Prognostic and pathological associations
Cervical	Expression of PD-L1 does not appear to affect prognosis [114]
Colon	PD-L1 expression associated with poorer prognosis [115]
Gastric	PD-L1 expression correlated with tumor size, invasion, lymph node metastasis, and reduced survival [116]
HBV-related HCC	PD-L1 on circulating leukocytes associated with tumor recurrence and shorter survival after cryoablation [117]
HCC	PD-L1 expression associated with tumor aggressiveness, postoperative recurrence, and worse prognosis [84]
Melanoma	PD-L1 expression associated with more advanced disease and shorter survival [82], or, more recently, longer survival [43] than patients with PD-L1 tumors
NSCLC	Tumor expression of PD-L1 correlates with adenocarcinoma (non-squamous) histology and shorter post-lobectomy survival [83]
Ovarian	Expression of PD-L1 associated with poor prognosis [118]
RCC	PD-L1 expression associated with increased tumor stage, risk of death from RCC, and shorter survival [119]

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; NSCLC: Non-small-cell lung cancer; RCC: Renal cell cancer.

PD-L1 and patient prognosis or predicted benefit from treatment [7,43]; many reports included both membranous and cytosolic expression of PD-L1 in their analysis [85]. It is presumed that membranous PD-L1 is the most relevant form of PD-L1 because PD-1 mediates downstream signaling cascades only when it has been ligated. The expression of PD-L1 (and in some patient subpopulations, PD-L2) by tumor cells and PD-1 by TILs and the correlation of these molecules with patient prognosis present a strong rationale for therapeutic targeting of the PD-1 signaling pathway in patients with cancer. It should be noted, however, that agents targeting PD-L1 will block the interactions of PD-L1 with PD-1 and CD80, whereas agents blocking PD-1 will block the interactions of PD-1 with PD-L1 and PD-L2, thus potentially resulting in distinct effects in patients with cancer.

5. PD-1-pathway-targeted agents: preclinical data

Bristol-Myers Squibb is developing fully human IgG4 anti-PD-1 (MDX-1106/BMS-936558/ONO-4538/nivolumab) and anti-PD-L1 (MDX-1105/BMS-936559) antibodies. Because both nivolumab and BMS-936559 are fully human, some of the murine preclinical experiments that served as proof-of-principle for further development were completed with surrogate murine antibodies. The addition of nivolumab to cultures of human, vaccine-induced, melanoma antigen-specific T cells and peptide-pulsed APCs resulted in improved T-cell proliferation, increased absolute numbers of T cells, augmented type 1 and 2 cytokine production, and enhanced cytotoxic T lymphocyte (CTL) recognition and lysis of melanoma targets [86]. The same antibody added to an *ex vivo* culture of human melanoma-specific CTLs reversed their Treg-mediated cytokine downregulation and induced their proliferation [87]. Treating melanoma- or colon cancer-bearing mice with an anti-PD-1 antibody and a GM-CSF-secreting tumor cell vaccine improved survival in comparison to mice treated with either agent alone. This extended survival correlated with antigen-specific T-cell responses, increased proinflammatory cytokine production, and the persistence of functional CTLs in the tumor microenvironment [88]. Mangsbo and colleagues found that treatment of mice with bladder carcinoma with chimeric rat/mouse anti-PD-1 and CpG oligodeoxynucleotide led to longer survival than treating mice with either agent alone [89]. This was associated with an increased number of circulating activated $CD8^+$ T cells. Finally and most recently, the combination of PD-1 and LAG-3 blockade appeared to synergize, leading to lasting tumor eradication in 70 – 80% of cancer-bearing mice treated with both agents [90].

Cure Tech is developing a humanized IgG1 anti-PD-1 monoclonal antibody (mAb) (pidilizumab, CT-011), and early reports support the rationale for its current clinical trials. A study from 2011 observed that NK cells from multiple myeloma patients express PD-1, whereas multiple myeloma cells express PD-L1. *In vitro* experiments demonstrated that addition of pidilizumab enhanced NK-cell migration toward and killing of autologous multiple myeloma cells. Furthermore, this phenomenon could be perpetuated by the addition of lenalidomide, which downregulates multiple myeloma cell expression of PD-L1 [91]. Rosenblatt and colleagues developed a vaccine in which tumor and DCs are fused, resulting in tumor antigen presentation accompanied by DC costimulation. Because multiple myeloma cells and fused multiple myeloma/DC cells expressed high levels of PD-L1, the investigators added pidilizumab to block PD-1/PD-L1 signaling in fusion cell/autologous T-cell cocultures. This improved the vaccine-induced polarization of T cells toward an activated T-helper-1 phenotype, decreased Treg numbers, and increased T-cell cytotoxicity against autologous multiple myeloma cells [92]. A related study reported that tumor-bearing mice treated with pidilizumab, a tumor vaccine, and low-dose cyclophosphamide (to deplete Tregs) experienced complete tumor regression more often and had longer survival than untreated animals. This combination of agents led to synergistic antigen-specific immune responses, including increases in tumor-bearing mouse spleen-cell IFNγ production, splenocyte killing of tumor target cells, and vaccine-induced TILs

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with a concurrent decrease in tumor-infiltrating Treg cells. This effect was dependent on both $CD8^+$ and $CD4^+$ T cells. Interestingly, pidilizumab administration lengthened the effect of cyclophosphamide on Treg inhibition [93].

MedImmune recently announced that it is developing MEDI4736, another mAb against PD-L1, with support from the Ludwig Institute for Cancer Research and the Cancer Research Institute. Cocultures of tumor cells and human T cells with MEDI4736 demonstrated T-cell-dependent killing of tumor cells. More than half of tumor-bearing mice treated with surrogate anti-mouse PD-L1 antibody and oxaliplatin achieved complete tumor regression [94].

Finally, Mkrtichyan and colleagues reported preclinical data available on the murine homolog of the PD-L2/IgG1 fusion protein AMP-224 in development at Amplimmune/GlaxoSmithKline [95]. Mice treated with the fusion protein in combination with cyclophosphamide and a vaccine had decreased PD-1^{hi} $CD4^+$ and $CD8^+$ T cells within their tumors [96]. Whether this phenomenon results from improved infiltration by and/or expansion of non-exhausted PD-1^{lo} T cells in the tumor microenvironment (and whether it can be recapitulated in human patients treated with AMP-224) remains to be confirmed.

There are other agents in development: Merck has a humanized IgG4 mAb against PD-1 termed MK-3475, and Roche and Genentech have partnered to pursue the clinical development of the anti-PD-L1 mAb MPDL3280A (RG7446). To our knowledge, there are no preclinical data publicly available regarding any of these agents.

6. PD-1-pathway-targeted agents: clinical data

6.1 Nivolumab

The first-in-human trial of the anti-PD-1 antibody nivolumab included 39 patients with advanced colorectal cancer (CRC), melanoma, NSCLC, RCC, or castration-resistant prostate cancer (CRPC). Initially, patients received a single dose (0.3, 1, 3, or 10 mg/kg) of nivolumab; responding patients could receive subsequent doses (NCT00441337) [54]. The investigators observed one complete response (CR) in a patient with CRC treated at 3 mg/kg (which was ongoing for 21 months at last follow-up) and two partial responses (PRs) in patients with RCC and melanoma, both treated at 10 mg/kg. In a repeat-dose expansion cohort (at the 10-mg/kg dose level) that included patients with stable disease (SD) or better, tumor responses were observed in patients with melanoma and RCC without a concordant increase in toxicity. One patient with RCC who received only 3 treatments with nivolumab achieved a PR lasting longer than 16 months. Additionally, one patient with NSCLC and one patient with melanoma had significant regressions in tumor burden not meeting criteria for a PR. No maximum tolerated dose (MTD) was identified and most AEs were immune-related, consistent with the presumed mechanism of action of nivolumab. No immune-related AEs of grades 3 – 4 were observed in the 4 weeks following the first dose of nivolumab. One serious treatment-related AE of inflammatory colitis occurred and improved after administration of infliximab and steroids. No patient developed anti-nivolumab antibodies during the study period. Mean peak receptor occupancy (RO) of nivolumab was 85% after a single dose, and RO appeared to be independent of dose level. Tumor biopsies from nine patients were available, and evaluation of PD-L1 expression suggested that patients whose tumors were PD-L1 positive might be more likely to achieve tumor regression after treatment with nivolumab ($p = 0.0476$). Equally notable was the lack of response in patients with PD-L1-tumors. Results from this study prompted a subsequent, larger investigation of nivolumab in patients with advanced cancer.

Interim data from this second Phase I trial were recently reported (NCT00730639) [7,97]. Three hundred and four patients with various advanced tumors, including melanoma, RCC, NSCLC, CRPC, and CRC, received 0.1 – 10 mg/kg doses of nivolumab on a biweekly schedule. Treatment continued (for 12 or fewer 8-week cycles) until patients developed progressive disease or achieved CRs. The evaluable patient population (as of July 2012) demonstrated objective responses (ORs) in 33/106 (31%) patients with melanoma, 10/34 (29%) patients with RCC, and 20/122 (16%) patients with NSCLC, a tumor type not typically perceived as responsive to immunotherapy. Twenty of 31 patients followed for a year or longer had responses of the same duration. Perhaps unsurprisingly, ORs to treatment with nivolumab followed two patterns: conventional and non-conventional [98]. Non-conventional responses to treatment with nivolumab included an initial increase in target lesion size followed by regression, and control of the target lesion with new lesion formation. In patients who did respond to nivolumab, tumor regression following treatment appeared to occur more rapidly when compared with responses observed in studies of patients treated with ipilimumab (personal observation) [7]. However, this remains to be firmly established by evaluation of both agents in a randomized, head-to-head trial.

Grade 3/4 AEs related to treatment with nivolumab occurred in 14% of patients and there were three deaths resulting from pneumonitis. The most common drug-related AEs, however, included pruritus, rash, fatigue, nausea, decreased appetite, and diarrhea; most of these (166/207 across grades) were of low grade. AEs with possible immune-related causes, termed AEs of special interest, occurred in 41% of patients and included vitiligo, hepatitis, pneumonitis, colitis, thyroiditis, and hypophysitis. In this report, the median PD-1 RO of nivolumab on circulating T-cells was between 64 and 70% across all dose levels. This ongoing trial also included a somewhat larger analysis of the prognostic significance of tumor PD-L1 expression (discussed below).

PK data on nivolumab were reported at American Society of Clinical Oncology (ASCO) 2012 [99]. Single-dose PK was approximately linear (over the dose range of 0.1 – 10 mg/kg)

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and there was a dose-proportional increase in peak plasma concentration (C_{max}) and geometric mean area under the curve (AUC[INF]) with moderate variability (20 – 44%) among patients. Geometric mean clearance was 0.13 – 0.19 mL/h/mg and did not vary across tumor types; however, clearance did vary according to gender. The mean volume of distribution V_z ranged from 83 to 113 mL/kg. Finally, the $t_{1/2}$ of nivolumab ranged from approximately 17 to 25 days. Five Phase III trials are currently open to further explore the activity of nivolumab in patients with melanoma, NSCLC, and RCC (www.clinicaltrials.gov).

6.2 Pidilizumab

A Phase I study evaluating safety, dose, and PK of the anti-PD-1 antibody pidilizumab was reported in 2008 [100]. Seventeen patients with advanced hematological cancers (acute myeloid leukemia, chronic lymphocytic leukemia, non-Hodgkin's lymphoma (NHL), Hodgkin's lymphoma, or multiple myeloma, with or without previous treatment) received escalating doses of pidilizumab ranging from 0.2 to 6 mg/kg. Although an MTD was not identified, clinical responses were observed in 33% of the patients, including one CR in a patient with NHL. Four patients experienced SD, and one had a mixed response. Diarrhea, the most common AE, was observed in two patients but was considered not likely associated with pidilizumab treatment. Pidilizumab PK appeared to be linear, and the mean clearance of pidilizumab ranged from 0.14 to 0.34 mL/h/kg. The V_z ranged from 53.1 to 90.3 mL/kg, whereas the median serum $t_{1/2}$ of pidilizumab was approximately 217 – 410 h (9 – 17 days), consistent with other mAb $t_{1/2}$ in humans. Gender and type of malignancy had no significant impact on pidilizumab PK. The investigators observed increased percentages of patient peripheral blood $CD4^+$ lymphocytes up to 21 days following pidilizumab treatment.

Although the clinical efficacy and safety results of a subsequent study (NCT00532259) are not yet public, an interim analysis presented at the 2009 American Society of Hematology (ASH) conference summarized lymphocyte differences in the first 30 patients with diffuse large B-cell lymphoma treated with pidilizumab (at 1.5 mg/kg, every 6 weeks for three doses) after autologous stem cell transplantation [101]. The median percentage (of total lymphocytes) in memory $CD4^+$ T-cell subsets was significantly increased from baseline levels after 16 weeks ($p < 0.05$). Additional data on pidilizumab were reported at ASH 2012 [102]. Patients with relapsed follicular lymphoma who had previously received rituximab were treated with pidilizumab (3 mg/kg every 4 weeks) and rituximab (375 mg/m^2 weekly, beginning 2 weeks after pidilizumab commencement). Of 29 evaluable patients, 19 achieved ORs; 15 (52%) had CRs and 4 (14%) had PRs; no grade 3 – 4 AEs were reported. Median progression-free survival was 21.1 months for the whole group, but was not reached for the 19 responding patients. The average time to response was 88 days.

6.3 MK-3475

Patnaik and colleagues reported interim data for a Phase I trial of MK-3475, an anti-PD-1 antibody, at ASCO 2012 (NCT01295827) [103]. Seventeen patients with advanced solid tumors were treated with one dose of MK-3475 at 1, 3, or 10 mg/kg, and then 4 weeks later, additional doses were administered every 2 weeks. No dose-limiting toxicities were reported; in this initial cohort there were no drug-related AEs of grade 3 or higher. It should be noted, however, that one case of pneumonitis was treated with corticosteroids (the patient improved clinically but was subsequently taken off study). The most common drug-related AEs were pruritus, fatigue, and nausea. Two patients with melanoma (treated for ≥ 6 months) achieved PRs (according to RECIST 1.1 criteria), and the investigators noted an unconfirmed PR in a patient with squamous NSCLC. In the melanoma expansion cohort (including 10 evaluable patients at the time of presentation) treated at 10 mg/kg, 4 unconfirmed ORs (3 PRs and 1 CR, according to RECIST 1.1) and 5 grade 3/4 drug-related AEs (including 1 patient with pneumonia and possible pneumonitis) were reported before the last follow-up date of May 18, 2012. This presentation also reported pharmacodynamic and PK data. High levels of PD-1 RO by MK-3475 were observed at all dose levels in an *ex vivo* whole blood stimulation assay. There was low clearance (2 – 5 mL/day/kg) and limited V_z (60 – 100 mL/kg). Both C_{max} and exposure increased with increasing dose, and the half-life of MK-3475 was 21 days. Additionally, there was no evidence of immunogenicity. Recently, additional data on this trial became available. Within 85 evaluable patients, ORs have been observed in 43 (51%); 8 of these patients achieved CRs. Eleven of 27 patients (41%) previously treated with ipilimumab had ORs, although no CRs were observed. Common AEs included cough, pruritis, arthralgia, rash, fever, diarrhea, fatigue, and nausea, and seven treatment-related grade 3/4 AEs with potentially immune-mediated causes were reported. This trial is ongoing [104].

6.4 BMS-936559

Brahmer and colleagues recently presented an interim analysis of a Phase I trial with the anti-PD-L1 antibody BMS-936559 (NCT00729664) [8]. Two hundred and seven patients with advanced solid tumors (NSCLC, melanoma, CRC, ovarian cancer, RCC, pancreatic cancer, gastric cancer, and breast cancer) were treated every 2 weeks, in 6-week cycles, with escalating doses of BMS-936559 (ranging from 0.3 to 10 mg/kg) until progressive disease, CR, or the completion of 16 cycles. An MTD was not identified and ORs (according to RECIST criteria) were observed in patients with melanoma (9/52), RCC (2/17), NSCLC (5/49), and ovarian cancer (1/17). Data for patients with breast, gastric, and pancreatic cancer were not mature at the time of this analysis. Eight out of 16 patients who underwent extended follow-up had responses that lasted for greater than 1 year.

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Table 3. Ongoing trials of PD-1-pathway-targeted agents.

Compound	Phase	Setting	NCT #	Sponsor	Description or control arm	Enrollment	Expected completion date
Pidilizumab	II	Metastatic melanoma	NCT01435369	Cure Tech, Ltd	1.5 vs 6 mg/kg	100	March 2014
With Rituximab	II	Relapsed follicular lymphoma	NCT00904722	Cure Tech, Ltd	3 mg/kg, single arm	30	June 2013
With FOLFOX	II	Metastatic CRC	NCT00890305	Cure Tech, Ltd	FOLFOX alone	168	September 2012
With DC/multiple myeloma vaccine	II	Multiple myeloma after stem cell transplantation	NCT01067287	Beth Israel Deaconess	Pidilizumab alone	35	March 2013
With DC/RCC vaccine	II	RCC	NCT01441765	Beth Israel Deaconess	Pidilizumab alone	44	November 2013
With DC/AML vaccine	II	AML	NCT01096602	Beth Israel Deaconess	DC/AML vaccine alone	35	May 2013
With Gemcitabine	II	Resected pancreatic cancer	NCT01313416	Georgia Health Sciences University	3 mg/kg pidilizumab plus 1000 mg/m^2 gemcitabine, single arm	29	February 2017
With Sipuleucel-T and cyclophosphamide	II	Advanced prostate cancer	NCT01420965	Georgia Health Sciences University	Sipuleucel-T only or Sipuleucel-T plus pidilizumab	57	December 2017
	II	Diffuse large B-cell lymphoma after ASCT	NCT00532259	Cure Tech, Ltd and Beth Israel Deaconess	1.5 mg/kg, single arm	70	August 2011 (final data not yet public)
MK-3475	I	Multiple solid tumors	NCT01295827	Merck	1, 3, and 10 mg/kg arms	439	March 2015
Low or high dose	II	Advanced melanoma	NCT01704287	Merck	Chemotherapy (investigator-choice)	510	March 2016
MEDI4736	II	Multiple solid tumors	NCT01693562	MedImmune, LLC	Dose escalation	110	November 2014
AMP-224	I	Multiple solid tumors	NCT01352884	Amplimmune/ GlaxoSmithKline	Dose escalation	63	July 2013
MPDL3280A/RG7446	I	Multiple solid tumors	NCT01375842	Genentech	Dose escalation	88	July 2013
With vemurafenib	I	Metastatic melanoma (BRAFV600-mutation +)	NCT01656642	Genentech	3 arms, no dose info	44	November 2014
With bevacizumab	I	Multiple solid tumors	NCT01633970	Genentech	Dose escalation with or without chemotherapy	68	March 2015
BMS-936559	I	Multiple solid tumors	NCT00729664	Bristol-Myers Squibb	Dose escalation	286	September 2013
Nivolumab	I	Multiple solid tumors	NCT00730639	Bristol-Myers Squibb	Dose escalation	290	October 2015
	I	Hepatocellular Carcinoma (±HCV or HBV)	NCT01658878	Bristol-Myers Squibb	Dose escalation, no infection versus HCV versus HBV	72	April 2014
With sunitinib or pazopanib	I	RCC	NCT01472081	Bristol-Myers Squibb	Nivolumab + pazopanib versus nivolumab + sunitinib	72	August 2013
With ipilimumab	I	Metastatic melanoma	NCT01024231	Bristol-Myers Squibb	Varying doses + ipilimumab or nivolumab alone	64	August 2014

AML: Acute myelogenous leukemia; DC: Dendritic cell; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NSCLC: Non-small-cell lung cancer; RCC: Renal cell cancer; tx: Treatment.

Table 3. Ongoing trials of PD-1-pathway-targeted agents.

Compound	Phase	Setting	NCT #	Sponsor	Description or control arm	Enrollment	Expected completion date
With various chemotherapies	I	NSCLC	NCT01454102	Bristol-Myers Squibb	Nivolumab alone or with gemcitabine/cisplatin, pemetrexed/cisplatin, paclitaxel/carboplatin, bevacizumab maintenance, ipilimumab or erlotinib	190	December 2013
With anti-KIR antibody	I	Multiple solid tumors	NCT01714739	Bristol-Myers Squibb	Dose escalation	150	August 2015
	I	RCC	NCT01358721	Bristol-Myers Squibb	0.3, 2, and 10 mg/kg (one group no previous tx)	80	August 2015
	I	Melanoma	NCT01621490	Bristol-Myers Squibb	3 mg/kg, single arm	80	December 2015
	I	Multiple solid tumors	NCT00836888	Ono Pharmaceutical Co.	Dose escalation	24	September 2010 (primary)
	I	Hematological malignancies	NCT01592370	Bristol-Myers Squibb	Dose escalation	110	February 2015
With vaccine	I	Resected melanoma	NCT01176474	H. Lee Moffit Cancer Center and Research Inst.	Nivolumab dose escalation + vaccine	34	July 2013 (primary)
With vaccine	I	Unresectable melanoma	NCT01176461	H. Lee Moffit Cancer Center and Research Inst.	Nivolumab dose escalation + vaccine	125	June 2014 (primary)
With IL-21	I	Multiple solid tumors	NCT01629758	Bristol-Myers Squibb	IL-21 dose escalation with 3 mg/kg nivolumab	160	September 2015
	II	Advanced squamous NSCLC	NCT01721759	Bristol-Myers Squibb	3 mg/kg, single arm	100	February 2014
	II	RCC	NCT01354431	Bristol-Myers Squibb	0.3, 2, and 10 mg/kg arms	150	November 2012
	III	Squamous NSCLC	NCT01642004	Bristol-Myers Squibb	Docetaxel	264	August 2014
	III	Non-squamous NSCLC	NCT01673867	Bristol-Myers Squibb	Docetaxel	574	November 2014
	III	Melanoma	NCT01721772	Bristol-Myers Squibb	Dacarbazine or placebo	410	June 2020
	III	Advanced melanoma	NCT01721746	Bristol-Myers Squibb	Dacarbazine or carboplatin + paclitaxel	390	May 2015
	III	Advanced or metastatic RCC	NCT01668784	Bristol-Myers Squibb	Everolimus	822	February 2016

AML: Acute myelogenous leukemia; DC: Dendritic cell; HBV: Hepatitis B virus, HCV: Hepatitis C virus; NSCLC: Non-small-cell lung cancer; RCC: Renal cell cancer; tx: Treatment

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Table 4. Direct comparison of anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies.

	Anti-PD-1	Anti-CTLA-4	Anti-PD-L1
Role of molecule in immune system	Induced upon TCR signaling; aids in maintenance of peripheral tolerance	Induced upon TCR signaling; controls magnitude of early T-cell activation	Induced via inflammatory signals (notably type I and II interferons)
Mechanism of action	Prevents binding of PD-1 to PD-L1 or PD-L2	Prevents binding of CTLA-4 to costimulatory molecule CD28	Prevents binding of PD-L1 to PD-1 or CD80
Response kinetics	Immune-related pattern, standard radiological pattern	Immune-related pattern, standard radiological pattern	Immune-related pattern, standard radiological pattern
Most common AEs	Fatigue, decreased appetite, diarrhea, nausea, cough	Dermatologic immune-related AEs, diarrhea, fatigue, nausea, GI immune-related AEs	Fatigue, infusion reactions, diarrhea, arthralgia, rash
Immune-related AEs	Diarrhea, rash, pruritus	Pruritus, rash, diarrhea	Infusion reaction, diarrhea, rash
Notable treatment-related deaths	Pneumonitis	Inflammatory colitis and bowel perforation	None

AEs: Adverse events; CTLA-4: Cytotoxic T lymphocyte antigen-4; GI: Gastrointestinal; TCR: T-cell receptor.

Most treatment-related AEs were of low grade; the most common were diarrhea, infusion reactions, fatigue, rash, headache, nausea, arthralgia, and pruritus. Nine percent of patients experienced treatment-related AEs of grade 3 or 4, and the most common of these included fatigue, lymphopenia, and infusion reaction. AEs of special interest (including rash, hypothyroidism, hepatitis, and one instance each of diabetes mellitus, sarcoidosis, myasthenia gravis, and endophthalmitis) consistent with possible immune-related causes occurred in 39% of patients. PK parameters reported in this study included the following: the AUCs (0 – 14 days) for 1, 3, and 10 mg/kg doses were 2,210, 7,750, and 36,620 μg/mL/h, respectively. Evaluation of population PK allowed an estimation of the $t_{1/2}$ of BMS-936559 of approximately 15 days. Median RO of peripheral blood T cells after one treatment cycle exceeded 65% across all dose levels. This trial is ongoing.

6.5 Other agents

Ongoing trials with AMP-224, MPDL3280A, MEDI4736, and the agents discussed above are presented in Table 3.

7. Biomarkers

Although there have been attempts to identify a pretreatment biomarker that would predict prognosis and/or response to ipilimumab [105-107], no single molecule has been pinpointed. Efforts continue (NCT00972933, NCT01489423) to establish a companion diagnostic. Presently, ipilimumab remains without a pretreatment biomarker indicating which patients are most likely to benefit.

However, both the studies led by Drs. Brahmer [54] and Topalian [7] describe preliminary data suggesting that tumor expression of PD-L1 might serve as a predictive or prognostic indicator of patient response to anti-PD-1 treatment. In the more recent clinical trial, pretreatment tumor biopsies were available from 42 patients (18 with melanoma, 10 with NSCLC, 7 with CRC, 5 with RCC, and 2 with CRPC), 25 of which were PD-L1 positive. Of these patients, 9/25 (36%) achieved PRs or CRs, whereas none of the patients with PD-L1 tumors had ORs. This is in line with the data demonstrating a correlation of PD-L1 expression in melanoma lesions with improved patient survival [43]; tumor PD-L1 expression may be induced by IFNγ-secreting TILs, thus indicating an ongoing antitumor response in the host. However, because the biopsies were from a non-random subset of patients in the nivolumab trial, this analysis is being repeated prospectively, in randomly selected populations, to confirm whether PD-L1 might serve as a prognostic or predictive biomarker (Phase III nivolumab trials, www.clinicaltrials.gov).

8. Expert opinion on the future of anti-PD-1 directed therapy

The potential is increasing for immunostimulatory antibodies (both those that release a "brake" on the immune system and those that stimulate T-cell activation pathways) to become key treatments in the therapy of patients with cancer. The recent FDA approval of ipilimumab has ushered in a new era of immunotherapy with clear and dramatic clinical efficacy. Trials evaluating anti-PD-1 antibodies and other immunostimulatory agents in early development (i.e., anti-OX40, anti-4-1BB, anti-CD40, anti-Killer-cell immunoglobulin-like receptor (KIR), and anti-IL-21) will demonstrate whether targeting other specific molecules and pathways in the immune system can also achieve clinically significant results. Furthermore, it is also rational to consider combining PD-1 pathway-targeted agents with agents or strategies designed to overcome other immunosuppressive mechanisms within the tumor microenvironment. Promising murine data on PD-1 and LAG3 or Tim-3 blockade [108,109] suggest that these agents may synergize in human patients resulting in improved anticancer immune responses.

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Although it may seem like certain tumor types respond to treatment with PD-1 pathway-targeted agents (NSCLC, melanoma, RCC) and others may not, it is important to note that the potential for response may be due to the presence of PD-L1 on tumors themselves. For example, Topalian and colleagues found that patients with PD-L1-positive tumors were more likely to respond to nivolumab. Only one patient (of seven) with CRC had a PD-L1+ lesion, so it will be instructive to determine the PD-L1 status of other CRC patients currently involved in clinical trials of anti-PD-1 agents before concluding whether they are capable of experiencing ORs. Thus far, a significant portion of patients – including some with cancers not historically observed to respond to immunotherapy – treated with anti-PD-1 antibodies have developed objective, durable tumor responses, suggesting the development and/or involvement of active antitumor immunosurveillance.

Although the safety profiles for the agents discussed in this report are largely encouraging, there is one AE meriting discussion. It is important that physicians administering PD-1-pathway-targeted agents are able to identify (via cough and shortness of breath) and treat pneumonitis rapidly and successfully. As previously published, mild-to-moderate pneumonitis accompanying treatment with anti-PD-1 antibodies has been managed with observation, treatment discontinuation, or via administration of corticosteroids (occasionally in combination with other immunosuppressive agents) [7,103]. It is our experience that pneumonitis is easily managed if it is identified early.

Finally, it might be presumptuous, given the earlier stage of development of PD-1-pathway-targeted agents, to conjecture that the combination of other therapies (including new indoleamine 2,3-dioxygenase and B-raf inhibitors) with anti-PD-1 might be more tolerable than combination of those same therapies with ipilimumab. However, the range and severity of inherent toxicities observed during treatment with ipilimumab and the encouraging safety profile of PD-1-pathway-targeted treatments will inevitably prompt such a comparison (Table 4). Another issue to be resolved will be the determination of optimal timing of PD-1-pathway-targeted treatment when administered in combination with other agents; a trial of ipilimumab and anti-PD-1 is ongoing in which both agents are administered in varying doses and schedules (NCT01024231). Table 3 presents planned and ongoing trials of PD-1-targeted agents alone and in combination. The results of these trials will establish the relative efficacy and safety of PD-1-pathway-targeted agents when given alone or with other therapies.

Declaration of interest

O Hamid has received research funding from Roche, Genentech, Merck, and Bristol-Myers Squibb (BMS), acted as a consultant for Merck, BMS, and Genentech, and served as a speaker for Genentech and BMS. R Carvajal states no conflict of interest and has received no payment in preparation of this manuscript. Professional medical writing and editorial assistance were provided by CM Wilke, at StemScientific, and was funded by Bristol-Myers Squibb.

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• **This editorial provides a summary of some of the newer checkpoint blockade clinical data and discusses the selection of patients who may have an increased likelihood of benefitting from PD-1 pathway-directed therapy.**

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ORIGINAL ARTICLE

Safety and Tumor Responses with Lambrolizumab (Anti–PD-1) in Melanoma

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ABSTRACT

BACKGROUND

The programmed death 1 (PD-1) receptor is a negative regulator of T-cell effector mechanisms that limits immune responses against cancer. We tested the anti–PD-1 antibody lambrolizumab (previously known as MK-3475) in patients with advanced melanoma.

METHODS

We administered lambrolizumab intravenously at a dose of 10 mg per kilogram of body weight every 2 or 3 weeks or 2 mg per kilogram every 3 weeks in patients with advanced melanoma, both those who had received prior treatment with the immune checkpoint inhibitor ipilimumab and those who had not. Tumor responses were assessed every 12 weeks.

RESULTS

A total of 135 patients with advanced melanoma were treated. Common adverse events attributed to treatment were fatigue, rash, pruritus, and diarrhea; most of the adverse events were low grade. The confirmed response rate across all dose cohorts, evaluated by central radiologic review according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, was 38% (95% confidence interval [CI], 25 to 44), with the highest confirmed response rate observed in the cohort that received 10 mg per kilogram every 2 weeks (52%; 95% CI, 38 to 66). The response rate did not differ significantly between patients who had received prior ipilimumab treatment and those who had not (confirmed response rate, 38% [95% CI, 23 to 55] and 37% [95% CI, 26 to 49], respectively). Responses were durable in the majority of patients (median follow-up, 11 months among patients who had a response); 81% of the patients who had a response (42 of 52) were still receiving treatment at the time of analysis in March 2013. The overall median progression-free survival among the 135 patients was longer than 7 months.

CONCLUSIONS

In patients with advanced melanoma, including those who had had disease progression while they had been receiving ipilimumab, treatment with lambrolizumab resulted in a high rate of sustained tumor regression, with mainly grade 1 or 2 toxic effects. (Funded by Merck Sharp and Dohme; ClinicalTrials.gov number, NCT01295827.)

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This article was published on June 2, 2013, at NEJM.org.

N Engl J Med 2013;369:134-44.
DOI: 10.1056/NEJMoa1305133
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Cancer evolves to exploit multiple mechanisms in order to avoid immune-cell recognition and antitumor effector functions, thereby limiting the clinical benefits of immunotherapy strategies. Antibodies that block the inhibitory receptor cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), such as ipilimumab, have been shown to release one of these negative immune regulatory pathways, leading to durable responses in a subgroup of patients with metastatic melanoma and an overall survival benefit in patients with metastatic melanoma.[1,2] The programmed cell death 1 (PD-1) receptor is another inhibitory receptor expressed by T cells preferentially with long-term exposure to antigens. Its primary ligand, PD-L1 (also known as B7-H1 or CD274), is frequently expressed within the tumor microenvironment, including cancer cells and tumor-infiltrating macrophages. The PD-1 receptor has a second ligand, PD-L2 (also known as B7-DC or CD273), that is preferentially expressed by antigen-presenting cells.[3] In tumor models, PD-1 negatively regulates the effector phase of T-cell responses after ligation of PD-L1 expressed within the tumor.[4] It has been postulated that antibodies that block the interaction between PD-1 and PD-L1 in tumors may preferentially release the cytotoxic function of tumor-specific T cells with fewer systemic toxic effects than those that are seen with other immune checkpoint inhibitors.[3,5,6]

Two large, dose-escalation, phase 1 clinical trials evaluating the safety of the anti–PD-1 antibody nivolumab (formerly known as BMS936558) and the anti–PD-L1 antibody BMS936559 showed significant antitumor activity in patients with advanced melanoma, lung carcinoma, and renal-cell carcinoma, among other cancers, thus validating the PD-1–PD-L1 axis as a therapeutic target.[7-9] Most tumor responses were durable beyond 1 year.[8,9] Toxic effects were generally of low grade.

Lambrolizumab (previously known as MK-3475) is a highly selective, humanized monoclonal IgG4–kappa isotype antibody against PD-1 that is designed to block the negative immune regulatory signaling of the PD-1 receptor expressed by T cells. The variable region sequences of a very-high-affinity mouse antihuman PD-1 antibody (dissociation constant, 28 pM) were grafted into a human IgG4 immunoglobulin with a stabilizing S228P Fc alteration. The IgG4 immunoglobulin subtype does not engage Fc receptors or activate complement, thus avoiding cytotoxic effects of the antibody when it binds to the T cells that it is intended to activate. In T-cell activation assays that used human donor blood cells, the 50% effective concentration was in the range of 0.1 to 0.3 nM (unpublished data). The first dose-escalation phase 1 study involving patients with solid tumors showed that lambrolizumab was safe at the dose levels tested (1 mg per kilogram of body weight, 3 mg per kilogram, and 10 mg per kilogram, administered every 2 weeks) without reaching a maximum tolerated dose. In addition, clinical responses were observed at all the dose levels.[10] We report here the safety and antitumor activity of three dosing regimens of lambrolizumab that we evaluated in patients with advanced melanoma.

METHODS

STUDY OVERSIGHT

This study was sponsored by Merck Sharp and Dohme, which provided the study drug and worked jointly with the senior academic authors to design the study, collect the data, and interpret the study results. The data were analyzed by a statistician employed by the sponsor and by the senior academic authors. All the authors made the decision to submit the manuscript for publication, vouch for the accuracy and completeness of the data, and attest that the study was conducted as specified in the protocol, which is available with the full text of this article at NEJM.org. The protocol and its amendments were approved by the relevant institutional review boards or ethics committees, and all participants provided written informed consent. All drafts of the manuscript were written by the corresponding author with input from the other authors. The sponsor provided assistance with the preparation of the manuscript. Aside from the authors and those listed in the acknowledgments, no others contributed to the preparation of the manuscript.

STUDY DESIGN

The primary objective of this study was to evaluate the safety profile of lambrolizumab. The secondary end point was a preliminary analysis of the antitumor activity of lambrolizumab, both in patients who had received prior treatment with ipilimumab and in those who had not. After dose escalation of lambrolizumab to a maximum

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administered dose of 10 mg per kilogram every 2 weeks,[10] an expansion cohort (Part B of the study) was initiated, with eligibility restricted to patients with advanced melanoma. In Part B of the study, which we report on here, the initial cohort of patients who were enrolled received lambrolizumab as a 30-minute intravenous infusion, every 2 weeks at a dose of 10 mg per kilogram; patients enrolled in additional cohorts in Part B received lambrolizumab as a 30-minute intravenous infusion every 3 weeks at a dose of 2 mg per kilogram or 10 mg per kilogram in sequential or concurrent cohorts without randomization. The study therapy was continued until disease progression was confirmed, unacceptable toxic effects developed, or consent was withdrawn. Patients in whom a scheduled scan showed initial disease progression were allowed to continue receiving treatment until a confirmatory scan was obtained at least 1 month later. Patients underwent a mandatory baseline biopsy and optional biopsies during the course of the trial for biomarker studies. Safety evaluations (clinical and laboratory) were performed at baseline and before each dose of lambrolizumab was administered. No premedications were administered before lambrolizumab infusions. The first scheduled assessment of tumor response was performed 12 weeks after the first dose of lambrolizumab and every 12 weeks thereafter. The evaluation of tumor response was made by investigators at the study site and by a central imaging vendor (Perceptive Informatics).

PATIENTS

Patients were eligible for participation in Part B of the study if they were 18 years of age or older, had measurable metastatic or locally advanced unresectable melanoma, and had adequate performance status and organ function (according to criteria listed in the protocol). The cohorts of patients who had not received prior treatment with ipilimumab were restricted to patients who had received no more than two prior regimens of systemic therapy. The cohorts of patients who had received prior therapy with ipilimumab included only patients who had full resolution of ipilimumab-related adverse events and no history of severe immune-related adverse events associated with ipilimumab therapy. Patients were allowed to enter the trial 6 weeks after the last dose of ipilimumab was administered. The protocol did not require patients who were asymptomatic to undergo screening brain imaging; however, patients with previously treated brain metastases were required to undergo baseline imaging by means of computed tomographic scanning or magnetic resonance imaging and to have had no evidence of central nervous system progression for 8 weeks. Major exclusion criteria were a melanoma of ocular origin, prior therapy with a PD-1 or PD-L1 blocking agent, current systemic immunosuppressive therapy, or active infections or autoimmune diseases.

PHARMACOKINETIC ANALYSIS

Peak-level and trough-level blood samples for pharmacokinetic analysis were obtained from patients at the initiation of treatment. Trough samples were also obtained approximately every 12 weeks for the first 12 months of the study and every 6 months thereafter. The serum concentration of lambrolizumab was quantified with the use of a validated electrochemiluminescent assay with a lower limit of quantification of 10 ng per milliliter.

STATISTICAL ANALYSIS

Data from 135 patients with melanoma who were enrolled and treated according to protocol amendments 02, 03, and 04 were used for the analysis of adverse events. Of the 135 patients, 117 had radiographically measurable disease as assessed by means of central radiologic review and were included in the efficacy analysis of responses according to central review. All other efficacy analyses (an analysis of response on the basis of assessment by the investigator, progression-free survival, and overall survival) were based on data from all 135 patients. Patients were included in the analysis if they received a first dose of study medication by September 6, 2012. Efficacy and safety data that were available as of February 1, 2013, were included in all the analyses. The efficacy analysis included two end points: overall responses derived from investigator-reported data, with assessment according to immune-related response criteria (135 patients)[11]; and overall responses derived from independent, central, blinded radiologic review, with assessment according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 (117 patients) (see Table S1 in the Supplementary Appendix, available at NEJM.org, for re-

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Table 1. **Baseline Characteristics of the Patients, According to Cohort.**

Characteristic	10 mg/kg Every 2 Wk		10 mg/kg Every 3 Wk		2 mg/kg Every 3 Wk	Total (N=135)
	No Prior Ipilimumab (N=41)	Prior Ipilimumab (N=16)	No Prior Ipilimumab (N=24)	Prior Ipilimumab (N=32)	No Prior Ipilimumab (N=22)	
	number (percent)					
Sex						
Male	23 (56)	9 (56)	16 (67)	17 (53)	14 (64)	79 (59)
Female	18 (44)	7 (44)	8 (33)	15 (47)	8 (36)	56 (41)
Age (yr)						
Mean	60.4	59.4	67	57.3	58.6	60.4
Range	25–94	29–87	37–87	32–77	30–79	25–94
Race*						
Asian	0	0	2 (8)	0	0	2 (1)
White	41 (100)	16 (100)	22 (92)	32 (100)	22 (100)	133 (99)
ECOG performance status†						
Unknown	1 (2)	0	0	0	0	1 (1)
0	32 (78)	13 (81)	18 (75)	21 (66)	13 (59)	97 (72)
1	8 (20)	3 (19)	6 (25)	11 (34)	9 (41)	37 (27)
BRAF mutation status						
Mutant	13 (32)	1 (6)	1 (4)	5 (16)	6 (27)	26 (19)
Nonmutant	23 (56)	14 (88)	21 (88)	21 (66)	14 (64)	93 (69)
Unknown	5 (12)	1 (6)	2 (8)	6 (19)	2 (9)	16 (12)
Brain metastasis						
Yes	3 (7)	3 (19)	0	4 (12)	2 (9)	12 (9)
No	38 (93)	13 (81)	24 (100)	28 (88)	20 (91)	123 (91)
Lactate dehydrogenase						
Normal	23 (56)	11 (69)	16 (67)	17 (53)	13 (59)	80 (59)
Elevated‡	13 (32)	5 (31)	6 (25)	7 (22)	5 (23)	36 (27)
Unknown	5 (12)	0	2 (8)	8 (25)	4 (18)	19 (14)
M staging of extent of metastasis						
MX	0	0	0	1 (3)	0	1 (1)
M0	7 (17)	2 (12)	2 (8)	3 (9)	1 (5)	15 (11)
M1a	1 (2)	3 (19)	6 (25)	3 (9)	1 (5)	14 (10)
M1b	11 (27)	3 (19)	7 (29)	5 (16)	2 (9)	28 (21)
M1c	20 (49)	8 (50)	9 (38)	18 (56)	18 (82)	73 (54)
Unknown	2 (5)	0	0	2 (6)	0	4 (3)
Previous treatment§						
No prior systemic treatment	16 (39)	0	12 (50)	0	14 (64)	42 (31)
Immunotherapy, excluding ipilimumab	11 (27)	4 (25)	5 (21)	10 (31)	4 (18)	34 (25)
Chemotherapy	11 (27)	8 (50)	9 (38)	14 (44)	5 (23)	47 (35)
BRAF inhibitor	4 (10)	0	1 (4)	4 (12)	1 (5)	10 (7)

* Race was self-reported.

† An Eastern Cooperative Oncology Group (ECOG) performance status of 0 indicates that the patient is fully active, 1 that the patient is restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, and 2 that the patient is ambulatory and capable of all self-care but unable to carry out any work activities.

‡ An elevated level was considered to be a level higher than the upper limit of the normal range.

§ This category included treatments for advanced disease. The numbers may add up to more than 100% since a patient may have received more than one type of oncologic therapy.

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Table 2. **Drug-Related Adverse Events.***

Drug-Related Event	All Grades (N=135)	Grade 3 or 4 (N=135)
	number (percent)	
Any	107 (79)	17 (13)
Hypothyroidism	11 (8)	1 (1)
Gastrointestinal disorder		
Diarrhea	27 (20)	1 (1)
Nausea	13 (10)	0
Abdominal pain	7 (5)	1 (1)
Generalized symptom		
Fatigue	41 (30)	2 (1)
Myalgia	16 (12)	0
Headache	14 (10)	0
Asthenia	13 (10)	0
Pyrexia	10 (7)	0
Chills	9 (7)	0
Decreased appetite	6 (4)	1 (1)
Increase in aminotransferase level		
AST	13 (10)	2 (1)
ALT	11 (8)	0
Renal failure	3 (2)	2 (1)
Respiratory disorder		
Cough	11 (8)	0
Dyspnea	6 (4)	0
Pneumonitis	6 (4)	0
Skin disorder		
Rash	28 (21)	3 (2)
Pruritus	28 (21)	1 (1)
Vitiligo	12 (9)	0

* Included are drug-related adverse events that occurred in at least five patients or drug-related grade 3 or 4 adverse events that occurred in at least two patients. ALT denotes alanine aminotransferase, and AST aspartate aminotransferase.

sponse criteria).[12] The overall response rate was defined as the number of patients with a complete or partial response divided by the total number of patients who had measurable disease at baseline and received at least one treatment dose. The overall response rate and exact two-sided 95% confidence interval were calculated. Toxic effects were graded with the use of the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.[13] Descriptive statistics were provided for the pharmacokinetic analysis of trough and peak samples according to treatment cohort.

RESULTS

BASELINE CHARACTERISTICS OF THE PATIENTS

Between December 1, 2011, and September 6, 2012, a total of 135 patients with advanced melanoma were enrolled in this multi-institutional, international, phase 1 expansion study. Initially, patients were enrolled in a cohort that received lambrolizumab at a dose of 10 mg per kilogram every 2 weeks. Subsequently, additional patients were enrolled in concurrent (not randomized) cohorts that received lambrolizumab at 10 mg per kilogram or 2 mg per kilogram every 3 weeks. A distinction was made between patients who had received prior treatment with ipilimumab (48 patients) and those who had not (87 patients) to provide preliminary data on the safety and antitumor activity of lambrolizumab on the basis of prior or no prior treatment with ipilimumab. The median time between the last dose of ipilimumab and the initiation of lambrolizumab was 23 weeks (range, 6 to 83). The majority of patients (38 of 48) were enrolled more than 12 weeks after the last dose of ipilimumab, and 90% (43 of 48) had received three or more infusions of ipilimumab. The baseline characteristics of the patients were similar across all the treatment groups (Table 1). Overall, more than 50% of the patients had visceral metastases (stage M1c), approximately 25% had an elevated lactate dehydrogenase level, and close to 9% had a history of brain metastases — all of which are recognized as poor prognostic factors in patients with advanced melanoma.

SAFETY

Table 2 shows the adverse events that were considered to be related to lambrolizumab therapy. Table S2 in the Supplementary Appendix provides further details of drug-related toxic effects according to the dosing cohort, and Table S3 in the Supplementary Appendix describes all adverse events regardless of the cause, according to the dosing cohort. Of the 135 patients who received at least one dose of lambrolizumab, 79% reported drug-related adverse events of any grade, and 13% reported grade 3 or 4 drug-related ad-

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verse events. Generalized symptoms, including fatigue and asthenia, fever and chills, myalgias, and headaches, were reported frequently but were of low grade in more than 95% of the cases. In addition to the data shown in the tables, there was one case of grade 1 infusion reaction. Rashes and pruritus were reported in 21% of the patients; grade 3 or 4 pruritus was reported in 1% of the patients, and grade 3 or 4 rash in 2%. Vitiligo was attributed to lambrolizumab in 9% of the patients. The highest incidence of overall treatment-related adverse events was seen among the patients who received 10 mg of lambrolizumab per kilogram every 2 weeks, as compared with the patients receiving 10 mg per kilogram every 3 weeks and those receiving 2 mg per kilogram every 3 weeks (23%, vs. 4% and 9%, respectively) (Table S2 in the Supplementary Appendix).

Adverse events of particular interest were of an inflammatory or autoimmune nature. Treatment-related pneumonitis was reported in 4% of the patients; none of the cases were grade 3 or 4. One patient, a 96-year-old man, died during the course of the study. Initial asymptomatic pneumonitis was identified on a scan, and lambrolizumab was discontinued. Subsequently, after shortness of breath developed, the patient received glucocorticoids. The clinical course was complicated when acute bronchopneumonia and pneumothorax due to bronchoscopy and biopsies were diagnosed. Although the pulmonary infiltrates were reduced with glucocorticoids, the patient died from a myocardial infarction and bronchopneumonia. Grade 3 or 4 elevations of aminotransferase levels were reported in 1% of the patients. Two cases of grade 3 renal failure were reported. Both cases were potentially immune-mediated, and the patients' renal function improved with glucocorticoid therapy along with the discontinuation of lambrolizumab. Although diarrhea was reported in 20% of the patients, a single case of grade 3 treatment-related diarrhea was reported. This case was managed with treatment of the symptoms, and the patient recovered promptly without glucocorticoid treatment. Hypothyroidism was reported in 8% of the patients and was effectively managed with thyroid-replacement therapy. In addition to the data shown in the tables, grade 3 hyperthyroidism and grade 2 adrenal insufficiency developed in one patient; these were managed with standard measures, and the patient continued in the study with a durable response. No other endocrinopathies were recorded.

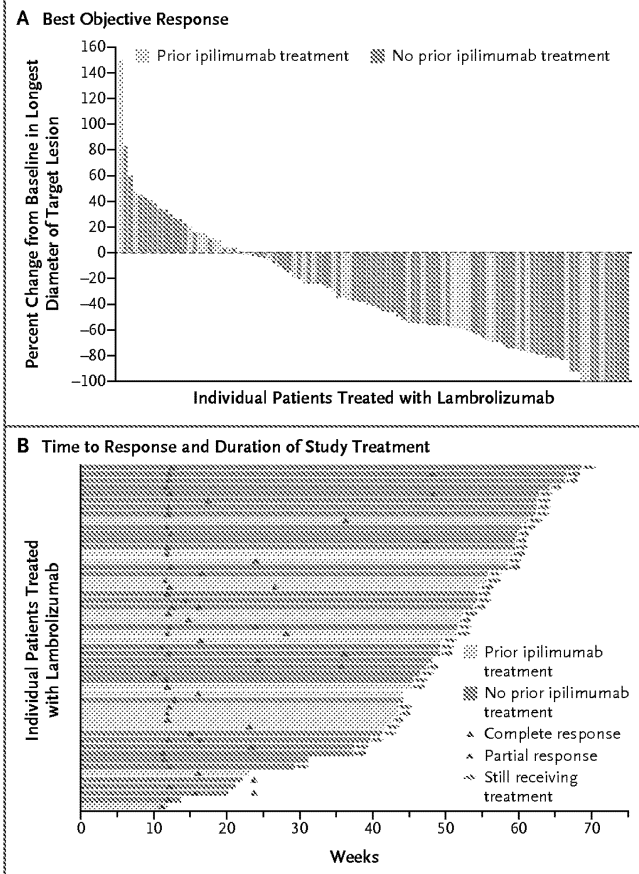


Figure 1. **Antitumor Activity of Lambrolizumab.**
Data on the antitumor activity of lambrolizumab, as assessed by independent, central radiologic review, is shown for the patients who could be evaluated. Panel A shows a waterfall plot of the best objective response according to prior treatment with ipilimumab, measured as the maximum change from baseline in the sum of the longest diameter of each target lesion. A total of 10 of 103 patients with radiographically measurable disease at baseline and at least one evaluation after treatment had a 100% reduction in target lesions. Panel B shows the time to response and the duration of study treatment. A total of 42 of the 52 patients who had a response were still receiving the study treatment at the time of the current analysis. Of the 10 patients who discontinued therapy, 5 discontinued owing to toxic effects, and 2 of these patients showed improvement in their response after discontinuation (denoted by the two triangles that are outside the bar of the on-treatment period).

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Table 3. **Objective Response Rate, According to Dosing Regimen and Status with Respect to Prior Therapy with Ipilimumab, as Assessed According to Two Criteria.***

Regimen and Ipilimumab Status	RECIST				Immune-Related Response	
	No. of Patients	Confirmed and Unconfirmed Objective Response	Confirmed Objective Response	Duration of Response†	No. of Patients	Confirmed Objective Response
		no. (% [95% CI])		*mo*		*no. (% [95% CI])*
10 mg/kg every 2 wk						
No prior ipilimumab	39	21 (54 [37–70])	19 (49 [32–65])‡	1.9–10.8	41	23 (56 [40–72])
Prior ipilimumab	13	8 (62 [32–86])	8 (62 [32–86])§	2.8–8.3	16	9 (56 [30–80])
Total	52	29 (56 [41–69])	27 (52 [38–66])	1.9–10.8	57	32 (56 [42–69])
10 mg/kg every 3 wk						
No prior ipilimumab	19	7 (37 [16–62])	5 (26 [9–51])	2.6–5.6	24	8 (33 [16–55])
Prior ipilimumab	26	9 (35 [17–56])	7 (27 [12–48])	2.8–8.3	32	7 (22 [9–40])
Total	45	16 (36 [22–51])	12 (27 [15–42])	2.6–8.3	56	15 (27 [16–40])
2 mg/kg every 3 wk, no prior ipilimumab	20	7 (35 [15–59])	5 (25 [9–49])¶	2.1–5.5	22	3 (14 [3–35])
Total‖	117	52 (44 [35–54])**	44 (38 [25–44])	1.9–10.8	135	50 (37 [29–45])

* The efficacy population of patients with measurable disease was assessed by means of an independent, central, blinded radiologic review with the use of the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, and by means of investigator assessment with the use of immune-related response criteria. The latter was the primary end point of the study. Responses based on immune-related response criteria included only those that were confirmed on two consecutive scans obtained at least 28 days apart.

† The duration of response was defined as the time from the first response to the time of documented progression or, in the case of censored data, the most recent tumor assessment. All the lower and upper ranges listed here are for censored data and refer to the time from the first response to the most recent tumor assessment, except for the lower range in the group with no prior ipilimumab, as well as the total cohort, receiving 10 mg per kilogram of body weight every 3 weeks; these two lower ranges refer to the time from first response to the time of documented progression. Only confirmed responses were included in the calculation of duration of response.

‡ Three of these patients had a complete response.

§ Two of these patients had a complete response.

¶ One of these patients had a complete response.

‖ The confirmed response rate, according to RECIST, version 1.1, was 38% (95% CI, 23 to 55) among patients who had received prior ipilimumab treatment and 37% (95% CI, 26 to 49) among patients who had not received prior ipilimumab treatment.

** Six patients with initial responses were awaiting confirmation of the response at the time of the data cutoff for this report. One response has since been confirmed, but since it was confirmed after the data cutoff for the current analysis, the data on overall response rate have not been modified.

PHARMACOKINETICS

Serum concentrations of lambrolizumab in samples obtained before and after administration of the drug were lower by a factor of approximately 5 in patients receiving 2 mg per kilogram every 3 weeks than in those receiving 10 mg per kilogram every 3 weeks; steady-state trough concentrations were 20% greater in the patients receiving 10 mg per kilogram every 2 weeks than in those receiving the same dose every 3 weeks (Table S4 in the Supplementary Appendix). The increase in trough serum concentrations over time is consistent with the half-life of lambrolizumab of about 2 to 3 weeks.[10]

CLINICAL ACTIVITY

We evaluated the response to therapy using two different criteria: investigator-assessed immune-related response criteria, which were designed to analyze the response to immunotherapy agents[11]; and RECIST,[12] as assessed by independent, central radiologic review, which is used routinely to assess responses to cytotoxic agents for cancer. The overall response rate during receipt of therapy, across all doses, on the basis of assessment by the investigator according to immune-related response criteria was 37%. The confirmed response rate across all doses, as assessed by central review according to RECIST, was 38% (44 of

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117 patients). There were an additional 8 unconfirmed responses. Six of these unconfirmed responses were in patients who had not yet undergone confirmatory scanning at the time of the data cutoff. Since then, 1 of these patients has been confirmed as having an objective response. The response rate, including confirmed and unconfirmed responses, across all doses was 44% (44 confirmed and 8 unconfirmed). The confirmed response rate, as assessed by central review according to RECIST, ranged from 25% in the cohort that received 2 mg per kilogram every 3 weeks to 52% in the cohort that received 10 mg per kilogram every 2 weeks. As shown in Figure 1A, 77% of the patients had a reduction in the tumor burden during the study, including 8 patients who were confirmed by central review as having stable disease for longer than 24 weeks. Responses did not vary according to prior exposure to ipilimumab (Table 3 and Fig. 1A).

Figure 1B shows the time to response and the treatment duration in the 52 patients who had an objective response (confirmed or unconfirmed) on the basis of central radiologic review according to RECIST. The majority of responses were seen at the time the first imaging was performed at 12 weeks. An additional 17 patients who had stable disease at an early assessment showed durable objective response with continued treatment, with 1 patient achieving a partial response according to RECIST after 48 weeks of treatment. The median duration of response had not been reached at the time of the analysis, at a median follow-up time of 11 months. A total of 81% of the patients who had a response were still receiving the study treatment at the time of the analysis in March 2013. Of the 52 patients with a response, 5 discontinued treatment owing to disease progression, and 5 discontinued treatment for other reasons (most commonly adverse events). The median progression-free survival among the 135 patients, as estimated with the use of a Kaplan–Meier analysis, was more than 7 months. The estimated median overall survival had not been reached.

Biopsied specimens of regressing lesions were densely infiltrated by CD8+ cytotoxic T lymphocytes (Fig. 2A and 2B), a finding that is consistent with the mechanism of action of lambrolizumab. As shown in Figures 2C and 2D, some patients may have had delayed responses after an initial period in which the tumor burden increased, a process consistent with an immune-related response.

DISCUSSION

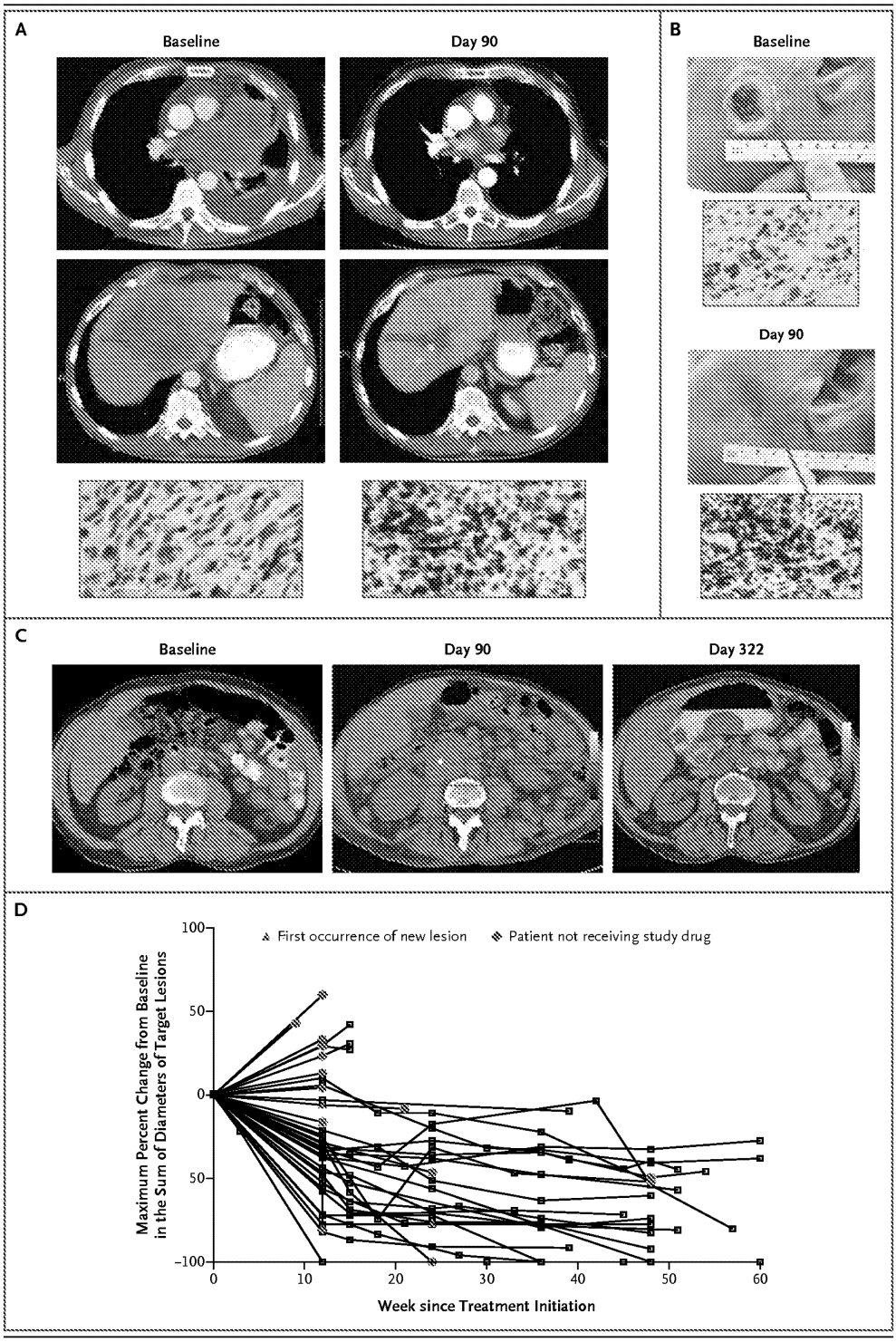
Immunotherapeutic agents, including high-dose interleukin-2, interferon alfa, and anti–CTLA-4 antibodies, have shown activity in patients with advanced melanoma; however, this is an infrequent event that is seen in 10 to 15% of patients.[6,14] This study provides evidence of a high response rate with lambrolizumab in patients with advanced melanoma. Most responses to lambrolizumab were durable — similar to the pattern of response with other immunotherapies[1,2,15,16] — and the majority of responses were ongoing at the time of the current analysis. The cohort with the maximum administered dose of lambrolizumab (10 mg per kilogram every 2 weeks) showed the highest response rate of 52%. This cohort also showed the highest rate of drug-related adverse events, although that may be due in part to a longer duration of therapy (Table S5 in the Supplementary Appendix). It is also possible that these nonrandomized cohorts had unmeasured confounders that could have led to different outcomes — although this is not readily apparent from an analysis of the baseline characteristics of the patients. Therefore, an additional randomized expansion of the cohort is ongoing to investigate the higher response rate observed in the cohort receiving 10 mg per kilogram every 2 weeks as compared with every 3 weeks.

Although cross-study comparisons of adverse-event rates should be viewed with caution, it seems that in comparison with anti–CTLA-4 therapy, lambrolizumab therapy was associated with a lower incidence and a different spectrum of immune-related adverse events, possibly owing to a distinct mechanism of action with a more targeted effect on tumor-specific T cells.[6]

Prior exposure to other immunotherapy strategies, most notably the use of the anti–CTLA-4 antibody ipilimumab or interleukin-2, did not have a major effect on the benefit from lambrolizumab treatment. Furthermore, the rate of immune-mediated or other toxic effects was not increased in patients who had received prior

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Figure 2 (facing page). **Tumor Responses with Lambrolizumab.**

Shown are examples of tumor responses in patients treated with lambrolizumab. Panel A shows images obtained from a patient with BRAF nonmutant metastatic melanoma who had symptomatic progression after biochemotherapy and treatment with high-dose interleukin-2 and ipilimumab; the patient had rapid resolution of symptoms and showed a partial response with lambrolizumab at the initial imaging on day 90. Arrows point to sites of melanoma metastases in the lung and liver. Immunohistochemical staining of biopsied specimens obtained before and after treatment show an increased CD8 T-cell infiltrate after treatment. Panel B shows the resolution of a local relapse of desmoplastic melanoma in a patient who had not received prior treatment with ipilimumab; an additional tumor response was observed in nodal and lung metastases (not shown). CD8 immunohistochemical staining of biopsy specimens obtained before and after treatment shows increased CD8 T-cell infiltrate. Panel C shows images from a patient without prior treatment with ipilimumab who had metastatic mucosal melanoma with significant progression at the initial 12-week imaging (red boxes), at which time lambrolizumab was discontinued. Without receiving any other therapy, the patient went on to have a nearly complete response that is ongoing more than 1 year after the start of the study. Panel D is a plot of the change in tumor burden (assessed as the longest dimension of the lesion) over time in patients with melanoma who had not received prior treatment with ipilimumab and who received lambrolizumab at a dose of 10 mg per kilogram of body weight every 2 weeks. In most patients who had an objective response, the responses were durable and were evident at the initial evaluation (12 weeks). Tumor regression followed both conventional and immune-related patterns of response, such as a prolonged reduction in the tumor burden in the presence of new lesions.

treatment with ipilimumab. In addition, a response to lambrolizumab was documented in patients who had previously had disease progression while receiving other forms of immunotherapy, chemotherapy, or BRAF-targeted therapy. The striking anticancer activity observed with lambrolizumab requires confirmation in larger studies. A randomized clinical trial involving patients who have ipilimumab-refractory disease (and if positive for the *BRAF V600* mutation have received treatment with an approved BRAF or MEK inhibitor) has commenced.

The ability to induce immune responses against cancer by abrogating an immune-system checkpoint that limits the antitumor activity of preexisting tumor-specific cytotoxic T cells points to the importance of focusing on immune regulatory events for cancer therapy. As first described with anti–CTLA-4 antibodies in preclinical studies[17] and in patients,[1,2,16] this study confirms the importance of releasing inhibitory immune regulation by PD-1 for effective antitumor immunity.[6]

Supported by Merck Sharp and Dohme.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Dr. Mary E. Hanson, Martha C. Vollmer, and Margaret Hodgson (Merck Sharp and Dohme) for assistance with preparation of the manuscript; Dr. Eric Rubin, Dr. Alise Reicin, Dr. Robert Iannone, Dr. Joseph E. Eid, Dr. Cong Chen, Anne Morosky, Maxine Giannotti, Kellie Celentano, and Amanda McDonald (Merck Sharp and Dohme) for critical review of the manuscript; Dr. Andrea Perrone and Linda Gammage (Merck Sharp and Dohme) for data collection and interpretation; and the following investigators and site personnel: Dr. John Glaspy, Denise Oseguera, Elizabeth Seja, Christine Kivork, Derek Chung, and Antonio J. Gutierrez (University of California, Los Angeles); Dr. Emilie Routier and Severine Roy (Institute Gustave Roussy); Dr. Georgina Long and Arthur Clements (Westmead Hospital and Melanoma Institute Australia, University of Sydney); Dr. Naiyer Rizvi and Nathan Gray (Memorial Sloan-Kettering Cancer Center); Ann Cross (Mayo Clinic, Jacksonville); Dr. Ragini Kudchadkar (H. Lee Moffitt Cancer Center); Dr. Alan H. Bryce and Dr. Peter Cohen (Mayo Clinic, Scottsdale); Joseph Ilagan, Karen Perdon, and Portia Velasquez (M.D. Anderson Cancer Center); David Hogg and Marcus Butler (Princess Margaret Cancer Centre); Dr. Anthony Tolcher, Dr. Drew Rasco, Dr. Kyriakos Papadopoulos, Dr. Muralidhar Beeram, Guillermo Espino, and Crystal Perez (South Texas Accelerated Research Therapeutics); Dr. John Kirkwood and Dr. Hussein Tawbi (University of Pittsburgh); and Dr. Lynn Schuchter, Dr. Ravi Amaravadi, Mary Carberry, Lydia Giles, and Naomi Haas (University of Pennsylvania).

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The NEW ENGLAND JOURNAL of MEDICINE

ESTABLISHED IN 1812 AUGUST 19, 2010 VOL. 363 NO. 8

Improved Survival with Ipilimumab in Patients with Metastatic Melanoma

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ABSTRACT

BACKGROUND

An improvement in overall survival among patients with metastatic melanoma has been an elusive goal. In this phase 3 study, ipilimumab — which blocks cytotoxic T-lymphocyte–associated antigen 4 to potentiate an antitumor T-cell response — administered with or without a glycoprotein 100 (gp100) peptide vaccine was compared with gp100 alone in patients with previously treated metastatic melanoma.

METHODS

A total of 676 HLA-A*0201–positive patients with unresectable stage III or IV melanoma, whose disease had progressed while they were receiving therapy for metastatic disease, were randomly assigned, in a 3:1:1 ratio, to receive ipilimumab plus gp100 (403 patients), ipilimumab alone (137), or gp100 alone (136). Ipilimumab, at a dose of 3 mg per kilogram of body weight, was administered with or without gp100 every 3 weeks for up to four treatments (induction). Eligible patients could receive reinduction therapy. The primary end point was overall survival.

RESULTS

The median overall survival was 10.0 months among patients receiving ipilimumab plus gp100, as compared with 6.4 months among patients receiving gp100 alone (hazard ratio for death, 0.68; P<0.001). The median overall survival with ipilimumab alone was 10.1 months (hazard ratio for death in the comparison with gp100 alone, 0.66; P=0.003). No difference in overall survival was detected between the ipilimumab groups (hazard ratio with ipilimumab plus gp100, 1.04; P=0.76). Grade 3 or 4 immune-related adverse events occurred in 10 to 15% of patients treated with ipilimumab and in 3% treated with gp100 alone. There were 14 deaths related to the study drugs (2.1%), and 7 were associated with immune-related adverse events.

CONCLUSIONS

Ipilimumab, with or without a gp100 peptide vaccine, as compared with gp100 alone, improved overall survival in patients with previously treated metastatic melanoma. Adverse events can be severe, long-lasting, or both, but most are reversible with appropriate treatment. (Funded by Medarex and Bristol-Myers Squibb; ClinicalTrials.gov number, NCT00094653.)

Drs. Hodi and O'Day contributed equally to this article.

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This article (10.1056/NEJMoa1003466) was published on June 5, 2010, and last updated on September 1, 2010, at NEJM.org.

N Engl J Med 2010;363:711-23.

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The incidence of metastatic melanoma has increased over the past three decades,[1,2] and the death rate continues to rise faster than the rate with most cancers.[3] The World Health Organization (WHO) estimates that worldwide there are 66,000 deaths annually from skin cancer, with approximately 80% due to melanoma.[4] In the United States alone, an estimated 8600 persons died from melanoma in 2009.[1] The median survival of patients with melanoma who have distant metastases (American Joint Committee on Cancer stage IV) is less than 1 year.[5,6] No therapy is approved beyond the first-line therapy for metastatic melanoma, and enrollment in a clinical trial is the standard of care. No therapy has been shown in a phase 3, randomized, controlled trial to improve overall survival in patients with metastatic melanoma.[6-9]

Regulatory pathways that limit the immune response to cancer are becoming increasingly well characterized. Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) is an immune checkpoint molecule that down-regulates pathways of T-cell activation.[10] Ipilimumab, a fully human monoclonal antibody (IgG1) that blocks CTLA-4 to promote antitumor immunity,[11-14] has shown activity in patients with metastatic melanoma when it has been used as monotherapy in phase 2 studies.[15-17] Ipilimumab has also shown activity when combined with other agents,[18,19] including cancer vaccines.[20,21] One well-studied cancer vaccine comprises HLA-A*0201–restricted peptides derived from the melanosomal protein, glycoprotein 100 (gp100). Monotherapy with this vaccine induces immune responses but has limited antitumor activity.[22] However, the results of a recent study suggest that gp100 may improve the efficacy of high-dose interleukin-2 in patients with metastatic melanoma.[23] With no accepted standard of care, gp100 was used as an active control for our phase 3 study, which evaluated whether ipilimumab with or without gp100 improves overall survival, as compared with gp100 alone, among patients with metastatic melanoma who had undergone previous treatment.

METHODS

PATIENTS

Patients were eligible for inclusion in the study if they had a diagnosis of unresectable stage III or IV melanoma and had received a previous therapeutic regimen containing one or more of the following: dacarbazine, temozolomide, fotemustine, carboplatin, or interleukin-2. Other inclusion criteria were age of at least 18 years; life expectancy of at least 4 months; Eastern Cooperative Oncology Group (ECOG) performance status of 0 (fully active, able to carry on all predisease performance without restriction) or 1 (restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, such as light housework or office work)[24]; positive status for HLA-A*0201; normal hematologic, hepatic, and renal function; and no systemic treatment in the previous 28 days. Exclusion criteria were any other cancer from which the patient had been disease-free for less than 5 years (except treated and cured basal-cell or squamous-cell skin cancer, superficial bladder cancer, or treated carcinoma in situ of the cervix, breast, or bladder); primary ocular melanoma; previous receipt of anti–CTLA-4 antibody or cancer vaccine; autoimmune disease; active, untreated metastases in the central nervous system; pregnancy or lactation; concomitant treatment with any nonstudy anticancer therapy or immunosuppressive agent; or long-term use of systemic corticosteroids.

The protocol was approved by the institutional review board at each participating institution and was conducted in accordance with the ethical principles originating from the Declaration of Helsinki and with Good Clinical Practice as defined by the International Conference on Harmonization. All patients (or their legal representatives) gave written informed consent before enrollment.

STUDY DESIGN AND TREATMENT

In this randomized, double-blind, phase 3 study, we enrolled patients at 125 centers in 13 countries in North America, South America, Europe, and Africa. Between September 2004 and August 2008, patients were randomly assigned to one of three study groups, with stratification according to baseline metastasis stage (M0, M1a, or M1b vs. M1c, classified according to the tumor–node–metastasis [TNM] categorization for melanoma of the American Joint Committee on Cancer), and receipt or nonreceipt of previous interleukin-2 therapy. The full original protocol, a list of amendments, and the final protocol, as well as the statistical analysis plan, are available with the full text of this article at NEJM.org.

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Patients were randomly assigned, in a 3:1:1 ratio, to treatment with an induction course of ipilimumab, at a dose of 3 mg per kilogram of body weight, plus a gp100 peptide vaccine; ipilimumab plus gp100 placebo; or gp100 plus ipilimumab placebo — all administered once every 3 weeks for four treatments. In the vaccine groups, patients received two modified HLA-A*0201–restricted peptides, injected subcutaneously as an emulsion with incomplete Freund's adjuvant (Montanide ISA-51): a gp100:209-217(210M) peptide, 1 mg injected in the right anterior thigh, and a gp100:280-288(288V) peptide, 1 mg injected in the left anterior thigh. Peptide injections were given immediately after a 90-minute intravenous infusion of ipilimumab or placebo. Treatment began on day 1 of week 1, and if there were no toxic effects that could not be tolerated, no rapidly progressive disease, and no significant decline in performance status, patients received an additional treatment during weeks 4, 7, and 10. Patients in whom new lesions developed or baseline lesions grew were allowed to receive additional treatments to complete induction. Patients with stable disease for 3 months' duration after week 12 or a confirmed partial or complete response were offered additional courses of therapy (reinduction) with their assigned treatment regimen if they had disease progression.

The original primary end point was the best overall response rate (i.e., the proportion of patients with a partial or complete response). The primary end point was amended to overall survival (with the amendment formally approved on January 15, 2009) in the ongoing blinded study, on the basis of phase 2 data and in alignment with another ongoing phase 3 trial of ipilimumab involving patients with metastatic melanoma.[25] The primary comparison in overall survival was between the ipilimumab-plus-gp100 group and the gp100-alone group. Prespecified secondary end points included a comparison of overall survival between the ipilimumab-alone and the gp100-alone groups and between the two ipilimumab groups, the best overall response rate, the duration of response, and progression-free survival. Subgroup comparisons of overall survival were performed across five prespecified categories: metastasis stage (M0, M1a, or M1b vs. M1c), receipt or nonreceipt of previous interleukin-2 therapy, baseline levels of serum lactate dehydrogenase (less than or equal to the upper limit of the normal range vs. higher than the upper limit of the normal range), age (<65 years vs. ≥65 years), and sex.

The trial was designed jointly by the senior academic authors and the sponsors, Medarex and Bristol-Myers Squibb. Data were collected by the sponsors and analyzed in collaboration with the senior academic authors, who vouch for the completeness and accuracy of the data and analyses and for the conformance of this report to the protocol, as amended. An initial draft of the manuscript was prepared by six of the academic authors in collaboration with the sponsor and a professional medical writer paid by the sponsor. All the authors contributed to subsequent drafts and made the decision to submit the manuscript for publication. All the authors signed a confidentiality disclosure agreement with the sponsor.

ASSESSMENTS

For the assessment of a patient's eligibility, each patient's HLA-A*0201 status was determined at a central laboratory. Patients who met the study criteria were assigned to receive treatment within 35 days after HLA typing and within 28 days after diagnostic imaging. Computed tomography with contrast material or magnetic resonance imaging of the brain, chest, abdomen, pelvis, and other anatomical regions, as clinically indicated, was performed. Cutaneous lesions were photographed. Tumor assessments were performed at baseline, and all patients who did not have documented early disease progression and who had stable disease or better at week 12 had confirmatory scans at weeks 16 and 24 and every 3 months thereafter. Tumor responses were determined by the investigators with the use of modified WHO criteria to evaluate bidimensionally measurable lesions.[26]

Adverse events were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 3.0. An immune-related adverse event was defined as an adverse event that was associated with exposure to the study drug and that was consistent with an immune phenomenon. Protocol guidelines for the management of immune-related adverse events included the administration of corticosteroids (orally or intravenously), a delay in a scheduled dose, or discontinuation of therapy.[15-17] Assigned doses were delayed in the case of nondermatologic immune-related adverse events of grade 2 or higher until the event im-

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proved to grade 1 or lower; if the event did not improve to grade 1 or lower, treatment was discontinued permanently. Monitoring of adverse events continued for at least 70 days after the last dose of study drugs had been administered or until any ongoing event resolved or stabilized. All patients, including those with low-grade changes in bowel frequency or stool consistency, were followed closely. A data and safety monitoring committee provided independent oversight of safety and the risk–benefit ratio.

During the study enrollment, the following stopping rule was in place: if 10% or more of the patients in any study treatment group, evaluated cumulatively every 3 months, had a nondermatologic-related toxic adverse event of grade 3 or higher that was attributable to the investigational agents and that could not be alleviated or controlled by appropriate care or corticosteroid therapy within 14 days after the initiation of supportive care or corticosteroid therapy, assignment of patients to that study group would be suspended until the sponsor and the data and safety monitoring committee had reviewed the events and determined the appropriate course of action.

STATISTICAL ANALYSIS

The original study sample size of 750 patients was determined on the basis of the primary end point of best overall response rate but was revised with the new primary end point of overall survival. We estimated that with 385 events (deaths) among a total of 500 patients randomly assigned to the ipilimumab-plus-gp100 and the gp100-alone groups, the study would have at least 90% power to detect a difference in overall survival, at a two-sided alpha level of 0.05, with the use of a log-rank test. A total of 481 events were required in all three groups (assuming that the events were distributed in a 3:1:1 ratio in the ipilimumab-plus-gp100, ipilimumab-alone, and gp100-alone groups, respectively). Therefore, all patients who were randomly assigned in the study were to be followed until at least 481 events had occurred in the study. Enrollment was completed on July 25, 2008, when more than 650 patients had been enrolled. A post hoc power analysis showed that the 219 events observed among a total of 273 patients randomly assigned to the ipilimumab-alone and gp100-alone groups provided at least 80% power to detect a difference in overall survival between the two groups, at a two-sided alpha level of 0.05, with the assumption that ipilimumab alone has the same treatment effect as the combination regimen of ipilimumab plus gp100.

Survival was defined as the time from randomization to death from any cause, and progression-free survival as the time from randomization to documented disease progression or death. Event-time distributions were estimated with the use of the Kaplan–Meier method. Cox proportional-hazards models, stratified according to metastasis status and receipt or nonreceipt of previous interleukin therapy, were used to estimate hazard ratios and to test for significance of the timing of events. All reported P values are two-sided, and confidence intervals are at the 95% level. Survival rates were based on Kaplan–Meier estimation, and confidence intervals were calculated with the use of the bootstrap method. Descriptive statistics were used for adverse events.

RESULTS

PATIENTS AND TREATMENT

Among 676 patients enrolled in the study, 403 were randomly assigned to receive ipilimumab plus gp100, 137 to receive ipilimumab alone, and 136 to receive gp100 alone (control group) (Fig. 1 in the Supplementary Appendix, available at NEJM.org). Included among these patients were 82 patients who had metastases in the central nervous system at baseline, of whom 77 received the study drug. The baseline characteristics of the patients are shown in Table 1. Efficacy analyses were performed on the intention-to-treat population, which included all patients who had undergone randomization (676 patients). The safety population included all patients who had undergone randomization and who had received any amount of study drug (643 patients). A total of 242 of 403 patients in the ipilimumab-plus-gp100 group (60.0%), 88 of 137 in the ipilimumab-alone group (64.2%), and 78 of 136 in the gp100-alone group (57.4%) received all four ipilimumab doses or placebo infusions. The most frequent reason for discontinuation of therapy was disease progression.

EFFICACY

All the analyses of the efficacy end points reported here were prespecified as per protocol.

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Table 1. **Baseline Characteristics of the Patients.***

Variable	Ipilimumab plus gp100 (N=403)	Ipilimumab Alone (N=137)	gp100 Alone (N=136)	Total (N=676)
Mean age — yr	55.6	56.8	57.4	56.2
Sex — no. (%)				
Male	247 (61.3)	81 (59.1)	73 (53.7)	401 (59.3)
Female	156 (38.7)	56 (40.9)	63 (46.3)	275 (40.7)
ECOG performance status — no. (%)†				
0	232 (57.6)	72 (52.6)	70 (51.5)	374 (55.3)
1	166 (41.2)	64 (46.7)	61 (44.9)	291 (43.0)
2	4 (1.0)	1 (0.7)	4 (2.9)	9 (1.3)
3	1 (0.2)	0	0	1 (0.1)
Unknown	0	0	1 (0.7)	1 (0.1)
M stage — no. (%)‡				
M0	5 (1.2)	1 (0.7)	4 (2.9)	10 (1.5)
M1a	37 (9.2)	14 (10.2)	11 (8.1)	62 (9.2)
M1b	76 (18.9)	22 (16.1)	23 (16.9)	121 (17.9)
M1c	285 (70.7)	100 (73.0)	98 (72.1)	483 (71.4)
Lactate dehydrogenase level — no. (%)				
≤Upper limit of the normal range	252 (62.5)	84 (61.3)	81 (59.6)	417 (61.7)
>Upper limit of the normal range	149 (37.0)	53 (38.7)	52 (38.2)	254 (37.6)
Unknown	2 (0.5)	0	3 (2.2)	5 (0.7)
CNS metastases at baseline — no. (%)	46 (11.4)	15 (10.9)	21 (15.4)	82 (12.1)
Received study drug	42 (10.4)	15 (10.9)	20 (14.7)	77 (11.4)
Had had previous treatment for CNS metastases	39 (9.7)	15 (10.9)	19 (14.0)	73 (10.8)
Previous systemic therapy for metastatic disease — no. (%)	403 (100.0)	137 (100.0)	136 (100.0)	676 (100.0)
Previous interleukin-2 therapy — no. (%)	89 (22.1)	32 (23.4)	33 (24.3)	154 (22.8)

* Percentages may not total 100 because of rounding. CNS denotes central nervous system.
† The Eastern Cooperative Oncology Group (ECOG) status ranges from 0 to 5, with higher scores indicating greater impairment (5 indicates death).
‡ The metastasis (M) stage was classified according to the tumor–node–metastasis (TNM) categorization for melanoma of the American Joint Committee on Cancer.

Patients were followed for up to 55 months, with median follow-up times for survival of 21.0 months in the ipilimumab-plus-gp100 group, 27.8 months in the ipilimumab-alone group, and 17.2 months in the gp100-alone group. The median overall survival in the ipilimumab-plus-gp100 group was 10.0 months (95% confidence interval [CI], 8.5 to 11.5), as compared with 6.4 months (95% CI, 5.5 to 8.7) in the gp100-alone group (hazard ratio for death, 0.68; P<0.001). The median overall survival in the ipilimumab-alone group was 10.1 months (95% CI, 8.0 to 13.8) (hazard ratio for death with ipilimumab alone as compared with gp100 alone, 0.66; P=0.003). No difference in overall survival was detected between the two ipilimumab groups (hazard ratio for death with ipilimumab plus gp100, 1.04; P=0.76) (Fig. 1). Analyses of survival showed that the rates of overall survival in the ipilimumab-plus-gp100 group, the ipilimumab-alone group, and the gp100-alone group, respectively, were 43.6%, 45.6%, and 25.3% at 12 months, 30.0%, 33.2%, and 16.3% at 18 months, and 21.6%, 23.5%, and 13.7% at 24 months. The

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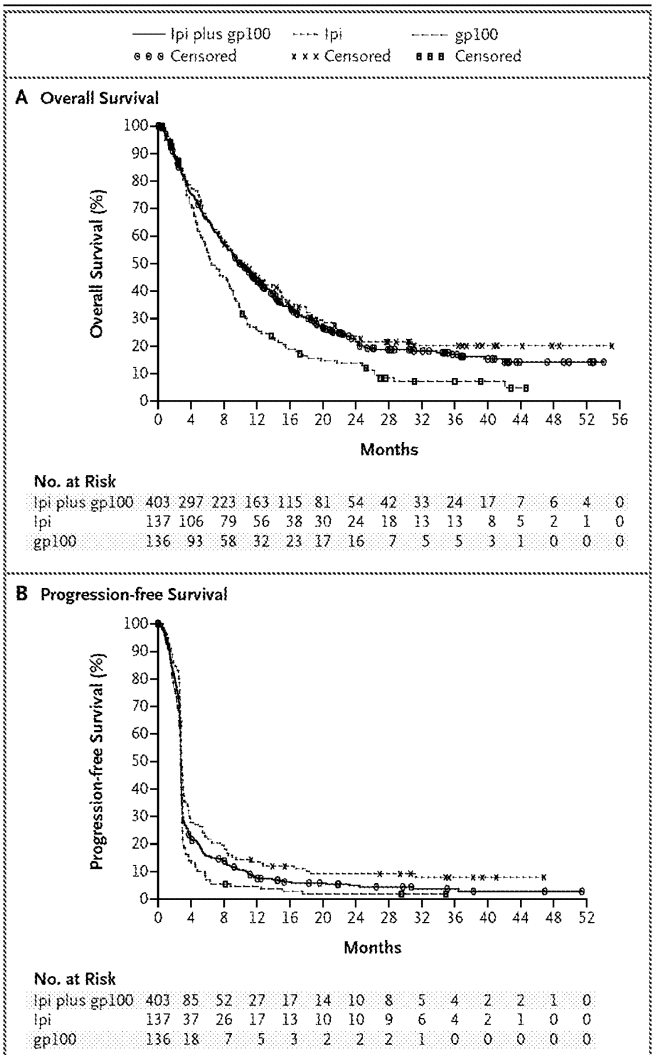


Figure 1. Kaplan–Meier Curves for Overall Survival and Progression-free Survival in the Intention-to-Treat Population.

The median follow-up for overall survival (Panel A) in the ipilimumab (Ipi)-plus-glycoprotein 100 (gp100) group was 21.0 months, and the median overall survival was 10.0 months (95% CI, 8.5 to 11.5); in the ipilimumab-alone group, the median follow-up was 27.8 months, and the median overall survival, 10.1 months (95% CI, 8.0 to 13.8); and in the gp100-alone group, the median follow-up was 17.2 months, and the median overall survival, 6.4 months (95% CI, 5.5 to 8.7). The median progression-free survival (Panel B) was 2.76 months (95% CI, 2.73 to 2.79) in the ipilimumab-plus-gp100 group, 2.86 months (95% CI, 2.76 to 3.02) in the ipilimumab-alone group, and 2.76 months (95% CI, 2.73 to 2.83) in the gp100-alone group. The rates of progression-free survival at week 12 were 49.1% (95% CI, 44.1 to 53.9) in the ipilimumab-plus-gp100 group, 57.7% (95% CI, 48.9 to 65.5) in the ipilimumab-alone group, and 48.5% (95% CI, 39.6 to 56.7) in the gp100-alone group.

effect of ipilimumab on overall survival was independent of age, sex, baseline serum lactate dehydrogenase levels, metastasis stage of disease, and receipt or nonreceipt of previous interleukin-2 therapy (Fig. 2).

A 19% reduction in the risk of progression was noted with ipilimumab plus gp100, as compared with gp100 alone (hazard ratio, 0.81; $P<0.05$), and a 36% reduction in risk of progression was seen with ipilimumab alone as compared with gp100 alone (hazard ratio, 0.64; $P<0.001$). The reduction in risk with ipilimumab plus gp100 was less than that with ipilimumab alone (hazard ratio with ipilimumab plus gp100, 1.25; $P=0.04$). The median values for progression-free survival were similar in all groups at the time of the first assessment of progression (week 12), after which there was a separation between the curves (Fig. 1B).

The highest percentage of patients with an objective response or stable disease was in the ipilimumab-alone group (Table 2); this group had a best overall response rate of 10.9% and a disease control rate (the proportion of patients with a partial or complete response or stable disease) of 28.5%. In the ipilimumab-alone group, 9 of 15 patients (60.0%) maintained an objective response for at least 2 years (26.5 to 44.2 months [ongoing]), and in the ipilimumab-plus-gp100 group, 4 of 23 patients (17.4%) maintained the response for at least 2 years (27.9 to 44.4 months [ongoing]). Neither of the two patients in the gp100-alone group who had a partial response maintained the response for 2 years. Responses to ipilimumab continued to improve beyond week 24: in the ipilimumab-plus-gp100 group, 3 patients with disease progression improved to stable disease, 3 with stable disease improved to a partial response, and 1 with a partial response improved to a complete response; in the ipilimumab-alone group, 2 patients with stable disease improved to a partial response and 3 with a partial response improved to a complete response. Among 31 patients given reinduction therapy with ipilimumab, a partial or complete response or stable disease was achieved by 21 (Table 2).

ADVERSE EVENTS

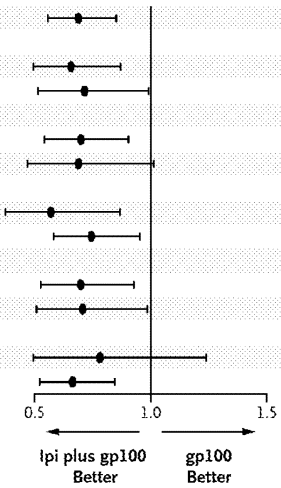
The adverse events reported in the safety population are listed in Table 3. The most common adverse events related to the study drugs were

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A

Subgroup	Ipi plus gp100	gp100	Hazard Ratio (95% CI)
	no. of deaths/no. randomized		
All patients	306/403	119/136	0.69 (0.56–0.85)
Sex			
Male	191/247	66/73	0.66 (0.50–0.87)
Female	115/156	53/63	0.72 (0.52–0.99)
Age			
<65 yr	219/291	81/94	0.70 (0.54–0.90)
≥65 yr	87/112	38/42	0.69 (0.47–1.01)
M stage at study entry			
M0, M1a, M1b	78/118	31/38	0.57 (0.38–0.87)
M1c	228/285	88/98	0.74 (0.58–0.95)
Baseline LDH			
≤ULN	178/252	66/81	0.70 (0.53–0.93)
>ULN	127/149	50/52	0.71 (0.51–0.98)
Prior use of interleukin-2			
Yes	68/89	25/33	0.78 (0.49–1.24)
No	238/314	94/103	0.66 (0.52–0.84)



B

Subgroup	Ipi	gp100	Hazard Ratio (95% CI)
	no. of deaths/no. randomized		
All patients	100/137	119/136	0.64 (0.49–0.84)
Sex			
Male	53/81	66/73	0.54 (0.37–0.77)
Female	47/56	53/63	0.81 (0.55–1.20)
Age			
<65 yr	69/95	81/94	0.65 (0.47–0.90)
≥65 yr	31/42	38/42	0.61 (0.38–0.99)
M stage at study entry			
M0, M1a, M1b	21/37	31/38	0.47 (0.27–0.82)
M1c	79/100	88/98	0.72 (0.53–0.97)
Baseline LDH			
≤ULN	52/84	66/81	0.56 (0.39–0.81)
>ULN	48/53	50/52	0.76 (0.51–1.13)
Prior use of interleukin-2			
Yes	19/32	25/33	0.50 (0.28–0.91)
No	81/105	94/103	0.69 (0.51–0.93)

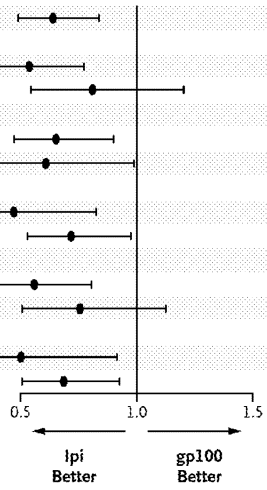


Figure 2. **Subgroup Analyses of Overall Survival.**

The prespecified analyses of overall survival among subgroups of patients, as defined by baseline demographic characteristics and stratification factors (metastasis [M] stage, classified according to the tumor–node–metastasis [TNM] categorization for melanoma of the American Joint Committee on Cancer; and receipt or nonreceipt of interleukin-2 therapy), showed that hazard ratios were lower than 1 (indicating a lower risk of death) for each subgroup in the ipilimumab (Ipi)-plus-glycoprotein 100 (gp100) group as compared with the gp100-alone group (Panel A) and for each subgroup in the ipilimumab-alone group as compared with the gp100-alone group (Panel B). Hazard ratios were estimated with the use of unstratified Cox proportional-hazards models. Horizontal lines represent 95% confidence intervals. LDH denotes lactate dehydrogenase, and ULN the upper limit of the normal range.

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Table 2. **Best Response to Treatment and Time-to-Event Data.***

Response and Time to Event	Ipilimumab plus gp100 (N=403)	Ipilimumab Alone (N=137)	gp100 Alone (N=136)
Overall survival			
Total no. of deaths	306	100	119
Comparison with gp100 alone			
Hazard ratio (95% CI)	0.68 (0.55–0.85)	0.66 (0.51–0.87)	—
P value by log-rank test	<0.001	0.003	—
Comparison with ipilimumab alone			
Hazard ratio (95% CI)	1.04 (0.83–1.30)	—	—
P value by log-rank test	0.76	—	—
Evaluation of therapy			
Induction			
Best overall response — no. (%)			
Complete response	1 (0.2)	2 (1.5)	0
Partial response	22 (5.5)	13 (9.5)	2 (1.5)
Stable disease	58 (14.4)	24 (17.5)	13 (9.6)
Progressive disease	239 (59.3)	70 (51.1)	89 (65.4)
Not evaluated	83 (20.6)	28 (20.4)	32 (23.5)
Best overall response rate — % (95% CI)	5.7 (3.7–8.4)	10.9 (6.3–17.4)	1.5 (0.2–5.2)
P value for comparison with gp100 alone	0.04	0.001	—
P value for comparison with ipilimumab alone	0.04	—	—
Disease control rate — % (95% CI)†	20.1 (16.3–24.3)	28.5 (21.1–36.8)	11.0 (6.3–17.5)
P value for comparison with gp100 alone	0.02	<0.001	—
P value for comparison with ipilimumab alone	0.04	—	—
Time to event — mo			
Time to progression — median (95% CI)	2.76 (2.73–2.79)	2.86 (2.76–3.02)	2.76 (2.73–2.83)
Time to response — mean (95% CI)	3.32 (2.91–3.74)	3.18 (2.75–3.60)	2.74 (2.12–3.37)
Duration of response — median (95% CI)	11.5 (5.4–NR)	NR (28.1–NR)	NR (2.0–NR)
Reinduction‡			
Best overall response — no./total no. (%)			
Complete response	0	1/8 (12.5)	0
Partial response	3/23 (13.0)	2/8 (25.0)	0
Stable disease	12/23 (52.2)	3/8 (37.5)	0
Progressive disease	8/23 (34.8)	2/8 (25.0)	1/1 (100.0)

* Of the 143 patients who could not be evaluated for a response, 33 patients did not receive any study drug and 110 patients did not have baseline or week-12 tumor assessments (or both). Percentages may not total 100 because of rounding. NR denotes not reached.

† The disease control rate is the percentage of patients with a partial or complete response or stable disease.

‡ A total of 40 patients (29 in the ipilimumab-plus-gp100 group; 9 in the ipilimumab-alone group, and 2 in the gp100-alone group) were given reinduction therapy, but 8 were not included in the efficacy analyses: 3 had major protocol violations and 5 were not eligible owing to the fact that they had had a best overall response of progressive disease during induction and were given reinduction therapy inadvertently.

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immune-related events, which occurred in approximately 60% of the patients treated with ipilimumab and 32% of the patients treated with gp100. The frequency of grade 3 or 4 immune-related adverse events was 10 to 15% in the ipilimumab groups and 3.0% in the gp100-alone group. All immune-related events occurred during the induction and reinduction periods; the immune-related adverse events most often affected the skin and gastrointestinal tract. The median time to the resolution of immune-related adverse events of grade 2, 3, or 4 was 6.3 weeks (95% CI, 4.3 to 8.4) in the ipilimumab-plus-gp100 group, 4.9 weeks (95% CI, 3.1 to 6.4) in the ipilimumab-alone group, and 3.1 weeks (95% CI, 1.1 to not reached) in the gp100-alone group.

The most common immune-related adverse event was diarrhea, which occurred at any grade in 27 to 31% of the patients in the ipilimumab groups. After the administration of corticosteroids, the median time to the resolution of diarrhea of grade 2 or higher was 2.0 weeks for 40 of 44 patients in the ipilimumab-plus-gp100 group and 2.3 weeks for 14 of 15 patients in the ipilimumab-alone group. In addition to corticosteroids, 4 patients received infliximab (anti–tumor necrosis factor α antibody) for diarrhea of grade 3 or higher or colitis. Among the 94 persons who survived for 2 years, residual effects of adverse events included those related to injection-site reactions (16 patients), vitiligo (12), diarrhea or colitis (e.g., proctocolitis with rectal pain) (4), and endocrine immune-related adverse events (e.g., inflammation of the pituitary) that required hormone-replacement therapy (8). Ongoing events in the persons who survived for 2 years included rash, pruritus, diarrhea, anorexia, and fatigue, generally of grade 1 or 2 (in 5 to 15% of the patients) and grade 3 leukocytosis (in one patient). There were 14 deaths related to the study drugs (2.1%), of which 7 were associated with immune-related adverse events.

DISCUSSION

This phase 3 study showed that ipilimumab, either alone or with gp100, improved overall survival as compared with gp100 alone in patients with metastatic melanoma who had undergone previous treatment. More than 70% of the patients had M1c disease (presence of visceral metastases) and more than 36% had elevated lactate dehydrogenase levels, both of which are associated with very poor survival.[27,28] The eligibility criteria for patients in this study included HLA-A*0201–positive status, on the basis of the mechanism of action of gp100. However, CTLA-4 blockade by ipilimumab is independent of HLA status, as indicated by efficacy and safety outcomes in earlier clinical trials that were similar between HLA-A*0201–positive and HLA-A*0201–negative patients[21] (and unpublished data).

In our study, the efficacy of ipilimumab was not improved by the addition of gp100. It is unlikely that this is due to a lack of gp100 expression in the tumors, because differentiation antigens have been shown to be strongly expressed in more than 90% of melanoma tumors, regardless of stage.[29] Some studies of adjuvant therapy for melanoma showed that patients who were administered non–gp100 vaccines had shorter survival than did patients in the control groups.[30,31] In contrast, phase 3 trials showed that in subgroups of patients with melanoma, vaccines had clinical activity when used as either adjuvant therapy or therapy for metastatic disease.[32,33] Cumulative data show that gp100-based vaccines have immunologic activity, although clinical activity is minimal when gp100 vaccines are administered as monotherapy.[22] In a randomized, phase 3 study involving patients with metastatic melanoma, a significant improvement in progression-free survival and response rate, and a nonsignificant improvement in overall survival, were seen with gp100-plus-high-dose interleukin-2, as compared with interleukin-2 alone.[23] Although gp100 appeared to attenuate ipilimumab responses in our study, it is important to consider the fact that some radiographic responses of immunotherapeutic agents are not captured by standard response criteria.[34] Regardless, such effects of gp100 did not translate into a difference in overall survival between the two ipilimumab groups.

The data in this study are consistent with the results of phase 2 trials of ipilimumab monotherapy in the same patient population.[15-17] The data from phase 2 studies suggest that there is a long-term survival effect of ipilimumab monotherapy; ipilimumab monotherapy at a dose of 3 mg per kilogram resulted in 1-year and 2-year survival rates of 39.3% and 24.2%, respectively.[16]

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Table 3. **Adverse Events in the Safety Population.***

Adverse Event	Ipilimumab plus gp100 (N=380)			Ipilimumab Alone (N=131)			gp100 Alone (N=132)		
	Total	Grade 3	Grade 4	Total	Grade 3	Grade 4	Total	Grade 3	Grade 4
	number of patients (percent)								
Any event	374 (98.4)	147 (38.7)	26 (6.8)	127 (96.9)	49 (37.4)	11 (8.4)	128 (97.0)	54 (40.9)	8 (6.1)
Any drug-related event	338 (88.9)	62 (16.3)	4 (1.1)	105 (80.2)	25 (19.1)	5 (3.8)	104 (78.8)	15 (11.4)	0
Gastrointestinal disorders									
Diarrhea	146 (38.4)	16 (4.2)	1 (0.3)	43 (32.8)	7 (5.3)	0	26 (19.7)	1 (0.8)	0
Nausea	129 (33.9)	5 (1.3)	1 (0.3)	46 (35.1)	3 (2.3)	0	52 (39.4)	3 (2.3)	0
Constipation	81 (21.3)	3 (0.8)	0	27 (20.6)	3 (2.3)	0	34 (25.8)	1 (0.8)	0
Vomiting	75 (19.7)	6 (1.6)	1 (0.3)	31 (23.7)	3 (2.3)	0	29 (22.0)	3 (2.3)	0
Abdominal pain	67 (17.6)	6 (1.6)	0	20 (15.3)	2 (1.5)	0	22 (16.7)	6 (4.5)	1 (0.8)
Other									
Fatigue	137 (36.1)	19 (5.0)	0	55 (42.0)	9 (6.9)	0	41 (31.1)	4 (3.0)	0
Decreased appetite	88 (23.2)	5 (1.3)	1 (0.3)	35 (26.7)	2 (1.5)	0	29 (22.0)	3 (2.3)	1 (0.8)
Pyrexia	78 (20.5)	2 (0.5)	0	16 (12.2)	0	0	23 (17.4)	2 (1.5)	0
Headache	65 (17.1)	4 (1.1)	0	19 (14.5)	3 (2.3)	0	19 (14.4)	3 (2.3)	0
Cough	55 (14.5)	1 (0.3)	0	21 (16.0)	0	0	18 (13.6)	0	0
Dyspnea	46 (12.1)	12 (3.2)	2 (0.5)	19 (14.5)	4 (3.1)	1 (0.8)	25 (18.9)	6 (4.5)	0
Anemia	41 (10.8)	11 (2.9)	0	15 (11.5)	4 (3.1)	0	23 (17.4)	11 (8.3)	0
Any immune-related event	221 (58.2)	37 (9.7)	2 (0.5)	80 (61.1)	16 (12.2)	3 (2.3)	42 (31.8)	4 (3.0)	0
Dermatologic	152 (40.0)	8 (2.1)	1 (0.3)	57 (43.5)	2 (1.5)	0	22 (16.7)	0	0
Pruritus	67 (17.6)	1 (0.3)	0	32 (24.4)	0	0	14 (10.6)	0	0
Rash	67 (17.6)	5 (1.3)	0	25 (19.1)	1 (0.8)	0	6 (4.5)	0	0
Vitiligo	14 (3.7)	0	0	3 (2.3)	0	0	1 (0.8)	0	0
Gastrointestinal	122 (32.1)	20 (5.3)	2 (0.5)	38 (29.0)	10 (7.6)	0	19 (14.4)	1 (0.8)	0
Diarrhea	115 (30.3)	14 (3.7)	0	36 (27.5)	6 (4.6)	0	18 (13.6)	1 (0.8)	0
Colitis	20 (5.3)	11 (2.9)	1 (0.3)	10 (7.6)	7 (5.3)	0	1 (0.8)	0	0
Endocrine	15 (3.9)	4 (1.1)	0	10 (7.6)	3 (2.3)	2 (1.5)	2 (1.5)	0	0
Hypothyroidism	6 (1.6)	1 (0.3)	0	2 (1.5)	0	0	2 (1.5)	0	0
Hypopituitarism	3 (0.8)	2 (0.5)	0	3 (2.3)	1 (0.8)	1 (0.8)	0	0	0
Hypophysitis	2 (0.5)	2 (0.5)	0	2 (1.5)	2 (1.5)	0	0	0	0
Adrenal insufficiency	3 (0.8)	2 (0.5)	0	2 (1.5)	0	0	0	0	0

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Increase in serum thyrotropin level	2 (0.5)	0	0	1 (0.8)	0	0	0	0	0	0
Decrease in serum corticotropin level	0	0	0	2 (1.5)	0	1 (0.8)	0	0	0	0
Hepatic	8 (2.1)	4 (1.1)	0	5 (3.8)	0	0	6 (4.5)	3 (2.3)	0	
Increase in alanine aminotransferase	3 (0.8)	2 (0.5)	0	2 (1.5)	0	0	3 (2.3)	0	0	
Increase in aspartate aminotransferase	4 (1.1)	1 (0.3)	0	1 (0.8)	0	0	2 (1.5)	0	0	
Hepatitis	2 (0.5)	1 (0.3)	0	1 (0.8)	0	0	0	0	0	
Other	12 (3.2)	5 (1.3)	0	6 (4.6)	2 (1.5)	1 (0.8)	3 (2.3)	1 (0.8)	0	

* The adverse events listed here were reported in at least 15% of patients. The most common immune-related adverse events and those of particular clinical relevance are also listed. Patients could have more than one adverse event. Included are all patients who received at least one dose of a study drug (643 patients). A total of 14 deaths (2.2%) were determined by the investigators to be related to the study drug (8 in the ipilimumab-plus-gp100 group, 4 in the ipilimumab-alone group, and 2 in the gp100-alone group). Seven of the 14 deaths related to the study drug were associated with immune-related adverse events: 5 in the ipilimumab-plus-gp100 group (1 patient had grade 3 colitis and septicemia; 3 patients had bowel perforation--inflammatory colitis, bowel perforation, or multiorgan failure--peritonitis; and 1 patient had Guillain–Barré syndrome, which is considered to be consistent with a neurologic immune-related adverse event) and 2 in the ipilimumab-alone group (1 patient had colic bowel perforation and the other had liver failure). Deaths related to the study drug that were not associated with immune-related adverse events included deaths from sepsis, myelofibrosis, and acute respiratory distress syndrome (3 patients in the ipilimumab-plus-gp100 group); severe infection--renal failure--septic shock, and vascular leak syndrome (2 patients in the ipilimumab-alone group), and cachexia and septic shock (2 patients in the gp100-alone group).

The long-term effect of ipilimumab in our study is shown by survival analyses at late time points, which showed 1-year and 2-year survival rates of 45.6% and 23.5%, respectively. In recent, randomized, phase 3 trials involving patients with unresectable stage III or IV melanoma who had received previous treatment, 1-year survival rates were reported to be 22% to 38% with various treatment regimens.[35,36] The median overall survival in these studies ranged from 5.9 to 9.7 months. Neither these nor other randomized, controlled trials had shown a significant improvement in overall survival.

The adverse-event profile of ipilimumab in this study is consistent with that reported in phase 2 trials,[15-17] with the majority of adverse events being immune-related and consistent with the proposed mechanism of action of ipilimumab.[11-14] As shown in phase 2 studies, prompt medical attention and early administration of corticosteroids are critical to the management of immune-related adverse events.[15-17] Management guidelines (algorithms) for immune-related adverse events involve close patient follow-up and the administration of high-dose systemic corticosteroids — which were used as necessary in our study — for grade 3 or 4 events.[37,38]

In conclusion, this randomized, controlled trial showed that there was a significant improvement in overall survival among patients with metastatic melanoma. In some patients, side effects can be life-threatening and may be treatment-limiting. Reinduction with ipilimumab at the time of disease progression can result in further clinical benefit. Overall, our findings suggest that the T-cell potentiator ipilimumab may be useful as a treatment for patients with metastatic melanoma whose disease progressed while they were receiving one or more previous therapies.

Supported by Medarex and Bristol-Myers Squibb.

All study sites and institutions received funding from Medarex or Bristol-Myers Squibb to cover the expenses of the investigators for undertaking this trial.

Dr. Hodi reports receiving consulting fees from Bristol-Myers Squibb–Medarex, Novartis, and Genentech; Dr. O'Day, receiving consulting fees, grants, honoraria, and fees for participation in speakers' bureaus from Bristol-Myers Squibb; Dr. McDermott, receiving consulting fees from Bristol-Myers Squibb–Medarex; Dr. Gonzalez, receiving honoraria from Bristol-Myers Squibb; Dr. Schadendorf, serving on a board for and receiving consulting fees, fees for expert testimony, and fees for participation in speakers' bureaus from Bristol-Myers Squibb; Dr. van den Eertwegh, receiving consulting fees from and serving on a board for Bristol-Myers Squibb; Dr. Lutzky, receiving consulting fees and honoraria from Bristol-Myers Squibb; Dr. Lorigan, receiving consulting fees from Bristol-Myers Squibb; Dr. Hogg, serving on a board for

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Bristol-Myers Squibb (pending); Dr. Ottensmeier, receiving honoraria and grant funding from Bristol-Myers Squibb; Dr. Lebbé, serving on a board for Bristol-Myers Squibb; Dr. Wolchok, serving on a board for Bristol-Myers Squibb; Dr. J.S. Weber, receiving consulting fees from Bristol-Myers Squibb; Drs. Tian, Yellin, and Nichol being former employees of Medarex; Dr. Hoos being currently employed by Bristol-Myers Squibb with stock or stock options; and Dr. Urba, receiving consulting fees from Bristol-Myers Squibb–Medarex. No other potential conflicts of interest relevant to this article were reported.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org

We thank the patients who volunteered to participate in this study and staff members at the study sites who cared for them; the members of the data and safety monitoring committee; and representatives of the sponsors who were involved in data collection and analyses (in particular, Tai-Tsang Chen, Xiaoping Zhu, Marianne Messina, and Helena Brett-Smith). Editorial and writing assistance was provided by Ward A. Pedersen of StemScientific, funded by Bristol-Myers Squibb.

APPENDIX

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Table (abstract 801).

	CAP	TAB		TAB			
	400 mg BID (cont)	400 mg BID (cont)	300 mg BID (cont)	200 mg TID (cont)	250 mg TID (int)	400 mg BID (int)	400 mg QD (cont)
Evaluable for safety, n	16*	17*	18*	16	15	16	15
Grade ⩾3 events, n (%)							
Anaemia	4 (22)	5 (29)	4 (22)	1 (6)	1 (7)	0	1 (7)
Vomiting	1 (6)	0	0	2 (13)	0	1 (6)	0
Dose modifications, n (%)							
Reductions	3 (17)	11 (65)	4 (22)	2 (13)	2 (13)	4 (27)	2 (13)
Interruptions	6 (33)	10 (59)	8 (44)	10 (63)	8 (53)	10 (67)	5 (33)
Evaluable for antitumour effect, n	20[†]		13	15	14	16	15
Least-squares mean change in tumour size at wk 8,[‡] %	−19.0	NA[§]	−19.9	−14.4	−5.4	−14.7	−3.3

*Includes ovarian and breast cancer pts with a germline *BRCA1/2* mutation.
[†]Includes pts from a second previously reported group.
[‡]Adjusted for baseline characteristics; [§]Dose not considered tolerable in a prior data review so analysis of antitumour effect not performed

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Administration of continuous/intermittent olaparib in ovarian cancer patients with a germline BRCA1/2 mutation to determine an optimal dosing schedule for the tablet formulation

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Background: Initial studies with the oral PARP inhibitor olaparib identified 400 mg BID as a tolerable and efficacious dose requiring 16 capsules (CAP)/day. We report final results of a study aimed at identifying an optimal dose of the more practical tablet (TAB) formulation, for which we previously reported a dose-response relationship. An expansion cohort was recruited to this multistage Phase I trial to evaluate alternative administration schedules (NCT00777582; sponsor, AstraZeneca).
Materials and Methods: In this expansion cohort, patients (pts) with *BRCA1/2* mutation and relapsed ovarian or primary peritoneal cancer were randomized 1:1:1:1 to one of two continuous (cont; 200 mg TID, 400 mg QD) or two intermittent (int; 250 mg TID: 2 wks on/1 wk off; 400 mg BID: 1 wk on/1 wk off) schedules. Primary objective was safety and tolerability. Secondary objectives included antitumour effect. Results were compared with previously reported groups receiving 400 mg CAP BID, 400 mg TAB BID or 300 mg TAB BID (all cont; Molife *et al* ASCO 2010, 2012).
Results: 62 patients were randomized. Median lines of prior chemotherapy was 3 (range: 1–11). 6 pts (10%) discontinued treatment due to toxicity. The table shows key safety and antitumour effect data.
Conclusions: 400 mg BID TAB (int and cont) were not considered tolerable for long-term use. 250 mg TID (int) and 400 mg QD (cont) failed to match the efficacy seen in prior cohorts. 200 mg TID did not improve tolerability vs 300 mg BID, so 300 mg BID (cont) is the recommended tablet dose for Phase III studies in *BRCA1/2*-mutant ovarian cancer, including the maintenance therapy setting. These findings simplify olaparib administration from 16 CAP to 4 TAB per day.
Conflict of interest: *Ownership: A. Fielding & K. Bowen are employees of AstraZeneca and own AstraZeneca stock. Advisory board: C. Gourley has been a consultant for Roche, Boehringer-Ingelheim, Schering-Plough, GlaxoSmithKline and Chugai. Corporate-sponsored research: C. Gourley has received research funding from AstraZeneca. Other substantive relationships: C. Gourley has received honoraria and other remuneration from Roche, Boehringer-Ingelheim, Schering-Plough, GlaxoSmithKline,Chugai and Pharma-Mar*

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MEDI4736, an anti-PD-L1 antibody with modified Fc domain: Preclinical evaluation and early clinical results from a phase 1 study in patients with advanced solid tumors

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Background: Tumors can evade immune detection by expressing the programmed cell death ligand 1 (PD-L1). Blockade of PD-L1 may help overcome immunosuppressive effects and restore T-cell activity against tumors. In nonclinical experiments, inhibition of PD-L1 was shown to produce durable antitumor activity as a single agent and in combination with other therapies. MEDI4736, a human monoclonal antibody that binds specifically to PD-L1, was engineered with a triple mutation in the Fc domain to abrogate Fc-mediated effector function, and is currently being evaluated in Phase 1 studies.
Materials and Methods: The properties of MEDI4736 were determined via *in vitro* assays, and anti-mouse PD-L1 was used in murine models of cancer to explore pharmacodynamic markers and combinations with other therapies. An ongoing Phase 1, multicenter, open-label study is evaluating the safety profile, pharmacokinetics, biomarkers, and antitumor activity of MEDI4736 administered IV in subjects with advanced solid tumors.
Results: MEDI4736 demonstrated potent and specific binding to PD-L1 with picomolar affinity. It blocks the interaction of PD-L1 with PD-1 and CD80 resulting in increased T-cell activation *in vitro*, but does not trigger cytokine release in whole blood assays. Three point mutations in the Fc domain have abrogated Fc-mediated effector function *in vitro*. Treatment of tumor-bearing mice with anti-mouse PD-L1 resulted in changes in peripheral immune markers and antitumor responses in a subset of mice. The combination of anti-mouse PD-L1 and CTLA-4 antibodies significantly enhanced this activity, leading to tumor regression in all mice treated.
As of 15 April, preliminary results from the Phase 1 study include 6 subjects (med. age 65 yrs, range 46–71), ECOG 0–1, with a median of 4 (3–10) prior treatments, received a median of 6 doses (1–13) of MEDI4736. No dose limiting toxicities or drug-related grade ⩾3 adverse events were reported (no pneumonitis or colitis of any grade). An early signal of clinical activity was observed as evidenced by RECIST-based responses and prolonged disease stabilization in different tumor types, including patients with extensive disease. Tumor shrinkage was observed as early as 7 weeks and was sustained at subsequent time points.
Conclusions: Preliminary safety and clinical activity data for MEDI4736 are encouraging at the initial dose levels explored and warrant further investigation of this molecule alone and in combination with other therapies.
Conflict of interest: *Corporate-sponsored research. Medimmune sponsored the study. SK, JL, NS, SA, JW are investigators and received research funding for the conduct of the study. ABH, RS, PR, AS, RI are employees of Medimmune. Other substantive relationships: ABH, RS, PR, AS, RI own stockstock options in Astrazeneca*

OncoImmunology 4:7, e1058597; July 2015; © 2015 Taylor & Francis Group, LLC

Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy?

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Keywords: CTLA4, ipilimumab, nivolumab, PD-1, PD-L1, pembrolizumab

The first study demonstrating that human colorectal carcinoma (CRC) is under robust immunosurveillance was published a decade ago. Today, it is clear that CRC patients with Stage III lesions abundantly infiltrated by effector memory T cells have a better prognosis than subjects with Stage I neoplasms exhibiting no or poor immune infiltration. Thus, immunological parameters have a superior prognostic value for CRC patients than TNM staging or the Dukes classification. In spite of the fact that CRC is the first neoplasia found to be under immunological control, most attempts made so far to cure this malignancy with immunotherapy have failed. With the exception of a minority of lesions characterized by microsatellite instability (MSI), CRC seems to be insensitive to the blockade of immunological checkpoints with monoclonal antibodies (mAbs) specific for cytotoxic T lymphocyte-associated protein 4 (CTLA4), programmed cell death 1 (PDCD1, best known as PD-1) and the PD-1 ligand CD274 (best known as PD-L1). Thus, CRC stands in contrast with an increasing number of malignancies that respond to checkpoint blockers. Efforts should therefore be dedicated to the development of strategies to (re)instate immunosurveillance in patients with MSI$^-$ CRC, perhaps based on the identification of novel, locally relevant immunological checkpoints.

Blocking the immunological checkpoints mediated by PD-1 and CTLA4 has recently emerged as a highly promising option for the treatment of an ever-increasing number of malignancies, including (but not limited to) melanoma, non-small cell lung carcinoma, bladder carcinoma, Hodgkin lymphoma, triple-negative breast carcinoma, as well as head and neck cancer.[1] Admittedly, only a fraction of individuals with these neoplasms respond to checkpoint blockers, and definitive cures are still an exception. However, robust and durable objective responses entailing the complete disappearance of neoplastic lesions and no relapse are not considered miraculous anymore. In other words, with the advent of checkpoint blockers, curing cancer has become an attainable - rather than a merely utopian - goal.[1]

Nonetheless, there are a few cancer types that appear to be rather refractory to checkpoint blockers, and CRC is one of them. As a notable exception, patients with mismatch repair-deficient CRC lesions obtain clinical benefits from the administration of a PD-1-targeting mAb.[2] Perhaps, this is because defects in mismatch repair favor MSI, a state of genomic instability that largely increases the incidence of somatic mutations and hence the immunogenicity of cancer cells.[3] The fact that CRC does not respond to checkpoint blockers appears somehow paradoxical, since the first sophisticated analyses of the immunological tumor microenvironment have been performed on CRC specimens, yielding the conclusion that the "immune contexture" has a critical impact on the fate of patients.[4,5] The term "immune contexture" refers to the density, distribution and function of the immune infiltrate, which globally constitutes the most robust prognostic parameter for overall survival in CRC patients undergoing standard surgery and/or chemotherapy. Thus, immunological variables including the so-called "immunoscore" supersede in importance all traditional classifications of MSI$^-$ CRCs, including TNM staging and the Dukes score.[6-9] Corroborating this notion, it has been found that oxaliplatin, a platinum derivative that is widely employed in adjuvant or neoadjuvant chemotherapeutic regimen against CRC,[10,11] exerts optimal effects only in the presence of a functional immune system.[12,13] Indeed, CRCs that develop in mice lacking T cells or Toll-like receptor-4 (Tlr4) fail to respond to oxaliplatin-based chemotherapy.[14,15] Moreover, CRC patients treated with oxaliplatin have a particularly high chance of experiencing disease relapse if they bear a

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Submitted: 06/01/2015; Accepted: 06/02/2015
http://dx.doi.org/10.1080/2162402X.2015.1058597

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loss-of-function allele of *TLR4*.[15] Thus, immunological parameters have not only a prognostic but also a predictive value for CRC patients treated with standard chemo- or radiotherapeutic regimens.

Based on the abovementioned preclinical and clinical findings, one may have predicted that mAbs targeting immunological checkpoints would be particularly efficient in CRC patients. However, neither the blockade of CTLA4 (with ipilimumab/Yervoy™) nor that of the PD-1/PD-L1 axis (with nivolumab/Opdivo™ or pembrolizumab/Keytruda™) has conferred any major clinical benefits to patients bearing mismatch repair-proficient CRC.[16-20] Rather, only mismatch repair-proficient MSI^+ CRC lesions (which generally display an abundant immune infiltrate) are likely to respond to pembrolizumab.[2] The etiology of mismatch repair-deficient MSI^+ CRCs is very different from that of their mismatch repair-proficient MSI^- counterparts. In particular, only the former are prone to accumulate somatic mutations, and this may significantly increase their immunogenicity (and hence explain their sensitivity to pembrolizumab, at least in part). Of note, other cytological events may lead to genomic instability, including tetraploidization. Supporting an etiological relationship between the amount of somatic mutations and immunogenicity, tetraploidization has also been shown to elicit immunosurveillance mechanisms (although it does not cause MSI).[21,22]

What might be the reason(s) why checkpoint blockers are not efficient in subjects with MSI^- CRC? There are several speculative answers to this question. First, in CRC lesions that are massively infiltrated by effector memory T cell, immunological checkpoints might be intrinsically inactive. In such a scenario, the exogenous administration of checkpoint blockers would simply be useless. Second, CRC lesions with limited T-cell infiltration may not respond to checkpoint blockers because they cannot be properly invaded, recognized or eliminated by the cellular immune system. This may reflect the antigenic properties of malignant cells, their inability to dispatch immunostimulatory danger signals in the course of oncogenic and/or chemotherapeutic stress, or the activation of yet to be discovered immunological checkpoints that actively suppress immunosurveillance against CRC.

Further preclinical and clinical studies are warranted to understand which among the aforementioned (and mutually non-exclusive) possibilities apply. Is immunosurveillance against CRC controlled by novel immunological checkpoints that are not regulated by CTLA4 and the PD-1/PD-L1 axis? Is it necessary to combine clinically approved checkpoint blockers with additional immunotherapeutic measures including immunostimulatory compounds,[23,24] therapeutic vaccines,[25] or immunogenic cell death inducers?[26-28] Is it a requirement to intervene on the gut microbiota, which shapes the local tumor microenvironment, to reinstate failed immunosurveillance?[29] Patients with MSI^- CRC are impatiently awaiting answers to these questions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

PD-1 Blockade in Tumors with Mismatch-Repair Deficiency

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ABSTRACT

BACKGROUND

Somatic mutations have the potential to encode "non-self" immunogenic antigens. We hypothesized that tumors with a large number of somatic mutations due to mismatch-repair defects may be susceptible to immune checkpoint blockade.

METHODS

We conducted a phase 2 study to evaluate the clinical activity of pembrolizumab, an anti–programmed death 1 immune checkpoint inhibitor, in 41 patients with progressive metastatic carcinoma with or without mismatch-repair deficiency. Pembrolizumab was administered intravenously at a dose of 10 mg per kilogram of body weight every 14 days in patients with mismatch repair–deficient colorectal cancers, patients with mismatch repair–proficient colorectal cancers, and patients with mismatch repair–deficient cancers that were not colorectal. The coprimary end points were the immune-related objective response rate and the 20-week immune-related progression-free survival rate.

RESULTS

The immune-related objective response rate and immune-related progression-free survival rate were 40% (4 of 10 patients) and 78% (7 of 9 patients), respectively, for mismatch repair–deficient colorectal cancers and 0% (0 of 18 patients) and 11% (2 of 18 patients) for mismatch repair–proficient colorectal cancers. The median progression-free survival and overall survival were not reached in the cohort with mismatch repair–deficient colorectal cancer but were 2.2 and 5.0 months, respectively, in the cohort with mismatch repair–proficient colorectal cancer (hazard ratio for disease progression or death, 0.10 [P<0.001], and hazard ratio for death, 0.22 [P=0.05]). Patients with mismatch repair–deficient noncolorectal cancer had responses similar to those of patients with mismatch repair–deficient colorectal cancer (immune-related objective response rate, 71% [5 of 7 patients]; immune-related progression-free survival rate, 67% [4 of 6 patients]). Whole-exome sequencing revealed a mean of 1782 somatic mutations per tumor in mismatch repair–deficient tumors, as compared with 73 in mismatch repair–proficient tumors (P=0.007), and high somatic mutation loads were associated with prolonged progression-free survival (P=0.02).

CONCLUSIONS

This study showed that mismatch-repair status predicted clinical benefit of immune checkpoint blockade with pembrolizumab. (Funded by Johns Hopkins University and others; ClinicalTrials.gov number, NCT01876511.)

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This article was published on May 30, 2015, at NEJM.org.

N Engl J Med 2015;372:2509-20.
DOI: 10.1056/NEJMoa1500596
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THE PROGRAMMED DEATH 1 (PD-1) PATHway is a negative feedback system that represses Th1 cytotoxic immune responses and that, if unregulated, can damage the host.[1-3] It is up-regulated in many tumors and in their surrounding microenvironment. Blockade of this pathway with antibodies to PD-1 or its ligands has led to remarkable clinical responses in patients with many different types of cancer, including melanomas, non–small-cell lung cancer, renal-cell carcinoma, bladder cancer, and Hodgkin's lymphoma.[4-10] The expression of PD-1 ligands (PD-L1 or PD-L2) on the surface of tumor cells or immune cells is an important — but not a definitive — predictive biomarker of response to PD-1 blockade.[4,6-8,11]

In reports of the effects of PD-1 blockade in human tumors, only 1 of 33 patients with colorectal cancer had a response to this treatment, in contrast to substantial fractions of patients with melanomas, renal-cell cancers, and lung tumors who have a response.[10,12] What was different about this single patient? We hypothesized that this patient had mismatch-repair deficiency, because mismatch-repair deficiency occurs in a small fraction of advanced colorectal cancers,[13,14] somatic mutations found in tumors can be recognized by the patient's own immune system,[15] and mismatch repair–deficient colorectal cancers have 10 to 100 times as many somatic mutations as mismatch repair–proficient colorectal cancers.[16-18] Moreover, mismatch repair–deficient cancers contain prominent lymphocyte infiltrates, a finding consistent with an immune response.[19-22] In addition, two of the tumor types that were most responsive to PD-1 blockade in a study by Topalian et al.[10] had high numbers of somatic mutations as a result of exposure to cigarette smoke (lung cancers) or ultraviolet radiation (melanomas).[23,24] Our hypothesis was correct: the tumor of the single patient with colorectal cancer who had a response to PD-1 blockade was mismatch repair–deficient.[25] Therefore, we hypothesized that mismatch repair–deficient tumors are more responsive to PD-1 blockade than are mismatch repair–proficient tumors.

To test this hypothesis, we initiated a phase 2 clinical trial to evaluate immune checkpoint blockade in patients whose tumors had or did not have mismatch-repair deficiency. Because mismatch-repair deficiency in tumors arises through two routes,[26-28] we recruited patients with hereditary nonpolyposis colorectal cancer (also known as the Lynch syndrome), which results from an inherited germline defect in one of four mismatch-repair genes followed by a second inactivating somatic change in the remaining wild-type allele. We also recruited patients with sporadic mismatch repair–deficient tumors, in which both alleles of a mismatch-repair gene are inactivated by somatic mutations or by epigenetic silencing.[29] In either case, the neoplasms that arise harbor hundreds or thousands of mutations.[16,18]

METHODS

PATIENTS

Patients with treatment-refractory progressive metastatic cancer were recruited from three centers for this phase 2 study (Table 1). Three cohorts were evaluated: cohort A included patients with mismatch repair–deficient colorectal adenocarcinomas, cohort B included patients with mismatch repair–proficient colorectal adenocarcinomas, and cohort C included patients with mismatch repair–deficient cancers of types other than colorectal.

STUDY OVERSIGHT

The protocol, available with the full text of this article at NEJM.org, was approved by the institutional review board at each site, and the study was conducted in accordance with the provisions of the Declaration of Helsinki and the International Conference on Harmonisation Good Clinical Practice guidelines. All the patients provided written informed consent before study entry. The first author (the principal investigator) and the last author (the Investigational New Drug sponsor) were responsible for oversight of the study. Merck donated the study drug and reviewed the final drafts of the protocol and of this manuscript before submission; they did not participate in the analysis of the data.

STUDY DESIGN

This phase 2 trial was conducted with the use of a Green–Dahlberg two-stage design and included the three parallel cohorts described above. The study agent, pembrolizumab, was administered intravenously at a dose of 10 mg per kilogram of body weight every 14 days (Fig. S1 in

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Table 1. **Demographic and Baseline Characteristics of the Patients.***

Characteristic	Mismatch Repair–Deficient Colorectal Cancer (N=11)	Mismatch Repair–Proficient Colorectal Cancer (N=21)	Mismatch Repair–Deficient Noncolorectal Cancer (N=9)	P Value†
Median age (range) — yr	46 (24–65)	61 (32–79)	57 (34–92)	0.02
Sex — no. (%)				0.72
Female	5 (45)	8 (38)	4 (44)	
Male	6 (55)	13 (62)	5 (56)	
Race — no. (%)‡				0.66
White	8 (73)	17 (81)	8 (89)	
Black	1 (9)	3 (14)	0	
Other	2 (18)	1 (5)	1 (11)	
ECOG performance status — no. (%)§				0.07
0	0	6 (29)	2 (22)	
1	11 (100)	15 (71)	7 (78)	
Cancer type — no. (%)				>0.99
Colon	9 (82)	18 (86)	0	
Rectal	2 (18)	3 (14)	0	
Ampullary or cholangiocarcinoma	0	NA	4 (44)	
Endometrial	0	NA	2 (22)	
Small bowel	0	NA	2 (22)	
Gastric	0	NA	1 (11)	
Histologic grade — no. (%)				0.20
Well or moderately differentiated	7 (64)	18 (86)	4 (44)	
Poorly differentiated	4 (36)	3 (14)	3 (33)	
Other	0	0	2 (22)	
Stage IV cancer — no. (%)	11(100)	21 (100)	9 (100)	>0.99
Liver metastases — no. (%)	6 (55)	11 (52)	6 (67)	>0.99
Median time since initial diagnosis (range) — mo	31 (6–95)	58 (27–192)	23 (2–105)	0.07
Previous therapies — no. (%)				0.89
1	0	0	1 (11)	
2	3 (27)	4 (19)	5 (56)	
3	3 (27)	5 (24)	1 (11)	
>4	5 (45)	12 (57)	2 (22)	
Detected germline mutation or known Lynch syndrome — no. (%)				<0.001
Yes	9 (82)	0	4 (44)	
No	2 (18)	21 (100)	4 (44)	
Unknown	0	0	1 (11)	
BRAF wild type — no. (%)				0.64
Yes	8 (73)	11 (52)	4 (44)	
No	0	1 (5)	0	
Unknown	3 (27)	9 (43)	5 (56)	
KRAS wild type — no. (%)				0.72
Yes	6 (55)	13 (62)	4 (44)	
No	5 (45)	8 (38)	1 (11)	
Unknown	0	0	4 (44)	

* NA denotes not applicable.
† P values are for the comparison between the cohort with mismatch repair–deficient colorectal cancer and the cohort with mismatch repair–proficient colorectal cancer.
‡ Race was self-reported.
§ Eastern Cooperative Oncology Group (ECOG) performance status is a measure of a patient's ability to perform activities of daily living; values range from 0 to 5, with higher scores indicating greater impairment.

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Supplementary Appendix 1, available at NEJM.org). Pembrolizumab is a humanized monoclonal anti–PD-1 antibody of the IgG4 kappa isotype that blocks the interaction between PD-1 and its ligands, PD-L1 and PD-L2 (Fig. S1 in Supplementary Appendix 1).

Safety assessments were performed before each treatment. At the start of each treatment cycle, the total tumor burden was assessed by means of measurement of serum biomarkers. Radiographic assessments were performed at 12 weeks and every 8 weeks thereafter. Further details concerning the clinical protocol are available at NEJM.org.

ANALYSIS OF MISMATCH-REPAIR STATUS

Tumors with genetic defects in mismatch-repair pathways are known to harbor hundreds to thousands of somatic mutations, especially in regions of repetitive DNA known as microsatellites. The accumulation of mutations in these regions of the genome is termed microsatellite instability.[26-28] Mismatch-repair status was assessed in tumors with the use of the MSI Analysis System (Promega), through the evaluation of selected microsatellite sequences that are particularly prone to copying errors when mismatch repair is compromised.[26-28] Additional details are provided in Supplementary Appendix 1.

GENOMIC AND BIOINFORMATIC ANALYSES

Primary tumor samples and matched normal peripheral-blood specimens were obtained from a subgroup of patients with mismatch repair–deficient carcinomas and a subgroup with mismatch repair–proficient carcinomas, for whom sufficient tumor tissue was available for exome sequencing[30] and HLA haplotyping. To assess the potential for mutant peptide binding, somatic exome data combined with each individual patient's major histocompatibility complex (MHC) class I HLA haplotype were applied to an epitope prediction algorithm.[31,32] This algorithm provided an estimate of the total number of mutation-associated neoantigens in each tumor. Additional details are provided in Supplementary Appendix 1.

STATISTICAL ANALYSIS

The primary end points for cohorts A and B were the immune-related objective response rate and the 20-week immune-related progression-free survival rate, assessed with the use of immune-related response criteria.[33] The primary end point for cohort C was the immune-related progression-free survival rate at 20 weeks (Fig. S1 in Supplementary Appendix 1). Immune-related criteria (i.e., one of the types of criteria used to evaluate immune-based therapies) are based on radiographic responses, and unlike Response Evaluation Criteria in Solid Tumors (RECIST), they capture newly developed lesions detected on radiography in the measurement of tumor burden; these criteria are defined and compared with RECIST, version 1.1, in Table S1 in Supplementary Appendix 1. The response rate and 20-week progression-free survival rate were evaluated and reported in this study with the use of both RECIST, version 1.1, and immune-related response criteria. Progression-free survival and overall survival were summarized by means of the Kaplan–Meier method. Details of the hypothesis, the decision rules for the rejection of the null hypotheses, decision rules for early discontinuation of the study in a cohort because of efficacy or futility, and statistical methods are provided in Supplementary Appendix 1.

RESULTS

PATIENTS

A total of 41 consecutive patients were enrolled in the study and treated during the period from September 2013 through January 2015 (Table 1). Recruitment included patients in pursuit of a clinical trial option who were known to have tumors with mismatch-repair defects or who had tumors of unknown status who were then tested. One patient in the cohort with mismatch repair–deficient colorectal cancer was enrolled under an institutional review board eligibility waiver allowing a grade 3 bilirubin level (i.e., higher than the cutoff specified in the inclusion criteria). A total of 32 patients with colorectal cancer were enrolled in cohorts A and B. All patients with colorectal cancer had received two or more previous chemotherapy regimens (a median of four regimens), except for 1 patient with mismatch repair–proficient cancer who had received one chemotherapeutic and one (non–PD-1–based) immunotherapeutic regimen.

Nine patients with mismatch repair–deficient solid tumors other than colorectal cancer were enrolled in cohort C. All patients in cohort C had

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Table 2. **Objective Responses According to RECIST Criteria.**

Type of Response	Mismatch Repair–Deficient Colorectal Cancer (N=10)	Mismatch Repair–Proficient Colorectal Cancer (N=18)	Mismatch Repair–Deficient Noncolorectal Cancer (N=7)
Complete response — no. (%)	0	0	1 (14)*
Partial response — no. (%)	4 (40)	0	4 (57)†
Stable disease at week 12 — no. (%)	5 (50)	2 (11)	0
Progressive disease — no. (%)	1 (10)	11 (61)	2 (29)
Could not be evaluated — no. (%)‡	0	5 (28)	0
Objective response rate (95% CI) — %	40 (12–74)	0 (0–19)	71 (29–96)
Disease control rate (95% CI) — %§	90 (55–100)	11 (1–35)	71 (29–96)
Median duration of response — wk	Not reached	NA¶	Not reached
Median time to response (range) — wk	28 (13–35)	NA¶	12 (10–13)

* The patient had a partial response at 12 weeks, which then became a complete response at 20 weeks.
† One patient had a partial response at 12 weeks.
‡ Patients could not be evaluated if they did not undergo a scan at 12 weeks because of clinical progression.
§ The rate of disease control was defined as the percentage of patients who had a complete response, partial response, or stable disease for 12 weeks or more.
¶ The median time to response was not applicable (NA) because no responses were observed among patients with mismatch repair–proficient colorectal cancer.

received one or more previous therapeutic regimens (a median of two regimens).

PRIMARY END POINT

The immune-related objective response rate in cohort A was 40% (4 of 10 patients; 95% confidence interval [CI], 12 to 74), and the immune-related progression-free survival rate at 20 weeks was 78% (7 of 9 patients; 95% CI, 40 to 97) (Table S2 in Supplementary Appendix 1); the corresponding rates in cohort C were 71% (5 of 7 patients; 95% CI, 29 to 96) and 67% (4 of 6 patients; 95% CI, 22 to 96). In cohort B, which included patients with mismatch repair–proficient colorectal cancers, the immune-related objective response rate was 0% (95% CI, 0 to 20), and the immune-related progression-free survival rate at 20 weeks was 11% (2 of 18 patients; 95% CI, 1 to 35). Both cohorts with mismatch repair–deficient cancers (cohorts A and C) reached the prespecified point at which the protocol indicated that the study reached its primary efficacy end point when 4 patients were free from disease progression at 20 weeks and objective responses on the basis of immune-related response criteria were observed in 4 patients (Table S2 and the Methods section in Supplementary Appendix 1).

The median follow-up was 36 weeks (range, 5 to 55) for patients with mismatch repair–deficient colorectal cancer (cohort A), 20 weeks (range, 4 to 52) for patients with mismatch repair–proficient colorectal cancer (cohort B), and 21 weeks (range, 0.1 to 49) for patients with mismatch repair–deficient noncolorectal cancer (cohort C). All patients for whom the 20-week immune-related progression-free survival rate could be evaluated were followed for at least 20 weeks.

RADIOGRAPHIC EVALUATION

Of the 10 patients with mismatch repair–deficient colorectal cancer (cohort A) who could be evaluated for RECIST, 4 (40%; 95% CI, 12 to 74) had objective responses according to these criteria (Table 2 and Fig. 1, and Fig. S2 in Supplementary Appendix 1). Patients were considered not to have been evaluated unless they underwent a radiographic scan at 12 weeks. The rate of disease control, which was defined as the percentage of patients who had an objective response or whose disease was stable, was 90% in cohort A (9 of 10 patients; 95% CI, 55 to 100). Of the 7 patients in cohort C who could be evaluated, 5 (71%; 95% CI, 29 to 96) had objective responses as defined by RECIST (Table 2 and Fig. 1, and Fig. S2 in Supplementary Appendix 1), and the rate of disease control was 71% (5 of 7 patients; 95% CI, 29 to 96).

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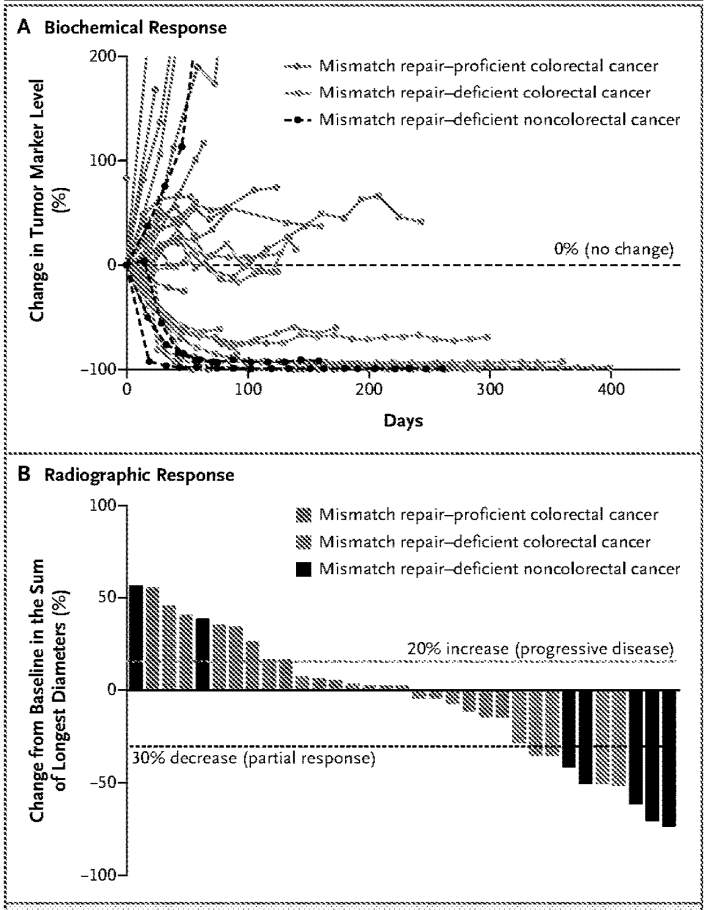


Figure 1. Clinical Responses to Pembrolizumab Treatment.

The biochemical responses to pembrolizumab treatment are shown in Panel A. Serum levels of protein biomarkers were measured at the start of each treatment cycle, and the values represent percentage changes from baseline. Each line represents one patient; patients were included if their baseline tumor marker values were higher than the upper limit of normal. CA-125 was used as the biomarker for one patient with endometrial cancer, CA19-9 was used for one patient with cholangiocarcinoma and one patient with ampullary cancer, and carcinoembryonic antigen (CEA) was used for all other patients. Radiographic responses to treatment with pembrolizumab, evaluated on the basis of Response Evaluation Criteria in Solid Tumors (RECIST), are shown in Panel B. Tumor responses were measured at regular intervals, and the values shown are the largest percentage change in the sum of longest diameters from the baseline measurements of each measurable tumor. Each bar represents one patient.

Patients in cohort C had faster responses than did patients in cohort A (median time to response according to RECIST, 12 weeks vs. 28 weeks; P=0.03). Furthermore, all 6 patients (100%) with mismatch repair–deficient tumors that were not associated with the Lynch syndrome had an objective response, whereas only 3 of 11 patients (27%) with tumors associated with the Lynch syndrome had a response (Table S3 in Supplementary Appendix 2) (P=0.009). No other baseline characteristics had a significant association with objective responses.

Among the 18 patients with mismatch repair–proficient colorectal cancers in cohort B, no objective responses as defined by RECIST were observed (Table 2 and Fig. 1, and Fig. S2 in Supplementary Appendix 1). In this group, the rate of disease control was 11% (2 of 18 patients; 95% CI, 1 to 35).

All the patients who had a response as defined by RECIST (Table 2) also had a response according to immune-related response criteria (Table S2 in Supplementary Appendix 1).

SURVIVAL

In the cohort of patients with mismatch repair–deficient colorectal cancer (cohort A), the median progression-free survival and median overall survival were not reached (Fig. 2). In contrast, among the patients with mismatch repair–proficient cancers (cohort B), the median progression-free survival was only 2.2 months (95% CI, 1.4 to 2.8), and the median overall survival was 5.0 months (95% CI, 3.0 to not estimable). In cohort C (patients with mismatch repair–deficient noncolorectal cancer), the median progression-free survival was 5.4 months (95% CI, 3 to not estimable), and the median overall survival was not reached. A post hoc comparison of the cohorts with mismatch repair–deficient and mismatch repair–proficient colorectal cancers showed hazard ratios for disease progression or death (0.10; 95% CI, 0.03 to 0.37; P<0.001) and for death (0.22; 95% CI, 0.05 to 1.00; P=0.05) that favored patients with mismatch repair–deficient colorectal cancer (Fig. 2).

To evaluate whether the difference in survival might be due to prognostic differences, we measured the time since the diagnosis of metastatic disease and the clinical performance of the regimen that patients had received before enrollment. We found that there was no significant difference between patients with mismatch repair–deficient colorectal cancer and patients with mismatch repair–proficient colorectal cancer with respect to the duration of metastatic disease (P=0.77 by the log-rank test) or the median progression-free survival while receiving their previous regimens (P=0.60 by the log-rank

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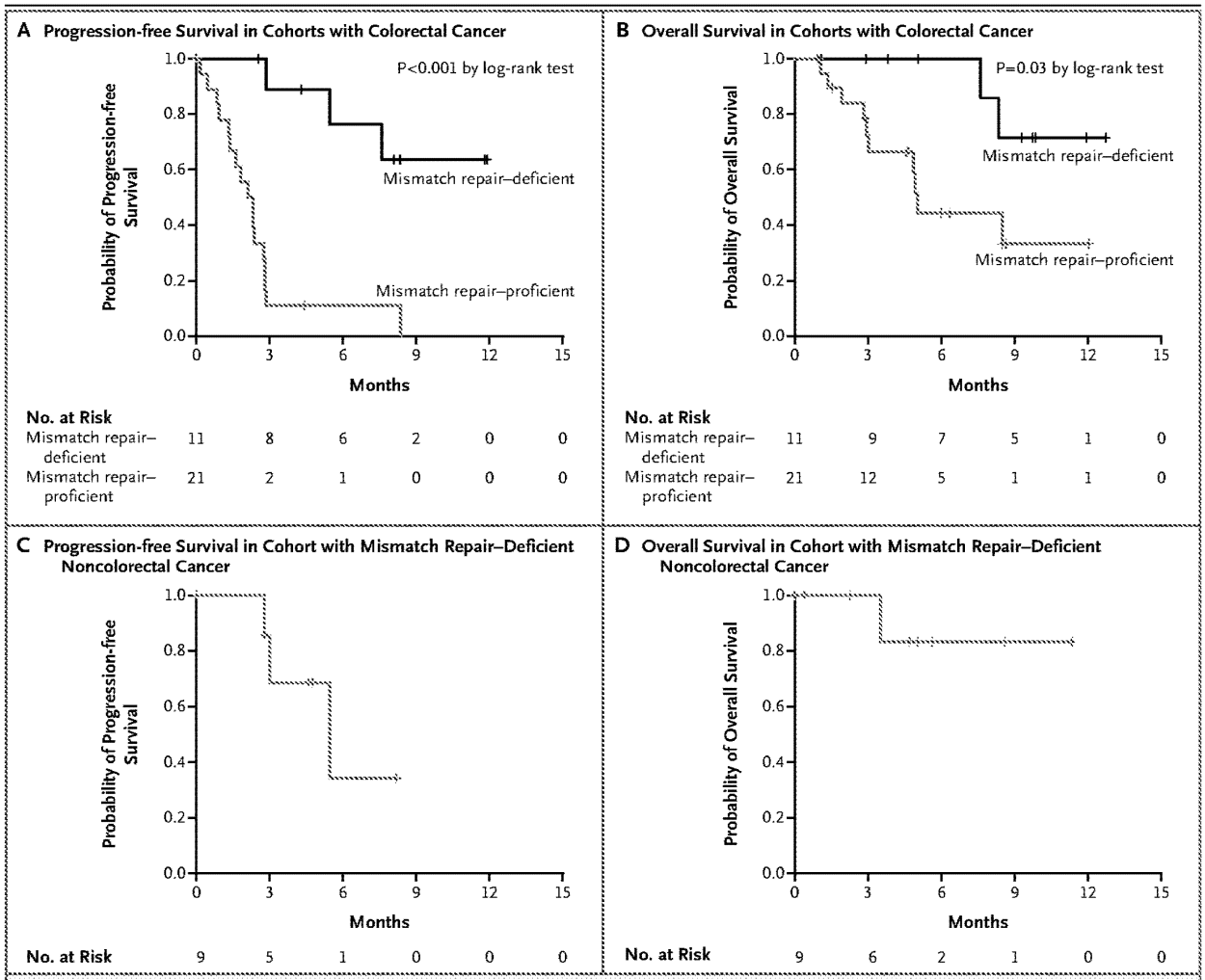


Figure 2. Clinical Benefit of Pembrolizumab Treatment According to Mismatch-Repair Status.

Kaplan–Meier curves are shown for progression-free survival in the cohorts with colorectal cancer (Panel A), overall survival in the cohorts with colorectal cancer (Panel B), progression-free survival among patients with mismatch repair–deficient noncolorectal cancers (Panel C), and overall survival among patients with mismatch repair–deficient noncolorectal cancers (Panel D). In both cohorts with mismatch repair–deficient tumors, median overall survival was not reached. Patients in the cohort with mismatch repair–proficient cancers had a median progression-free survival of 2.2 months (95% CI, 1.4 to 2.8) and a median overall survival of 5.0 months (95% CI, 3.0 to not estimable). Patients with mismatch repair–deficient noncolorectal cancers had a median progression-free survival of 5.4 months (95% CI, 3 to not estimable).

test) (Fig. S3 in Supplementary Appendix 1). We also performed an additional multivariate analysis of progression-free and overall survival to examine the difference in outcomes between mismatch repair–deficient colorectal cancer and mismatch repair–proficient colorectal cancer, adjusting for elapsed time since the initial diagnosis. The magnitude of the hazard ratios for disease progression or death (hazard ratio, 0.04; 95% CI 0.01 to 0.21; P<0.001) and for death (hazard ratio, 0.18; 95% CI, 0.03 to 1.01; P=0.05), representing the differing effects of pembrolizumab between mismatch repair–deficient tumors and mismatch repair–proficient tumors, was maintained after adjustment for this potential difference.

SAFETY ASSESSMENT

Adverse events occurring in more than 5% of patients are listed in Table 3. Events of clinical

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interest included rash or pruritus (24%); thyroiditis, hypothyroidism, or hypophysitis (10%); and asymptomatic pancreatitis (15%). Although the numbers were small, thyroid-function abnormalities were limited to the cohorts with mismatch repair–deficient cancer (Table 3).

TUMOR MARKERS

In the two cohorts with colorectal cancer, it was possible to evaluate levels of carcinoembryonic antigen (CEA) before enrollment; in 29 of 32 patients, these levels were above the upper limit of normal (3 mg per deciliter). Substantial decreases in CEA level occurred in 7 of the 10 patients with mismatch repair–deficient colorectal cancer and in none of the 19 patients with mismatch repair–proficient colorectal cancer in whom CEA could be evaluated (Fig. 1, and Fig. S4 in Supplementary Appendix 1). Among patients with mismatch repair–deficient noncolorectal cancer, levels of tumor markers (CEA, CA19-9, or CA-125) were elevated above the upper limit of normal in 4 patients. Declines in CA19-9 or CA-125 of more than 70% occurred in 3 of these 4 patients. Tumor marker kinetics in all three cohorts are shown in Figure 1. The degree of CEA decline after one dose (between day 14 and day 28) of pembrolizumab was predictive of both progression-free survival ($P=0.01$) and overall survival ($P=0.02$). The CEA response occurred well in advance of radiographic confirmation of disease control (range, 10 to 35 weeks). In contrast, patients who had disease progression had rapid biomarker elevation within 30 days after the initiation of therapy. Thus, changes in CEA levels significantly preceded and correlated with ultimate radiographic changes.

GENOMIC ANALYSIS

The analysis of whole-exome sequences showed a mean of 1782 somatic mutations per tumor in patients with mismatch repair–deficient cancer (nine patients), as compared with 73 mutations per tumor in patients with mismatch repair–proficient cancer (six patients) ($P=0.007$ by nonparametric Wilcoxon test) (Fig. S5 in Supplementary Appendix 1 and Table S3 in Supplementary Appendix 2). Most of these mutations (63%) are predicted to alter amino acids.

These mutations were then assessed for their immunogenic potential in the context of each patient's MHC haplotype. We identified a mean of 578 potential mutation-associated neoantigens from the tumors of patients with mismatch repair–deficient cancers; 21 such neoantigens were identified in tumors from patients with mismatch repair–proficient cancers (Table S3 in Supplementary Appendix 2). The percentage of potential mutation-associated neoantigens among all somatic mutations was similar in the two cohorts (a mean of 32% in patients with mismatch repair–deficient cancer and 29% in patients with mismatch repair–proficient cancer). High numbers of somatic mutations and potential mutation-associated neoantigens were associated with longer progression-free survival and with a trend toward objective response (Fig. S5 and Table S4 in Supplementary Appendix 1).

IMMUNOHISTOCHEMICAL ANALYSIS

The expression of CD8 and PD-L1 was evaluated within the tumor and at the invasive fronts of the tumor in an immunohistochemical analysis in the 30 cases in which tumor tissue was available (Fig. S6 in Supplementary Appendix 1). Tumors from patients in cohorts A and C contained a greater density of CD8-positive lymphoid cells than did tumors from patients in cohort B ($P=0.10$) (Fig. S7 in Supplementary Appendix 1), and CD8 labeling was associated with a trend toward objective response and stable disease (Fig. S8 and Table S5 in Supplementary Appendix 1). This CD8-positive lymphoid infiltrate was especially prominent at the invasive fronts of the tumors ($P=0.04$) (Fig. S7 in Supplementary Appendix 1). Membranous PD-L1 expression occurred only in patients with mismatch repair–deficient cancer and was prominent on tumor-infiltrating lymphocytes and tumor-associated macrophages located at the invasive fronts of the tumor ($P=0.04$) (Fig. S7 in Supplementary Appendix 1). The expression of CD8 and PD-L1 was not significantly associated with progression-free survival or overall survival (Table S5 in Supplementary Appendix 1).

DISCUSSION

The data from this small phase 2 trial of pembrolizumab for the treatment of tumors with and tumors without mismatch-repair deficiency support the hypothesis that mismatch repair–deficient tumors are more responsive to PD-1 blockade than are mismatch repair–proficient tumors. Mismatch-repair deficiency occurs in many cancers, including those of the colorec-

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tum, uterus, stomach, biliary tract, pancreas, ovary, prostate, and small intestine.[18,34-42] It is possible that patients with mismatch repair–deficient tumors of these types may also benefit from anti–PD-1 therapy, as may patients whose tumors contain other DNA repair deficiencies, such as those with mutations in *POLD*, *POLE*, or *MYH*.[18,43,44]

The hypothesis that mismatch repair–deficient tumors stimulate the immune system is not a new idea[45]; it has been supported by observations of the dense immune infiltration and Th1-associated cytokine-rich environment in mismatch repair–deficient tumors.[19-22,46] A recent study refined these classic observations by showing that the mismatch repair–deficient tumor microenvironment strongly expressed several immune checkpoint ligands, including PD-1, PD-L1, CTLA-4, LAG-3, and IDO, which indicates that their active immune microenvironment is counterbalanced by immune inhibitory signals that resist tumor elimination.[47] The most likely explanation for both the old and new findings was that the immune infiltrate associated with mismatch repair–deficient carcinomas was directed at neoantigens. The correlation of a higher mutational load and a higher rate of response to anti–CTLA-4 in melanoma[41] and anti–PD-1 in lung cancer[48] provides further support for the idea that mutation-associated neoantigen recognition is an important component of the endogenous antitumor immune response.

On the basis of the results of the current and previous studies, we suggest that the greatly increased number of mutation-associated neoantigens resulting from mismatch-repair deficiency (more than 20 times higher than in tumors without this deficiency) (Table S4 in Supplementary Appendix 1) is the basis for the enhanced anti–PD-1 responsiveness of this genetically defined subset of cancers. Although our estimates of the number of mutation-associated neoantigens in tumors are based only on in silico predictions of binding affinity, this suggestion is consistent with the observation that mismatch repair–proficient tumors have far less infiltration of lymphocytes than do mismatch repair–deficient tumors (Fig. S6 and S7 and Table S5 in Supplementary Appendix 1). Recent studies[49,50] have shown that only a tiny proportion of predicted neo-epitopes are actually presented on the cell surface with MHC and are targets of endogenous T cell responses. It seems likely, however,

Table 3. **Adverse Events.***

Event	All Grades	Grade 3 or 4
	no. of patients (%)	
Any	40 (98)	17 (41)
Blood or lymphatic		
Anemia	8 (20)	7 (17)
Lymphopenia	8 (20)	8 (20)
Sinus tachycardia	4 (10)	0
Dermatologic		
Dry skin	5 (12)	0
Rash or pruritus	10 (24)	0
Thyroiditis, hypothyroidism, or hypophysitis	4 (10)	0
Gastrointestinal		
Abdominal pain	10 (24)	0
Anorexia	4 (10)	0
Constipation	8 (20)	0
Diarrhea	10 (24)	2 (5)
Dry mouth	5 (12)	0
Nausea	5 (12)	0
Bowel obstruction	3 (7)	3 (7)
Hepatobiliary		
Elevated alanine aminotransferase	3 (7)	2 (5)
Pancreatitis†	6 (15)	0
Metabolism and nutrition		
Hypoalbuminemia	4 (10)	4 (10)
Hyponatremia	3 (7)	3 (7)
Musculoskeletal		
Arthralgia	7 (17)	0
Myalgia	6 (15)	0
Nervous system		
Dizziness	4 (10)	0
Headache	7 (17)	0
Insomnia	3 (7)	0
Respiratory‡		
Allergic rhinitis	12 (29)	0
Cough	4 (10)	0
Dyspnea	6 (15)	0
Upper respiratory infection	3 (7)	0
Cold intolerance	6 (15)	0
Edema	4 (10)	0
Fatigue	13 (32)	0
Fever	5 (12)	0
Pain	14 (34)	0

* Included are adverse events occurring in more than 5% of patients. A total of 41 patients were included in the analysis.
† All cases of pancreatitis were asymptomatic.
‡ One case of pneumonitis occurred (2%).

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that the number of predicted mutation-associated neoantigens is proportionate to the number of actual mutation-associated neoantigens, and tumors with a high number of actual mutation-associated neoantigens are more likely to stimulate the immune system to react against the tumor.

Alternative mechanisms underlying the difference in anti–PD-1 responsiveness between mismatch repair–deficient tumors and mismatch repair–proficient tumors should also be considered. For example, different signaling pathways activated in the two types of tumors may result in differences in the secretion of soluble factors that could result in differential activation of the PD-1 pathway within the tumor microenvironment.[26-28] Genetic differences could affect epigenetic differences that alter the expression of tumor-associated self-antigens; in turn, these could alter the antigenicity of the tumor. Experimental analyses of antigen-specific immune responses and of changes in immune microenvironments should help to define the relative contribution of these factors to the striking responsiveness of mismatch repair–deficient tumors to PD-1 antibodies.

Several other notable observations were made during the course of this study. First, changes in serum levels of protein biomarkers, such as CEA, corresponded with clinical benefit after a single dose of therapy. Declines in CEA levels preceded objective radiographic evidence of treatment benefit by several months; perhaps other biomarkers, such as circulating tumor DNA, would also be beneficial as surrogate markers of early response.[51-53] Second, our results suggest that the evaluation of tumor genomes can help guide immunotherapy. They support the view that the number and type of alterations may prove to be valuable for judging the potential usefulness of immune checkpoint inhibitors, even in mismatch repair–proficient cancers.[41,48,54] Most importantly, our results show an approach for the treatment of a specific class of tumors that is based solely on genetic status — that is, without regard to the underlying tumor type.

Supported by Swim Across America Laboratory at Johns Hopkins University, the Banyan Gate Foundation, the Lustgarten Foundation for Pancreatic Cancer Research, the Commonwealth Fund, the Virginia and D.K. Ludwig Fund for Cancer Research, the Sol Goldman Pancreatic Cancer Research Center, a Gastrointestinal Specialized Programs of Research Excellence (SPORE) grant (P50CA062924), and grants (P30CA006973, CA163672, CA43460, CA67941, CA16058, and CA57345) from the National Institutes of Health.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Kathy Helwig and Mary Savage for administrative support; Cheryl Blair, Katherine Judge, Tierra Brown, and Suping Chen for technical assistance; and Eric H. Rubin and Alise S. Reicin for review of an earlier version of the manuscript.

APPENDIX

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Trends in Molecular Medicine

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Review

Mismatch Repair and Colon Cancer: Mechanisms and Therapies Explored

Stephen K.H. Li[1] and Alberto Martin[1,*]

Colorectal cancer (CRC) remains one of the most prevalent cancers worldwide. In sporadic CRC, mutations frequently occur in the DNA mismatch repair (MMR) pathway. In addition, germline MMR mutations have been linked to Lynch syndrome, the most common form of hereditary CRC. Although genetic mutations, diet, inflammation, and the gut microbiota can influence CRC, it is unclear how MMR deficiency relates to these factors to modulate disease. In this review, the association of MMR to the etiology of CRC is examined, particularly in the context of microRNAs (miRNAs), inflammation, and the microbiome. We also discuss the most current targeted therapies, methods of prevention, and molecular biomarkers against MMR-deficient CRC, all of which are encouraging advancements in the field.

Colorectal Cancer: An Urgent Need for Targeted Therapies

Colorectal cancer (CRC) is the third most common cancer worldwide, resulting in over 690 000 deaths annually [1]. The overwhelming morbidity and mortality caused by CRC impacts on the global economy through loss of productivity and the burden on the healthcare system. In the USA alone, CRC is projected to exceed $17 billion in medical care by the year 2020 [2]. Consequently, researchers have expended considerable resources to develop treatments and preventative strategies to reduce the incidence of CRC. Large-scale sequencing as well as genome, microbiome, proteome, and transcriptome analyses have allowed researchers to better characterize and understand the plethora of CRC subtypes. The majority of CRC cases are sporadic, with no clear hereditary connection. By contrast, ~30% of reported CRC cases are familial, but only a small fraction of these conditions have been well characterized [3]. Examples of hereditary CRCs include familial adenomatous polyposis coli, MUTYH-associated polyposis, juvenile polyposis syndrome, Peutz–Jeghers syndrome, and Lynch syndrome. Mutations in the DNA MMR pathway are a frequent occurrence in sporadic CRC, and germline MMR mutations are associated with Lynch syndrome. Although genetic mutations, diet, inflammatory processes, and the gut **microbiota** have been associated with the etiology of CRC (Figure 1), it is unclear how MMR deficiency relates to these factors to influence CRC initiation and/or progression. There is increasing evidence linking **microRNAs** (miRNAs or miRs; see Glossary), inflammation, and the gut microbiota to tumorigenesis. This review will focus on recent advances in understanding the associations of MMR mutations in CRC, particularly in the context of inflammation and the microbiome. In addition, the most recent developments in targeted therapies, preventative methods, and molecular biomarkers against colon tumorigenesis will be discussed. With a multitude of existing CRC diseases, it is clear that the development of fruitful therapeutic strategies is urgently needed. As such, novel interventions against MMR-deficient CRC may indeed yield promising results in the field of cancer therapy.

Trends

Somatic and germline mutations in genes within the MMR pathway occur frequently in CRC.

The MMR pathway is involved in multiple processes that are independent of DNA repair, including the processing of miRNAs and inducing apoptosis. The lack of MMR-mediated DNA repair could promote tumorigenesis.

Microbe-induced inflammation or DNA damage can promote CRC and alter MMR expression.

The microbiota can promote tumorigenesis in MMR-deficient CRC models.

Anti-PD1 Immunotherapy and aspirin for chemoprevention have been demonstrated to be effective against MMR-deficient CRC.

Novel next-generation sequencing approaches (DNA, miRNA, and microbiome analyses) are currently being developed for CRC prevention and detection in susceptible individuals.

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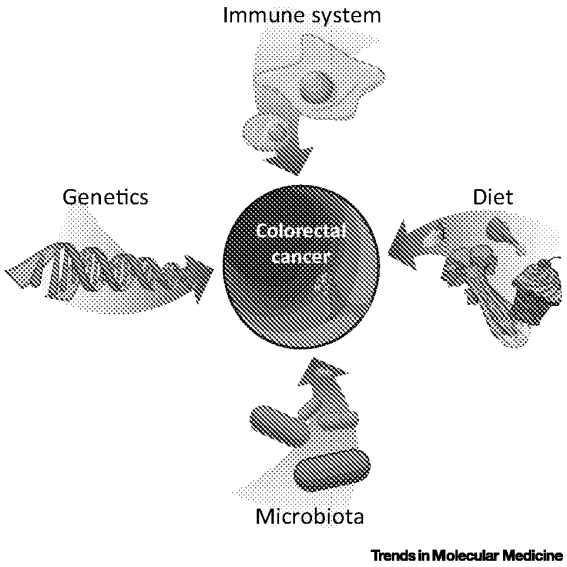


Figure 1. Etiologic Factors of Colorectal Cancer (CRC). Genetic mutations, diet, the immune system, and the microbiota are all associated with CRC development and progression. It is unclear how mismatch repair (MMR) deficiency relates to these factors to influence CRC. Associations of MMR mutations in CRC, particularly in the context of inflammation and the microbiome, are depicted.

Mismatch Repair Defects and CRC

The development of CRC is a multistep process whereby normal colon epithelial cells (CECs) develop into colorectal adenoma (polyps) and, over time, can give rise to colorectal adenocarcinoma. Genetic lesions have been identified as central contributors to CRC development, and inactivation of the DNA MMR pathway contributes to genetic mutations (Figure 2). Deficiency in MMR caused by germline mutations manifests as Lynch syndrome in humans, which occurs in 1 in 35 total CRC cases, making it the most common form of familial CRC [4]. Loss of MMR function also occurs in ~15–20% of sporadic CRC cases [5,6]. Moreover, loss of MMR function increases the risk of acquiring endometrial, gastric, and hematopoietic cancers [7–9], and these phenotypes are recapitulated in mouse models with germline or tissue-specific deletions of MMR genes (reviewed in [10]).

Mismatch repair is an evolutionarily conserved mechanism that corrects mutations arising during DNA replication or damage. The Nobel Prize in Chemistry was awarded to Paul Modrich in 2015 for his contributions towards understanding MMR, which allowed researchers to link mutations in MMR genes to CRC [11,12]. In humans, germline mutations in MMR genes, most commonly *MSH2, MLH1, MSH6*, and *PMS2*, have been identified as genetic contributors to Lynch syndrome [4,5,13,14]. Mutations in MMR genes typically result in a hallmark phenotype known as **microsatellite instability** (MSI), which is characterized by alterations in the length of tandem nucleotide repeats located throughout the genome [15–19]. Changes in tandem repeats can result in a temporary self-complementary insertion–deletion loop (IDL) in DNA during replication. Such DNA mismatches or IDLs not corrected by MMR can generate mis-sense or frameshift mutations in coding genes during subsequent rounds of DNA replication, resulting in dysfunctional proteins during translation [5]. Furthermore, elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) are another form of MSI detected in 50–60% of sporadic CRC cases, and loss of MSH3 function (MSH3 protein) is associated with EMAST [20,21]. Of note, CRC patients diagnosed with both MSI and EMAST tumors have a reduction in overall survival compared to MSI tumors with no EMAST [22].

Glossary

***Apc*$^{Min/+}$:** a mouse line containing a point mutation in one allele of the *Apc* gene. These mice spontaneously develop intestinal adenomas and are commonly used as a model for sporadic CRC.
Azoxymethane (AOM): a common mutagen used to induce CRC in animals. AOM is metabolized into methylazoxymethanol by cytochrome P450 2E1, generating DNA mutations.
***Cdx2*^{Cre} *Apc*$^{lox/lox}$:** a mouse model where *Apc* is deleted specifically in the intestinal epithelium. The *Apc* gene is 'floxed', and the gene is excised by Cre recombinase which is driven by the expression of the gut-specific *Cdx2* transcription factor.
Dextran sulfate sodium (DSS): a salt commonly used in animal studies to induce intestinal inflammation by disrupting the permeability of the intestinal epithelium.
Dysbiosis: broad shifts in the populations of microorganisms within the body which can result from extrinsic factors such as antibiotics or diet, or from intrinsic genetic abnormalities.
Epigenetic: a modification to DNA that does not involve base changes but regulates gene expression. Examples include DNA methylation and acetylation.
Germ-free: animals which are raised in environments devoid of microorganisms to prevent their natural colonization.
Microbiota: the collective presence of microorganisms, including bacteria, viruses, fungi, and protozoa, present within an environment.
Microsatellite: tandem repetitive DNA sequences found throughout the genome which range from 2–5 base pairs and usually have an upper repeating limit of up to 50-fold, but longer elements can be detected.
Microsatellite instability (MSI): abnormal changes in the length of repetitive tandem DNA repeats found throughout the genome.
MicroRNA (miRNA or miR): short non-coding RNAs which are transcribed and processed to function as gene silencers.
Somatic hypermutation: mutations induced in the variable region of immunoglobulin genes by the enzyme activation-induced deaminase (AID), generating diversity in the antibody repertoire.

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Somatic and **epigenetic** changes in MMR genes have also been associated with CRC. Somatic mutations in *MSH2* and *MLH1* are frequently detected in CRC patients who do not have germline mutations in MMR genes [23]. Moreover, somatic and germline mutations in *MSH3* and exonuclease 1 (*EXO1*) have been detected in specific cohorts of Lynch syndrome patients [24–27]. In addition, both *MSH3* and *MSH6* are frequently mutated in human CRC with high levels of MSI [28], suggesting that multiple components of the MMR pathway are important in maintaining genomic stability. Promoter regions of genes which contain dense CpG-rich regions (CpG islands) can be methylated, resulting in gene silencing. High levels of CpG island methylation at the promoters of genes involved in cell cycle regulation, apoptosis, and DNA repair (including MMR) exhibit a CpG island methylator phenotype (CIMP) which can result in CRC (reviewed in [29–31]). CIMP can result in a similar phenotype to MSI because hypermethylated promoters of *MSH2* and *MILH1* are frequently observed in CRC [28,32]. Interestingly, there have been reported cases of epigenetic *MSH2* and *MLH1* alterations being heritable [33,34], providing an additional level of complexity in classifying subtypes of CRC. For example, deletion of *EPCAM*, a cell adhesion molecule expressed in most normal epithelial cells, has been demonstrated to contribute to Lynch syndrome through epigenetic silencing of *MSH2* in view of the close genomic proximity between the two genes [35]. It has also been demonstrated that the epigenetic histone modification H3K36me3 is required to recruit MSH2 and MSH6 onto chromatin for mismatch recognition *in vivo* [36]. Short hairpin RNA knockdown of the methyltransferase SETD2 in DLD-1 or HeLa cells was shown to reduce the interaction between H3K36me3 and MSH6, resulting in both elevated spontaneous mutations and MSI, thus providing evidence that histone modifications can directly regulate MMR function [36].

Although germline mutations in the adenomatous polyposis coli (*APC*) gene are typically associated with FAP, somatic mutations in *APC* have also been detected in individuals with Lynch syndrome [37–39]. Loss of APC function can lead to hyperproliferation of CECs through constitutive WNT/β-catenin signaling, as observed in human *APC*-deficient CRC cell lines [40]. Moreover, *APC* is frequently mutated in CRCs containing high levels of MSI [28], indicating that loss of APC function also contributes to MMR-deficient tumorigenesis. Overall, mutations in MMR genes and *APC* can enhance the MSI phenotype or lead to accumulated genetic lesions over time.

Mechanism of Mammalian MMR

MMR is a multistep process involving key proteins at each stage to maintain genomic stability (Figure 3). Initiation of MMR begins when a heterodimer of the MutS homolog (MSH), either MSH2–MSH6 (MutSα) or MSH2–MSH3 (MutSβ), binds to mismatched DNA. MutSα recognizes base–base mismatches and 1–2 base pair (bp) IDLs, whereas MutSβ recognizes IDLs longer than 2 bp [41]. Following mismatch or IDL recognition, a heterodimer of MutL homolog (MLH) consisting of MLH1 and the post-meiotic segregation 2 protein (PMS2) is recruited to MutSα or MutSβ [42]. The 'DNA clamp' proliferating cell nuclear antigen (PCNA) is loaded by replication factor C (RFC), after which PCNA interacts with MLH1–PMS2 (MutLα) to enable PMS2 to exert its endonuclease activity [43]. The endonuclease activity of MutLα is required for 3′-mediated mismatch excision and is promoted by PCNA–RFC, whereas PCNA–RFC has a limited effect in 5′-mediated excision [44]. Because PCNA is also part of the DNA replication machinery, MMR recognition can occur either coupled to DNA replication or independently [42]. Excision of mismatched DNA is mediated by a well-characterized exonuclease 1 (Exo1)-dependent interaction, or by an unclear Exo1-independent mechanism [45]. Replication protein A (RPA) binds to and protects the single-stranded DNA that is generated by the exonuclease, and promotes excision termination [46]. Lastly, the excised bases are filled in by DNA polymerase δ and ligated by DNA ligase I [46,47].

Toll-like receptor (TLR): receptors involved in innate immunity which recognize conserved structures associated with viruses, bacteria, protozoa, fungi, and their nucleic acids. TLRs are expressed on a variety of epithelial and immune cells, and their activation results in a signaling cascade which activates transcription factors associated with proliferation, inflammation, and survival.

Warburg effect: the shift in metabolism from oxidative respiration to aerobic glycolysis which occurs commonly in cancerous cells.

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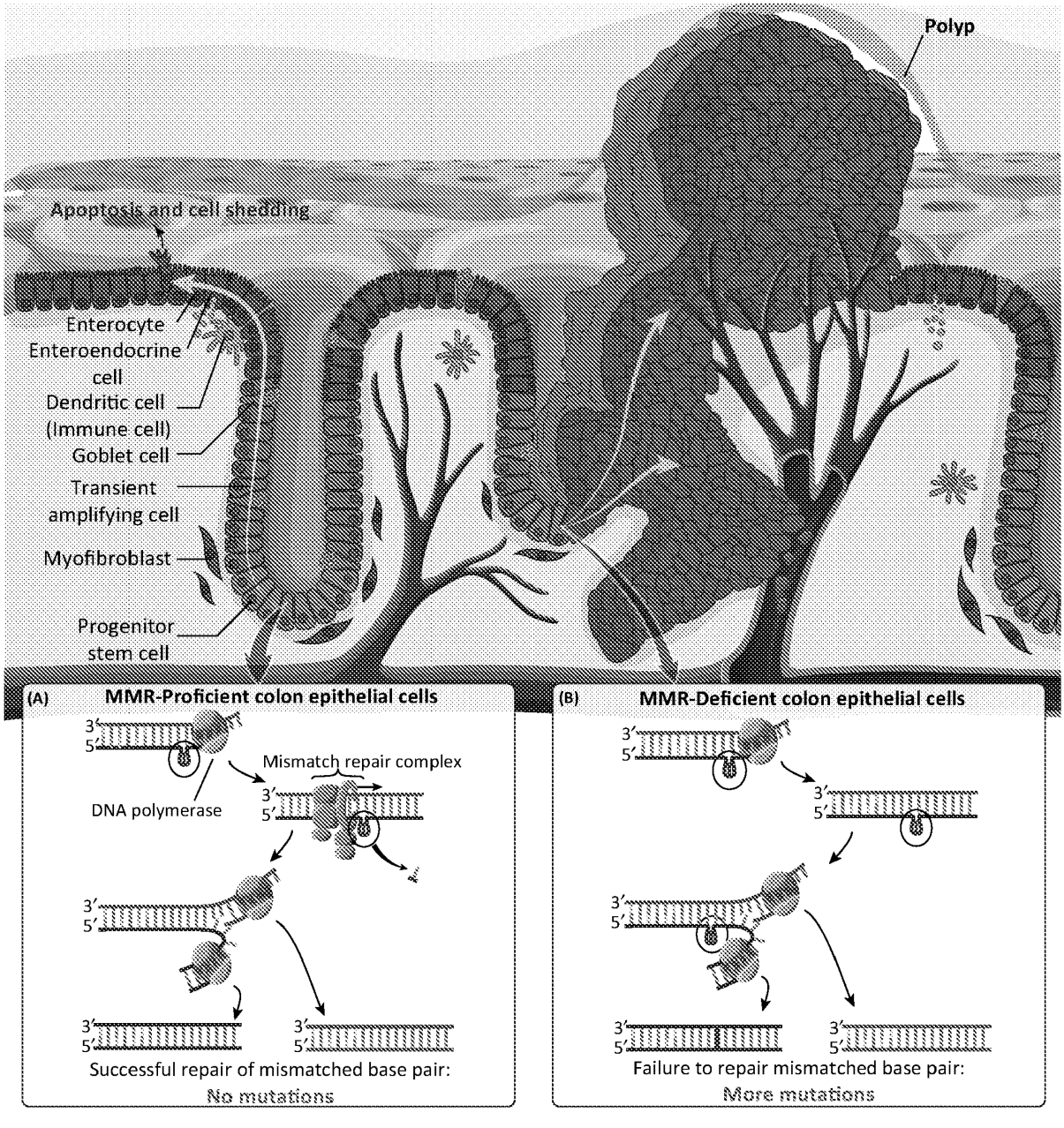
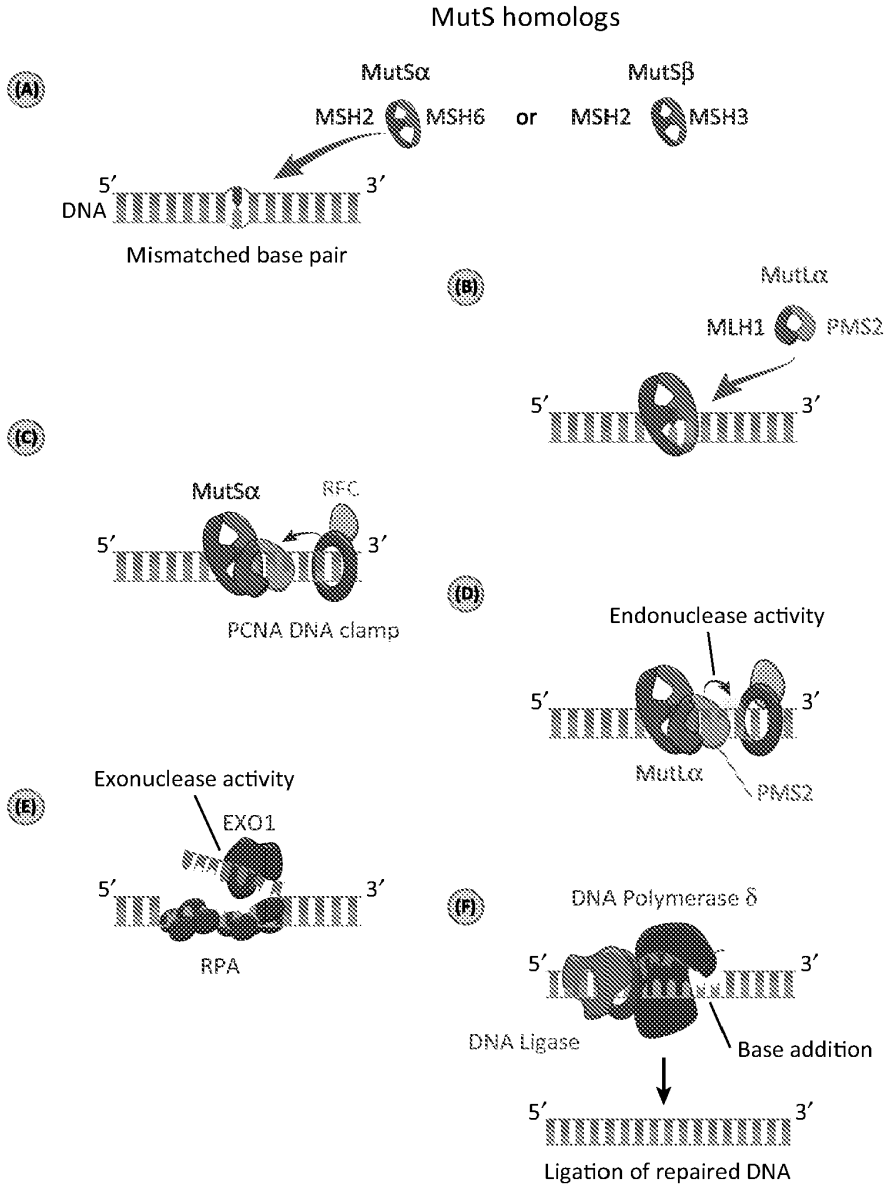


Figure 2. Genetic Mutations Resulting from MMR Deficiency Contribute to CRC Development. Shown is a representation of the cellular organization of colon epithelial and immune cells within a normal intestinal crypt, relative to a malignant polyp. (A) In MMR-proficient colon epithelial cells (CECs), mismatched base pairs in DNA (circled) are effectively repaired by the MMR pathway, not leading to DNA mutations. (B) In MMR-deficient CECs, the recognition or repair of mismatched DNA base pairs (circled) does not occur, resulting in somatic mutations.

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Figure 3. Mechanism of Mammalian MMR. (A) Mismatched DNA base pairs are recognized by either MSH2–MSH6 (MutSα) or MSH2–MSH3 (MutSβ). (B) Recruitment of MLH1–PMS2 (MutLα) to MutSα or MutSβ following mismatch recognition. (C) PCNA interacts with MutLα after being loaded onto DNA by RFC. (D) PCNA–MutLα interaction mediates the endonuclease activity (arrow) of PMS2. (E) EXO1 mediates excision of the mismatched DNA. RPA binds to the single-stranded DNA generated by excision to protect the strand, and also promotes excision termination. (F) DNA polymerase synthesizes new DNA to fill the excised bases, and the newly synthesized strand is sealed by DNA ligase.

Non-canonical Roles of MMR in CRC

Although MMR is able to recognize and repair mismatched DNA base pairs and IDLs, there are additional biological roles for MMR which are independent of DNA replication. Discussed below are a few non-canonical roles of MMR which could contribute to the progression of CRC.

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MMR-Mediated Mutagenesis

Counter-intuitively, MMR is involved in processes which induce more mutations. MMR is involved in processing mutations caused by the enzyme activation-induced deaminase (AID) during **somatic hypermutation** of immunoglobulin genes in activated mature B lymphocytes [48]. In this context, MMR in conjunction with a post-translationally modified mono-ubiquitinated PCNA (Ub-PCNA) functions to recruit the error-prone polymerase η (Pol η). This in turn, induces mutations at dA:dT base pairs in immunoglobulin gene variable regions (V-regions) in both mice and humans [49,50]. Moreover, the processing of other DNA analogs, such as O^6-methyl-guanine (meG) caused by the DNA-alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), also requires MMR and occurs in other cell lines such as human embryonic kidney 293, ovarian A2780 carcinoma, and CRC HCT-116 cells [51]. This study showed that, in contrast to MLH1-deficient HCT-116 cells, MLH1-expressing HCT-116 cells contained more mutations when stimulated *in vitro* with MNNG, thus suggesting a role for MMR as a source of genomic instability activated by DNA lesions [51]. MNNG-induced meG recruited Ub–PCNA and Pol η together with MMR proteins to chromatin in MLH1-expressing human HCT-116 CRC cells, resulting in increased mutagenesis [51]. Moreover, the recruitment of MMR proteins appeared to occur independently of DNA replication [51]. Because induced mutations are a common hallmark of cancer cells, the replication-uncoupled recognition of some DNA analogs by MMR could represent another mechanism by which DNA mutagenesis occurs in CRC.

MMR and Apoptosis

MMR is involved in inducing apoptosis during DNA damage. Overexpression of MLH1 or MSH2 in human cell lines promotes apoptosis [52], but the complete mechanism is unclear. Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) are a family of protein kinases involved in apoptosis and cell-cycle regulation during DNA damage. MSH2 contributes to the recruitment of ATR upon DNA damage, which promotes apoptosis via activation of the transcription factor p53 [53]. In addition, ATM is involved in the accumulation of MLH1 during DNA damage, and MLH1 contributes to the activation of p53 [54]. MLH1 is cleaved by caspase-3, a member of the family of cysteine proteases crucial for cell death, and nuclear translocation of the C-terminal fragment of MLH1 induces apoptosis [55]. Consequently, a reduction of MLH1 or MSH2 expression could contribute to CRC progression through reduced rates of apoptosis.

MMR and miRNAs

Recently, MMR has also been implicated in the regulation of miRNA processing [56]. miRNAs are short non-coding RNAs capable of modulating the expression of protein-coding genes. Primary miRNAs (pri-miR) transcripts are first processed into precursor miRNAs (pre-miR) in the nucleus, and then are further processed into functional miRNAs in the cytosol [57]. The transformation of pri-miR into pre-miR requires the 'Microprocessor' complex, comprising the RNase III enzyme Drosha and its RNA-binding partner DGCR8 [58]. Co-immunoprecipitation and electromobility shift assays performed on HeLa cells have demonstrated that MutLα directly interacts with Drosha and DGCR8, suggesting that MutLα could potentially function within the Microprocessor complex to facilitate miRNA processing directly [56]. Moreover, processing of miR-422a and miR-16-1 from pri-miR to pre-miR formation has been shown to involve MutLα in MLH1-expressing HCT-116 cells, but not in HCT-116 cells lacking MLH1 expression, demonstrating that MLH1 can contribute to miRNA processing [56]. Functional miR-422a can also inhibit MLH1 expression by binding to the *MLH1* 3′-untranslated region (UTR), suggesting that there is a regulatory feedback mechanism between MMR and miRNA expression [56]. Furthermore, a few groups have demonstrated downregulation of miR-422a and miR-16 expression in CRC tissues or serum relative to controls (adjacent healthy tissues or healthy controls, respectively) [59–61]. In addition, although the MMR status was not examined in these studies, the findings suggest that aberrant miRNA expression could be a consequence of reduced MutLα expression.

There are several examples where miRNAs regulate MMR expression. For instance, transient overexpression of miR-21 in Colo-320DM and SW620 CRC cell lines *in vitro* was shown to reduce MSH2 and MSH6 protein levels, whereas inhibition of endogenous miR-21 (transfection of a locked nucleic acid against miR-21) into those same CRC cell lines led to increased MSH2 and MSH6 protein expression [62]. These findings are relevant because, in humans, miR-21 is significantly overexpressed in CRC tissues harboring MSI relative to adjacent non-CRC tissues [63]. MMR expression can also be regulated by miR-155. Indeed, as reported in luciferase assays performed in human CRC Colo-320 DM cells, *MLH1*, *MSH2*, and *MSH6* transcripts have been shown to directly bind to miR-155, resulting in reduced expression of their respective encoded proteins [64]. In addition, through length analysis of microsatellite markers, overexpression of miR-155 in human CRC cell lines has been shown to exacerbate an MSI phenotype [64]. Moreover, in human CRC patients, overexpressed miR-155 has been detected in tumor biopsies and was shown to correlate with reduced MSH2 and MLH1 protein expression [64]. Together, these studies suggest that regulatory feedback mechanisms between miRNAs and MMR proteins may very well be perturbed in MMR-deficient CRC tumor cells, and could contribute to dysregulated gene profiles seen in CRC.

Effects of Inflammation and Oxidative Damage in CRC

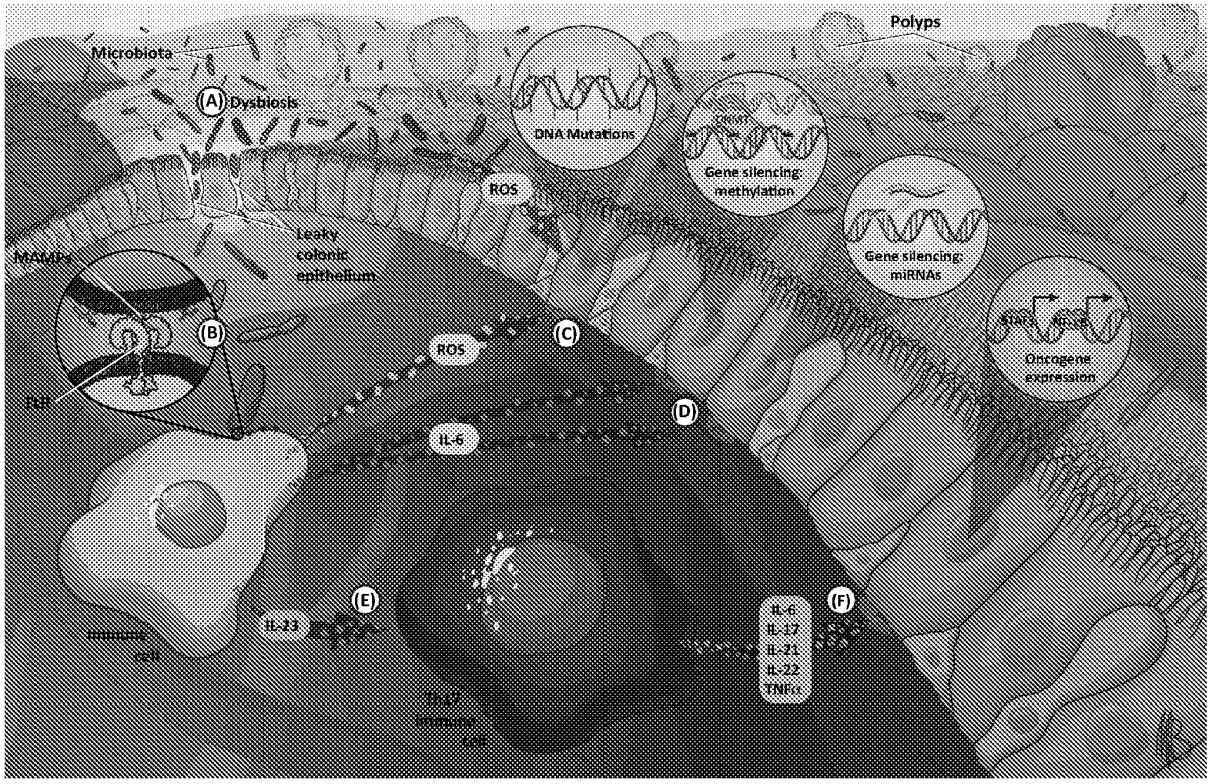
Inflammation and CRC

Genetic and epigenetic alterations are observed in CRC, and these changes could be promoted by inflammation in the intestinal tract. Inflammation in the colon can be triggered by the gut microbiota and their metabolites or byproducts [65]. Immune cells and CECs produce regulatory signals so as to maintain homeostatic levels of inflammation. The intestinal microbiota is recognized by pattern recognition receptors, such as **Toll-like receptors** (TLRs) expressed on CECs and immune cells, which are bound by conserved structures of bacterial cell wall and nucleic acids collectively [66]. The binding of ligands to TLRs activates downstream signaling cascades, which can result in the activation of the transcription factor NF-κB, a key player in cell survival, proliferation, and inflammatory processes [67].

Cytokines have the potential to promote tumorigenesis by activating oncogenes or through gene silencing (Figure 4). In fact, inflammatory cytokines have been demonstrated to promote CRC development *in vivo*. For instance, in mice containing either germline or tissue-specific *Apc* mutations (***Apc*$^{Min/+}$** or ***Cdx2*^{Cre} *Apc*$^{lox/lox}$**, respectively) and which sporadically develop CRC, as well as in **azoxymethane** (AOM) + **dextran sulfate sodium** (DSS)-induced mouse models of CRC, interleukin (IL)-17 or IL-23 signaling has been shown to contribute to tumorigenesis by preventing apoptosis or enhancing proinflammatory cytokine production [68–70]. Indeed, in mice harboring *Apc* gene mutations in addition to germline deletions of genes encoding the IL-17 receptors *Il17ra* and *Il17re*, reduced CRC tumor growth has been observed [68,69]. Moreover, IL-17A signaling has been reported to induce CEC proliferation *ex vivo* through activation of the transcription factor NF-κB and mitogen-activated protein kinase p38 signaling pathways [70]. In addition, IL-23 contributes to tumor growth by maintaining a tumorigenic T-helper IL-17-producing (T_H17) response, subsequently stabilizing the production of IL-23R, IL-17A, and RORγt [68]. Of note, the production of multiple cytokines including IL-23, IL-6, IL-17A, IL-17C is reduced in the absence of TLR signaling via germline deletion of *Tlr2*, *Tlr4*, *Tlr9*, or the adapter protein *Myd88*, indicating the importance of microbe-driven cytokine production in CRC [68,69].

From another perspective, the proinflammatory cytokine IL-6 has multiple roles in tumorigenesis. Studies have shown that human CRCs can express elevated levels of the DNA methyltransferase DNMT1, an enzyme which contributes to gene silencing by methylating DNA [71]. DNMT1 is overexpressed in inflammatory bowel disease-associated CRC compared to sporadic CRC [71]. Treatment of IL-6 has been shown to induce DNMT1 stabilization in HCT-116 CRC cell lines

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Figure 4. Effects of Inflammation and Oxidative Damage in CRC. (A) Dysbiosis can compromise the structural integrity of the intestinal epithelium, resulting in microbes or their products crossing freely into the lamina propria. (B) Microbe-associated molecular patterns (MAMPs), or PAMPs can bind to and activate Toll-like receptors (TLRs) expressed by immune and epithelial cells, resulting in the production of reactive oxygen species (ROS) or the downstream activation of various transcription factors, including NF-κB. The activation of NF-κB can result in the production of multiple proinflammatory cytokines including IL-6, IL-17, IL-23, and TNF-α. (C) ROS is produced by activated immune cells or directly by bacteria. ROS can mediate DNA damage, reduce MMR protein expression through gene silencing, and promote the exclusion of MMR proteins from the nucleus. Failure to repair ROS-mediated DNA damage as a result of MMR deficiency could contribute to additional mutations leading to CRC. (D) The proinflammatory cytokine IL-6 can contribute to gene silencing through the activity of miRNAs and DNMTs. (E) IL-23 signaling contributes to tumorigenesis by maintaining a T_H17 response. (F) Inflammatory cytokines can influence CRC progression by activating oncogenes. Lymphocytes producing elevated levels of IL-6, IL-17, IL-21, IL-22, and TNF-α can promote cell proliferation by activating the transcription factors NF-κB and STAT3.

via Akt signaling, and lead to altered methylation of gene promoters implicated in tumor suppression and apoptosis resistance, such as *IL4*, *SERPINB5* (Maspin), *IRF7*, *BIRC5* (Survivin), *NES1*, and *HOXA2* [71]. In another study, when HCT-116 or SW480 CRC cells were stimulated with IL-6, both cell lines upregulated and secreted miR-21 [72]. In subsequent co-culture and miRNA transfection experiments, it was suggested that CRC-produced miR-21 could induce human monocyte THP-1 cells to secrete additional IL-6, thereby generating a feedback loop to sustain IL-6 production [72]. IL-6 has also been demonstrated to perturb MMR function by disrupting MMR protein localization: it was reported that activation of STAT3 through IL-6 signaling could disrupt the nuclear localization of MSH3 in SW480 cells [73]. The shift of MSH3 from the nucleus into the cytosol inhibited its ability to function in nuclear MMR, thus resulting in EMAST in CRC cells [74]. Therefore, these findings collectively suggest that IL-6 has the potential to enhance CRC tumorigenesis via mechanisms that include gene silencing, feedback augmentation of the tumor-promoting cytokine IL-6, and altering the localization of MMR proteins away from their functional sites.

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Cytokines produced by immune cells might also influence CRC progression. For example, tumor-infiltrating lymphocytes (TILs) produce elevated levels of IL-6, IL-17A, IL-17F, IL-21, IL-22, and TNF-α, which promote the proliferation of DLD-1 and HIT-29 CRC cell lines by activating transcription factors NF-κB and STAT3 [75]. Of clinical relevance, MMR-deficient patients have elevated levels of peritumoral lymphocytes [76], and their presence, together with that of TILs, is associated with an increased risk of CRC incidence in MMR-deficient individuals [77]. However, in *Msh2*-deficient $Apc^{Min/+}$ mice lacking lymphocytes because of a *Rag1* deletion ($Apc^{Min/+}$ $Msh2^{-/-}$ $Rag1^{-/-}$), the number of colonic polyps is not further enhanced relative to controls [78]. This suggests that tumor progression might not necessarily depend on lymphocyte-produced cytokines in MMR-deficient CRC. In summary, cytokine networks induced by inflammation can contribute to promoting CRC, but further studies to determine the precise role of these cytokines in MMR-deficient CRC are required.

Oxidative Damage and CRC

Elevated levels of reactive oxygen species (ROS) are produced by intestinal epithelial cells and immune cells in response to TLR activation [79,80]. The induction of ROS stimulates hyperproliferation of mouse CECs [81], and can also induce DNA damage. It has been reported that DNMTs are recruited to sites of oxidative damage. By using DLD1 CRC cells which lack MSH6 expression, it was demonstrated that transient expression of MSH6 in DLD1 cells recruited DNMT1 to H_2O_2-damaged chromatin [82]. Oxidative damage resulted in reduced *MLH1* expression, which was dependent on MSH6 and DNMT1 [82]. Therefore, MutSα might potentially contribute to methylation-induced silencing of *MLH1* following excessive oxidative damage, whereas loss of MSH2 or MSH6 function could ablate the ability of CECs to repair ROS-mediated lesions. In support, H_2O_2-treated SW480 CRC cells resulted in MSH3 translocation from the nucleus to the cytosol, suggesting that oxidative stress might be able to promote EMAST in CRCs as a result of nuclear exclusion of MSH3 [73]. Thus, although further studies are warranted, failure to repair ROS-mediated DNA damage as a result of MMR deficiency could represent an additional contributing mechanism to colon tumorigenesis.

The Gut Microbiota and CRC

The availability of high resolution next-generation sequencing (NGS) technologies has begun to elucidate the etiologic complexity of the relationship between the microbiome and CRC. It is currently well accepted that the intestinal microbiota forms a symbiotic relationship between its host and has broad functions in metabolism and immunity [83,84]. NGS analyses on human CRC tissues have allowed researchers to determine the bacterial composition of the microbiome associated with subtypes of CRC. For example, numerous reports have demonstrated an increase in the association of *Fusobacterium* with CRC (Box 1). A study examining AOM-treated IL-10-deficient mice suggested that inflammation could promote tumorigenesis through the expansion of genotoxic bacteria [85]. Colonizing mice with *Escherichia coli* NC101, which contained the polyketide synthase (*pks*) gene cluster, induced DNA damage in CECs and increased tumor multiplicity [85]. By contrast, genotoxicity and tumor counts were reduced when the mice were colonized with *pks*-deleted *E. coli* mutants [85]. It is unclear whether the MMR pathway might be able to repair the DNA damage caused by *E. coli* containing *pks*. If it can, the failure to repair damaged DNA might potentially lead to CRC. Moreover, **dysbiosis** has been associated with multiple diseases including CRC [86–89]. Numerous murine CRC models have demonstrated that animals develop fewer polyps in the colon when given antibiotics or when housed in **germ-free** environments [68,78,90]. The absence of the gut microbiota can alter the expression of genes related to metabolism, proliferation, and immune responses in the intestinal epithelium of the host [91]. Likewise, dysbiosis can contribute to CRC by altering homeostatic levels of inflammation and metabolism, which can subsequently give rise to opportunistic pathogens and/or alter microbe-induced signaling in CECs.

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Box 1. *Fusobacterium* Is Associated with MMR-Deficient CRC

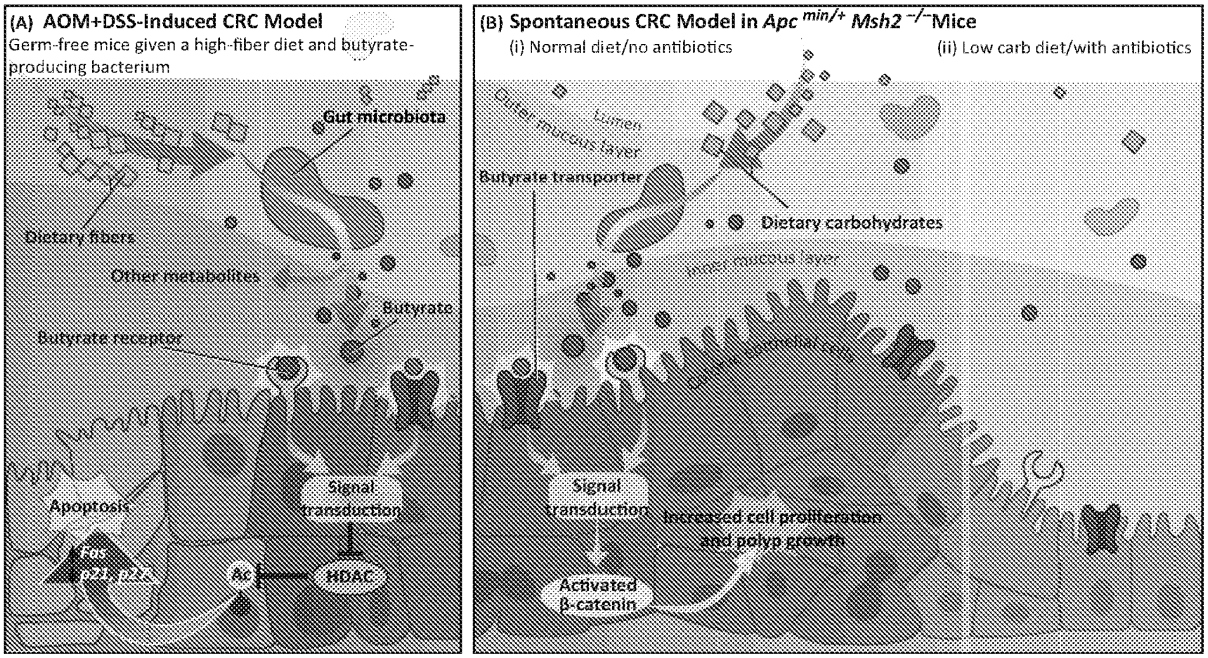
In numerous reports, the bacterial genus *Fusobacterium* has been found to be over-represented in colorectal tumors compared to normal tumor adjacent tissue or healthy controls [86–88,110]. Studies have been performed in mice to determine the mechanism(s) by which *Fusobacterium* species can contribute to CRC. There has been particular interest in determining whether *Fusobacterium nucleatum* is a cause or a consequence of CRC. *F. nucleatum* is an opportunistic oral commensal bacterium associated with a range of human diseases [111]. In the sporadic CRC model of $Apc^{Min/+}$ mice, animals inoculated with *F. nucleatum* developed more colonic tumors compared to Streptococci-colonized $Apc^{Min/+}$ mice. *F. nucleatum*-colonized mice exhibited a proinflammatory phenotype without exacerbated colitis [113]. Multiple virulence factors expressed by *F. nucleatum* have been linked to CRC progression in mouse models, including the adhesion proteins FadA and Fap2. FadA promoted tumor growth via activated WNT/β-catenin signaling when bound to human CRC cells transplanted into mice [113]. In addition, Fap2 is involved in tumor immune evasion by binding to T cell immunoglobulin and ITIM domain (TIGIT), an inhibitory receptor expressed on natural killer (NK) and T cells [114]. A few studies have also analyzed the relationship between mutational status of CRC samples and the presence of Fusobacteria. Interestingly, it has been reported that hypermethylation of MLH1 and MSI are associated with high levels of Fusobacteria, suggesting that they may play a pathogenic role in this subset of CRC [115,116]. In addition, the amount of *F. nucleatum* in the colon was associated with a high MSI status in CRC, independently of other CRC-associated mutations, namely *CIMP* and *BRAF* [116]. Therefore, *F. nucleatum* might be considered as a prognostic indicator for particular MMR-deficient subsets of CRC.

Butyrate Production by Gut Microbiota and its Contribution to MMR-deficient CRC

Diet is one of the risk factors for CRC, and even short-term changes in diet can alter the microbial communities in the gut [92]. The metabolic products of the gut microbiota influence the development of CRC [65]. Short-chain fatty acids, including acetate, propionate, and butyrate, are produced within the intestinal lumen by bacterial fermentation. Normal CECs utilize bacterially produced butyrate as a primary energy source [93]. However, butyrate has contrasting proliferative effects between normal and cancerous CECs; proliferation is enhanced by low concentrations of butyrate in normal CECs, whereas it is suppressed by butyrate treatment in colon cancer cells due to the **Warburg effect** [94]. Contrasting effects of butyrate have also been reported between AOM plus DSS (AOM+DSS)-induced CRC in germ-free BALB/c mice, and in $Apc^{Min/+}$ $Msh2^{-/-}$ mice where CRC occurs spontaneously [78,95] (Figure 5). Butyrate-producing bacteria colonized in germ-free BALB/c mice exerted a protective role against AOM +DSS-induced CRC [95]. Owing to the Warburg effect, butyrate has been shown to accumulate in cancerous murine CECs, prompting its function as a histone deacetylase (HDAC) inhibitor [94,96]. The inhibition of HDACs has been shown to lead to decreased mRNA expression of the cell-cycle regulators *p21* and *p27*, and increased mRNA expression of the proapoptotic gene *Fas*, thus contributing to reduced cell proliferation and increased apoptosis [95]. By contrast, butyrate-producing bacteria or rectally infused butyrate were reported to accelerate CEC hyperproliferation in MMR-deficient mice [78]. These results suggest a difference in the butyrate effect on CRC progression depending on the genetic background or the mouse model used. For example, the former model is a colitis model of CRC [95], and butyrate has been shown to impact on inflammatory responses [96]; the latter model does not involve inflammatory processes in disease etiology [78]. The contrasting effects of butyrate in these mouse studies point to an underlying need to stratify treatments or interventions based on CRC subtypes. In a human randomized control study involving individuals with Lynch syndrome, increased concentrations of butyrate in the colon were achieved in patients supplemented with resistance starch or (as opposed to a placebo control) [97]. However, no differences in CRC development were detected between the two groups. Hence, it is conceivable that butyrate may not be universally protective against the development of all CRC subtypes. Future studies should focus on further clarifying this point.

Anti-PD-1 Immunotherapy Against MMR-Deficient CRC

Tumor cells can evade the immune system by expressing inhibitory ligands and receptors. For example, the surface receptor programmed death ligand 1 (PD-L-1) is expressed by many tumor



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Figure 5. Effects of Butyrate Produced by the Gut Microbiota in MMR-proficient and MMR-deficient CRC Murine Models. (A) In germ-free BALB/c mice fed with a high-fiber diet, inoculation with butyrate-producing bacteria was protective against AOM+DSS-induced CRC [95]. Butyrate acts as a HDAC inhibitor to promote apoptosis (increased Fas expression) and inhibit cell proliferation (reduced p21 and p27 expression). (B) (i) In $Apc^{Min/+}$ $Msh2^{-/-}$ mice, butyrate might promote CRC by inducing proliferation in CECs. (ii) Administering antibiotics or feeding a low-carbohydrate diet to $Apc^{Min/+}$ $Msh2^{-/-}$ mice can reduce CEC proliferation and polyp growth.

cells and binds to its ligand programmed death 1 (PD-1) on T cells, blocking T cell-mediated immunity. As such, there has been increased interest in developing immunotherapies that block these inhibitory interactions to enable T cell effector function against tumors. With respect to MMR-deficient CRCs, the increased mutation load in the tumors leads to the production of neo-epitopes which could stimulate an inflammatory response. This was the rationale employed by a recent Phase II study which demonstrated that blocking the PD-1 pathway with pembrolizumab, an anti-PD-1 humanized monoclonal antibody, decreased the likelihood of disease progression, or even death, in MMR-deficient CRC patients, compared to MMR-proficient CRC patients [76]. Indeed, relative to MMR-proficient tumor microenvironments, MMR-deficient CRCs expressed elevated levels of PD-L1 at the junction between normal and tumorigenic colon tissue [76]. However, the mechanism of anti-PD-1 therapy in MMR remains unclear, and protective effects could be potentially influenced by the gut microbiota (Box 2). Nonetheless, anti-PD-1 therapy remains a promising adjuvant treatment for Lynch syndrome patients and should continue to be explored.

NSAIDs in CRC Chemoprevention

The chemopreventive effects of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (acetylsalicylic acid; ASA) on colorectal cancer have been supported by experimental data in mice and humans [98,99]. Mice with conditionally deleted *Msh2* in the intestinal epithelium (*Villin–Cre* $Msh2^{lox/lox}$) that were administered ASA in their food exhibited a 20% increase in median survival time when compared to untreated controls [99]. In an AOM-induced CRC model using $Msh2^{-/-}$ mice, the NSAID sulindac reduced tumor frequency by more than twofold in the distal

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Box 2. A Role for the Gut Microbiota in Immunotherapy

Antibody-based immunotherapies depend on the gut microbiota for the treatment of subcutaneously established melanoma and CRC models in mice. In one study, inhibition of CTLA-4 (a negative regulator of T cell activation) with the anti-CTLA-4 antibody ipilimumab was demonstrated to have antitumor effects dependent on the presence of specific *Bacteroides* species, including *B. thetaiotaomicron* and *B. fragilis* [117]. By contrast, no anti-CTLA-4-dependent antitumor effects were observed when mice were treated with antibiotics or when housed under germ-free conditions [117]. Another group determined that *Bifidobacterium* promoted antitumor effects upon anti-PD-L1 therapy [118]. When mice lacking *Bifidobacterium* in their intestinal microbiota were inoculated with select *Bifidobacterium* species, these mice became significantly more responsive to anti-PD-L1 therapy against established melanomas in a mouse melanoma tumor model [118]. These reports suggest an appreciable role for the gut microbiota in modulating the efficacy of immunotherapies in mice. Whether this phenomenon is applicable to the clinical setting remains to be tested.

colon relative to controls [100]. However, sulindac also induced proinflammatory lesions in the proximal colon, suggesting that treatment with this NSAID can lead to side effects [100]. In a human double-blind randomized control study, Lynch syndrome patients administered 600 mg of aspirin daily had a significant reduction in CRC incidence compared to placebo-administered controls [98]. In support, a retrospective epidemiological study found that MMR gene mutation carriers who had continuously taken NSAIDs for longer than one month presented a reduced risk of developing CRC relative to controls [101]. However, the optimal dose, long-term safety, and cost/benefit analysis of using aspirin for CRC will require further validation. Moreover, the exact mechanism by which aspirin reduces CRC remains unclear (Box 3). Nevertheless, aspirin may be contemplated as a potential therapeutic strategy which can be effective in reducing the CRC risk in individuals who are carriers of MMR mutations.

Molecular Biomarkers for CRC Detection

Tumor biomarkers have been studied in relation to genetics, immunity, and the microbiota. Investigations into the interactions between different etiologic factors of CRC have been incorporated into an interdisciplinary field termed 'molecular pathological epidemiology' (MPE). Determining molecular associations and biomarkers within specific subsets of CRC can then be associated with host and environmental factors to improve personalized risk assessment [102–104].

There are currently few noninvasive tests for effective CRC detection. Lately, laboratories have been developing CRC diagnostic strategies based on NGS technologies, and several of these are now commercially available (reviewed in [105]). A growing number of NGS applications are being developed for CRC detection, including DNA, miRNA, and rRNA sequencing. For DNA sequencing-based assays, panels of genes associated with the development of CRC are sequenced in parallel, reducing the cost and turnaround time of traditional methods. ColoSeq™ was one of the early adopters for screening hereditary CRC by NGS. In this method, all exons, non-repeating intronic sequences, and promoters of selected CRC-associated genes are sequenced after being specifically capture-targeted with biotinylated complementary RNA [106]. It has proved to be highly specific and sensitive (>99%) in detecting mutations in Lynch syndrome-related genes [106]. DNA sequencing can be performed on stool samples, and therefore is an effective noninvasive method for detecting genetic mutations associated with hereditary and possibly sporadic CRC.

Another NGS application for CRC detection currently in development is miRNA sequencing. Several miRNAs capable of regulating genes associated with CRC have been demonstrated to present altered expression patterns in CRC tissues (reviewed in [107]). Screening for miRNA expression differences by miRNA sequencing, followed by RT-qPCR validation in patient sera, can discriminate between healthy individuals, colorectal adenomas, and colorectal adenocarcinomas [51]. Furthermore, when detected via miRNA microarrays, CRC with MSI have distinct

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Box 3. Potential Aspirin-Mediated Antitumor Mechanisms in MMR-Deficient CRC

Aspirin can act as a cyclooxygenase (COX) inhibitor on tumor cells [119]. Combined treatment with aspirin and anti-PD-1 in a mouse CT26 colon tumor model reduced tumor growth, and completely eradicated the tumor in 30% of mice, whereas treatment with aspirin or anti-PD-1 alone demonstrated low efficacy [119]. In MMR-deficient models, administering a COX-2 inhibitor to *Msh2*-deficient mice carrying a mutant *Apc* allele resulted in reduced polyp formation in both small intestine and colon [120]. Moreover, in HCT116 and HT-29 CRC cell lines, aspirin could induce acetylation of the tumor-suppressing transcription factor p53, potentially increasing its stability and DNA-binding activity, which could also induce increased p21 protein levels [121]. Aspirin could also affect the microbiota directly because it has been shown to inhibit *in vitro* the growth of intestinally isolated bacteria [122,123]. The growth inhibition observed in bacteria for some NSAIDs is caused by preventing the interactions between DNA polymerase III β subunit and DNA polymerase III δ and/or α subunits, thereby inhibiting DNA replication [124]. Consequently, the protective role of aspirin in CRC could also stem from a combined effect against the microbiota as well as against cancer-initiating cells.

miRNA profiles compared to microsatellite stable CRC or normal colonic tissue [108]. In fact, the differences in miRNA profiles have been used to discriminate between Lynch syndrome and sporadic CRCs [108]. miRNA profiling not only serves as a potential diagnostic marker but will very likely provide insight into the molecular pathogenesis of CRC.

Because the gut microbiota can contribute to CRC progression in mice, there has been growing interest in identifying specific bacterial species or profiles linked to various stages of CRC. The composition of the gut microbiota can be analyzed using 16S rRNA sequencing from the stool of CRC patients, and significant differences in the gut microbiota from CRC patients versus healthy individuals have been detected [109]. However, it is unclear whether these microbes are a direct cause of the disease because CRC can take years to develop, and it is unclear whether these specific microbes are present at disease onset. Nonetheless, using the microbiota as a diagnostic test for polyp detection has had greater sensitivity than testing for fecal hemoglobin [109]. As NGS technologies improve and become better standardized for clinical diagnostics, they will undoubtedly serve as powerful tools in the personalized identification of specific CRC subtypes.

Concluding Remarks

MMR-deficient CRC is clinically distinct from other subtypes of CRC. The relationship between MMR status, inflammation, and the intestinal microbiota will need to be further examined and validated (see Outstanding Questions). Inflammation and oxidative damage mediated by microbes are only a few of the many mechanisms which could promote tumorigenesis, and there are likely many additional bacterial species which influence or are associated with MMR-deficient CRC. Presently, there are few human case–control studies involving MMR-deficient CRC patients, but none have controlled for variations in miRNAs, cytokine profiles, or intestinal microbiota. Presumably, differences in these factors could exist between sporadic and hereditary loss of MMR function. It will be informative to assess, among other factors, additional differences in miRNAs, cytokines, and microbiomes of MMR-deficient individuals. This will facilitate the development of novel biomarkers for MMR-deficient CRC. Murine models of sporadic CRC have so far been useful in determining mechanisms of CRC progression. However, additional studies will be necessary in MMR-deficient models to elucidate the extrinsic and intrinsic mechanisms of MMR in suppressing tumorigenesis. By understanding the ways in which MMR deficiency contributes to disease, novel pathways may be exploited for therapeutic intervention and/or disease prevention.

Acknowledgments

We would like to thank Ursula Flojanczyk (Figures 1 and 3), Lauren Huff (Figure 2), Matan Berson (Figure 4), and Cheng Jou (Ruth) Chang (Figure 5) for their contributions to the illustrations. We also thank Thergiory Irrazabal and Mingsong Kang for critically reading the manuscript. This work is funded by the Canadian Cancer Society (grant 703185).

Outstanding Questions

What genes are altered in MMR-deficient CECs? MMR can alter gene expression through miRNA processing or DNMT1 recruitment. Hence, MMR-deficient CECs could present altered gene expression profiles compared to MMR-proficient CECs. Determining these differences could provide insight into genes potentially regulated by various MMR pathways.

How does MMR deficiency influence cytokine networks? Although inflammation contributes to CRC, few studies have compared the cytokine profiles between MMR-deficient and MMR-proficient samples. Differences in cytokine signaling could alter MMR localization within the cell or gene expression profiles.

What member(s) of the gut microbiota are associated with the development of CRC in MMR-deficient individuals? Although Fusobacteria have been associated with MMR-deficient CRC and other CRCs, there are likely other bacteria which could be involved in its pathogenesis. It is possible that the microbiota is altered between the various subtypes of CRC, including those with MMR mutations. Determining differences in the microbiota between MMR-deficient and MMR-proficient CRCs could identify specific bacteria as causative agents.

Which microbe-produced metabolite(s) influence MMR-deficient CRC? Although butyrate has been suggested to influence CEC proliferation in MMR-deficient mice, further research to show if it can cause CRC in these mice is required. It is also likely that other bacterial metabolites can contribute to CRC in MMR-deficient models. Modulating the composition of the gut microbiota might prove to be an effective preventative measure in Lynch syndrome patients.

What are the mechanisms of anti-PD1 immunotherapy and NSAID chemoprevention against MMR-deficient CRCs? Anti-PD1 and ASA are effective interventions for MMR-deficient CRC, but the exact mechanisms of action are unclear. Elucidating the effects of these agents could provide insight into other pathways involved in MMR-deficient tumorigenesis which could be exploited for novel treatments.

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Author Manuscript
Cancer Discov. Author manuscript; available in PMC 2015 July 01.

Published in final edited form as:
Cancer Discov. 2015 January ; 5(1): 43–51. doi:10.1158/2159-8290.CD-14-0863.

The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints

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Abstract

We examined the immune microenvironment of primary colorectal cancer (CRC) using immunohistochemistry, laser capture microdissection/qRT-PCR, flow cytometry and functional analysis of tumor infiltrating lymphocytes. A subset of CRC displayed high infiltration with activated $CD8^+$ CTL as well as activated Th1 cells characterized by IFN-γ production and the Th1 transcription factor Tbet. Parallel analysis of tumor genotypes revealed that virtually all of the tumors with this active Th1/CTL microenvironment had defects in mismatch repair, as evidenced

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Conflict of Interest Disclosure: The authors disclose no potential conflicts of interest

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by microsatellite instability (MSI). Counterbalancing this active Th1/CTL microenvironment, MSI tumors selectively demonstrated highly up-regulated expression of multiple immune checkpoints, including five – PD-1, PD-L1, CTLA-4, LAG-3 and IDO – currently being targeted clinically with inhibitors. These findings link tumor genotype with the immune microenvironment, and explain why MSI tumors are not naturally eliminated despite a hostile Th1/CTL microenvironment. They further suggest that blockade of specific checkpoints may be selectively efficacious in the MSI subset of CRC.

Keywords

PD-1; LAG-3; IFN-γ; Tumor microenvironment; Immunotherapy

Introduction

An increasing body of research has revealed that the immune microenvironment of cancer is unique and complex; indeed, tumors can be viewed as distinct immunologic organs. Pathologists have long recognized the diversity of immune infiltration into tumors, invoking terms like "lymphocyte poor" and "inflammatory". For some cancer types, it is becoming clear that simple quantitation of lymphocyte infiltration has prognostic significance, suggesting that lymphocyte infiltration is not passive but may actively promote or inhibit tumor growth (1). In the case of colorectal cancer (CRC), Galon and colleagues demonstrated that levels of lymphocyte infiltration into primary tumors represented a strong independent predictor of relapse and overall survival, with high lymphocyte infiltration being a positive prognostic factor (2). Using expression profiling of CRCs, they further defined the relevance of specific immune signatures, demonstrating that T helper1 cells (Th1) type, IFN-γ dominant immune profiles signified an improved prognosis while Th17 type, IL-17 dominant immune profiles signified a poor prognosis (3). These findings are in concordance with multiple studies in murine models demonstrating that Th1 / cytotoxic T lymphocyte (CTL) dominant immune responses are anti-tumorigenic (4) while Th17 dominant responses are typically pro-carcinogenic (5).

The relevance of molecular regulation of lymphocyte function in the tumor microenvironment (TME) has been further highlighted by the emerging clinical experience in treatment of cancer with antibody blockade of the PD-1 pathway. Anti-PD-1 and anti-PD-L1 antibodies have been demonstrated to induce significant durable tumor regressions in patients with melanoma, renal cancer and non-small cell lung cancer (6-9). The finding that expression of PD-L1 in tumor biopsies is a predictor of response to PD-1 pathway blocking antibodies (6) supports conclusions from murine studies that this pathway plays a major role in blocking anti-tumor immune responses directly within the TME (10). With increasing efforts to develop antibody blockers of multiple additional immune inhibitory ligands and receptors (termed checkpoints) for cancer immunotherapy, understanding the expression patterns and functions of these molecules in the context of the TME will be critical in selecting patient populations most likely to benefit from their application (11). In contrast to melanoma, renal and lung cancer, CRC cohorts demonstrated a very low response rate to PD-1 or PD-L1 blockade and have been considered a non-responding histology to the PD-1

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pathway blockade (6-8). However, there were insufficient patients to potentially define subsets of CRC that might be more amenable to checkpoint blockade.

With these considerations in mind, we have undertaken a comprehensive analysis of the immune microenvironment of CRC, using a combination of immunohistochemistry (IHC), laser capture microdissection combined with quantitative reverse transcription PCR (LCM/qRT-PCR), multiparameter flow cytometry (MFC) and functional analysis of purified tumor infiltrating lymphocytes (TIL) from a large set of surgically resected primary CRC. These analyses revealed, in an unbiased fashion, that a subset of CRC possesses a highly activated Th1- and CTL-rich microenvironment. Through concomitant genetic analysis, we found that this subset of tumors harbored microsatellite instability (MSI), indicating that they were mismatch repair deficient (12). This finding was in accordance with numerous prior studies indicating that MSI tumors are highly infiltrated with T-cells including CTLs (13-16). How could tumors persist in this hostile microenvironment? To find out, we carried out an extensive investigation of the regulatory factors operative in this microenvironment. We found that there was a dramatic overexpression of immune checkpoint-related proteins in the microenvironment of MSI tumors, thus explaining a long-standing enigma and suggesting that immunotherapeutic interventions involving checkpoint blockade might be selectively effective in this important subset of cancers. Based on these findings, clinical trials have been initiated to test PD-1 blockade in CRC patients selected to be MSI.

Results

We began our analysis of primary sporadic CRC by performing quantitative IHC for standard T cell subsets using antibodies to CD3, CD4, CD8 and Foxp3. We distinguished three compartments – true TIL representing lymphocytes intercalated within the glandular or medullary epithelial component of the tumor, T cells in the tumor stroma surrounding the epithelial component of the tumor and the invasive front where the tumor invades into the lamina propria (**Fig. S1**). Infiltration by these T cell types in each of the compartments was quantitated numerically by blinded analysis of 5 high power fields (hpf; 0.0028mm^2/hpf) and displayed as average number of stained cells per hpf. We found a subset of tumors (roughly 20%) that had large infiltrates of total ($CD3^+$ T cells) as well as $CD4^+$ and $CD8^+$ T cells in all three compartments with $CD8^+$ T cells most dramatically increased. We wondered whether this subset of tumors was MSI, given prior studies indicating lymphocyte infiltrations in such tumors (13-16). Indeed, analysis of microsatellites showed that nearly all of the tumors with the infiltrates were MSI. We therefore focused on differences between MSI versus MSS tumors **(Table S1)** for the in-depth analysis of the immune TME of CRC.

Figures 1A & B show typical examples of IHC staining and **Fig.1C** shows the quantitative analyses demonstrating statistical differences between MSI and MSS tumors for the total $CD3^+$ T cells and $CD4^+$ T cells among TIL and invasive front as well as $CD8^+$ T cells among all three compartments. Although it did not reach statistical significance in tumor stroma and the invasive front, we observed higher $Foxp3^+$ cell infiltrates, representative of regulatory T cells (Treg), in MSI compared to MSS tumors. Of note, a single outlier included in our MSS cohort displayed a high infiltration of each T cell subset, similar in range to the one of MSI tumors **(Fig. 1C, S2 and S3A)**.

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We further explored the nature of the T cell infiltrates by performing LCM on MSI and MSS tumors, separately dissecting the TIL, stroma and invasive front compartments (**Fig. S1**) and then performed qRT-PCR (**Fig. 2A-C**) for selected genes encoding signature T cell cytokines as well as core transcription factors for each of the three major T helper subsets, Th1/Tc1 (type 1 CTL; TBX21 and IFNG are common to Th1 and Tc1), Th2 and Th17. We additionally analyzed genes associated with CTL and Treg and also general inflammatory cytokines. Finally, we analyzed expression of genes encoding both co-inhibitory membrane ligands and receptors (commonly termed checkpoints) and metabolic enzymes that have been shown to regulate lymphocyte activity; these serve feedback inhibitory functions in normal physiology but can represent important mechanisms of adaptive immune resistance by tumors in the face of an endogenous anti-tumor T cell repertoire (11).

We found that the expression of the gene encoding IFN-γ (*IFNG*) the canonical Th1/Tc1 cytokine is higher in all three compartments of MSI compared to MSS tumors (**Fig. 2A-C**; the differences reach statistical significance in the TIL and invasive front areas with Wilcoxon test p values = 0.041, for both of them). The expression of *TBX21* encoding Tbet, the Th1/Tc1 canonical transcription factor is similarly increased in MSI tumors, though differences did not reach statistical significance among the cohort analyzed. The *CD8A* gene, mainly expressed by CTL is highly differentially expressed in the TIL regions of MSI relative to MSS tumors (**Fig. 2A**; p=0.004), in concordance with significantly higher CD8 infiltration observed by IHC (**Fig. 1C**). In marked contrast to Th1 and CTL genes, expression of Th17 genes is virtually identical between MSI and MSS tumors for all compartments. IL-13 and IL-4 (the canonical Th2-type cytokines) were undetectable in most of the MSI and MSS samples for each of the TME regions analyzed and GATA3 (Th2 transcription factor) expression was not different between MSS and MSI (data not shown). Treg-associated genes, including *FOXP3*, were similar between MSI and MSS tumors. Gene group comparison analysis using Wilcoxon-Mann-Whitney Permutation test confirms that Th1/Tc1 (*TBX21* and *IFNG*) and CTL (*CD8A*, *GZMB*, *PRF1*, *IL21*) groups but not the Th17 (*RORC, IL17A* and *IL23*) group show statistical differences in their representation between TIL compartments of MSI and MSS CRC (**Fig. 2D**). In summary, MSI tumors have a selective Th1 and CTL infiltration and activation relative to MSS tumors. The highest value for *IFNG* and *TBX21* expression in TIL from a single MSS sample (**detailed in Fig. S3B**) represents the same outlier observed in the quantitative analysis of CD4 and CD8 TIL infiltration from **Fig. 1C**. Among genes encoding inflammatory cytokines, *IL18*, which is generally associated with Th1 responses and selectively promotes IFN-γ production by T cells, is more highly expressed in MSI tumors in all three compartments ($p<0.05$) whereas, genes encoding IL-1 and IL-6, which selectively promote Th17 responses, are not (**Fig. 2A-C**). The expression of *PTGS2* (encoding COX2), *IL12A* (encoding IL12p35) and *TNF* (encoding TNF-α) did not differ between MSS and MSI specimens.

We next analyzed the expression of genes encoding checkpoint receptors. We found that all three of the clinically targeted checkpoint receptors - CTLA-4 (*CTLA4*), PD-1 (*PDCD1*) and LAG-3 (*LAG3*) - were expressed at considerably higher levels in MSI compared to MSS tumors ($p<0.05$ in all compartments for *CTLA4*, in TIL and invasive front for *LAG3*; $p>0.05$ in all compartments for *PDCD1*). The gene encoding PDL1 (*CD274*), a major IFN-γ-

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inducible PD-1 ligand expressed in the epithelial cells of many solid tumors (17) was unexpectedly expressed in TIL and stroma of MSI tumors (Wilcoxon test p values are 0.009 and 0.015, respectively; **Fig. 2A&B**). Among inhibitory metabolic enzymes, the IFN-γ-induced gene encoding indolamine 2'3' dioxygenase (*IDO*), which metabolizes tryptophan to kynurenine, demonstrates significantly higher expression in TIL of MSI tumors (p=0.041; **Fig. 2A**). In contrast, arginase (ARG1), a myeloid enzyme induced by the Th2 cytokines IL-4 and IL-13 and up-regulated in M2 macrophages, was generally not detectable in either MSI or MSS tumors (data not shown). Thus, along with the Th1 and CTL genes, multiple immune-inhibitory genes, including a number that are IFN-γ-induced, are preferentially expressed in the TILs and stroma of MSI tumors. To note, these results reflect a combination of higher immune infiltration and cellular upregulation in MSI compared to MSS tumors.

Because differences in checkpoints expression could have significant implications in defining patient subgroups potentially responsive to checkpoint blockade, we sought to determine whether differences at the RNA level were mirrored at the protein level. Indeed, IHC for both PD-1 and LAG-3 demonstrated a robust expression of each of them in lymphocytes of MSI tumors, whereas very little was observed in MSS tumors (**Fig. 3A-B**). The qRT-PCR/LCM analyses shown in **Fig. 2** therefore underestimated the differences between MSI and MSS tumors with respect to PD-1 protein surface expression by TIL. Multi-parameter flow cytometry of freshly isolated lymphocytes from tumors demonstrated that a large proportion of both $CD4^+$ and $CD8^+$ T cells infiltrating MSI tumors express high levels of PD-1 (**Fig. 4A-B**). This PD-1^{hi} population was largely absent in MSS tumors (except two MSS specimens, one of which was the highly infiltrated MSS outlier described above) and the normal mucosa adjacent to MSI tumors. The T-bet downregulation and PD-1 upregulation by T cells is typically found in chronic viral infection and termed "exhaustion" (18). Since the presence of PD-1^{hi} T cell infiltrate in MSI tumors is concomitant with the detection of high IFN-γ (MFC in **Fig.4C** and qRT-PCR in **Fig.2**) and high T-bet (qRT-PCR in **Fig.2**), further investigation should be conducted to determine the co-expression of these molecules at the single cell level and formally rule out the classic "exhaustion" phenotype of these TILs. Of note, the two MSS tumors exhibiting an unusually strong proportion of $PD1^{hi}$ $CD8^+$ T cells (**Fig.4B**), were also characterized by a high proportion of IFN-γ-producing $CD8^+$ T cells (**Fig.S3** and data not shown). Our findings suggest that a small proportion of MSS tumors are characterized by the concordant detection of Th1/CTL infiltration and immune checkpoint expression that is found in all MSI tumors.

Finally, we analyzed PD-L1 protein expression by IHC. MSI tumors demonstrated much higher PD-L1 expression than MSS tumors (**Fig. 4D**). Surprisingly, in contrast to other cancers such as melanoma, renal cancer and lung cancer (19), there was virtually no discernable PD-L1 expression on tumor cells of MSI tumors by IHC; rather, co-staining with CD163 demonstrated that the majority of PD-L1 expression was by myeloid cells. There were large numbers of PD-$L1^+$ myeloid cells at the invasive front and in the stroma and some were intercalated between epithelial cells in the tumor nests of MSI specimens (**Fig. 4D**). Histological scoring of $PDL1^+$ cells in TIL and the invasive front regions confirmed the high expression of PD-L1 in MSI tumors (**Fig. 4E**). Omitting the MSS outlier patient (high CD8 infiltration and IFN-γ production), that also demonstrated high PD-L1

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expression, we found that the difference between MSS and MSI specimens reached statistical significance in the TIL area ($p<0.05$, Mann Whitney U test). MFC analysis performed on freshly dissociated MSI tumors confirmed high levels of PD-L1 expression on viable $CD11b^{+}HLA\text{-}DR^{lo}CD15^{-}CD14^{+}CD33^{+}$ myeloid cells (**Fig. 4F**). Because most human cancers appear to up-regulate PD-L1 as an adaptive response to IFN-γ (17), the lack of clear PD-L1 expression on tumor cells in the MSI specimens as assayed by IHC was unexpected, particularly given the high IFN-γ level in these tumors. Of note, while PD-L1 was up-regulated on a number of both MSI and MSS colon tumor cell lines after incubation with IFN-γ, this up-regulation was, in general, much less than the one observed in melanoma cell lines (**Fig. S4**). Interestingly, cell lines with the weakest PD-L1 induction also showed weak MHC II induction after IFN-γ treatment, suggesting that colon tumors may have relatively dampened Stat1 signaling.

MSI tumors have a much higher mutational load (and thus potentially more neoantigens) than MSS tumors, and this could be related to the presence of the vigorous immune microenvironment in this genotypic subset of CRC. We therefore wondered whether the highly infiltrated MSS tumor described above had more mutations than expected for an MSS tumor. To address this question, we performed exome sequencing on the microdissected tumor of this individual. We found only 49 nonsynonymous, somatic mutations in this tumor, a number well within the typical range for MSS CRC and much less than in MSI tumors (20). Additionally, no mutations in *POLE* and *POLD1* genes were identified; tumors with mutations in these genes also contain a high mutational load despite having stable microsatellites (21). Thus, in this individual tumor, the prominent T cell infiltration was not due to an unusually high mutational load (**Table S2**).

Discussion

Direct analysis of the immune microenvironment of tumors is emerging as the most important means of understanding the relationship between patients' immune systems and their cancer (1). These analyses are bearing fruit in informing prognosis and guiding immunotherapy, particularly using antibody blockade of immune checkpoints. We demonstrate here that the immune microenvironment of DNA-repair deficient MSI CRC contains a strong Th1 and CTL component not found in the vast majority of DNA repair sufficient MSS tumors, in keeping with previous reports of high T cell infiltration in MSI tumors (13-16). More importantly, we address a vexing question in the field: why are human tumors, which are apparently strongly immunogenic, as judged by histopathologic criteria, not rejected by the host? The numbers of infiltrating activated lymphocytes and CTLs in some tumors are huge, in some cases larger than the number of neoplastic cells. The answer to this question was found through the analysis of immune checkpoints. Multiple key immune inhibitory ligands, receptors and metabolic enzymes, including those that are IFN-γ inducible (PD-L1, IDO), were highly up-regulated in MSI tumors relative to MSS (**Fig. 2**). We suggest that MSI represents a classic example of adaptive resistance in which an active immune Th1/CTL microenvironment results in compensatory induction of checkpoints that protect the tumor from killing (11).

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The mechanistic basis for the link between MSI genetic status and a high Th1/CTL microenvironment in CRC is not known. However, one important factor may be the increased number of tumor "neoantigens" in DNA repair-deficient tumors created by the high mutational load, typically between 10 and 50 times that of DNA repair-sufficient tumors (14, 16). The notion is that mutation-generated neoantigens are truly tumor specific and may not induce immune tolerance to the same extent as self-antigens, even those up-regulated in tumors due to epigenetic dysregulation (22). Ultimately, an altered amino acid due to a coding mutation is only relevant as a tumor neoantigen for T cells if it can be processed and presented on self-MHC. Exomic sequencing and application of a multifactorial algorithm to the mutations in the MSS with an active Th1/Tc1 microenvironment may predict a number of potential neoepitopes capable of being presented on the tumor's HLA class I alleles (23). The multiple variables involved in antigen processing and presentation suggest that while mutational density may correlate with neoantigenicity on a population level, individual tumors with lower mutational load can none-the-less generate good T cell neoepitopes if the mutations are appropriately positioned. Additionally, many other mechanisms besides neoantigenicity (i.e. production of immune regulatory cytokines and chemokines by the tumor and non-transformed stromal cells) likely contribute to the character of the immune microenvironment.

Among the many immune inhibitory ligand-receptor pairs (checkpoints) and metabolic enzymes discovered to date, CTLA-4, PD-1, PD-L1, LAG-3 and IDO are of particular interest because inhibitory antibodies or drugs (in the case of IDO) are currently in active clinical testing to enhance therapeutic anti-tumor responses. Of note, all of these were highly elevated in all three compartments (TIL, stroma and invasive front) of MSI tumors. IHC of PD-1, PD-L1 and LAG-3 confirmed the gene expression findings and demonstrated that MSS tumors and their infiltrating and invasive front lymphocytes (with the exception of the outlier) express very little of these checkpoint molecules. Recent findings point to the elevated expression of these checkpoints in tumors (particularly PD-L1 and IDO) as an adaptive response to cytokines such as IFN-γ that are part-and-parcel of an active intratumoral immune response (24, 25). This mechanism appears to be operative in MSI tumors. What is strikingly different in MSI CRC relative to melanoma, renal or lung cancer is that there is little PD-L1 expressed by tumor cells; rather it is expressed predominantly by infiltrating myeloid cells. The modest up-regulation of PD-L1 by CRC cell lines in response to in vitro incubation with IFN-γ suggests that adaptive PD-L1 expression in CRC is predominantly at the level of infiltrating myeloid cells, though low levels of PDL1 could be expressed by tumor cells in situ that is below the detection level of IHC. Regardless, a very important prediction of our findings on differential expression of immune inhibitory ligands, receptors and metabolic enzymes is that MSI tumors will respond to checkpoint blockade with agents such as anti-PD-1 or anti-PD-L1 antibodies, while MSS tumors will be much less responsive. Indeed, based on these findings, 2 clinical trials testing anti-PD-1 antibodies in selected MSI patients have been initiated (*NCT01876511 and NCT02060188).*

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Materials and Methods

Patient selection, tumor samples and cell lines

Tumor tissues were collected at Johns Hopkins Hospital (Baltimore, MD, USA) from patients with primary sporadic CRC and free of prior chemotherapy. Demographic, pathologic (tumor location, TNM grade) and genetic status are detailed in **Table S1**. This study was approved by the Johns Hopkins Institutional Review Board. All samples were obtained in accordance with the Health Insurance and Accountability Act.

Assessment of microsatellite instability (MSI) was done using the length of a panel of microsatellite markers in the tumor and a normal reference (either normal mucosa or germline) by using fragment analysis of PCR products labeled with fluorescent dyes. Fragment analysis determined the expression level of the proteins in charge of maintaining the integrity of microsatellite tracts. Differences in the length of 2 or more markers (the standard Bethesda panel uses 5 markers) was indicative of MSI status. Eleven patients tested as MSI positive and fourteen patients as MSS (**Table S1**).

Analysis of mutations on the MSS outlier sample was performed using DNA exomic sequencing from formalin-fixed, paraffin-embedded (FFPE) tissues. Identification of tumor-specific mutations involved comparison with normal matched cells derived from FFPE sections that contained no tumor. Genomic DNA libraries were prepared following Illumina's suggested protocol with small modification. Briefly, the ends of sheared genomic DNA fragments are blunted and Illumina adaptors are ligated. The fragment library was amplified by 15 cycles of PCR. Human exome capture is performed following a modified protocol from Agilent's SureSelect Paired-End Version 2.0 Human Exome Kit (Agilent, Santa Clare, CA). The genomic library is hybridized to the SureSelect probes. After washes, the genomic regions captured by the probes are eluted. The captured region covers 38 Mb of exomic sequences. Following PCR amplification of 6 cycles the library is hybridized to an Illumina flow cell and eventually sequenced according to Illumina's protocols. Mismatched bases were identified as a mutation based on rigorous criteria described previously. Mutations were further confirmed to not be variants present in the normal population according to the NCBI dbSNP database.

For analysis of cell lines, three microsatellite instable (SW48, HCT116 and DLD1) and 3 chromosomal instable (HT29, Caco-2 and SW480) human CRC cell lines were used. These cell lines were obtained from the Genetic resources core facility (GRCF) - Institute of Genetic Medicine - Johns Hopkins School of Medicine, which purchased these cells from the authenticated ATCC (American Type Culture Collection) repository. The cell lines were maintained in culture in 10% FCS DMEM supplemented with L-Glutamine, non-essential amino acids, sodium pyruvate and antibiotics.

Immunohistochemistry

FFPE specimens were cut into 5-μm sections and mounted on glass slides. For each specimen, CD3 (clone PS1, Leica Biosystems, Buffalo Grove, IL), CD4 (clone Sp35, Ventana, Tuscon, AZ), CD8 (clone C8144B, Cell Marque, Rocklin, CA), Foxp3 (236A/E7, Abcam, Cambridge, MA), CD163 (clone Novacastra10D6, Leica Biosystems) stainings

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were performed according to standard protocols. IHC for PD-L1 (clone 5H1), PD-1 (clone M3) stainings were performed according to a protocol previously reported (24). Positive cells were counted in 5 HPF (0.028 mm/HPF; Olympus BX41) by two blinded pathologists (R.A.A and M.C) in three distinct tumor areas denominated TIL, tumor stroma and Invasive Front. LAG3 IHC was performed using clone 17B4 (LS Bio, Seattle, WA).

Laser capture microdissection and Taqman quantitative RT-PCR

5um FFPE and H&E stained tissue sections were used for LCM procedure using the Leica LMD 7000 system (Leica, Buffalo Grove, Il). For each patient, tissues were microdissected from the 3 previously defined areas and directly collected in tissue lysis buffer for RNA extraction. RNA was isolated following the manufacturer instructions (High pure paraffin kit, Roche). RNA was converted to cDNA using high capacity RNA to cDNA kit (Life Technologies, Grand Island, NY) and a step of preamplification was first done using a pool of primers and preamplification master mix kit (Life technologies,) followed by Taqman RT-PCR (list of primers in **Table S3**). Data are expressed as $2^{-\Delta Ct}$ where ΔCt represents Ct_{gene}-Ct_{ctrl}. For our calculation Ct_{ctrl} is the average of Ct for 2 ubiquitous genes (GAPDH, GUSB). When undetectable, a value of 40 was assigned as Ct.

Tumor processing and flow cytometry

Tumor specimens were collected and freshly dissociated using enzymatic cocktail (0.1% DNaseI and Liberase 400u/ml, Roche). Leukocytes were enriched by Percoll density gradient (GE healthcare, US) and cells were banked in liquid nitrogen until further analysis. MFC was performed with a LSR II cytometer (BD Biosciences). Samples were stained with anti-CD45 (2D1, BD Biosciences) anti-CD3 (UCHT1, BD Biosciences), anti-CD4 (OKT4, BD Bioscience), anti-CD8 (RPA-T8, BD Biosciences), anti-PD-1 (EH12.1, BD Biosciences), anti-Foxp3 (259D/C7, BD Biosciences). Cytokine intracellular staining (ICS) for IFN-γ (B27, BD Biosciences) and IL-17 (SCPL1362, BD Biosciences) was performed following 3 hr -*in vitro* stimulation in the presence of stimulation cocktail (PMA +ionomycin; Ebioscience) and GolgiStop (Monensin; BD Biosciences) according to the manufacturer instructions. Data were analyzed using DIVA 6.1 Software (BD Biosciences). Myeloid cells were stained using anti-CD45, anti-CD11b (ICRF44, BD Biosciences), anti-DR (G46.6, BD Biosciences), anti-CD15 (HI98, BD Biosciences), anti-CD14 (M5E2, BD Biosciences), anti-CD33 (WM53, BD Biosciences), anti-CD11c (B-Ly6, BD Biosciences) and anti-PD-L1 (29E.2A3, Biolegend, San Diego, CA). Cell lines were cultured for 3 days in the presence or absence of IFN-γ (500IU/ml). Cells were stained with DR and B7-H1/PD-L1 mAb prior to analysis by flow cytometry.

Statistical analysis

IHC scoring in each of the three histological areas (i.e., TIL, stroma and invasive front) and MFC data were summarized using scatter plots and compared between MSI and MSS patients using means and non-parametric Mann Whitney U test. For the gene expression analysis, scatter plots and geometric means were used to characterize MSI/MSS patient groups for each of the three locations. Genes were grouped by lineage and/or function (Th1/Tc1, CTL, Th17, Treg, Pro-inflammation, Metabolism) and to distinguish which groups of

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genes were differentially expressed based on MSS/MSI status, a re-sampling based permutation test was conducted based on the maximum Wilcoxon Mann Whitney test statistic within the gene group. Individual gene expression was also compared across MSI/MSS status using the Wilcoxon-Mann-Whitney test. All tests are descriptive and no multiplicity adjustment was considered. Statistical analysis were performed using the R statistical package (version 2.15.1)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank K. Judge and C. Blair for her excellent management and coordination of CRC patient recruitment and specimen banking. We acknowledge Robert Scharpf (Department of Biostatistics, Johns Hopkins University School of Medicine) for his helpful expertise in genomic data analysis.

Financial Support: This work was supported by National institutes of Health through P50 CA062924 (GI SPORE to FH), RO1 CA151393 (CLS and DMP), K08 DK087856 (ECW), 5T32CA126607-05 (EMH), P30 DK089502 (GI Core), P30 CA006973 (SKCCC core). Additional support was provided by GSRRIG-015 (American Society of Colon and Rectal Surgeons to EMH), the Mérieux Foundation (CLS and DMP), a Stand Up To Cancer–Cancer Research Institute Cancer Immunology Dream Team Translational Research Grant SU2C-AACR-DT1012 (Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research), a Melanoma Research Alliance award (to DMP), the Commonwealth Foundation (KWK, NP, and BV), The Virginia and D.K. Ludwig Fund for Cancer Research and the Ludwig Institute (KWK and BV).

Abbreviations list

ARG	arginase
CRC	colorectal cancer
CTL	cytotoxic T lymphocytes
FFPE	formalin-fixed paraffin-embedded
H&E	hematoxylin and eosin
IDO	indolamine 2'3' dioxygenase
IHC	immunohistochemistry
LCM	laser capture microdissection
MSI	microsatellite instable
MSS	microsatellite stable
MFC	multiparameter flow cytometry
Th	T helper
TIL	tumor-infiltrating lymphocyte
TME	tumor microenvironment
Treg	regulatory T cells

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Significance

The findings reported in this article are the first to demonstrate a link between a genetically defined subtype of cancer and its corresponding expression of immune checkpoints in the tumor microenvironment. The mismatch repair defective subset of CRC selectively up-regulates at least 5 checkpoint molecules that are targets of inhibitors currently being clinically tested.

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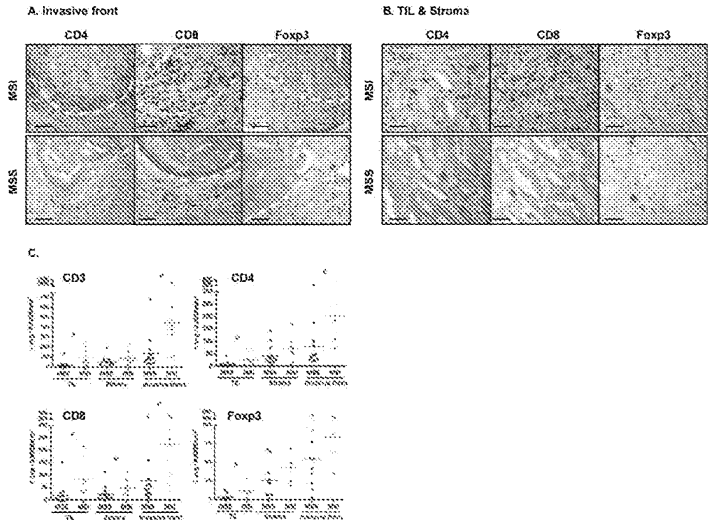


Figure 1. Geographic distribution *in situ* of MSI and MSS CRC-infiltrating lymphocytes

FFPE tissue sections were characterized by IHC for $CD4^+$, $CD8^+$ and $Foxp3^+$ cell infiltration. Three distinct histological areas designated as tumor infiltrating lymphocytes (TIL), tumor stroma and invasive front (where tumor invaded normal lamina propria) were histologically identified and separately analyzed. Invasive front (**A**) and TIL/Stroma (**B**) areas of a representative MSS (bottom panel) and MSI (top panel) are shown (20X magnification). Dashed lines in (**A**) delineate the invasive front with the tumor tissue on the top side and the normal tissue on the bottom side. Red stars and blue arrows in (**B**) indicate the tumor stroma and tumor epithelium-infiltrating immune cells, respectively. Scale bars, 100μm. **C**, cell density was scored in 14 MSS (blue) and 9 MSI (red) specimens by determining the average number of stained cells in 5 distinct hpf ($0.0028mm^2$/hpf). The graphs display the mean for each group and * indicates statistically significant differences between MSS and MSI ($p<0.05$ using Mann-Whitney U test).

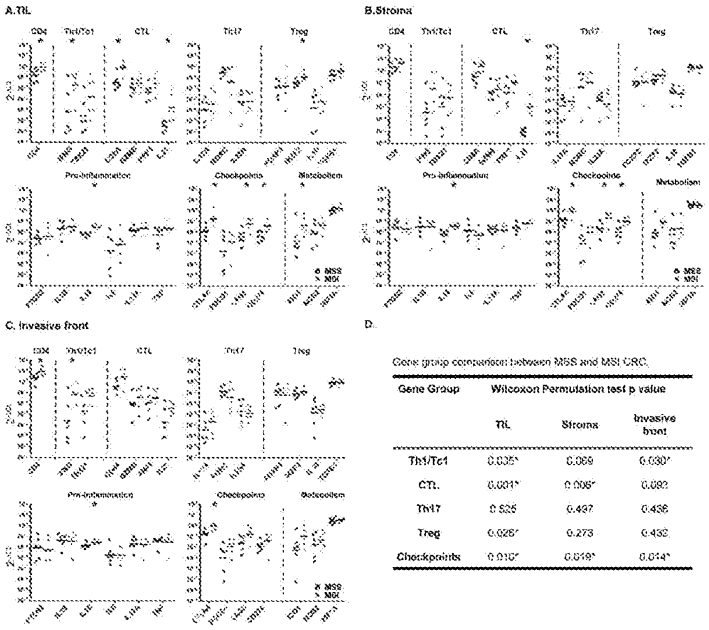


Figure 2. Th1 and CTL based immune signature and elevated checkpoint expression in MSI CRC

RNA was extracted from tissue samples laser-microdissected representing TIL in tumor nests (**A**), stroma surrounding tumor (**B**) and invasive front (**C**) areas of MSS (blue squares) and MSI (red circles) CRC specimens. Immune-related gene expression profiles were assessed using Taqman-based qRT-PCR for selected genes. Sets of genes were defined by functional relevance (Th1/Tc1, CTL, Th17, Treg, pro-inflammation, immune checkpoints and metabolism). The Y axis represents an arbitrary unit of expression $2^{-\Delta Ct}$, ΔCt representing cycle threshold (Ct) of the gene of interest normalized by Ct of ubiquitous genes (*GUSB*, *GAPDH*). The graphs display the geometric means. Their differential representation between MSS and MSI specimens was analyzed using adjusted Wilcoxon Mann Whitney test as described in *Methods* section. *, Wilcoxon p-value < 0.05. **D**, gene group comparison in TIL, tumor stroma and invasive front areas between MSS and MSI specimens.Permutation Test Results based on the maximum Wilcoxon Mann Whitney test statistic within the gene groups Th1/Tc1, CTL, Th17 and immune checkpoints. * indicates statistically significant differences between MSS and MSI ($p<0.05$).

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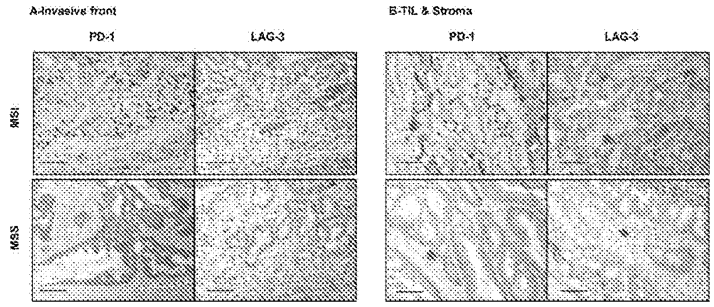


Figure 3. PD-1 and LAG-3 expression in MSI and MSS CRC specimens

IHC analysis of PD-1 and LAG-3 expression in invasive front (**A**) and TIL/Stroma (**B**) areas was performed on FFPE tissue sections of a representative set of MSI (top panel) and MSS (bottom panel) CRC specimens. Magnification, X20; Scale bars, 100μM; Red stars and blue arrows in (**B**) indicate the tumor stroma and tumor epithelium infiltrating immune cells, respectively.

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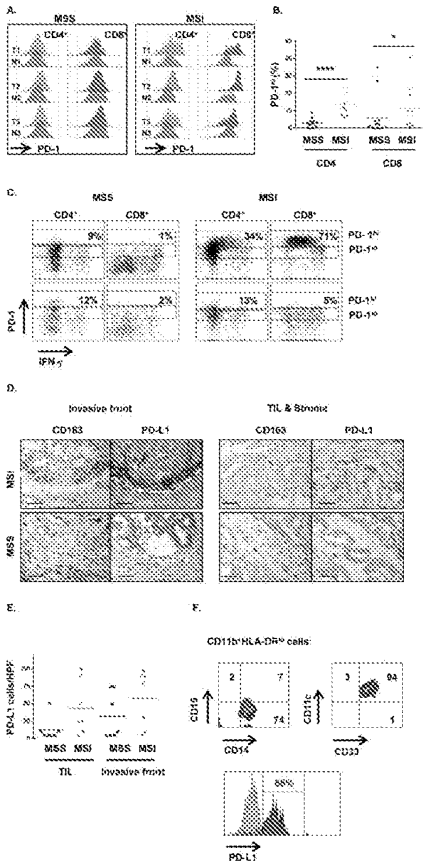


Fig. 4. MSI CRC are characterized by IFN-γ-producing PD1^{hi} TIL and PDL-1^{+} tumor-infiltrating myeloid cells

A, Freshly dissociated MSS and MSI colon tumors (**T**) as well as patient-matched normal tissue (**N**) were assessed by MFC for the expression of PD-1 on infiltrating CD4^{+} and CD8^{+} T cells. PD-1 expression in tumor was normalized to the normal tissue ran simultaneously and both histograms were aligned to delineate in tumor samples the PD1^{hi} cells when compared to normal tissue. **B**, proportion of PD-1^{hi} CD4^{+} and CD8^{+} cells among CD3^{+} lymphocytes infiltrating MSS (blue squares) and MSI (red circles) specimens. In each group the mean is indicated and * represents statistically significant differences between MSS and MSI (*p<0.05, ****p<0.0001; non-parametric Mann-Whitney U test). **C**, representative intracellular staining for IFN-γ production by *in vitro* PMA/ionomycin activated T cells (3hrs). The dot plots show the co-expression of PD-1 and IFN-γ in CD4^{+} T cells and CD8^{+} T cells in a representative set of MSS (Left) and MSI (Right) CRC (Top) and patient-matched normal (Bottom) specimens. The gates delineate PD1^{hi} and PD1^{lo} cells. **D,** co-localization of CD163 and PD-L1 expression in invasive front (left panel) and TIL/Stroma (right panel) areas of a representative set of MSS (lower panels) and MSI (upper panel) CRC specimens were assessed by IHC; x20 magnification. Scale bars, 100μM. Red stars indicate the tumor stroma. **E**, PD-L1 expression scores in 7 MSS (blue) and 7 MSI (red) CRC specimens (average of 5 hpf per sample). **F**, MFC analysis of PD-L1 expression on MSI

CRC-infiltrating myeloid cells. Dot plots represent the expression of myeloid associated markers on $CD11b^{+}HLA\text{-}DR^{-/low}$ cells. Infiltrating myeloid cells were characterized as $CD15^{-}CD14^{+}CD33^{+}CD11c^{+}$ cells. PD-L1 expression (dark gray) is overlaid with corresponding isotype control (light gray).

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REVIEW

PD-1 as a potential target in cancer therapy

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Keywords
Cancer, immune tolerance, immunotherapy, nivolumab, programmed cell death-1 receptor, programmed cell death-1-ligand 1

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Funding Information
Supported in part by the DF/HCC Kidney Cancer SPORE, P50 CA101942-01. Professional medical writing and editorial assistance was provided by Caitlin Moira Wilke, PhD, at StemScientific and was funded by Bristol-Myers Squibb

Received: 14 May 2013; Revised: 31 May 2013; Accepted: 3 June 2013

Cancer Medicine 2013; 2(5): 662–673

doi: 10.1002/cam4.106

Abstract

Recently, an improved understanding of the molecular mechanisms governing the host response to tumors has led to the identification of checkpoint signaling pathways involved in limiting the anticancer immune response. One of the most critical checkpoint pathways responsible for mediating tumor-induced immune suppression is the programmed death-1 (PD-1) pathway, normally involved in promoting tolerance and preventing tissue damage in settings of chronic inflammation. Many human solid tumors express PD ligand 1 (PD-L1), and this is often associated with a worse prognosis. Tumor-infiltrating lymphocytes from patients with cancer typically express PD-1 and have impaired antitumor functionality. Proof-of-concept has come from several preclinical studies in which blockade of PD-1 or PD-L1 enhanced T-cell function and tumor cell lysis. Three monoclonal antibodies against PD-1, and one against PD-L1, have reported phase 1 data. All four agents have shown encouraging preliminary activity, and those that have been evaluated in larger patient populations appear to have encouraging safety profiles. Additional data are eagerly awaited. This review summarizes emerging clinical data and potential of PD-1 pathway–targeted antibodies in development. If subsequent investigations confirm the initial results, it is conceivable that agents blocking the PD-1/PD-L1 pathway will prove valuable additions to the growing armamentarium of targeted immunotherapeutic agents.

Introduction

Multiple immunotherapeutic approaches to cancer treatment have been evaluated over the past several decades. Although the results of many of these early efforts have been disappointing, the ability to produce durable remissions of solid tumors with high-dose interleukin-2 (HD IL-2), interferon-α, and vaccines has nevertheless provided evidence of immunotherapy's potential [1–3]. Recent data have provided a clearer understanding of the factors that limit an antitumor immune response, leading to the development of various agents targeting immune costimulatory and inhibitory ("checkpoint") pathways. One of the key checkpoint molecules that mediates tumor-induced immune suppression is programmed death-1 (PD-1).

Traditional costimulation is delivered by the signaling of antigen-presenting cell (APC) CD80/86 through T-cell CD28, the so-called "second signal" required for T-cell activation. In addition to CD28, other immune costimulatory molecules include inducible costimulator [4], CD137 (also known as 4-1BB), and OX40 [5]. Conversely, several negative regulatory checkpoint molecules function to prevent, or "check," overstimulation of immune responses and contribute to the maintenance of immune tolerance to self-antigens [6]. These molecules include cytotoxic T-lymphocyte antigen-4 (CTLA-4) as well as the PD-1 receptor and its ligands. CTLA-4 acts as a signal dampener that acts largely within the lymph nodes to regulate the magnitude of early activation of naïve and memory T cells. PD-1, by contrast, is induced on T cells after activation in response to inflammatory signals and limits T-cell function in various peripheral tissues, largely in the context of infection or tumor progression [7]. As the T-cell response builds, these negative regulatory molecules are induced, limiting the magnitude and duration of the response to prevent healthy tissue damage.

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Tumors are capable of exploiting the homeostatic mechanisms regulated by these checkpoint molecules. They can overwhelm the immune system via multiple strategies, including alterations in antigen expression, interference with T-cell priming, and a spectrum of effects referred to as "immune editing," whereby tumors manipulate their microenvironment during development to escape immune detection and eradication [8]. Limiting antitumor T-cell responses via exploitation of checkpoint pathways (such as those involving CTLA-4 or PD-1) serves to prevent significant tumor destruction and leads to an equilibrium between the tumor and immune system that typically progresses to tumor escape. New immunotherapies for cancer focus on shifting the balance from a pro-tumor to an antitumor microenvironment, thus allowing the immune system to mount an effective antitumor response; consequently, negative regulatory pathways are key targets. The anti–CTLA-4 monoclonal antibody (mAb) ipilimumab improved survival in a phase 3 trial in patients with metastatic melanoma (MEL) [9] and was subsequently approved by the United States Food and Drug Administration for the treatment of patients with advanced MEL. A recent report of an early-stage trial has provided preliminary evidence of activity of ipilimumab in patients with castrate-resistant prostate cancer (CRPC) [10]. The fully human anti–PD-1 mAb BMS-936558/MDX-1106/ONO-4538 (nivolumab), tested in renal cell cancer (RCC), MEL, CRPC, non–small cell lung cancer (NSCLC), and colorectal cancer (CRC), has demonstrated antitumor activity in phase I/II studies [11]. The humanized anti–PD-1 antibody MK-3945 (lambrolizumab) has also demonstrated antitumor activity in patients with solid cancers in a phase 1 study [12]. CT-011 (pidilizumab), a humanized anti–PD-1 antibody, has been evaluated in multiple hematologic malignancies, demonstrating potential clinical activity in patients with non-Hodgkin's lymphoma, chronic lymphocytic leukemia, Hodgkin's lymphoma, multiple myeloma, and acute myeloid leukemia [13]. Finally, the anti–PD ligand 1 (PD-L1) mAb BMS-936559 has shown preliminary antitumor activity in various solid cancers [14]. PD-1 pathway–targeted agents in development are summarized in Table 1.

This review examines the role of the PD-1 negative regulatory pathway in antitumor immune responses and outlines the rationale for targeting PD-1 and PD-L1 in the treatment of patients with cancer.

Role of the PD-1 Pathway in the Immune Response

PD-1 (CD279), a member of the B7-CD28 family [15], is a cell surface coinhibitory receptor expressed on T cells, B cells, monocytes, and natural killer T cells, following activation [16]. PD-1 has two known ligands: PD-L1 (B7-H1) [17] and PD-L2 (B7-DC) [18], which have distinct expression profiles. Both ligands are expressed on APCs, including dendritic cells (DCs); in addition, PD-L1, thought to be the principal mediator of PD-1–dependent immunosuppression [19], is expressed on some nonhematopoietic cells. Binding of PD-L1 or PD-L2 to PD-1 inhibits T-cell receptor signaling and downregulates the expression of certain antiapoptotic molecules (including B-cell lymphoma-extra large [Bcl-xL]) and proinflammatory cytokines [16]. The interaction of PD-1 with its ligands also affects the cell cycle, preventing progression through G1 phase by increasing expression levels of p15 and suppressing transcription of *SKP2*, a component of the ubiquitin ligase responsible for degrading p27 [20]. PD-L1 itself has also been observed to serve as an antiapoptotic factor on tumor cells [21]. Interestingly, PD-L1 expressed on APCs can also bind T-cell CD80, which curtails T-cell activation and cytokine production. The PD-1 pathway is an important regulator of the induction and maintenance of peripheral tolerance (and tolerance to malignant "self" cells within the tumor microenvironment) [22, 23], by upholding a balance between T-cell activation and the protection of healthy tissues from immune-mediated damage.

The critical role of the PD-1 pathway in blunting T-cell responses was first demonstrated by the various autoimmune phenotypes observed in PD-1 knockout mice [16]. In addition, PD-L1 expression on nonhematopoietic cells was shown to inhibit immune-mediated tissue damage [21, 24]. The PD-1/PD-L1 pathway also participates in

Table 1. PD-1 pathway–targeted agents in development.

Target	Name	Description	Sponsor	Phase
PD-1	Nivolumab	Fully human IgG4 monoclonal antibody	Bristol-Myers Squibb	3
	Lambrolizumab	Humanized IgG4 monoclonal antibody	Merck	3
	Pidilizumab	Humanized IgG1 monoclonal antibody	CureTech	2
	AMP-224	B7-DC/IgG1 fusion protein	GlaxoSmithKline/Amplimmune	1
PD-L1	BMS-936559	Fully human IgG4 monoclonal antibody	Bristol-Myers Squibb	1
	RG7446/MPDL3280A	Monoclonal antibody	Roche/Genentech	1
	MEDI4736	Monoclonal antibody	MedImmune	1

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the regulation of immunity to chronic infections. Persistent T-cell activation in the setting of chronic infection produces sustained, high-level PD-1 expression and results in functionally exhausted T cells. Such T cells are unable to proliferate and destroy invading microorganisms, thus allowing infections to persist; however, this inactivity of T cells also reduces collateral immune-related damage to host cells [25]. Cancers exploit multiple immunoregulatory pathways to evade elimination by infiltrating, activated tumor-specific T cells, including the production of immunosuppressive cytokines (transforming growth factor-β [TGF-β], interleukin-10 [IL-10]), the expression of immunosuppressive enzymes (indoleamine-2,3-dioxygenase), and the conversion of "normal" APCs and T cells to immunosuppressive cell populations (e.g., regulatory T cells) [26]. Similarly, tumor upregulation of PD-L1 expression, commonly induced by infiltrating T-cell release of interferon-γ (IFN-γ) [27], leads to inactivation of T cells expressing PD-1, further enabling tumor cell evasion of immune destruction (Fig. 1).

Role of the PD-1 Pathway in Cancer

Gajewski and colleagues have recently observed that certain tumors exhibit an "inflammatory" gene signature, suggesting the existence of an ongoing immune response; moreover, patients with "inflamed" tumors appear to respond better to immunotherapy. Intriguingly (and perhaps counter-intuitively), "inflamed" tumors contained higher expression levels of the immunosuppressive molecules PD-L1, FoxP3 (the transcription factor controlling regulatory T-cell [Treg] development) and indoleamine-2,3-dioxygenase (an enzyme critically involved in peripheral tolerance) [28]. The PD-1 pathway, therefore, may have a key role in the interaction of tumor cells with the host immune response, and tumor cell PD-L1 expression may serve as a mechanism of adaptive immune resistance.

Expression of PD-1 by tumor-infiltrating lymphocytes (TILs) is associated with impaired effector function (cytokine production and cytotoxic efficacy against tumor cells) and/or poor outcome in several tumor types [29–32]. Moreover, a variety of tumors, including RCC, MEL, as well as stomach, breast, ovarian, pancreatic, and lung cancers, have been shown to express PD-L1, potentially contributing to immune suppression and evasion [33]. Of note, PD-L1 expression on tumor cells has been shown to correlate with poor prognosis in patients with RCC (Fig. 2) [34], MEL, and breast, pancreatic, stomach, bladder, lung, liver, and ovarian cancers (Table 2) [33, 35–38]. However, a recent report has shown that not all

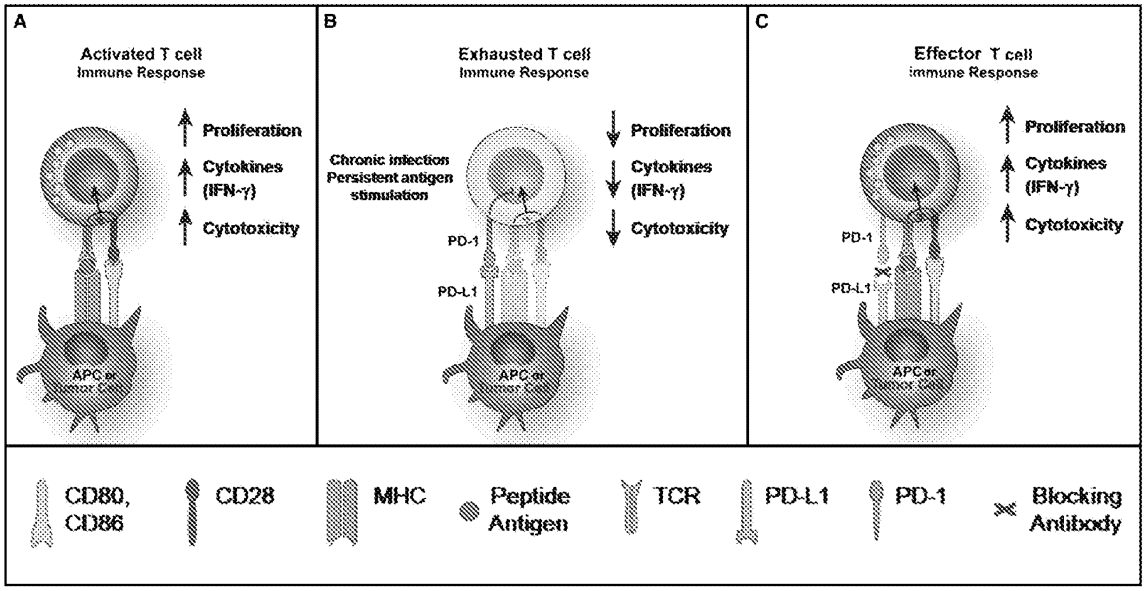


Figure 1. PD-1 in T-cell activation, exhaustion, and effector function. (A) T cells are activated via (1) binding of MHC plus peptide on an APC to the TCR and then (2) binding of APC CD80/86 to T-cell CD28. In patients with cancer, tumor cells can also serve as APCs. Upon T-cell activation, PD-1 expression is induced. (B) In situations of chronic infection or persistent stimulation, PD-L1 signals through T-cell PD-1 to "turn off" T cells in order to minimize damage to healthy tissue. Tumor cells can upregulate PD-L1 in order to "turn off" T cells that might destroy them. (C) Blocking the PD-1/PD-L1 signaling pathway allows T cells to maintain their effector functions. In patients with cancer, activated tumor-specific T cells can kill tumor cells and secrete cytokines that activate/recruit other immune cells to participate in the antitumor response. APC, antigen-presenting cell; IFN-γ, interferon gamma; MHC, major histocompatibility complex; PD-1, programmed death-1; PD-L1, PD ligand 1; TCR, T-cell receptor.

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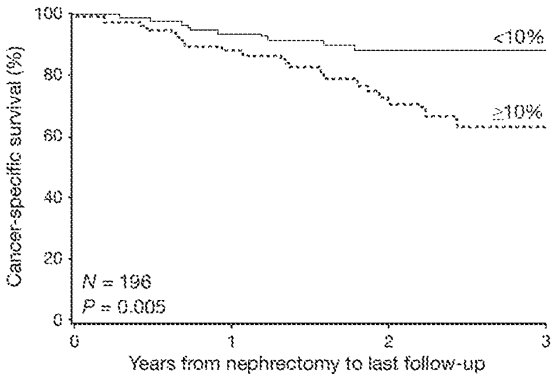


Figure 2. Increased PD-L1 expression (≥10% vs. <10%) diminishes survival in RCC. From Thompson et al. [34]. Copyright 2012 National Academy of Sciences, U.S.A. Reprinted with permission.

Table 2. PD-L1 expression and clinical outcomes in cancer.

Disease	PD-L1 expression associations
RCC	Poor prognosis [30]
NSCLC	Decreased survival [35]
MEL	Increased [24] or decreased [32] survival
Breast	Tumor size, stage, and HER2 expression [30]
Gastric	Tumor size, metastasis, and poor survival [30]
Ovarian	Poor prognosis [30]
Pancreatic	Decreased TILs and poor prognosis [30]
HCC (HBV related)	Poor postcryoablation prognosis[1] [33]
HCC	Tumor aggressiveness and recurrence after resection [34]

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MEL, melanoma; NSCLC, non–small cell lung cancer; RCC, renal cell cancer
[1]Associated with PD-L1 expression on circulating leukocytes.

tumor PD-L1 expression confers a worse prognosis [27]. In this study, patients with PD-L1$^+$ MEL survived significantly longer than patients with PD-L1$^-$ MEL, spurring investigators to hypothesize that TILs may actually prompt their own inhibition by secreting cytokines (such as IFN-γ) that drive tumor PD-L1 expression. However, because of the significant number of patients who received subsequent immune-based therapies, care must be taken interpreting these data. It is, however, possible that induction of the PD-L1/PD-1 pathway represents a mechanism of adaptive resistance exerted by tumors infiltrated by effector T cells, and is therefore a marker of an ongoing antitumor immune response. Similarly, PD-L2 has been observed to be upregulated in a subset of human tumors and has occasionally been linked to poor outcome [39]. Consequently, therapies that block the PD-1 pathway may unleash antitumor immunity and be particularly beneficial to patients with PD-L$^+$ tumors.

Ahmadzadeh and colleagues showed that PD-1 expression on TILs in MEL lesions was significantly higher than expression on T cells isolated from the peripheral blood (PB) or noncancerous tissue from the same patients or healthy donors [31]. In this study, PD-1$^+$ TILs had impaired effector function, as measured by IFN-γ secretion, indicating that PD-1 expression on TILs limits their capacity to mount an effective immune response. Patients with MEL also have higher levels of PD-1 expression on TILs than on PB lymphocytes [40]. In addition, blockade of the PD-1/PD-L1 pathway increased IFN-γ secretion by T cells in response to stimulation by antigen-loaded DCs. Overall, these findings suggest that inhibition of the PD-1/PD-L1 pathway can restore effector functions of exhausted T cells, which may translate into improved antitumor immune responses. Thus, the PD-1/PD-L1 pathway represents a logical target for cancer immunotherapy.

Preclinical Studies of PD-1 Blockade

Blockade of either PD-1 or its ligands has shown consistent immune-potentiating effects in a range of preclinical models. Antibodies against PD-1 or PD-L1 can enhance or restore T-cell effector function, including cytolytic activity against tumor cells [40–42]. Moreover, PD-L1 blockade promoted infiltration of tumor-reactive CD8+ T cells into established tumors in a mouse model of pancreatic cancer [43], while PD-L2 blockade decreased the numbers of tumor-infiltrating regulatory T cells [44]. PD-1 blockade inhibited the metastatic spread of MEL and colon cancer cells in mice [45]. Experiments in mice lacking PD-1 showed that hematogenous tumor spread was inhibited via several T-cell potentiating mechanisms, including enhanced induction of effector T cells in the spleen, augmented homing of these cells to tumor sites, and improved T-cell proliferation and cytotoxicity [45]. Several preclinical studies have shown that PD-1 or PD-L1 blockade improves the immune response to, and/or efficacy of, other immunotherapies, including anti–CTLA-4 mAbs [41–43, 46]. PD-L1 inhibition was also synergistic with chemotherapy in a mouse model of pancreatic cancer [43]. These observations have important implications for the development of potential combination treatment strategies for patients with cancer.

Clinical Studies Investigating PD-1/PD-L1 Blockade

Because of the varied negative signals resulting from interactions between PD-1/PD-L1/PD-L2 and PD-L1/CD80, there are opportunities to target this checkpoint pathway from two directions: PD-1 blockade inhibits

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negative signaling induced by PD-L1 and PD-L2 ligation, whereas PD-L1 blockade inhibits negative signaling via PD-1 and CD80. MAbs that target these interactions are being evaluated in clinical studies and hold promise as an important immunotherapeutic approach in various malignancies. Data from clinical trials targeting the interactions between PD-1 and its ligands have recently become available.

PD-1 Blockade with Anti–PD-1 Antibodies

Nivolumab

The first phase 1 trial of varying doses (0.3–10 mg/kg) of nivolumab, a fully human IgG4 anti–PD-1-blocking antibody, in 39 patients with advanced MEL, CRC, CRPC, NSCLC, or RCC was reported in 2010 [19]. One complete response (CR) (CRC 3 mg/kg) (21+ months at last follow-up) and two partial responses (PRs) (RCC and MEL [both 10 mg/kg]) were observed. No maximum tolerated dose (MTD) was identified. Most adverse events (AEs) reported for nivolumab were immune-related, consistent with the mechanism of action of anti–PD-1 antibodies. Flow cytometric analysis showed sustained occupancy of the majority of PD-1 molecules on circulating T cells isolated from patient plasma samples for ≥3 months following a single dose of nivolumab. In an expansion cohort at the 10-mg/kg dose level, responses were seen in patients with MEL and RCC without increased toxicity. One patient with RCC who had received several prior therapies achieved a PR that lasted 16+ months despite receiving only three nivolumab infusions. Additionally, two patients (NSCLC, MEL) achieved significant regression of metastatic lesions that did not meet PR criteria [19]. Follow-up of three patients from this study has demonstrated long-term responses after discontinuation of therapy [47].

The activity seen in the initial study prompted an exploration of a biweekly schedule of nivolumab in a separate phase 1 trial [11, 48]. Interim data from 304 patients with RCC, MEL, CRPC, NSCLC, or CRC who received 0.1 mg/kg to 10 mg/kg nivolumab every 2 weeks in 8-week cycles have recently been reported. Patients received treatment (≤12 cycles) until they experienced CR or progressive disease. Of 294 response-evaluable patients, there were objective responses (ORs) in 20/122 patients with NSCLC, 33/106 patients with MEL, and 10/34 patients with RCC. No ORs were noted in patients with CRPC or CRC. At last follow-up, 28 patients to date have had responses lasting ≥1 year. Stable disease (SD) lasting 24+ weeks was observed in an additional six evaluable patients with MEL, 11 with NSCLC, and nine with RCC. Additional patients with each disease experienced tumor responses to nivolumab according to immune-related response criteria; one pattern is typified by an initial increase and then decrease in tumor size (Fig. 3). Antitumor activity was observed at 1- to 10-mg/kg dose levels; some patients appeared to continue to exhibit tumor response beyond 96 weeks, the protocol-defined treatment stoppage point. The most common treatment-related AEs included fatigue, rash, nausea, diarrhea, decreased appetite, and pruritus. Grade 3/4 treatment-related AEs occurred in 15% of patients, and there were three deaths, all attributed to pulmonary toxicity. Drug-related AEs of special interest (AEOSIs, AEs with potentially immune-related etiology) included vitiligo, pneumonitis, hepatitis, colitis, thyroiditis, and hypophysitis. Should this preliminary efficacy and relatively favorable toxicity profile be confirmed in future trials, PD-1

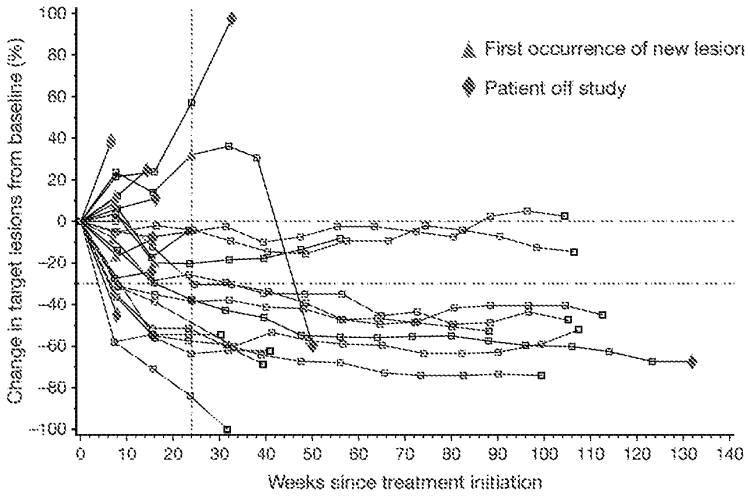


Figure 3. Durable responses in a cohort of patients with MEL treated with 1 mg/kg nivolumab. From Topalian et al. [11]. Copyright 2012 Massachusetts Medical Society. Reprinted with permission.

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pathway blockade may be an approach that can produce a durable benefit with fewer toxicities than with other immunotherapies (e.g., HD IL-2 or ipilimumab). Nivolumab is currently being evaluated in several ongoing clinical trials of patients with RCC, MEL, and various other malignancies (clinicaltrials.gov).

Pidilizumab

The humanized, anti–PD-1 mAb pidilizumab was evaluated in a phase 1 clinical trial of patients with advanced hematologic malignancies [13]. Seventeen patients received escalating doses of pidilizumab ranging from 0.2 to 6 mg/kg. Treatment with pidilizumab generally was well tolerated, and no MTD was defined. Only one patient reported toxicity, specifically weakness, and flushing, which were possibly treatment related. Clinical benefit was observed in 33% of patients. An apparent response to treatment was observed in six patients, including one CR, four SD, and one mixed response; the CR was maintained for ≥68 weeks. Sustained elevation in PB $CD4^+$ lymphocyte numbers was observed up to 21 days following treatment with pidilizumab [13]. The activity of pidilizumab in solid tumors is now being explored (clinicaltrials.gov).

Lambrolizumab

Patnaik and colleagues reported the results of a small, open-label dose-escalation study examining lambrolizumab, a humanized IgG4 mAb against PD-1 [12]. Cohorts of three to six patients with various advanced solid tumors were treated with 1–10 mg/kg initially and then additional doses every 2 weeks. No MTD was identified, and there were no grade ≥3 drug-related AEs; one patient developed pneumonitis, which was treated with corticosteroids. Two patients with MEL treated for 6+ months had PRs, and there was an unconfirmed PR in a patient with squamous NSCLC. In the MEL expansion cohort treated with 10 mg/kg, one unconfirmed CR, three unconfirmed PRs, and five grade 3/4 AEs were reported (NCT01295827) [12]. Additional interim data on 85 evaluable patients were recently presented at the Society for Melanoma Research Congress [49]. Forty-three patients (51%) experienced ORs, including eight CRs. 27 patients had previously been treated with ipilimumab and 11 of these (41%) had ORs. Seven treatment-related grade 3/4 AEs with potentially immune-mediated causes were reported. There were three instances of grade 1/2 pneumonitis; one patient died of myocardial infarction while being treated for pneumonitis/pneumonia. Common AEs included arthralgia, cough, diarrhea, fatigue, fever, nausea, pruritus, and rash.

Patterns of response to PD-1 blockade

While a subset of melanoma and lung cancer patients treated with PD-1-pathway–targeted agents have experienced encouraging ORs, fewer have experienced SD. It is possible that the response to PD-1 pathway blockade is "all or nothing." In comparison, while only a small percentage of patients treated with ipilimumab achieve ORs, this agent improved median OS in phase 3 trials, suggesting a clinical benefit in patients who did not meet response criteria [9, 50]. It is possible that PD-1-pathway–directed agents may impart a survival benefit only to those patients on the "tail" of the survival curve. Whether these agents will improve median OS in unselected patients will be determined by ongoing, randomized phase 3 trials.

It should be noted that select responding patients who discontinued PD-1 antibody therapy subsequently demonstrated disease progression, suggesting that the optimal duration of anti–PD-1 agent dosing may vary from patient to patient. However, some patients who progressed after discontinuation of anti–PD-1 therapy have demonstrated durable responses after retreatment [47]. The optimal duration of PD-1-pathway–targeted agent treatment is still being determined; if shorter treatment durations are proven efficacious, this approach would be more cost-effective. The ultimate benefit of immunotherapeutics (e.g., IL-2, ipilimumab) is their ability to produce remissions that are durable when therapy is discontinued. Although this has been observed in select patients treated with anti–PD-1 agents, the percentage of patients who achieve durable remissions remains to be determined. Nonetheless, the potential benefits for patients who experience "treatment-free survival" include decreased treatment-associated toxicity, improved quality of life, and decreased cost to the healthcare system. While this endpoint is not currently standard for approving therapies, its value to the patient merits consideration in future studies.

PD-1 Blockade with PD-1 Ligand-targeted Agents

BMS-936559

Interim data from a phase 1 trial (NCT00729664) of BMS-936559, an anti–PD-L1 mAb, have recently been disclosed [14]. As of 24 February 2012, 207 patients (with NSCLC, MEL, CRC, RCC, and ovarian, pancreatic, or breast cancer) had received BMS-936559 (at escalating doses ranging from 0.3 to 10 mg/kg) every 2 weeks in 6-week cycles. This continued for 16 cycles or until progressive disease or CR; the MTD was not identified. In

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the evaluable population, ORs were observed in patients with MEL (9/52), RCC (2/17), NSCLC (5/49), and ovarian cancer (1/17). In the 16 patients with ≥1 year of follow-up, responses in half lasted for ≥1 year. SD (≥24 weeks) was observed in 14 patients with MEL, six patients with NSCLC, three patients with ovarian cancer, and seven patients with RCC. Most AEs were low grade; the most common treatment-related AEs included fatigue, diarrhea, infusion reactions, rash, arthralgia, headache, nausea, and pruritus. Grade 3/4 treatment-related toxicities occurred in 9% of patients. Thirty-nine percent of patients experienced AEOSIs, including rash, hepatitis, hypothyroidism, and one case each of diabetes mellitus, endophthalmitis, sarcoidosis, and myasthenia gravis. The data collected to date demonstrate that BMS-936559 has a relatively good safety profile and can induce durable responses in patients with MEL, NSCLC, and RCC.

Other agents

Genentech and Roche are evaluating a mAb targeting PD-L1 (RG7446/MPDL3280A), and Amplimmune and GlaxoSmithKline have partnered in the development of a PD-L2/IgG1 fusion protein (AMP-224) that blocks PD-1 signaling. More recently, MedImmune announced that it would pursue development of the anti–PD-L1 mAb MEDI4736 [51]. All three of these compounds are currently in phase 1 testing (clinicaltrials.gov).

Targeting PD-1 versus PD-L1

Without a randomized, head-to-head trial of these drugs, no conclusive statements can be made regarding the comparative safety and activity of agents targeting PD-1 versus those targeting PD-L1. It appears, however, that targeting only PD-L1 may be accompanied by less toxicity, but also may be less effective than targeting PD-1 and thus blocking signaling via both PD-L1 and PD-L2.

Differences from anti–CTLA-4 antibodies

The anti–PD-1 data we currently have are from phase 1 trials, and no randomized, head-to-head studies of anti–PD-1 and anti–CTLA-4 antibodies have been completed. However, the existence of these agents in the same immunotherapeutic category will no doubt prompt comparisons. Both PD-1 and CTLA-4 are checkpoint molecules; however, they participate in different phases of the immune response. Murine experiments suggest that CTLA-4 is centrally involved in the development of tolerance and the prevention of autoimmunity, while the role of PD-1 is to prevent bystander tissue damage at sites of chronic infection and inflammation [52–54]. Additionally, given the body of data on PD-L1 and PD-1 as potential prognostic and predictive biomarkers, it seems that this pathway may be more important in the tumor microenvironment. Preclinical data [46] suggest that the combination of inhibitors of PD-1 and CTLA-4 may present a promising approach, which is currently being explored in the clinical setting [NCT01454102, NCT01024231].

Future Directions

Predictive biomarker development

The correlation between tumor PD-L1 expression and prognosis supports the hypothesis that this molecule may serve as a predictive biomarker. Although immune infiltration of antitumor lymphocytes leading to PD-L1 upregulation might establish a scenario predictive of benefit for any effective immunotherapy, this is particularly true of agents targeting the PD-1 pathway. As mentioned previously, expression of PD-1 by TILs is associated with poor prognosis in multiple cancers [29, 32]. In patients with RCC, the presence of PD-1$^+$ TILs was associated with more aggressive tumors and shortened survival [29]. In addition, patients with PD-1$^+$ TILs were more likely than patients without PD-1$^+$ TILs to have larger tumors, tumors of higher nuclear grade, advanced tumor node-metastasis stage, coagulative tumor necrosis, and sarcomatoid differentiation. In RCC and other cancers, tumor expression of PD-L1 is also associated with poor prognosis and more aggressive disease [33, 34]. However, the significance of PD-L1 expression in the context of active immunotherapy, particularly PD-1/PD-L1 pathway blockade, is less clear. Given that tumor PD-L1 expression is induced by T-cell expression of IFN-γ, it may signify an effort by the tumor to evade an ongoing immune response. Therefore, blockade of the PD-1/PD-L1 pathway with an anti–PD-1 or anti–PD-L1 antibody may enable the unmasking of antitumor immunity patients with PD-L1$^+$ tumors. Preliminary data suggesting that tumor PD-L1 expression could be a predictive biomarker of response to nivolumab [19] were later supported by an observation that 0/17 nivolumab-treated patients with PD-L1$^-$ tumors experienced ORs, whereas 9/25 patients with PD-L1$^+$ tumors (36%) achieved ORs ($P = 0.006$). If these data are confirmed, PD-L1 positivity may be a way to identify and enrich the population of patients who benefit from PD-1 pathway–targeted therapy. Further evaluation of PD-L1 as a potential predictive marker is warranted.

Opportunities for combination therapy

Even at this stage of PD-1–targeted agent development, it is important to consider opportunities for combination

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therapy. Given the observation that multiple factors can induce PD-L1 expression, it may be feasible to treat patients with these molecules (e.g., IFN-α, -β, and -γ [16]; cisplatin [55]) to induce PD-L1 expression in the tumor microenvironment and expand the population of patients who might benefit from treatment with PD-1-pathway–directed agents. In addition, tumors can evade detection and destruction by manipulating the immune system: downregulating costimulatory molecules on tumor cells, increasing expression of immunosuppressive molecules, and dysregulating T cells and APCs. Therefore, the implementation of rational, multiagent treatments that target distinct pathways may circumvent these tumor survival strategies.

Dual checkpoint blockade

The combination of CTLA-4 and PD-1 blockade may also enable more persistent immune activation while avoiding the major toxicities associated with HD IL-2. Hypothetically, combining two agents that target T-cell activation at different stages of the immune response will be a more potent anticancer treatment than therapy with each agent alone. Ipilimumab removes a physiological brake on T cells during activation, whereas anti–PD-1 removes a brake on activation during T-cell effector function. This combination may also overcome resistance to CTLA-4 blockade mediated by tumor PD-L1 expression or resistance to PD-1 blockade mediated by T-cell downregulation through the coexpression of CTLA-4. A phase 1 trial of nivolumab and ipilimumab in patients with MEL is ongoing (NCT01024231). Another potential checkpoint combination therapy might include blockade of PD-1 and LAG-3, a molecule also involved in the regulation of T-cell activation. Combination therapy in mice has shown impressive activity [56].

Targeted therapy combinations

The combination of agents that block immune downregulation with genetic or cell-targeted therapies may prove particularly effective in select patients. Following this line of thought, pidilizumab is being tested in combination with rituximab, an anti-CD20 antibody, in a phase 2 trial of patients with relapsed follicular lymphoma (NCT00904722). The combination of PD-1–targeted therapy with agents effective against genetic mutations may also demonstrate activity. Trials examining vemurafenib, an inhibitor of B-raf enzymes with the V600E mutation, and other BRAF and epidermal growth factor receptor–targeted agents, in combination with PD-1 pathway inhibitors are under way (NCT01454102, NCT01656642). Various chemotherapies are also being evaluated in combination with anti–PD-1 agents (NCT00890305, NCT01454102).

T-cell stimulating agent combinations

IL-2 is a cytokine that supports T-cell survival and proliferation. The combination of HD IL-2 and ipilimumab has demonstrated manageable toxicity and impressive efficacy (CR rate of 17%) in patients with advanced MEL [57]. Thus, it is conceivable that the combination of HD IL-2, to induce T-cell expansion, and PD-1 blockade, to eliminate tumor-induced immune suppression, might prove equally or more efficacious in select patients.

Vaccine combinations

The PD-1/PD-L1 pathway may play an important role in blunting immune response to tumor vaccines. Study findings suggest that stimulation with dendritic/tumor fusion cells increases T-cell expression of PD-1, which may blunt the host response to vaccination [58]. In ex vivo studies, stimulation of T cells with a DC/tumor fusion vaccine in the presence of PD-1 blockade resulted in increased cytokine production, decreased Tregs, and enhanced tumor killing [58]. As such, combining DC-based tumor vaccines with PD-1 blockade may be an effective means of enhancing immunologic and clinical response to vaccination. Interestingly, two trials evaluating the efficacy of nivolumab in combination with multipeptide melanoma vaccines are ongoing (NCT01176474, NCT01176461). Thus far, confirmed PRs have been reported in patients with unresectable melanoma at each of the treating dose levels (two at 1 mg/kg, four at 3 mg/kg, and one at 10 mg/kg), and the most common drug-related AEs are low-grade injection site reactions and nausea/vomiting [59]. Pidilizumab is being tested in combination with DC fusion vaccines in patients with multiple myeloma after stem cell transplantation (NCT01067287), patients with RCC (NCT01441765), and patients with acute myeloid leukemia (NCT01096602).

VEGFR tyrosine kinase inhibitor (TKI) combinations

The anti–CTLA-4 mAb tremelimumab administered with the vascular endothelial growth factor receptor (VEGFR) TKI sunitinib produced PRs in 9/21 evaluable patients with RCC, but was associated with acute renal toxicity, which the authors proposed might be immune related [60]. It is conceivable that combinations involving VEGFR TKIs with PD-1/PD-L1 pathway blockade may be better tolerated. Thus, an ongoing trial is assessing the safety and tolerability of nivolumab when administered

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with sunitinib or pazopanib in patients with RCC (NCT01472081) [61].

VEGF-targeted agent combinations

There is evidence that VEGF decreases DC function [62], and by extension, antigen presentation and T-cell activation. Concurrently restoring DC and T-cell functionality may improve the antitumor T-cell response and lead to improved clinical activity. As proof-of-concept, Hodi and colleagues have observed durable PRs in patients with MEL treated with a combination of ipilimumab and the anti–VEGF-A antibody bevacizumab [63]. Trials evaluating the combination of nivolumab (NCT01454102) or ipilimumab (NCT00790010) with bevacizumab are ongoing (clinicaltrials.gov).

Various benefits and drawbacks accompany the use of broad spectrum versus more specific VEGF inhibitors. Broad-spectrum agents are indeed pleiotropic and, among various "off-target effects" can impact immune system function. For instance, murine experiments have shown that sunitinib can decrease Treg populations [64]; other TKIs are associated with immune system-potentiating effects [65]. If the pleiotropic effects of a given VEGFR TKI have a net positive effect on the immune system and are not overwhelmingly detrimental to other physiological processes, it may indeed be worth exploring in combination with anti–PD-1-pathway–directed agents. In contrast, VEGF-targeted agents like bevacizumab and VEGF-trap are more specific in their inhibition, but are not typically accompanied by immune-potentiating effects. As high levels of VEGF are associated with impeded dendritic cell function, lowering environmental VEGF concentrations might benefit this aspect of the antitumor immune response. In recent phase 3 studies, bevacizumab administered in combination with IFNα was more effective than IFNα alone [66], suggesting that combination therapy of PD-1–directed agents and VEGF-binding antibodies merits future study.

Conclusions

Although first-generation immunotherapies were of limited efficacy, they provided proof-of-concept that, in some patients, the immune balance in the tumor could be shifted in favor of tumor elimination. Expanding knowledge and understanding of the immune system's role in cancer has revealed multiple mechanisms by which tumors evade immune destruction. In particular, negative regulatory pathways involved in the T-cell-mediated response, including interaction of PD-1 and PD-L1, are being investigated to determine their role in tumor development. Various solid tumors express PD-L1, often associated with poor prognosis, whereas TILs from these patients express PD-1 and have impaired antitumor functionality. Preclinical proof-of-concept for PD-1/PD-L1 blockade has been demonstrated by many studies: improvements in various antitumor T-cell functions have been observed. Several PD-1 and PD-L1-blocking mAbs have now completed early clinical development with encouraging activity and safety. The potential for these agents in the treatment of patients with advanced cancers, including their incorporation into combination regimens (e.g., PD-1- plus CTLA-4-blocking antibodies), is significant, and further data are eagerly awaited.

Acknowledgments

Supported in part by the DF/HCC Kidney Cancer SPORE: P50 CA101942-01. Professional medical writing and editorial assistance was provided by Cailin Moira Wilke, PhD, at StemScientific and was funded by Bristol-Myers Squibb.

Conflict of Interest

Dr. Atkins has served as a consultant for BMS, Prometheus, and Merck; he also is a member of the Scientific Advisory Board for CoStim Pharmaceuticals Inc., and eCam Biotherapeutics. Dr. McDermott has participated in a BMS Advisory Board and has served as a consultant for BMS, Genentech, Prometheus, Pfizer, and Curetech.

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Colorectal Cancer—Patient Version

Health Professional Version

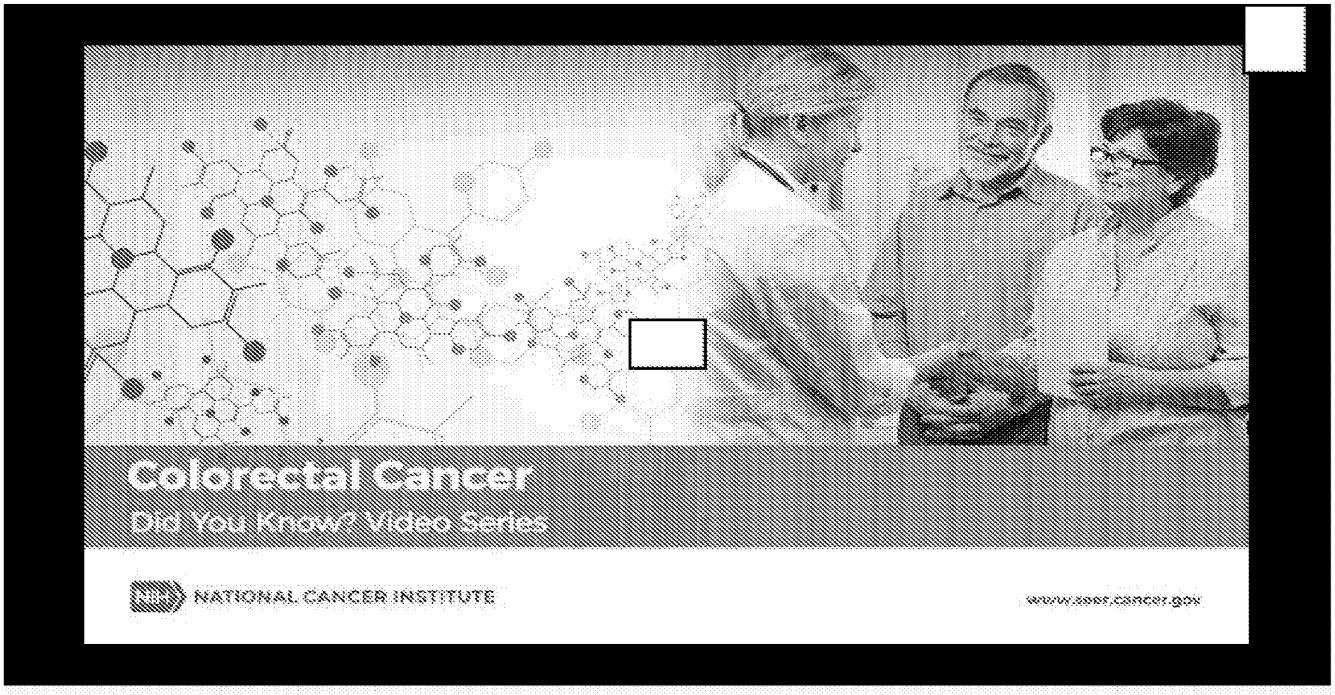
OVERVIEW

Colorectal cancer is cancer that starts in the colon or rectum. The colon and the rectum are parts of the large intestine, which is the lower part of the body's digestive system. During digestion, food moves through the stomach and small intestine into the colon. The colon absorbs water and nutrients from the food and stores waste matter (stool). Stool moves from the colon into the rectum before it leaves the body.

Most colorectal cancers are adenocarcinomas (cancers that begin in cells that make and release mucus and other fluids). Colorectal cancer often begins as a growth called a polyp, which may form on the inner wall of the colon or rectum. Some polyps become cancer over time. Finding and removing polyps can prevent colorectal cancer.

Colorectal cancer is the third most common type of cancer in men and women in the United States. Deaths from colorectal cancer have decreased with the use of colonoscopies and fecal occult blood tests, which check for blood in the stool.

Colorectal Cancer Statistics | Did You Know?



Key statistics about colorectal cancer from the SEER Cancer Statistics Review, 1975-2010.

TREATMENT

PDQ Treatment Information for Patients

- Colon Cancer Treatment
- Rectal Cancer Treatment

More Information

- Unusual Cancers of Childhood Treatment (PDQ®)
- Late Effects of Treatment for Childhood Cancer (PDQ®)
- Drugs Approved for Colon and Rectal Cancer
- Clinical Trials to Treat Colon Cancer
- Clinical Trials to Treat Rectal Cancer

RESEARCH

- Immunotherapy Targets Common Cancer Mutation
- Colorectal Cancer Survival Linked to Primary Tumor Location
- Survival Disparities Identified in Young African Americans with Colorectal Cancer

- Drug Combo Shrinks Duodenal Polyps in FAP

View more research

CAUSES & PREVENTION

PDQ Prevention Information for Patients

- Colorectal Cancer Prevention

More Information

- Obesity
- Clinical Trials to Prevent Colon Cancer
- Clinical Trials to Prevent Rectal Cancer

SCREENING

PDQ Screening Information for Patients

- Colorectal Cancer Screening

More Information

- Tests to Detect Colorectal Cancer and Polyps
- Clinical Trials to Screen for Colorectal Cancer

STATISTICS

Colorectal cancer statistics based on data from large groups of patients to be used as a general guide.

GENERAL RESOURCES ON COPING

Supportive information on managing the physical and emotional effects of cancer and treatment for both patients and caregivers.

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Cancer of Unknown Primary (See Occult Primary)

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Cervical Cancer

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Chronic Myeloid Leukemia

Colon/Rectal Cancer

- Colon Cancer
- Rectal Cancer

Cutaneous Melanoma (See Melanoma)

Endometrial Cancer (See Uterine Neoplasms)

Esophageal and Esophagogastric Junction Cancers

Fallopian Tube Cancer (See Ovarian Cancer)

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Malignant Pleural Mesothelioma

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- Melanoma (cutaneous)
- Uveal Melanoma NEW

Multiple Myeloma/Other Plasma Cell Neoplasms

- Multiple Myeloma
- Systemic Light Chain Amyloidosis
- Waldenström's Macroglobulinemia / Lymphoplasmacytic Lymphoma

Myelodysplastic Syndromes

Myeloproliferative Neoplasms

Neuroendocrine and Adrenal Tumors

Non-Hodgkin's Lymphomas

- Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (See Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma)
- B-Cell Lymphomas
- Hairy Cell Leukemia (See Hairy Cell Leukemia)
- Primary Cutaneous B-Cell Lymphomas
- T-Cell Lymphomas

Non-Melanoma Skin Cancers

- Basal Cell Skin Cancer
- Dermatofibrosarcoma Protuberans
- Merkel Cell Carcinoma
- Squamous Cell Skin Cancer

Non-Small Cell Lung Cancer

Occult Primary

Ovarian Cancer

Pancreatic Adenocarcinoma

Penile Cancer

Primary Peritoneal Cancer (See Ovarian Cancer)

Prostate Cancer

Small Cell Lung Cancer

Soft Tissue Sarcoma

Systemic Mastocytosis NEW

Testicular Cancer

Thymomas and Thymic Carcinomas

Thyroid Carcinoma

Uterine Neoplasms

Vulvar Cancer

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- Breast Cancer Risk Reduction
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- Colorectal Cancer Screening
- Genetic/Familial High-Risk Assessment: Colorectal

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Prostate Cancer Early Detection

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Cancer- and Chemotherapy-Induced Anemia

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Cancer in People Living with HIV NEW

Older Adult Oncology

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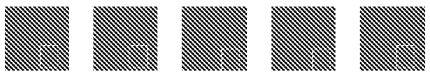
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Browse: A B C D E F G H I J K L M N O P Q R S T U V W X Y Z # All

anti-PD-1 fusion protein AMP-224

A recombinant B7-DC Fc-fusion protein composed of the extracellular domain of the PD-1 ligand

programmed cell death ligand 2 (PD-L2, B7-DC) and the Fc region of human immunoglobulin (Ig) G1, with potential immune checkpoint inhibitory and antineoplastic activities. Anti-PD-1 fusion protein AMP-224 specifically binds to PD-1 on chronically stimulated T-cells and reduces their proliferation. This may restore immune function and may result in the activation of cytotoxic T-cells and cell-mediated immune responses against tumor cells. PD-1, a transmembrane protein of Ig superfamily and inhibitor receptor expressed on activated T-cells, negatively regulates T-cell activation and effector function when activated by its ligands, and plays an important role in tumor evasion from host immunity. AMP-224 does not bind normal activated T-cells. Check for active clinical trials using this agent. (NCI Thesaurus)

Code name:	AMP-224

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anti-PD-1 monoclonal antibody MEDI0680

A humanized immunoglobulin (Ig) G4 monoclonal antibody directed against the negative immunoregulatory

human cell surface receptor programmed cell death 1 (PD-1), with potential immune checkpoint inhibitory and antineoplastic activities. Upon administration, anti-PD-1 monoclonal antibody MEDI0680 binds to and inhibits PD-1 and its downstream signaling pathways. This may restore immune function through the activation of T-cells and cell-mediated immune responses against tumor cells. PD-1, a transmembrane protein in the Ig superfamily expressed on T cells, functions as an immune checkpoint that negatively regulates T-cell activation and effector function when activated by its ligands programmed cell death ligand 1 (PD-L1) or 2 (PD-L2); it plays an important role in tumor evasion from host immunity. Check for active clinical trials using this agent. (NCI Thesaurus)

Synonym:	antprogrammed cell death 1 monoclonal antibody AMP-514
Code name:	AMP-514 MEDI0680

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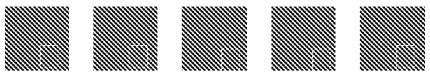
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pembrolizumab Patient Information

A humanized monoclonal immunoglobulin (Ig) G4 antibody directed against human cell surface receptor

PD-1 (programmed death-1 or programmed cell death-1) with potential immune checkpoint inhibitory and antineoplastic activities. Upon administration, pembrolizumab binds to PD-1, an inhibitory signaling receptor expressed on the surface of activated T cells, and blocks the binding to and activation of PD-1 by its ligands, which results in the activation of T-cell-mediated immune responses against tumor cells. The ligands for PD-1 include programmed cell death ligand 1 (PD-L1), overexpressed on certain cancer cells, and programmed cell death ligand 2 (PD-L2), which is primarily expressed on APCs. Activated PD-1 negatively regulates T-cell activation and plays a key role in in tumor evasion from host immunity. Check for active clinical trials using this agent. (NCI Thesaurus)

Synonym:	anti-PD-1 monoclonal antibody MK-3475 humanized mouse monoclonal (228-L-proline(H10-S>P))gamma 4 heavy chain (134-218')-disulfide with humanized mouse monoclonal kappa light chain dimer (226-226'':229-229'')-bisdisulfide immunoglobulin G4, anti-(human programmed cell death 1)
US brand name:	Keytruda
Code name:	MK-3475 SCH 900475

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Disease Markers 20 (2004) 199–206
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Colorectal carcinogenesis: MSI-H versus MSI-L

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Abstract. Microsatellite instability (MSI) is a well-recognized phenomenon that is classically a feature of tumors in the hereditary non-polyposis colorectal syndrome. Ten to 15% of sporadic colorectal cancers, however, will have MSI. Microsatellite unstable tumors can be divided into two distinct MSI phenotypes: MSI-high (MSI-H) and MSI-low (MSI-L). MSI sporadic colorectal cancers with a high level of MSI (MSI-H) form a well defined group with distinct clinicopathologic features characterized by an overall better long-term prognosis. These sporadic MSI-H colorectal tumors most often arise from the epigenetic silencing of the mismatch repair gene MLH1. In contrast, MSI-L colorectal tumors have not been shown to differ in their clinicopathologic features or in most molecular features from microsatellite stable (MSS) tumors. Unlike MSI-H tumors, MSI-L tumors appear to arise through the chromosomal instability carcinogenesis pathway, similar to MSS tumors. Some groups have reported more frequent mutations in K-ras and in the methylation of methylguanine transferase in MSI-L tumors, but others have questioned these findings. Therefore, although the use of the MSI-L category is widespread, there continues to be some debate as to whether a discrete MSI-L group truly exists. Rather, it has been suggested that MSI-L tumors differ quantitatively from MSS tumors but do not differ qualitatively. Future studies will need to evaluate the specific mutations in non-MSI-H tumors in an attempt to sub-classify MSI-L tumors with regard to MSS tumors so that subtle differences between these two sub-groups can be identified.

1. Introduction

Colorectal cancer is both the third most common type of cancer, as well as the third leading cause of cancer-related deaths in North America [1]. In 2003, an estimated 145,000 new cases of colorectal cancer will be diagnosed with an associated 58,000 colorectal deaths [1]. The malignant transformation associated with colorectal carcinogenesis involves the stepwise accumulation of multiple genetic alterations that allow the growth of neoplastic cells [2–4]. Currently, adenocarinomas of the colon and rectum are believed to arise from two distinct molecular genetic pathways: one involving chromosomal instability (CIN) and the other involving microsatellite instability (MSI) [5–8]. CIN results from a series of genetic changes that involve the activation of proto-oncogenes such as K-ras, and inactivation of tumor-suppressor genes, such as APC, TP53, DCC, SMAD2, SMAD4 [2,9–12]. CIN tumors are characterized by a high frequency of allelic imbalance (most commonly involving chromosomal arms 5q, 8p, 17p, and 18q), chromosomal amplifications, and translocations [2]. Seventy-five to 80% of sporadic cases of colorectal carcinoma arise through the CIN pathway [9,10].

The second pathway of colorectal carcinogenesis is the MSI pathway. In the MSI mutational pathway, colorectal cancers display increased rates of intragenic mutations of short, tandemly repeated DNA sequences known as microsatellites [13–15]. The phenomenon of MSI results from inactivation, mutational and/or epigenetic silencing of mismatch repair (MMR) genes. The primary function of the postreplicative MMR is to eliminate base-base mismatches and insertion/deletion loops that arise as a consequence of DNA polymerase slippage during DNA synthesis [10, 16,17]. Mutation rates in tumor cells with MMR deficiency are 100- to 1,000-fold more common as com-

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pared to normal cells [18,19]. The vast majority of hereditary non-polyposis colorectal cancer (HNPCC) patients will have microsatellite high (MSI-H) tumors. HNPCC is an autosomal-dominant condition that accounts for approximately 2–3% of all colorectal carcinomas. In most cases, the genetic basis for instability in MSI tumors is an inherited germline alteration in any one of the five human MMR genes: MSH2, MLH1, MSH6, PMS2, and PMS1 [20–23]. More specifically, germline mutations in MSH2 and MLH1 are responsible for most HNPCC families, while MSH6 is less common and PMS2 and PMS1 are rare [20,22].

MSI-H is also seen in 10–15% of sporadic non-HNPCC colorectal cancers. In patients with MSI-H sporadic colorectal cancer, the basis for instability is usually an acquired hypermethylation of the MLH1 promoter with subsequent transcriptional silencing [24–28]. MSI-H sporadic colorectal cancers do not exhibit gross cytogenetic abnormalities, infrequently display allelic losses at tumor suppressor loci, and are usually not aneuploid [13,29–31]. Instead, MSI-H sporadic colorectal tumors accumulate mutations at microsatellite sequences in coding regions of specific genes that are implicated in tumor progression, such as TGFβRII, IGFIIR, MSH3, MSH6, and BAX [32–35].

2. Microsatellite Instability: High versus low

Criteria and guidelines used to define MSI have evolved and, to some degree, remain somewhat elusive. MSI was initially described in independent publications from three groups in 1993, each who used unique descriptors for their findings [13,15,36]. Since that time, there has been a concerted effort to develop international criteria for the determination of MSI in colorectal cancer [37]. Despite this, the number, type, and identity of the microsatellites which should be used for MSI assessment remain unclear and the criteria for scoring MSI continue to vary among studies [38]. There is, however, a consensus that some markers, including mononucleotide repeats such as BAT25, BAT26, and BAT40 and the dinucleotide repeats D5S346, D2S123, and D17S250, are particularly useful for the identification of MSI tumors [39–42].

More recently, several studies have shown that colorectal cancers demonstrating the MSI phenomenon can be divided into two distinct MSI tumor phenotypes: MSI-high (MSH-H) and MSI-low (MSI-L). This division depends on the proportion of markers showing MSI and on the type of microsatellite marker that is utilized. In the MSI-H group, the majority of tested markers exhibit instability, whereas in the MSI-L group only a minority of markers show evidence of MSI. The distinction between these two groups is highly dependent on both the type and the number of microsatellites analyzed. For example, dinucleotide markers demonstrate both MSI-H and MSI-L, whereas mononucleotide markers such as BAT26 and BAT40 are relatively specific for MSI-H cancers [42,43]. Based on this, some groups have distinguished between the type of tandem repeat as a reliable means of defining MSI-L, or have used specific markers, such as the complex repeat MYCL, which is believed to be more specific for MSI-L tumors [42,44,45]. With regard to the number of markers demonstrating instability, most groups have defined MSI-H as those tumors with more than about 30–40% of markers unstable and MSI-L as a level of instability lower than this threshold [37,38,42,46].

Compared to MSI-L or microsatellite stable (MSS) tumors, MSI-H tumors have a distinct clinicopathologic phenotype [47–49]. MSI-H tumors tend to be poorly differentiated, right-sided, mucinous, have an expansile growth pattern, histologic heterogeneity, and increased tumor infiltrating lymphocytes with a prominent inflammatory reaction [5,50–53]. In addition, the carcinogenic pathway underlying MSI-H tumors (MMR repair defect) is believed to be distinct from the CIN pathway underlying non-MIS-H cancer. Based on these phenotypic as well as genetic differences, the distinction between MSI-H and non-MSI-H tumors has come to be universally accepted. In contrast, the MSI-L phenotype is not associated with distinct clinicopathological features or altered MLH1 or MSH2 expression and the biological basis of the MSI-L phenotype remains uncertain [28,37,42,44,54]. Given this, considerable controversy and debate remains around the validity and clinical utility of separating MSI-L tumors from MSS tumors.

3. MSI-L: A distinct sub-group of sporadic colorectal carcinoma

Some authors have argued that although MSI-L cancers may overlap more with MSS than MSI-H tumors, MSI-L still represents a distinct intermediate subset of colorectal cancers [43,55]. Although the MSI-L subset may not show distinct clinical, morphological, or molecular differences from the remaining MSS cancers (at least not to the degree seen between MSI-H and

non-MSI-H tumors), there are certain molecular features that are distributed in a nonrandom basis within the non-MSI-H subset of colorectal cancers [56]. In order to establish MSI-L as a distinct subset of non-MSI-H cancers, these molecular features would need to be identified in a group of tumors (i.e. MSI-L tumors) at a rate more than would be expected by chance. Jass and others have argued that when a panel of relatively sensitive markers is used, approximately 8% of sporadic colorectal cancers can indeed be designated as MSI-L [55,56]. Futhermore, some markers (D2S123, MYCL) are mutated at a much higher rate outside the MSI-H subset [57]. It is also evident that some colorectal cancers show more extensive MSI-L than would be expected by chance while others are "superstable" [58]. Therefore, nonrandomly distributed features among non-MSI-H cancers – including chromosomal alterations, DNA methylation, and the K-ras mutation – are hard to simply explain away by claiming that they are due to "more cell divisions" [55,59–62].

Kambara et al. examined the molecular events in early colorectal cancer with regard to MSI [60]. These investigators noted a significantly higher frequency of the low-level MSI (MSI-L) phenotype in early colorectal cancers (51.0%) compared with advanced colorectal cancers (25.9%; $p = 0.0001$) [60]. In early colorectal cancers, tumors with MSI-L phenotype were significantly different from MSS tumors with respect to loss of heterozygosity (LOH) at 1p32 (48.6% versus 18.9%) and 8p12-22 (71.1% versus 39.0%). In advanced colorectal cancers, the frequency of LOH at 1p32, 2p16, 7q31, 8p12-22, and 17q11 was significantly higher in MSI-L than that in MSS cancers. In addition, a high frequency of cancers with MSI-L phenotype was observed in early colorectal cancers (51.0%) compared with advanced lesions (25.9%) [60]. K-ras mutation was also more frequently observed in advanced MSI-L colorectal cancers, whereas no such correlation was observed in early colorectal tumors with MSI-L [60]. Others have corroborated this finding of more frequent K-ras mutations in MSI-L as compared to MSS tumors [55,59]. In each of 3 independent studies, K-ras mutation was found to be significantly more frequent in MSI-L (41% to 50%) versus MSS cancers (18% to 30%) [55,59,60]. The high frequency of K-ras mutation appears to occur in non-MSI-H cancers with the CpG island methylator phenotype (CIMP) and be associated with methylation of the DNA repair gene methylguanine transferase (MGMT) [61,63]. Whitehall et al. has reported that methylation of MGMT is associated with MSI-L colorectal cancers with instability at the D2S123 marker [44]. Loss of expression of the MGMT gene may represent the underlying molecular mechanism for MSI-L tumors [44]. Jass et al. has postulated that inactivation of MGMT may result in excessive production of methylG:T mismatches which would increase the probability of errors during DNA resynthesis that escape the mismatch repair system [56].

Based on the common features of MSI-L, K-ras mutation and serrated morphology, Jass and others have suggested that the family of serrated polyps (i.e. serrated adenomas, mixed polyps, and hyperplastic polyps) and MSI-L colorectal cancer might be related histogenetically [43,64]. Given that the adenoma-carcinoma sequence in sporadic MSI-H colorectal cancer is found only rarely and that MSI in adenomas occurs mainly at a low level (MSI-L), Jass and co-workers have postulated an alternative colorectal tumorigenesis pathway termed the "serrated pathway" [64–67]. It has been observed that MSI-L status is frequent in serrated polyps showing dysplasia, but not within traditional adenomas [64]. Due to the presence of MSI-L in some hyperplastic polyps and the lack of sporadic MSI-H adenomas, Jass has suggested that many sporadic MSI-H cancers may arise along an accelerated "serrated" pathway: hyperplastic polyp to serrated adenoma to cancer. All serrated or hyperplastic polyps, however, do not appear to be at the same risk of malignant transformation. Rather, a heterogeneity in hyperplastic polyps may exist, whereby left sided and distal hyperplastic polyps may continue to be viewed as innocuous, while proximal lesions need to be considered at increased risk for colorectal carcinogenesis [43,68].

In the model proposed by Jass, proximal serrated polyps may progress to MSI-H colorectal cancer through the inactivation of HPP1 in a background of disordered methylation [43]. The precise role of HPP1 is unknown, but it appears to be involved in the regulation of TGFβ, which is known to be involved in regulating growth, differentiation and cell migration [69, 70]. Those hyperplastic polyps with the MSI-L phenotype are thereby "primed" for the subsequent loss of MLH1 expression and the high mutator pathway [43]. Methylation of the DNA mismatch repair gene MLH1 would result in the MSI-H phenotype with progression to malignancy occurring rapidly through the acquisition of DNA instability [43,68]. This, in part, could explain why a paucity of MSI-H adenomas are clinically encountered.

Recently, a high frequency of BRAF mutation has been documented in serrated polyps, including both serrated adenomas and hyperplastic polyps [71]. BRAF

is one of three RAF genes that encodes a cytoplasmic serine/threonine kinase which is regulated by binding to RAS and can subsequently lead to malignant transformation. Mutation of BRAF is very infrequent in conventional adenomas. BRAF mutation, however, is known to be associated with MSI-H colorectal cancer and more recently this association has been narrowed done exclusively to the sporadic MSI-H subset [72, 73]. Since serrated polyps and sporadic MSI-H colorectal cancer are the principal colorectal lesions that have BRAF mutation, the link between the two seems certain. The high incidence of BRAF mutations in hyperplastic polyps and serrated adenomas is consistent with the notion that the majority of colorectal cancers carrying BRAF mutations have progressed through the serrated carcinoma pathway [71]. BRAF mutation is also found in the non-MSI-H subset of colorectal cancers with extensive DNA methylation, though not to the same extent as the sporadic MSI-H subset (personal correspondence). This suggests that serrated polyps may be the precursors of CIMP colorectal cancers that may be non-MSI-H as well as MSI-H.

BRAF is immediately downstream of K-ras, which is also associated with serrated polyps and CIMP colorectal cancers [74]. BRAF and K-ras mutations, however, are mutually exclusive [72]. It seems that mutation of one or the other of these closely related oncogenes is associated with CIMP status and can perhaps synergize with a gene (or genes) that are silenced by methylation. This may provide a mechanism for the early initiation of the serrated pathway. The fact that the pathway can bifurcate does not necessarily diminish its importance, but rather may increase its significance in terms of its potential overall contribution to tumorigenesis.

Recently, Whitehall et al. examined a small series of heavily methylated non-MSI-H colorectal cancers and showed that most of these colorectal cancers were MSI-L [75]. MSI-L status, however, does not appear to be restricted to the non-MSI-H CIMP-high subset of colorectal cancers. Rather, colorectal cancers with the MSI-L phenotype are more heterogeneous than the MSI-H group. In other words, some may be classified with the sporadic MSI-H subset (e.g. showing extensive methylation, either BRAF or K-ras mutation, and an origin in serrated polyps), whereas other will not. Being a small and heterogeneous subset, it will only be possible to characterize fully MSI-L colorectal cancers by applying a multilayered and meticulous approach to a very large series of cases.

One such novel approach comes from recent data derived from the use of the cDNA microarray, which supports the use of MSI-L as a natural sub-grouping of colorectal cancers [76]. To evaluate the impact of MSI status on global molecular phenotypes and to determine the importance of MSI relative to other molecular and clinical features, Mori et al. examined 41 colon cancers using cDNA microarray [76]. Interestingly, MSI-L cancers were clearly distinguishable from non-MSI-L tumors by principle component 10 (the "MSI-L separator"). Of note, several genes identified on the basis of high loading values in the MSI-L separator component have previously been implicated in the biology of MSI-L tumors. For example, the loading value of the DNA repair gene MGMT in component 10 was negative, consistent with decreased expression of MGMT in MSI-L tumors due to promoter hypermethylation [44, 76]. Another relevant gene was selenoprotein P, which has previously been postulated as a possible cause of disrupted DNA MMR in MSI-L tumors [77]. This data, therefore, appears to substantiate the distinction between MSS and MSI-L tumors based on molecular phenotypes. Of note, the impact of the MSI-L distinction was more subtle than that of the MSI-H on molecular phenotype, which corresponds to the known clinicopathologic similarities between MSS and MSI-L tumors [28,78].

4. Continuing questions and controversies

In spite of the reports noted above, there is still controversy over whether MSI-L tumors exist as a distinct sub-group of colorectal cancer. Some authors have argued that because microsatellite repeat slippage occurs about every 1,000 to 10,000 divisions in normal cells, the clonal nature of cancers would make this slippage detectable and therefore account for at least some MSI-L tumors [58]. That is, given the use of enough makers, it may be that all colorectal cancers will exhibit some level of MSI [37]. In two independent studies examining sporadic colorectal carcinomas, 68% and 79% of the non-MSI-H tumors exhibited some MSI and therefore could nominally be classified as MSI-L [57,58]. In the study by Halford et al., however, no evidence for a discrete MSI-L group was identified. No association was found between MSI-L (or the level of MSI) and any clinicopathological or molecular variable, including K-ras mutation and LOH at APC [57]. A recent meta-analysis of those studies investigating the relation of K-ras mutation and MSI-L similarly failed to show a significantly higher frequency of K-ras in MSI-L cancers. Rather a significant heterogeneity was

found in the frequency of K-ras mutations in MSI-L and MSS tumors, presumably some of which was due to the different methods used to assess MSI in each group [38]. Critics of the MSI-L sub-classification suggest that the greater variation in the non-MSI-H group may be more due to MSI-L cancers being quantitatively different from MSS tumors in some way (i.e. larger number of cell divisions, somatic mutational load, or germ-line genotype), rather than there actually being a true qualitative distinction from MSS cancers [38,58]. In this regard, Gonzalez-Garcia et al. specifically considered whether the frequency of MSI in the cancers they examined better fit a bimodal (MSI positive, MSS) or trimodal (MSI-H, MSI-L, and MSS) distribution and found no evidence for a separate category for MSI-L cancers [38,79].

Finally, some authors have questioned the validity of the "serrated adenoma pathway" and the role of MSI-L in sporadic colorectal carcinogenesis. Although a "serrated pathway" of colorectal carcinogenesis may exist, critics of MSI-L's role in tumorigenesis suggest that an alternative explanation for the reason that MSI-L is found very rarely in sporadic, non-serrated adenomas is that MSI-H do develop from classical adenomas, but that MSI is not acquired until after malignant progression [38]. In support of this, Sawyer et al. analyzed 39 serrated adenomas from 27 patients to define the molecular characteristics of serrated adenomas of the colorectum [80]. MSI was found in $< 5\%$ of cases and there was no loss of expression of MMR proteins. Instead, a variety of genetic defects including APC mutation/LOH, β-catenin mutation and CRAC1 LOH were found in a number of serrated adenomas. Given these findings, the authors concluded that no "serrated pathway" per se existed, but rather the pathway of colorectal tumorigenesis for serrated adenomas appeared to be heterogeneous and could not be accounted for by changes in known genes [80].

5. MSI status and prognostic implications

It is clear that MSI status has prognostic implications. Retrospective and population-based studies have been published confirming an improved survival in patients with MSI-H versus non-MSI-H colorectal tumors. In the Ontario population-based study, a series of 607 patients were diagnosed with colorectal cancer at age 50 or younger. Multivariate analysis revealed that MSI-H was an independent prognostic factor, along with stage at diagnosis, histology of tumor, and tumor grade [46]. Furthermore, regardless of the depth of tumor invasion, colorectal cancers with high-frequency MSI had a decreased likelihood of metastasizing to regional lymph nodes or distant organs [46]. Based on the findings of this study, high-frequency MSI in colorectal cancer appears to be independently predictive of a relatively favorable outcome [46]. Similarly, in the Utah and Northern California population-based study of 1,026 patients, there was a relationship between MSI-H and improved prognosis independent of stage, site, tumor grade, and age [81]. In fact, the authors reported that there was a 60% decrease in colorectal-related deaths in patients with MSI-H tumors. Most of the risk reduction occurred in Stage III patients whose tumors behaved as Stage II if the tumor was MSI-H [81]. A retrospective analysis of 460 patients with Stage III colorectal cancer, enrolled in five national trials of adjuvant 5-fluorouracil (FU)-based chemotherapy, revealed that patients with MSI tumors and mutations in the type II receptor for TGF-β1 had a better prognosis [82]. It therefore appears that not all MSI-H tumors behave the same. In one study, mutation of the gene for the type II receptor for TGF-β1 in cancers with high levels of microsatellite instability had a more favorable outcome [81,82].

Even though the majority of the published data suggest that colorectal cancer patients with MSI-H tumors have an improved prognosis, there are still some conflicting reports. Investigators from Scotland recently reported that patients less than 30 years of age at diagnosis who had MSI-H colorectal cancer had a worse chance of survival than those patients whose tumors were MSS [83]. Younger patients had excess MSI tumors, mucinous tumors, and more advanced disease [83]. The relative risk of death associated with tumor MSI was 0.87 in the cohort of patients < 30 years of age as compared to 0.11 for the control cohort of older patients [83].

MSI status also appears to play a critical role in the responsiveness of colorectal tumors to certain types of chemotherapy [84]. Recently a study by Ribic et al. showed that fluorouracil-based adjuvant chemotherapy benefited patients with stage II or stage III colon cancer with MSS or MSI-L, but not those with tumors exhibiting MSI-H [84]. In the absence of adjuvant chemotherapy, the patients with tumors displaying high-frequency microsatellite instability had significantly longer overall survival than patients with tumors exhibiting microsatellite stability or low-frequency microsatellite instability. When the analysis was limited to the group receiving adjuvant chemotherapy, however, patients with MSI-H tumors did not have a significant increase in

overall survival as compared with patients with MSS or MSI-L tumors [84]. Therefore, it appeared that, opposed to the patients with non-MSI-H tumors, the MSI-H group did not benefit from the adjuvant chemotherapy.

Although there is a preponderance of data indicating a significant difference in the prognosis of MSI-H versus non-MSI-H tumors, currently there is no data to support a distinction between MSI-L and MSS cancers with regards to prognosis. Rather, tumors with low-frequency microsatellite instability have been found to have similar clinical and prognostic features to MSS tumors [46,53,79,84]. In multiple studies, a bimodal distribution of MSI (MSI-H versus non-MSI-H), rather than a trimodal distribution (MSI-H versus MSI-L versus MSS) has been shown to more accurately reflect the prognosis and natural history of colorectal tumors [46, 79,84].

6. Conclusion

MSI is a well-recognized and accepted phenomenon. The existence of MSI-H tumors as a unique subclassification of colorectal tumors has been unequivocally established both phenotypically and genetically. What remains to be determined is whether an intermediate classification of MSI-L tumors exists between those tumors classified as MSS and MSI-H. To date, the MSI-L subset has not shown any distinct clinical or morphological differences from the remaining MSS cancers. Given this, some may wonder why any debate around the existence of MSI-L status is even clinically relevant or warranted. MSI-L status is important insofar as MSI-L might serve as the by-product of a DNA repair defect. Identifying this defect, however subtle it may be, could hold potential importance as it may define a subgroup of tumors associated with a distinct natural history and an altered response to chemotherapeutic agents. Because of this, the possibility that a "mild mutator" pathway may be driven by a DNA repair defect in MSI-L tumors warrants additional scientific and clinical research. It is imperative that future prospective studies not only evaluate the role of MSI as a prognostic factor, but that these studies also examine specific mutations in an attempt to sub-classify MSI-L with regard to MSS tumors so that subtle differences in prognosis and natural history between these two sub-groups can be identified.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
3338055PC01		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2017/035822	2 June 2017 (02-06-2017)	3 June 2016 (03-06-2016)
Applicant		
BRISTOL-MYERS SQUIBB COMPANY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

Form PCT/ISA/210 (first sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/035822

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K39/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUNG T. LE ET AL: "PD-1 Blockade in Tumors with Mismatch-Repair Deficiency", NEW ENGLAND JOURNAL OF MEDICINE, THE - NEJM -, vol. 372, no. 26, 25 June 2015 (2015-06-25), pages 2509-2520, XP055390373, ISSN: 0028-4793, DOI: 10.1056/NEJMoa1500596	1-7
Y	the whole document	8-15
X	WO 2016/077553 A1 (UNIV JOHNS HOPKINS [US]) 19 May 2016 (2016-05-19)	1-7
Y	the whole document	8-15

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2017	01/08/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hix, Rebecca

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Anonymous: "An Investigational Immuno-therapy Study of Nivolumab, and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread - Full Text View - ClinicalTrials.gov", , 18 December 2013 (2013-12-18), XP055390376, Retrieved from the Internet: URL:https://clinicaltrials.gov/ct2/show/NCT02060188 [retrieved on 2017-07-12]	1-5,7
Y	the whole document	8-15

X	Anonymous: "Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors - Full Text View - ClinicalTrials.gov", , 10 June 2013 (2013-06-10), XP055390377, Retrieved from the Internet: URL:https://clinicaltrials.gov/ct2/show/NCT01876511 [retrieved on 2017-07-12]	1-6
Y	the whole document	7-15

Y	GUIDO KROEMER ET AL: "Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy?", ONCOIMMUNOLOGY, vol. 4, no. 7, 5 June 2015 (2015-06-05), page e1058597, XP055389458, DOI: 10.1080/2162402X.2015.1058597 the whole document	1-15

Y	N. J. LLOSA ET AL: "The Vigorous Immune Microenvironment of Microsatellite Instable Colon Cancer Is Balanced by Multiple Counter-Inhibitory Checkpoints", CANCER DISCOVERY, vol. 5, no. 1, 1 January 2015 (2015-01-01) , pages 43-51, XP055349318, US ISSN: 2159-8274, DOI: 10.1158/2159-8290.CD-14-0863 the whole document	1-15

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MICHAEL A. POSTOW: "Managing Immune Checkpoint-Blocking Antibody Side Effects", AMERICAN SOCIETY OF CLINICAL ONCOLOGY EDUCATIONAL BOOK, vol. 35, 1 June 2015 (2015-06-01), pages 76-83, XP055320216, ISSN: 1548-8748, DOI: 10.14694/EdBook_AM.2015.35.76 the whole document	1-15
Y	J. DURAISWAMY ET AL: "Dual Blockade of PD-1 and CTLA-4 Combined with Tumor Vaccine Effectively Restores T-Cell Rejection Function in Tumors", CANCER RESEARCH, vol. 73, no. 12, 15 June 2013 (2013-06-15) , pages 3591-3603, XP055218335, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-12-4100 the whole document	1-15
Y	J. C. DUDLEY ET AL: "Microsatellite Instability as a Biomarker for PD-1 Blockade", CLINICAL CANCER RESEARCH, vol. 22, no. 4, 14 February 2016 (2016-02-14), pages 813-820, XP055389482, US ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-15-1678 the whole document	1-15
Y	MATTHEW W ROSENBAUM ET AL: "PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes", MODERN PATHOLOGY, vol. 29, no. 9, 20 May 2016 (2016-05-20), pages 1104-1112, XP055389478, GB ISSN: 0893-3952, DOI: 10.1038/modpathol.2016.95 the whole document	1-15

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI STEPHEN K H ET AL: "Mismatch Repair and Colon Cancer: Mechanisms and Therapies Explored", TRENDS IN MOLECULAR MEDICINE, ELSEVIER CURRENT TRENDS, GB, vol. 22, no. 4, 9 March 2016 (2016-03-09), pages 274-289, XP029490434, ISSN: 1471-4914, DOI: 10.1016/J.MOLMED.2016.02.003 the whole document	1-15
A	ZUMWALT TIMOTHY J ET AL: "Immunotherapy of Metastatic Colorectal Cancer: Prevailing Challenges and New Perspectives.", HHS Public Author Manuscript , 1 June 2015 (2015-06-01), pages 1-28, XP055389454, Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4591512/ [retrieved on 2017-07-19] abstract Published in final edited form as: Curr Colorectal Cancer Rep. 2015 Jun 1; 11(3): 125-140. Published online 2015 Jun 29. doi: 10.1007/s11888-015-0269-2	1-15
X,P	MARK J. SELBY ET AL: "Preclinical Development of Ipilimumab and Nivolumab Combination Immunotherapy: Mouse Tumor Models, In Vitro Functional Studies, and Cynomolgus Macaque Toxicology", PLOS ONE, vol. 11, no. 9, E0161779, 9 September 2016 (2016-09-09), pages 1-19, XP055389440, DOI: 10.1371/journal.pone.0161779 the whole document	1-15
X,P	WO 2016/197067 A1 (H LEE MOFFITT CANCER CT & RES [US]) 8 December 2016 (2016-12-08) the whole document	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/035822

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016077553 A1	19-05-2016	AU 2015346295 A1	25-05-2017
		CA 2966660 A1	19-05-2016
		SG 11201703541P A	30-05-2017
		WO 2016077553 A1	19-05-2016
WO 2016197067 A1	08-12-2016	CN 106714836 A	24-05-2017
		WO 2016197067 A1	08-12-2016

Form PCT/ISA/210 (patent family annex) (April 2005)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2017/035822	02.06.2017	03.06.2016

International Patent Classification (IPC) or both national classification and IPC
INV. C07K16/28 A61K39/00

Applicant
BRISTOL-MYERS SQUIBB COMPANY

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☒ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Fax: +31 70 340 - 3016	see form PCT/ISA/210	Hix, Rebecca Telephone No. +31 70 340-0

Form PCT/ISA/237 (Cover Sheet) (January 2015)

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	8-15
	No:	Claims	1-7
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-15
Industrial applicability (IA)	Yes:	Claims	1-15
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43*bis*.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43*bis*.1 and 70.9)

see form 210

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Item V:

Reasoned statement under Rule 43bis1(a)(i) with regard to novelty, inventive step or industrial applicability.

For the assessment of the present claims involving the medical use; Claims 1 to 15 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 39.1(iv) / 67.1(iv) PCT. The patentability can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound in medical treatment, but may allow claims to a product, in particular substances or compositions for use in a first or further medical treatment.

The following **documents** are referred to in this communication;

D1	DUNG T. LE ET AL: "PD-1 Blockade in Tumors with Mismatch-Repair Deficiency", NEW ENGLAND JOURNAL OF MEDICINE, THE - NEJM -, vol. 372, no. 26, 25 June 2015 (2015-06-25), pages 2509-2520, XP55390373, ISSN: 0028-4793, DOI: 10.1056/NEJMoa1500596
D2	WO 2016/077553 A1 (UNIV JOHNS HOPKINS [US]) 19 May 2016 (2016-05-19)
D3	Anonymous: "An Investigational Immuno-therapy Study of Nivolumab, and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread - Full Text View - ClinicalTrials.gov", , 18 December 2013 (2013-12-18), XP055390376, Retrieved from the Internet: URL:https://clinicaltrials.gov/ct2/show/NCT02060188 [retrieved on 2017-07-12]
D4	Anonymous: "Phase 2 Study of MK-3475 in Patients With Microsatellite Unstable (MSI) Tumors - Full Text View - ClinicalTrials.gov", , 10 June 2013 (2013-06-10), XP055390377, Retrieved from the Internet: URL:https://clinicaltrials.gov/ct2/show/NCT01876511 [retrieved on 2017-07-12]

D5	GUIDO KROEMER ET AL: "Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy?", ONCOIMMUNOLOGY, vol. 4, no. 7, 5 June 2015 (2015-06-05), page e1058597, XP055389458, DOI: 10.1080/2162402X.2015.1058597
D6	N. J. LLOSA ET AL: "The Vigorous Immune Microenvironment of Microsatellite Instable Colon Cancer Is Balanced by Multiple Counter-Inhibitory Checkpoints", CANCER DISCOVERY, vol. 5, no. 1, 1 January 2015 (2015-01-01), pages 43-51, XP55349318, US ISSN: 2159-8274, DOI: 10.1158/2159-8290.CD-14-0863
D7	MICHAEL A. POSTOW: "Managing Immune Checkpoint-Blocking Antibody Side Effects", AMERICAN SOCIETY OF CLINICAL ONCOLOGY EDUCATIONAL BOOK, vol. 35, 1 January 2015 (2015-01-01), pages 76-83, XP55320216, ISSN: 1548-8748, DOI: 10.14694/EdBook_AM.2015.35.76
D8	J. DURAISWAMY ET AL: "Dual Blockade of PD-1 and CTLA-4 Combined with Tumor Vaccine Effectively Restores T-Cell Rejection Function in Tumors", CANCER RESEARCH, vol. 73, no. 12, 15 June 2013 (2013-06-15), pages 3591-3603, XP55218335, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-12-4100

NOVELTY (Art. 33(2) PCT)

D1 and D2 discloses a phase 2 study to evaluate the clinical activity of pembrolizumab, an anti-programmed death 1 (anti-PD-1) immune check point inhibitor. Prembrolizumab was administered intravenously at a dose of 10mg per kilogram of body weight every 14 days in patients with mismatch repair deficient colorectal cancers. D1, Figure 2, page 2515 shows the results for progression free survival of the patients with colorectal cancer. The results indicate that mismatch repair deficient tumors are more responsive to PD-1 blockade than mismatch repair proficient tumors.

In the light of the disclosures of D1 and D2, the subject-matter of claims 1 to 7 is not novel according to Article 33(2) PCT.

D3 describes a Phase 2 clinical trial investigating the treatment of the anti-PD-1 antibody nivolumab on patients with microsatelite unstable colorectal cancer.

In the light of the disclosure of D3, the subject-matter of claims 1 to 5 and 7 is not novel according to Article 33(2) PCT.

D4 also describes a Phase 2 clinical trial investigating the treatment of the anti-PD-1 antibody MK-3475 on patients with microsatelite unstable colorectal cancer.

In the light of the disclosure of D4, the subject-matter of claims 1 to 6 is not novel according to Article 33(2) PCT.

The present application does not meet the requirements of Article 33(2) PCT because the subject-matter of claims 1 to 7 is not new.

In view of the prior art cited, claims 8 to 15 appear to be novel and meet therefore the requirements of Art. 33(2) PCT.

INVENTIVE STEP (Art. 33(3) PCT)

Documents D1 and D2 are considered to represent the most relevant state of the art.

The subject-matter of claims 8 to 15 differ from the prior art as the treatment of a subject afflicted with a tumor derived from a colorectal cancer, wherein the tumor exhibits a high degree of microsatellite instability ("MSI-H") using an anti-PD-1 antibody (nivolumab) combined with the administration of an anti-CTLA-4 antibody (ipilimumab) is not disclosed in the state of the art.

The effect of said difference is shown in the description page 47, Figure 6 and Table 7, where 45.9% of patients receiving 3 mg/kg nivolumab monotherapy exhibited progression-free survival at 6 months, while **66.6% of patients receiving 3 mg/kg nivolumab in combination with 1 mg/kg ipilimumab exhibited progression-free survival at 6 months.**

Table 8 shows that 75.0% of patients receiving 3 mg/kg nivolumab monotherapy exhibited progression-free survival at 6 months, while **85.1% of patients receiving 3 mg/kg nivolumab in combination with 1 mg/kg ipilimumab exhibited progression-free survival at 6 months**. At 9 months, 65.6% of patients receiving 3 mg/kg nivolumab monotherapy exhibited progression-free survival, while **85.1% of patients receiving 3 mg/kg nivolumab in combination with 1 mg/kg ipilimumab exhibited progression free survival**.

The problem to be solved by the present invention may therefore be regarded as providing an alternative treatment of a subject afflicted with a tumor derived from a colorectal cancer, wherein the tumor exhibits a high degree of microsatellite instability.

The proposed solution is a combination therapy involving administration of an anti-PD-1 antibody (nivolumab) combined with the administration of an anti-CTLA-4 antibody (ipilimumab).

The prior already discusses the blockade of immune checkpoints such as CTLA-4 and PD-1 as showing promise in treating cancer patients, D2, page 2, [06].

D5, page 2, left column states;

> "Based on the abovementioned preclinical and clinical findings, one may have predicted that mAbs targeting immunological check ponts would be particularly efficient in CRC patients. However, neither the blockade of CTLA4 (with ipilimumab/Yervoy™) nor that of the PD-1/PD-L1 axis with nivolumab/Opdivo™) or pembrolizumab/Keytruda™) has conferred any major clinical benefits to patients bearing mismatch repair proficient CRC."

D6 describes the link between the subgroup of colon cancer characterised by microsatellite instability and the corresponding expression of immune check point such as PD-1 and CTLA-4.

D7 discloses the combined use of the blockade of CTLA-4 and PD-1 in the successful treatment of melanoma, page 79, right column.

D8 also describes the dual blockade of both PD-1 and CTLA-4 in tumor models including CT26 for colon cancer which achieves increased proliferation of antigen-specific effector CD8+ and CD4+ T-cells, antigen specific cytokine release, inhibition of suppressive functions of Tregs and upregulation of key signaling molecules critical for T-cell function.

Taking the above prior art into account, the person skilled in the art would consider the known treatments of patients with microsatelite unstable colorectal cancer with anti-PD-1 antibodies, D1 and D2, combined as suggested in the state of the art, D8, with the expectation of achieving successful treatment and an extension of progression-free survival.

In view of the above, the present application does not meet the requirements of Article 33(3) PCT, because the subject-matter of claims 8 to 15 does not involve an inventive step.

However the particular combination of the therapeutic regime involving the combined administration of **3 mg/kg nivolumab in combination with 1 mg/kg ipilimumab administered according to the dosing schedule of the application to patients with metastatic colorectal cancer with high microsatellite instability**, appears to result in the surprising result of prolonged progression free survival compared to those patient receiving nivolumab monotherapy which is considered to involve an inventive step according to Article 33(3) PCT.

Item VI:

Certain documents cited

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO2016/197067	08.12.2016	03.06.2016	5.06.2015

Item VIII:

Certain observations on the international application

CLARITY (Art.6 PCT)

It is clear from the description that the particular combination of the therapeutic regime involving the combined administration of **3 mg/kg nivolumab in combination with 1 mg/kg ipilimumab administered according to the dosing schedule of the application to patients with metastatic colorectal cancer with high microsatellite instability** is essential to the performance of the invention. Since independent claim 1 does not contain this feature it does not meet the requirement following from Article 6 PCT that any independent claim must contain all the technical features essential to the invention.

Managing Immune Checkpoint-Blocking Antibody Side Effects

Michael A. Postow, MD

OVERVIEW

Immune checkpoint-blocking antibodies that enhance the immune system's ability to fight cancer are becoming important components of treatment for patients with a variety of malignancies. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) was the first immune checkpoint to be clinically targeted, and ipilimumab, an inhibitor of CTLA-4, was approved by the U.S. Food and Drug Administration (FDA) for patients with advanced melanoma. The programmed cell death-1 (PD-1) receptor and one of its ligands, PD-L1, more recently have shown great promise as therapeutic targets in a variety of malignancies. Nivolumab and pembrolizumab recently have been FDA-approved for patients with melanoma and additional approvals within this therapeutic class are expected. The use of anti-CTLA-4 and anti-PD-1/PD-L1 antibodies is associated with side effects known as immune-related adverse events (irAEs). Immune-related adverse events affect the dermatologic, gastrointestinal, hepatic, endocrine, and other organ systems. Temporary immunosuppression with corticosteroids, tumor necrosis factor-alpha antagonists, mycophenolate mofetil, or other agents can be effective treatment. This article describes the side-effect profile of the checkpoint-blocking antibodies that target CTLA-4 and PD-1/PD-L1 and provides suggestions on how to manage specific irAEs.

The immune system plays an important role in controlling and eradicating cancer. Recently, strategies that enhance T-cell function by blocking negative regulatory components on T cells, which are called checkpoints, have led to remarkable success for patients with many different malignancies. CTLA-4 was the first T-cell checkpoint to be clinically targeted. Ipilimumab, an anti-CTLA-4 antibody, was approved by the FDA for patients with advanced melanoma based on an overall survival benefit.[1,2] A second immunologic checkpoint, known as PD-1, and its predominant ligand, PD-L1, also is demonstrating incredible promise as a therapeutic target. Nivolumab and pembrolizumab (anti-PD–1 blocking antibodies) have been FDA-approved for patients with advanced melanoma.[3,4] Nivolumab and pembrolizumab, and additional antibodies that target the PD-1 axis, are also effective in additional cancers such as non-small cell lung cancer, renal cell cancer, bladder cancer, and Hodgkin lymphoma.[5-9]

Although these antibodies can be associated with substantial benefits, by increasing immune system function, immune-checkpoint blockade can lead to inflammatory side effects called immune-related adverse events (irAEs). Immune-related adverse events can affect any organ system, but they typically involve the skin, gastrointestinal, hepatic, and endocrine systems. Temporary use of immunosuppressive medications can suppress these side effects without eliminating the possibility of a favorable antitumor response.

This review focuses on irAEs associated with antibodies that block the immunologic checkpoints, CTLA-4 and PD-1, including antibodies that block the ligand of PD-1/PD-L1. These agents have been studied most extensively in patients with melanoma, and experience and recommendations are based primarily on data obtained from studies involving patients with melanoma. Nevertheless, the principles of irAE recognition and management are relevant across the oncologic spectrum and will become increasingly important as the use of these antibodies expands.

SPECIFIC TYPES OF IMMUNE-RELATED ADVERSE EVENTS AND MANAGEMENT

Rash and Mucosal Irritation

The most common and typically earliest onset (Fig. 1) irAE associated with checkpoint inhibitors is dermatologic toxicity.[10] Nearly 50% of patients treated with ipilimumab will experience rash and/or pruritus. Rashes associated with checkpoint blockade often appear as faintly erythematous, reticular, and maculopapular. Typically, the trunk and extremities are involved.[11] In one case, neutrophilic infiltration, diagnostic of Sweet's Syndrome, was reported.[12] Vitiligo also can be seen, although it typically does not appear until months after the initiation of checkpoint blockade.

Topical corticosteroid creams can be used to treat rash induced by checkpoint blockade. Oral antipruritics (hy-

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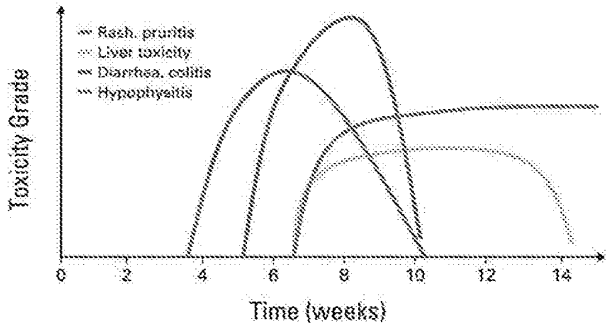
Disclosures of potential conflicts of interest are found at the end of this article.

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FIGURE 1. Kinetics of Appearance of Immune-Related Adverse Events



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droxyzine HCl or diphenhydramine HCl) can help if pruritus is problematic. Severe rashes (grade 3 and above) should be managed with oral corticosteroids. Consideration of permanent discontinuation of checkpoint blockade because of reactions such as Stevens-Johnson syndrome/toxic epidermal necrolysis rarely have been reported. Such reactions require hospitalization for intravenous corticosteroids, dermatologic evaluation, and fluid/electrolyte management.

Antibodies that block PD-1/PD-L1 also can result in dermatologic/mucosal toxicity. Anecdotal cases of patients with oral mucositis and/or dry mouth symptoms have been described with PD-1/PD-L1 treatment. In a large phase I trial of nivolumab, 6.5% of patients had symptoms of dry mouth; one patient had symptoms of grade 3 dry mouth.[13] Oral corticosteroid rinses or lidocaine can treat this symptom effectively. Since many patients with this complaint may be receiving concomitant immunosuppression to treat another irAE, oral candidiasis remains in the differential diagnosis.

KEY POINTS

- Immune checkpoint-blocking antibodies can cause immune-mediated adverse events involving the skin, liver, gastrointestinal, endocrine, neurologic, and other organ systems.
- If appropriate immunosuppressive treatment is used, patients generally completely recover from immune-mediated adverse events.
- The use of immunosuppressive treatment to treat an immune-mediated adverse event does not impair the efficacy of immune-checkpoint blockade.
- There does not appear to be a strong correlation between the occurrence of an immune-mediated adverse event and long-term outcomes to immune checkpoint-blocking antibody therapy.
- Patients who stop immunotherapy because of side effects can still have excellent long-term outcomes.

DIARRHEA/COLITIS

Diarrhea is common in patients undergoing treatment with checkpoint-blocking antibodies. However, there is a much higher incidence of diarrhea in patients receiving CTLA-4 blocking antibodies compared to those targeting PD-1/PD-L1. When considering the occurrence of this irAE, distinguishing diarrhea (increase in frequency of stool) from colitis (abdominal pain, radiographic or endoscopic findings of colonic inflammation) is important. For patients with melanoma undergoing CTLA-4 blockade with ipilimumab, approximately 30% had diarrhea of any grade and less than 10% had severe (grade 3/4) diarrhea.[1] Rates of grade 3/4 colitis have been found to affect only approximately 5% of treated patients.[1,14] Patients should be informed that diarrhea/colitis does not typically begin with initiation of checkpoint blockade; instead, it usually begins approximately 6 weeks into treatment (Fig. 1).[15]

Diarrhea/colitis with CTLA-4 blockade is more common than with PD-1/PD-L1 blockade. The rate of grade 3/4 diarrhea in patients treated with PD-1/PD-L1 agents is very low (1% to 2%).[8,13,16] Although the precise safety profile is still under evaluation, patients who had significant diarrhea/colitis during CTLA-4 blockade have been treated with PD-1 therapy without diarrhea/colitis recurrence.[17] Nonetheless, ongoing trials and clinical experience are necessary to more fully understand the safety of PD-1/PD-L1 blockade in this setting.

When a patient presents with mild diarrhea, clinicians should consider other etiologies that may be responsible, such as *Clostridium difficile* infection or other bacterial/viral pathogens. Patients should be counseled on the importance of maintaining oral hydration. Some clinicians find that the American Dietary Association's colitis diet and antimotility agents (oral diphenoxylate HCl and atropine sulfate 4 times a day) can be helpful. If symptoms persist for more than 3 days, or increase, and/or no infectious causes are readily identified, the use of oral or intravenous corticosteroids are required.

In severe cases or situations in which symptoms do not improve with oral corticosteroids, hospitalization for intravenous corticosteroids, hydration, and electrolyte management is required. A colonoscopy is not necessarily indicated unless the diagnosis is unclear. If intravenous corticosteroids (up to 2 mg/kg methylprednisolone twice a day) do not lead to symptom resolution, infliximab (Remicade; Janssen Biotech, Horsham, PA) at a dose of 5 mg/kg, once every 2 weeks can be helpful.[18-20] The use of infliximab in this setting is based on its use in patients with inflammatory bowel diseases.[21] In very rare cases, colitis can result in bowel perforation that can potentially require colostomy.

Unfortunately, there are no proven treatments to prevent the occurrence of diarrhea. In one study, prophylactic use of the matrix-release corticosteroid budesonide was not found to be helpful.[22] Nevertheless, some clinicians find budesonide to be helpful in early treatment for mild noninfectious

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diarrhea symptoms that persist but do not escalate after 2 to 3 days of dietary modification and antimotility agents.

HEPATOTOXICITY

Hepatitis, as determined by elevations in aspartate aminotransferase (AST), aminotransferase (ALT) and less commonly, total bilirubin, occasionally is seen in patients treated with checkpoint blockade. Although most episodes present only as asymptomatic laboratory abnormalities, some patients have an associated fever. Rates of AST and ALT elevations with CTLA-4 blockade vary among clinical trials, but they typically have been reported in less than 10% of patients.[14,23,24] In large trials of PD-1–blocking antibodies, the rates of hepatitis were similarly low (below 5%) and grade 3/4 toxicity was even rarer.[3,13,16]

Among patients who develop hepatitis, the most common onset is 8 to 12 weeks after initiation of treatment, although early or delayed events also may be seen (Fig. 1).[19] Radiographic findings are not typical. In severe cases, however, findings on CT scans may include mild hepatomegaly, periportal edema, or periportal lymphadenopathy.[25] Liver biopsies have described pathologic changes that include severe panlobular hepatitis with prominent perivenular infiltrate with endothelialitis or a primary biliary pattern with mild portal mononuclear infiltrate around bile ductules.[25,26]

Hepatic function (transaminases and bilirubin) should be monitored before each dose of ipilimumab. If AST and ALT increase, viral and other drug-induced causes of hepatitis should be excluded. As with treating other irAEs, if no other cause is obvious, prompt treatment with corticosteroids is necessary. In rare cases, elevations in AST and ALT are steroid-refractory and 500 mg every 12 hours of mycophenolate mofetil (CellCept; Genentech, South San Francisco, CA) may be helpful. The use of antithymocyte globulin therapy also was described in a case report.[27] Unlike for patients with diarrhea/colitis, infliximab should not be given to patients with hepatitis because infliximab carries a risk of hepatotoxicity. Hepatitis may persist for quite some time and require prolonged or repeated corticosteroid tapers (minimum of 3 weeks suggested) and/or additional immunosuppression.

ENDOCRINOPATHY

Immune-related adverse events that affect the pituitary, adrenal, and thyroid glands often present with nonspecific symptoms such as nausea, headache, and fatigue. The incidence of endocrinopathy has been difficult to precisely determine because of the variable methods of assessment, diagnosis, and monitoring in each clinical trial. Nonetheless, hypophysitis (pituitary inflammation) and hypothyroidism are the most common endocrinopathies and are typically believed to occur in up to 10% of patients treated with CTLA-4 blockade.[28,29]

When hypophysitis is suspected, all or some of the hormones released by the pituitary may be reduced (adrenocorticotropic hormone [ACTH], thyroid stimulating hormone [TSH], follicle stimulating hormone, luteinizing hormone, growth hormone, prolactin). Typically, hypophysitis is diagnosed by clinical symptoms of fatigue and headache, radiographic findings (enhancement and enlargement of the pituitary[30,31]), and biochemic evidence of pituitary dysfunction (low ACTH and TSH). Biochemical tests associated with hypophysitis are distinct from primary adrenal insufficiency (low cortisol or inappropriate cortisol stimulation test; high ACTH) and primary hypothyroidism (low free T4; high TSH).

When hypophysitis is suspected, some clinicians have anecdotally described that a course of high-dose corticosteroids (1 mg/kg of prednisone daily) given during the acute phase can reverse the inflammatory process and prevent the need for longer-term hormone replacement. In almost all patients, however, longer-term supplementation of affected hormones is necessary because of secondary hypothyroidism (treated with levothyroxine) or secondary hypoadrenalism (treated with replacement doses of hydrocortisone, typically 20 mg each morning and 10 mg each evening). Some authors have described that patients can be successfully weaned from replacement corticosteroids over time, but this is likely the exception.[32] The immunologic mechanisms of hypophysitis are unknown, but they may be related to the development of humoral (antibody) immunity against the pituitary gland and subsequent complement activation.[33]

Since routine monitoring of thyroid function tests (TSH) is required before each dose of ipilimumab, patients often are diagnosed with thyroid abnormalities (hyperthyroidism and hypothyroidism) as a result of checkpoint blockade. Hypothyroidism is believed to occur far more commonly than hyperthyroidism. When patients are evaluated for fatigue that is possibly a result of endocrinopathy, it is important to distinguish primary hypothyroidism (low free T4 and high TSH) from hypophysitis, which can result in secondary hypothyroidism (low free T4 and low TSH). Management of hypothyroidism involves replacement with thyroid hormone (levothyroxine).

The most emergent endocrinopathy is an adrenal crisis associated with dehydration, hypotension, and electrolyte imbalances such as hyperkalemia and hyponatremia. When this occurs, intravenous corticosteroids and immediate hospitalization is required. Consultation with an endocrinologist, aggressive hydration, and evaluation for sepsis is critical.

The frequency of endocrinopathy in patients treated with PD-1/PD-L1 agents is not known yet, but it may differ from those seen with CTLA-4 blockade. Hypophysitis rarely has been described in published trials of PD-1 blockade for patients with advanced melanoma.[3,13] Thyroid disorders have been described in less than10% of patients.[3,16] Some cases, however, can be severe.[34] Treatment of endocrinopathy with PD-1/PD-L1 agents is approached in a way similar to treating patients undergoing CTLA-4 blockade.

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LESS FREQUENTLY INVOLVED ORGANS

Lung

Several pulmonary inflammatory conditions have been seen in patients treated with ipilimumab, including sarcoidosis[35,36] and organizing inflammatory pneumonia.[37] Pneumonitis also has been described in patients treated with PD-1 blocking agents (< 10%)[3,5,16,38] but with occasional fatal consequence in early trials.[5] In any patient presenting with pulmonary symptoms, such as an upper respiratory infection, new cough, or shortness of breath, pneumonitis should be considered and evaluated with imaging. In moderate to severe cases, a bronchoscopy should be performed to exclude infectious etiologies before starting immunosuppression. In severe cases, treatment should consist of high doses of corticosteroids such as 2 mg/kg of intravenous methylprednisone. Additional immunosuppression with infliximab, mycophenolate mofetil, or cyclophosphamide is reasonable.

Eye

Inflammation of components of the eye has been described with CTLA-4 blockade. These include episcleritis, conjunctivitis, and uveitis. Typically, the incidence is believed to be less than1%, and symptoms can include photophobia, pain, dryness of the eyes, and blurry vision.[39] Consultation with an ophthalmologist is recommended, and treatment with topical intraocular corticosteroids such as 1% prednisolone acetate suspension may be helpful. Oral corticosteroids can be used for more severe (grade 3/4 or refractory) cases. The incidence of ophthalmologic toxicity in patients treated with PD-1/PD-L1 blockade is less-well described, but it is likely an infrequent side effect.

Kidney

Several case reports have described patients treated with ipilimumab who have developed renal insufficiency believed to be related to treatment. Histopathologic analyses of kidney biopsies have described several different pathologic processes, including acute granulomatous interstitial nephritis[40] and lupus membranous nephropathy.[41] Treatment with oral or intravenous corticosteroids in these cases has been associated with improvement in renal function.[42,43] Renal insufficiency with PD-1 agents and the combination of CTLA-4 and PD-1 blockade similarly has been reported in several patients with anecdotal findings of interstitial nephritis and response to corticosteroids.[16,44]

Pancreas

The routine monitoring of amylase and lipase values in otherwise asymptomatic patients treated with checkpoint blockade is not recommended. Similarly, corticosteroids are not indicated in patients with asymptomatic elevations in amylase/lipase without other symptoms of pancreatitis. Nevertheless, when pancreatitis is suspected clinically, amylase and lipase should be checked since immune-related pancreatitis has been reported in patients treated with CTLA-4 and PD-1 blockade.[38,45] The high rate of asymptomatic elevations in amylase/lipase in clinical trials of patients receiving CTLA-4 and PD-1/PD-L1 blockade is of uncertain clinical significance since these patients did not meet the diagnostic criteria for pancreatitis.[46]

Neurologic Syndromes

Neurologic syndromes have been associated with checkpoint blockade with ipilimumab. These include posterior reversible encephalopathy syndrome,[47] aseptic meningitis,[48] enteric neuropathy,[49] and transverse myelitis.[50] Cases of Guillain-Barre syndrome are particularly notable[51] because one case resulted in a treatment-related death in a postsurgical adjuvant study of ipilimumab.[52] As with other irAEs, corticosteroids can be helpful. In consultation with a neurologist, plasmapheresis and intravenous immunoglobulin may be considered.

Hematologic Syndromes

Red cell aplasia,[53] neutropenia,[54] and acquired hemophilia A[55] also have been described in patients treated with ipilimumab, as has thrombocytopenia.[56] Similar to many of the irAEs described above, the standard approach remains initial immunosuppression with corticosteroids. A bone marrow biopsy may be necessary in some cases, particularly when the diagnosis remains unclear.

IMMUNE-RELATED ADVERSE EVENTS ASSOCIATED WITH COMBINATION OF CONCURRENT CTLA-4 AND PD-1 BLOCKADE

The distinct action mechanisms of CTLA-4 and PD-1 blockade have led to investigations treating patients with both CTLA-4 and PD-1/PD-L1 in those who develop a variety of malignancies. Published data on the combination of ipilimumab and nivolumab exist for patients with advanced melanoma.[44] The rate of published grade 3/4 treatment-related adverse events related to therapy in patients receiving this treatment is approximately 50%, which is numerically higher than rates described for patients receiving either CTLA-4 or PD-1/PD-L1 agents as single agents.[1,3,5,8,16] Many grade 3/4 irAEs in this trial, however, were asymptomatic, abnormal laboratory values. This included a high rate of patients with asymptomatic elevations in lipase (over 10%), none of whom developed pancreatitis. No new toxicities were attributed to the combination of ipilimumab and nivolumab that have not been seen with ipilimumab or nivolumab alone.

The combination of ipilimumab and nivolumab may have a different safety profile in patients with other advanced malignancies, and this combination remains under active investigation. Other studies seek to test similar combinations using different CTLA-4 and PD-1/PD-L1–blocking antibodies (NCT01975831, NCT02089685, NCT01928394).

COMBINATION OF IMMUNE CHECKPOINT-BLOCKING ANTIBODIES AND TARGETED THERAPY

The treatment of many malignancies has improved with the discovery of oncogenic proteins amenable to targeted inhibi-

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tion. One of these momentous advances has been targeting mutant *BRAF* and the mitogen-activated protein (MAP) kinase pathway in patients with melanoma.[57-62] Based on preclinical evidence that suggests RAF inhibitors can have positive immunologic effects,[63-68] there has been great interest in exploring combinations of targeted agents that inhibit mutant *BRAF* with immune checkpoint- blocking antibodies.

The toxicity profile of combinations of targeted agents and immune-checkpoint blockade, however, is just beginning to be explored. In the only published prospective study, treatment with vemurafenib and ipilimumab was associated with a high rate of grade 3 transaminase elevations that required cessation of further exploration of this combination as concurrent therapy.[69] Rash was also an important problem with this combination, which is consistent with similar findings when vemurafenib was administered soon after completing ipilimumab.[70]

The irAE profile of ipilimumab in combination with RAF inhibitors may vary by specific RAF inhibitor. In a separate phase I study, dabrafenib was combined with ipilimumab and preliminarily, no major safety concerns with this doublet combination have been seen.[71] The safety of ipilimumab with dabrafenib and trametinib, however, may be more problematic because some patients treated with this triplet had severe colitis with perforation.

Future investigations of targeted agents and immune checkpoint-blocking antibodies should continue in patients who develop multiple diseases, and that dose and schedule will be critical to the safety and possibly efficacy of these combination approaches. It is expected that combinations of targeted agents with PD-1/PD-L1 antibodies may show a more favorable side-effect profile than combinations with ipilimumab; however, this is the subject of ongoing investigation.

COMBINATION OF IPILIMUMAB AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

Granuloycte-Macrophage Colony-Stimulating Factor (GM-CSF) (Leukine; Genzyme, Ridgefield, NJ) currently is not indicated to prevent or treat irAEs. Nonetheless, combining GM-CSF with immune-checkpoint blockade is of interest based on favorable preclinical data suggesting GM-CSF–secreting tumor vaccines enhanced the activity of CTLA-4 blockade.[72] The combination of GM-CSF and ipilimumab was tested in patients with advanced melanoma in a phase II randomized study (ipilimumab 10 mg/kg plus GM-CSF vs. ipilimumab 10 mg/kg alone). Interim results indicate that the combination prolonged survival and was associated with fewer irAEs than ipilimumab 10 mg/kg alone.[73]

Reasons for the decreased rate of side effects are unclear, but GM-CSF has been implicated in the pathogenesis of inflammatory bowel disease.[74] Whether GM-CSF would result in similar effects in patients treated with the commercially available dose of ipilimumab (3 mg/kg) is unknown. Additional studies are necessary before GM-CSF is routinely recommended for patients being treated with ipilimumab.

OPPORTUNISTIC INFECTIONS IN IMMUNOSUPPRESSED PATIENTS

Since prolonged immune suppression is occasionally used to treat irAEs, patients can be at risk for unusual or opportunistic infections. Though anecdotal cases of these infections, such as *Aspergillus* pneumonia, have been reported,[75] the true incidence of these opportunistic infections remains unknown. *Pneumocystis jiroveci* prophylaxis with cotrimoxazole, atovaquone, or pentamidine should be considered in patients treated with 20 mg of prednisone equivalent daily for at least 4 weeks, based on the National Comprehensive Cancer Network guidelines for the Prevention and Treatment of Cancer-Related Infections (Category 2B recommendation). The role of prophylactic antiviral or antifungal therapy in this setting is unclear.

INVESTIGATIONS CORRELATING IMMUNE-RELATED ADVERSE EVENTS WITH EFFICACY

The correlation between efficacy of checkpoint-blocking antibodies and the occurrence of irAEs is controversial.[76,77] Patients can benefit from checkpoint-blocking antibodies without developing irAEs. Any potential association between PD-1/PD-L1–blockade and irAEs will be hard to determine as the incidence of significant irAEs is low.

SAFETY OF IMMUNE-CHECKPOINT INHIBITION IN PATIENTS WITH UNDERLYING AUTOIMMUNE CONDITIONS

The safety of immune-checkpoint inhibitors in patients with an unrelated autoimmunity disorder (e.g., rheumatoid arthritis, systemic lupus erythematosus) is unknown. Both CTLA-4 and PD-1 have an important function in maintaining immunologic homeostasis, and there is theoretic concern that therapeutic blockade of these receptors could lead to exacerbations of underlying autoimmune conditions. Preclinical models suggest CTLA-4 blockade can exacerbate autoimmune diseases.[78,79] Since patients with underlying autoimmune conditions were not included in clinical trials of checkpoint-blocking antibodies, clinical evidence of the safety of this approach requires further study. Anecdotally, some patients with underlying autoimmune conditions have been treated safely with ipilimumab,[80,81] but one patient with multiple sclerosis reported worsening symptoms after ipilimumab.[82] Given the profound potential benefits of these checkpoint- blocking antibodies in patients with life-threatening malignancies, clinicians should engage patients with autoimmune diseases in thoughtful discussion about the possible benefits and risks of immunologic checkpoint blockade.

CONCLUSION

Immunologic-checkpoint inhibition targeting CTLA-4 and PD-1/PD-L1 has dramatically improved the care of patients

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with many advanced malignancies. Treatment is associated with typically transient irAEs, but irAEs occasionally can be severe and fatal. Rapid identification of these side effects and initiation of systemic immunosuppression can improve outcomes without compromising the efficacy of immune-checkpoint inhibition. Although no prospective data exist to guide recommendations for the best specific immunosuppressive treatment, adherence to established guidelines based upon collective clinical experience is recommended. Clinical experience with immune-checkpoint inhibitors in a variety of disease settings and in novel combinations ultimately will refine knowledge and management of irAEs and provide the opportunity to obtain the full therapeutic potential of this promising treatment modality.

Disclosures of Potential Conflicts of Interest

Relationships are considered self-held and compensated unless otherwise noted. Relationships marked "L" indicate leadership positions. Relationships marked "I" are those held by an immediate family member; those marked "B" are held by the author and an immediate family member. Institutional relationships are marked "Inst." Relationships marked "U" are uncompensated

Employment: None. **Leadership Position:** None. **Stock or Other Ownership Interests:** None. **Honoraria:** None. **Consulting or Advisory Role:** Michael A. Postow, Bioconnections LLC, FirstWord, Millennium Takeda. **Speakers' Bureau:** None. **Research Funding:** Michael A. Postow, Bristol-Myers Squibb (Inst). **Patents, Royalties, or Other Intellectual Property:** None. **Expert Testimony:** None. **Travel, Accommodations, Expenses:** Michael A. Postow, Bristol-Myers Squibb. **Other Relationships:** None.

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Modern Pathology (2016) 29, 1104–1112

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PD-L1 expression in colorectal cancer is associated with microsatellite instability, *BRAF* mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes

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Programmed cell death 1 (PD-1) and its ligand (PD-L1) are key suppressors of the cytotoxic immune response. PD-L1 expression on tumor cells may be induced by the immune microenvironment, resulting in immune escape (adaptive immune resistance), and an adverse prognosis in many malignancies. In colorectal carcinoma the response to PD-1/PD-L1 inhibition is correlated with microsatellite instability. However, little is known about the clinicopathologic, molecular, and prognostic characteristics of colorectal carcinoma with PD-L1 expression. We performed immunohistochemistry for PD-L1 on 181 cases of colorectal carcinoma with known microsatellite instability and mutational status, and correlated PD-L1 expression with clinicopathologic features including tumor-infiltrating lymphocyte burden/immunophenotype, tumor mutational profile, and disease-specific survival. PD-L1 was expressed in tumors from 16 patients (9%) who were more often older ($P=0.006$) and female ($P=0.035$), with tumors exhibiting a larger size ($P=0.013$), but lower stage ($P<0.001$). PD-L1 expression was associated with increased CD8 and TBET-positive tumor-infiltrating lymphocytes, medullary phenotype, poor differentiation, microsatellite instability, *BRAF* mutation ($P<0.001$ for each), and a lower frequency of *KRAS* mutation ($P=0.012$). On multivariate analysis, PD-L1 expression was associated with medullary morphology and frequent CD8-positive tumor-infiltrating lymphocytes, suggesting adaptive immune resistance. PD-L1 positivity was not predictive of survival in the entire cohort, but it was associated with a lower disease-specific survival within the microsatellite-instability high cohort. PD-L1 expression in colorectal carcinoma is associated with clinicopathologic and molecular features of the serrated pathway of colorectal carcinogenesis, and is associated with a worse outcome within microsatellite-unstable tumors. These findings support the role of PD-L1 expression in providing normally immunogenic colorectal carcinoma a means of immune evasion and a more aggressive biology, provide a potential mechanistic explanation for the favorable response of microsatellite-unstable colorectal carcinoma to PD-1/PD-L1 pathway blockade, and suggest potential predictive and prognostic roles of PD-L1 immunohistochemistry in colorectal carcinoma.
Modern Pathology (2016) **29**, 1104–1112; doi:10.1038/modpathol.2016.95; published online 20 May 2016

Programmed cell death ligand 1 (PD-L1) is a key immunoregulatory molecule that, upon interacting with its receptor, PD-1, suppresses the CD8 cytotoxic immune response in both physiologic and pathologic pathways.[1] PD-L1 and PD-1 have come under close scrutiny in recent years as potential targets for cancer therapy, and there are multiple ongoing clinical trials with anti-PD-1 or PD-L1 agents. PD-L1 expression on tumor cells has been linked to a weakened host immune response and consequent poor prognosis in several malignancies, including melanoma and esophageal, gastric, hepatocellular and urothelial carcinomas, among others.[2,3] The role of PD-L1 expression in colorectal carcinoma is less clear, with some published studies reporting conflicting results as to whether PD-L1 expression indicates a better or worse prognosis.[4,5]

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Received 18 February 2016; revised 6 April 2016; accepted 15 April 2016; published online 20 May 2016

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In contrast to some malignancies such as melanoma, renal, and lung cancers, colorectal carcinoma generally demonstrates a very low response rate to PD-1 or PD-L1 blockade.[6–8] However, a subset of colorectal carcinomas harboring microsatellite instability and increased tumor-infiltrating lymphocytes has been shown to respond to an anti-PD-1 agent, although microsatellite-stable tumors did not.[9] However, there are conflicting results regarding the association of microsatellite instability with PD-L1 expression in colorectal carcinoma. Some studies have shown PD-L1 expression on colorectal carcinoma tumor cells to be rare in both microsatellite-unstable and microsatellite-stable tumors,[9,10] whereas others have reported strong PD-L1 expression in up to one third of both microsatellite-unstable and microsatellite-stable tumors.[4] In addition, the clinicopathologic features of colorectal carcinoma with PD-L1 expression are not well defined.

In this study we sought to evaluate clinicopathologic and molecular characteristics, including microsatellite instability, and prognosis of tumors harboring PD-L1 expression in a large cohort of resected colorectal carcinomas.

Materials and methods

This study was approved by the institutional review board of the Massachusetts General Hospital, Boston, MA, USA. Patients with colorectal carcinoma who underwent surgical resection of primary or metastatic tumors and clinical molecular testing between the years of 1997 and 2012 were identified from the pathology database. We selected 181 cases with available tissue blocks, including resections of primary tumors ($n=138$) and metastatic foci ($n=43$), including both pre- ($n=124$) and post- ($n=57$) chemotherapy specimens. For the purpose of this study, the tissue microarrays were enriched for cases with microsatellite instability through the inclusion of 31 cases from 2011 through 2012 that were known to be microsatellite unstable.

Hematoxylin and eosin-stained sections of all formalin-fixed paraffin-embedded cases were reviewed by two pathologists (MM-K and JRB) to confirm the presence of adequate lesional tissue for tissue microarray construction and to evaluate pathological features including tumor histology, grade, abundance of tumor-infiltrating lymphocytes, lymphatic, vascular, and perineural invasion, and pattern of advancing border (pushing or infiltrating). Tissue microarrays were constructed using two to three (median three) 2-mm cores of representative tumor tissue from each case, along with multiple cores of non-neoplastic colonic tissue as controls.

The tumor mutation status had been determined before the study as part of the clinical work-up by a multiplex PCR-based assay (SNaPshot platform; Applied Biosystems) to detect a panel of commonly mutated genes implicated in oncogenesis, including *BRAF*, *KRAS*, *APC*, and *TP53*, among others, as previously described.[11] Microsatellite-instability testing by PCR and/or by immunohistochemistry for mismatch-repair proteins (MLH1, MSH2, PMS2, and MSH6) was also conducted.

Immunohistochemistry was performed on 5-μm sections cut from tissue microarray blocks using an automated stainer (Bond Rx, Leica Microsystems, Bannockburn, IL) and the following antibodies in accordance with the manufacturer's recommendations for each antibody: PD-L1 (clone:E1L3N #13684, 1:200, Cell Signaling Technology, MA, USA), PTEN (clone:138G6 # 9559S 1:100, Cell Signaling Technology), CD8 (clone: IgG2b 4B11, RTU, Leica, IL), GATA3 (clone: L50-823# CM405A 1:250, Biocare, Concord, CA), and TBET (clone: D6N8B XP Rabbit mAb, 1:100, Cell Signaling Technology).

PD-L1 expression on tumor cells was evaluated using a three-tiered grading system: $0 = < 5\%$ of the tumor cells; $1 = 5–49\%$ of tumor cells; and $2 = \geq 50\%$ tumor cells with membranous staining of any intensity. Cytoplasmic staining was not considered in this study. Scores of 1 and 2 were considered to be positive for PD-L1 expression (Figure 1a–c). Cytoplasmic and/or nuclear PTEN staining was semi-quantitatively evaluated as either lost (0: $< 10\%$ of tumor cells), reduced (1: 10–90% of tumor cells), or preserved (2: $> 90\%$ of the tumor cells with positive staining; Figure 1d–f). PTEN expression was then dichotomized as lost ($< 10\%$ of tumor cells with expression of PTEN) or retained ($\geq 10\%$ of tumor cells with expression).

Expression of CD8, TBET (a Th1 pathway transcription factor), and GATA3 (a Th2 pathway transcription factor) in tumor-infiltrating lymphocytes was semi-quantitatively evaluated on a scale of 0–3 based on the number of positive lymphocytes infiltrating tumor cells. The score was defined based on the percentage of tumor cells with overlying positively-staining T cells: 0 = none or rare; 1+ = $< 5\%$; 2+ = $\geq 5\%$ and $< 25\%$; 3+ = $\geq 25\%$ (Figure 1g–j). Subsequently, the grades were dichotomized into high (scores 2–3) and low/negative (scores 0–1) for tumor-infiltrating lymphocytes.

Three of the authors (MWR, JRB, and MM-K), blinded to the clinical and pathological data, independently evaluated the immunohistochemical stains. In cases with discrepant scores between cores of an individual case or between observers, a final score was recorded based on a consensus assessment at a multi-head microscope.

Statistical Methods

Statistical analysis was performed using Stata (Version 14.0, StataCorp, College Station, Texas). Univariate analysis of categorical variables was done using a Pearson χ^2-test or a Fisher exact test, where appropriate; continuous variables were evaluated using a Wilcoxon rank-sum test. Multivariate

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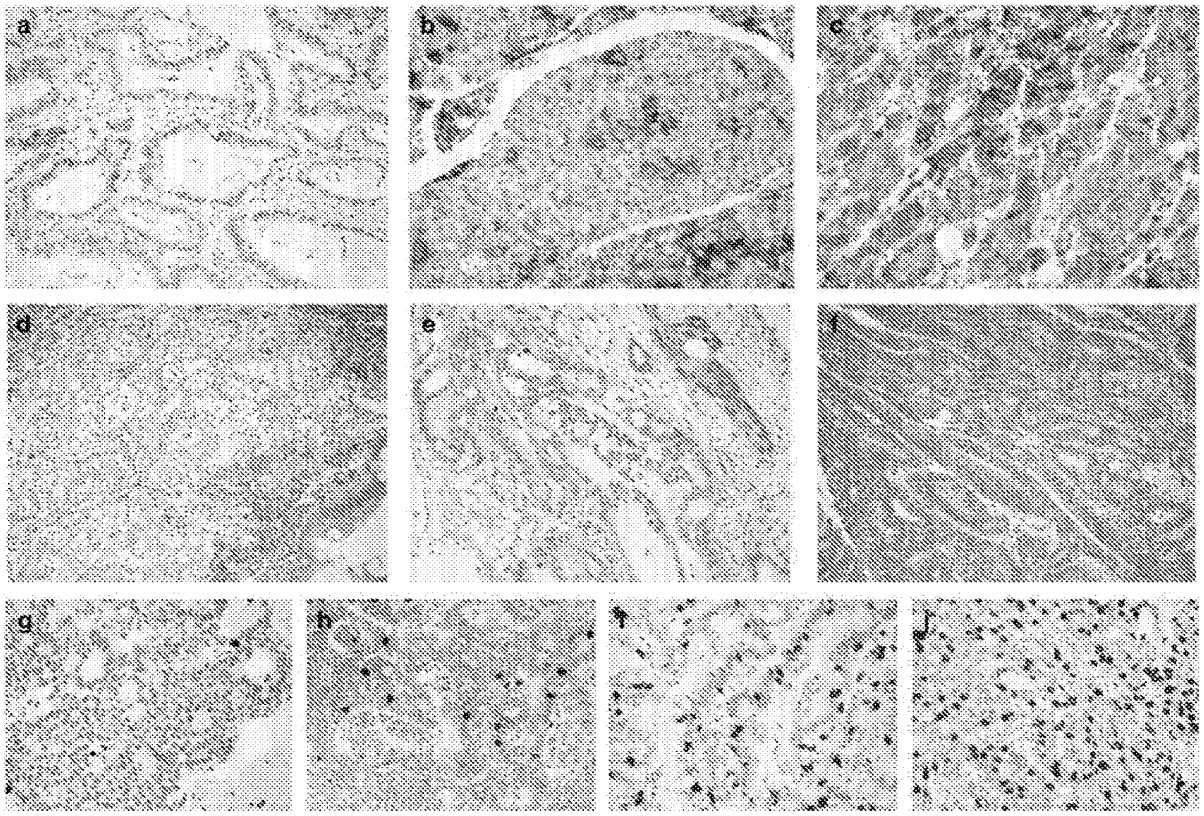


Figure 1 Immunohistochemical scoring of PD-L1 (**a–c**), PTEN (**d–f**) and CD8 (**g–j**). PD-L1 expression was scored on a scale of 0–2: 0 = < 5% (**a**), 1+ = 5–49% (**b**), and 2+ = ≥ 50% (**c**) of the tumor cells exhibiting membranous staining of any intensity. Cytoplasmic and/or nuclear PTEN staining was semi-quantitatively evaluated as either lost (0) = < 10% of tumor cells with positive staining ((**d**) negative tumor cells in the background of positive normal epithelium and stromal cells), reduced (1) = 10–90% of tumor cells with positive staining (**e**) or preserved (2) = > 90% of the tumor cells with positive staining (**f**). PTEN expression was then dichotomized as lost (< 10% of tumor cells with expression of PTEN) or retained (≥10% of tumor cells with expression). Tumor-infiltrating CD8+, TBET+, or GATA3+ lymphocytes were scored on a scale of 0–3 based on the percentage of tumor cells with overlying positive cells: 0 = none or rare (**g**), 1 + = < 5% (**h**), 2+ = ≥ 5% and < 25% (**i**), and 3+ = ≥ 25% (**j**). CD8, TBET, and GATA3 scores were subsequently dichotomized into cases with high (scores 2+ to 3+) and low (scores 0 and 1+) expression. PD-L1, programmed cell death ligand 1.

analysis was performed to identify independent predictors of PD-L1 positivity using exact logistic regression. Variables with P-value < 0.1 on univariate analysis were considered in the multivariate model, and the most parsimonious model including the covariates with the highest biological and clinical relevance was selected. The covariates included in the final model were age, sex, CD8+ tumor-infiltrating lymphocytes, microsatellite instability, *BRAF* mutation, and medullary histology. To limit confounding by prior treatment, survival analysis was performed on a subset of the cohort in which PD-L1 expression was examined on untreated, primary tumors using the Kaplan–Meier method, log-rank tests, and the Cox proportional hazards model. Odds ratios (OR) and 95% confidence intervals (CI) were obtained. Statistical significance was set at $\alpha = 0.05$.

Results

The study cohort ($n = 181$) had a mean age of 62 years (range: 26–91), and 53% were female. PD-L1 expression was scored as 0, 1, and 2 in 165 (91%), 4 (2%), and 12 (7%) cases, respectively. Using the 5% cut-off (PD-L1 score 1–2), 16 cases (9%) were deemed positive for PD-L1 expression. Clinicopathologic and molecular characteristics of the study cohort stratified by PD-L1 status are shown in Table 1. PD-L1 expression was significantly associated with older age at the time of resection ($P = 0.006$) and female gender ($P = 0.035$). Interestingly, PD-L1 expression was only seen in resections of the primary tumor ($P = 0.014$), mostly in cases without neoadjuvant therapy ($P = 0.023$). Although the median tumor size was larger in the PD-L1 positive tumors ($P = 0.013$), this did not reach significance in terms of T-staging. Histologically, the PD-L1 positive

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Table 1 Clinicopathological characteristics

	PD-L1 positive, n *(%)*	*PD-L1 negative*, n *(%)*	P-*value*
Age, years, median (range)	74.5 (50–92)	61 (24–91)	0.006
Sex			
Male	3 (19)	78 (47)	0.035
Female	13 (81)	87 (53)	
Primary or recurrence/metastasis			
Primary	16 (100)	122 (74)	0.014
Recurrence/ metastasis	0	43 (26)	
Prior treatment			
No	15 (94)	109 (66)	0.023
Yes	1 (6)	56 (34)	
Tumor size, cm, median (range)	6.4 (2.9–13)	4.5 (0.8–13.9)	0.013
pT			
1	0	8 (5)	0.97
2	2 (12.5)	18 (12)	
3	10 (62.5)	83 (53)	
4	4 (25)	46 (30)	
pN			
0	7 (44)	70 (45)	0.32
1	6 (37)	35 (23)	
2	3 (19)	49 (32)	
Stage			
I	1 (6)	14 (9)	0.001
II	6 (38)	37 (22)	
III	8 (50)	33 (20)	
IV	1 (6)	81 (49)	
Tumor differentiation			
Well/moderate	2 (13)	112 (72)	< 0.001
Poor	14 (87)	43 (28)	
Mucinous histology			
Yes	3 (19)	33 (20)	1
No	13 (81)	128 (80)	
Medullary histology			
Yes	12 (75)	23 (7)	< 0.001
No	4 (25)	149 (93)	
Tumor-infiltrating lymphocytes			
Yes	12 (80)	34 (26)	< 0.001
No	3 (20)	98 (74)	
Small vessel invasion			
Yes	9 (56)	77 (54)	1
No	7 (44)	65 (46)	
Large vessel invasion			
Yes	6 (40)	66 (46)	0.79
No	9 (60)	77 (54)	
Perineural invasion			
Yes	6 (37)	58 (42)	0.71
No	10 (63)	79 (58)	
Tumor border			
Pushing	7 (47)	35 (26)	0.13
Infiltrating	8 (53)	100 (74)	

Abbreviation: PD-L1, programmed cell death ligand 1.

tumors were more likely to be poorly differentiated ($P < 0.001$) with a medullary phenotype ($P < 0.001$), and were more likely to exhibit prominent tumor-infiltrating lymphocytes ($P < 0.001$) (Figure 2), although angiolymphatic and perineural invasion had no bearing on PD-L1 expression.

Regarding the subsets of tumor-infiltrating lymphocytes (Table 2), 10 (62%) PD-L1-positive tumors contained a high number of CD8-positive tumor-infiltrating lymphocytes (five each of scores 2+ and 3+) and the remaining six (38%) PD-L1-positive tumors had score 1+ CD8-positive tumor-infiltrating lymphocytes. Only five (3%) of 164 PD-L1-negative tumors in which immunohistochemistry for tumor-infiltrating lymphocytes could be examined had a high number of CD8-positive tumor-infiltrating lymphocytes (all five with a score of 2+) and tumor-infiltrating lymphocytes were significantly more frequent in PD-L1-positive tumors ($P < 0.001$). Although no cases were scored 3+ for TBET-positive tumor-infiltrating lymphocytes, PD-L1-positive tumors had a significantly higher proportion of score 2+ TBET-positive tumor-infiltrating lymphocytes (62% *vs* 3% of PD-L1-negative tumors, $P < 0.001$). There was no association between PD-L1 expression and GATA3-postitive tumor-infiltrating lymphocytes.

Of 181 cases, 54 (30%) were determined to be microsatellite-instability high by immunohistochemistry combined with PCR ($n = 39$), or immunohistochemistry alone ($n = 15$). Of those, four (7%) patients were considered to have hereditary nonpolyposis colorectal cancer by clinical history and genetic testing. As for molecular testing, 118 patients exhibited one or more mutations with 83 harboring one mutation, 30 with two, and 5 with three. These included 44 (24%) with *BRAF* mutations, 44 (24%) with *KRAS* mutations and 37 (20%) with *TP53* mutations (Supplementary Table 1). In 11 microsatellite-unstable tumors *BRAF* mutation status was not known. Loss of PTEN expression by immunohistochemistry (< 10% of tumor cells staining) was seen in 35 (20%) of the 178 evaluated cases. PD-L1 expression was present in 12 (22%) of the 54 microsatellite-instability high tumors, 14 (32%) of the 44 BRAF-mutated tumors, three (8.1%) of the 37 *TP53* mutated tumors, the one *IDH* mutated tumor, as well as six (17%) of the 35 tumors with loss of PTEN protein expression on immunohistochemistry. Interestingly, the four hereditary nonpolyposis colorectal cancer cases did not exhibit PD-L1 expression. Of note, nine (75%) of the 12 microsatellite-unstable tumors with PD-L1 expression and nine (64%) of the 14 *BRAF*-mutated tumors with PD-L1 expression also showed increased CD8+ tumor-infiltrating lymphocytes (Figure 2). Overall, PD-L1 positive tumors were more likely to harbor microsatellite-instability ($P < 0.001$) and *BRAF* mutations ($P < 0.001$), but were less likely to exhibit *KRAS* mutations ($P = 0.012$). Furthermore, there was a trend towards tumors with PD-L1 expression exhibiting loss of PTEN expression

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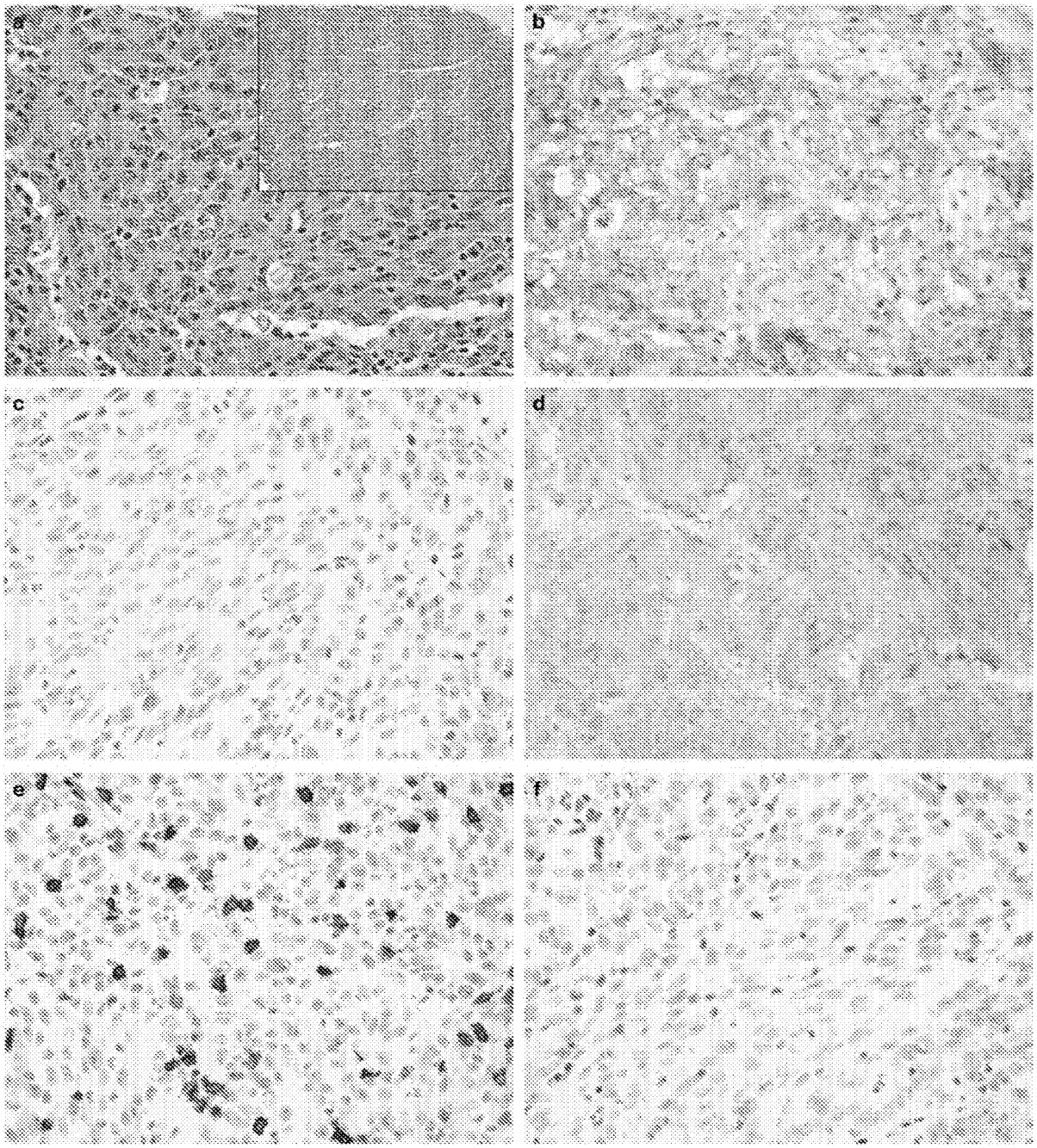


Figure 2 (**a**–**f**) Colorectal adenocarcinoma with PD-L1 expression. Poorly differentiated adenocarcinomas (**a**) with high PD-L1 expression (**b**) often demonstrated features of a medullary phenotype including syncytial growth and tumor-infiltrating lymphocytes. PD-L1 expression was associated with microsatellite instability ((**c**) loss of MLH1 nuclear expression in the tumor cells with preserved nuclear expression in the stromal cells) and *BRAF* mutation ((**d**) positive staining with BRAF V600E immunohistochemistry). These tumors also demonstrated high numbers of intraepithelial CD8+ (**e**) and TBET+ (**f**) small lymphocytes.

by immunohistochemistry ($P=0.092$), but not with *PTEN* mutation (Table 3).

By multivariate analysis using exact logistic regression, a high number of CD8-positive tumor-infiltrating lymphocytes (odds ratio (OR) 8.67, 95% CI 1.29, 77.93; $P=0.022$) and medullary histology (OR 10.33, 95% CI 1.18, 155.17; $P=0.032$) were independent predictors of PD-L1 expression after adjusting for other covariates in the model (Table 4). Age, microsatellite instability, and female gender

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Table 2 Tumor-infiltrating lymphocyte immunophenotype

	PD-L1 positive, n *(%)*	*PD-L1 negative*, n *(%)*	P-*value*
CD8			
Low	6 (37.5)	159 (97)	< 0.001
High	10 (62.5)	5 (3)	
TBET			
Low	6 (37.5)	157 (97)	< 0.001
High	10 (62.5)	5 (3)	
GATA3			
Low	16 (100)	161 (100)	1
High	0	0	

Abbreviation: PD-L1, programmed cell death ligand 1.
Low: scores 0–1. High: scores 2–3.

Table 3 Microsatellite instability and mutational status

	PD-L1 positive, n *(%)*	*PD-L1 negative*, n *(%)*	P-*value*
MSI status			
Stable	4 (25)	120 (74)	< 0.001
Instable	12 (75)	42 (26)	
BRAF			
WT	2 (13)	121 (80)	< 0.001
Mutant	14 (87)	30 (20)	
KRAS			
WT	15 (100)	102 (70)	0.012
Mutant	0	44 (30)	
PIK3CA			
WT	15 (100)	130 (91)	0.62
Mutant	0	13 (9)	
PTEN			
WT	15 (100)	139 (97)	1
Mutant	0	4 (3)	
PTEN IHC			
Retained	10 (63)	133 (82)	0.09
Lost	6 (37)	29 (18)	
APC			
WT	15 (100)	133 (93)	0.60
Mutant	0	10 (7)	
IDH			
WT	14 (93)	143 (100)	0.10
Mutant	1 (7)	0	
NRAS			
WT	15 (100)	137 (96)	1
Mutant	0	6 (4)	
TP53			
WT	12 (80)	109 (76)	1
Mutant	3 (20)	34 (24)	

Abbreviations: IHC, immunohistochemistry; MSI, microsatellite instability; PD-L1, programmed cell death ligand 1.

were not associated with PD-L1 expression in this analysis. Although it did not reach significance, there was a strong trend towards BRAF-mutated cancers having an independent predictive value for PD-L1 expression (OR 11.74, 95% CI 0.90, 203.93; $P = 0.064$).

Table 4 Multivariate analysis, predictors of PD-L1 positivity

	OR (95% CI)	P-*value*
Sex (female)	1.97 (0.21, 26.8)	0.83
Age (≥62 y/o)	0.14 (0.004, 2.07)	0.23
CD8 TIL	8.67 (1.29, 77.93)	0.022
BRAF mutation	11.74 (0.90, 203.93)	0.064
Medullary histology	10.33 (1.18, 155.17)	0.032
MSI	0.91 (0.03, 17.51)	1

Abbreviations: CI, confidence interval; MSI, microsatellite instability; OR, odds ratio.

Table 5 Multivariate survival analysis (Cox proportional hazards model)

	HR (95% CI)	P-*value*
Sex (male *vs* female)	1.6 (0.6–4.2)	0.38
Age	1 (0.96–1.04)	0.92
pT	1.9 (0.9–4.0)	0.12
pN	1.9 (1.0–3.5)	0.038
PD-L1 score 1 (*vs* 0)	1.3 (0.1–13.6)	0.84
PD-L1 score 2 (*vs* 0)	16.9 (1.0–297.7)	0.053
CD8 TIL	0.14 (0.12–1.8)	0.13
BRAF mutation	1.8 (0.5–6.8)	0.39
KRAS mutation	2.4 (0.8–7.1)	0.11
Medullary histology	0.13 (0.01–3.7)	0.23
MSI	4.8 (0.8–29.6)	0.094

Abbreviations: CI, confidence interval; HR, hazard ratio; MSI, microsatellite instability; PD-L1, programmed cell death ligand 1.

Among the patients who had undergone resection of the primary tumor without neoadjuvant therapy, the 3-year disease-specific survival rate for the PD-L1-positive ($n = 15$) and -negative ($n = 98$) groups were 76% and 72%, respectively ($P = 0.1896$). Interestingly, among the microsatellite-instability-positive patients, PD-L1 expression was associated with reduced disease-specific survival (3-year survival rate of 72% *vs* 97% of the PD-L1-negative group, $P = 0.0319$). By multivariate survival analysis using Cox proportional hazards model (Table 5), when individual PD-L1 expression scores (0, 1, and 2) were considered, nodal status was significantly associated with decreased survival (hazard ratio 1.9, 95% CI 1.04, 3.51, $P = 0.038$), and tumors with the highest PD-L1 expression (score 2) had marginally significant but markedly reduced disease-specific survival (hazard ratio 16.9, 95% CI 0.96, 297.67, $P = 0.053$) compared with those with negative PD-L1 expression (score 0). The significance of this association was reduced when PD-L1 expression was dichotomized into high (scores 1–2) *vs* low (score 0; $P = 0.096$). CD8-positve tumor-infiltrating lymphocytes, medullary histology, mutation status, and microsatellite-instability status were not independently prognostic.

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Discussion

PD-L1 expression by tumor cells has been shown to provide a mechanism of immune evasion through downregulation of the active T-cell-mediated immune response.[12,13] In this study, we demonstrate that although PD-L1 expression is infrequent in sporadic colorectal carcinomas, it is associated with signatures of the serrated pathway of colorectal carcinogenesis, including *BRAF* mutation, microsatellite instability, poor differentiation (with medullary morphology), and frequent tumor-infiltrating lymphocytes.[14] By multivariate analysis, PD-L1 expression is independently associated with medullary morphology and frequent tumor-infiltrating lymphocytes, and shows a trend towards association with *BRAF* mutation. In addition, the majority of PD-L1 positive tumors contained an increased number of TBET-positive tumor-infiltrating lymphocytes, indicative of activated Th1 pathway but not GATA3-positive tumor-infiltrating lymphocytes, which are characteristic of Th2 pathway activation.

To date, two different mechanisms of PD-L1 upregulation on tumor cells have been reported: innate immune resistance and adaptive immune resistance.[1] The former refers to the upregulation of PD-L1 expression as a result of constitutive oncogenic signaling within tumor cells, as seen in *ALK*-rearranged and *EGFR*-mutant lung cancer.[15,16] By contrast, adaptive immune resistance refers to the induction of PD-L1 expression on tumor cells in response to local inflammatory signals (eg, interferons) produced by active anti-tumor immune response (cytotoxic T cell and/or Th1 pathway activation).[13] This in turn induces PD-1 expression on T cells.[17] When engaged by PD-L1 or other ligands, PD-1 inhibits kinases that are involved in T-cell activation through the phosphatase SHP250, which leads to the apoptosis of T cells, although additional signaling pathways are likely also induced.[1,16,19] Our findings suggest that upregulation of PD-L1 in colorectal carcinoma is due to an adaptive immune response based on the association of PD-L1 expression and frequent tumor-infiltrating lymphocytes including CD8-positive and TBET-positive tumor-infiltrating lymphocytes.

In contrast to other tumor types, colorectal carcinoma in general shows a low response rate to PD-1 or PD-L1 blockade.[6–8] However, two clinical trials of anti-PD-1 antibodies for patients with colorectal carcinoma selected for microsatellite-instability high status have been initiated (NCT01876511 and NCT02060188), and a recent report from one of these trials found a significantly higher response rate and survival in colorectal carcinomas with microsatellite instability compared with their microsatellite-stable counterparts.[20] That study also revealed a greater than tenfold higher rate of somatic mutations in microsatellite-instability high tumors compared with microsatellite-stable colorectal carcinomas, as might be expected in tumors with global DNA-repair defects.[10] It has been hypothesized that this higher mutational burden may result in the formation of more neo-antigens in microsatellite-unstable tumors, leading to a robust anti-tumor immune response. Upregulation of PD-L1 expression in tumor cells within this environment may lead to abrogation of the active immune response and more aggressive tumor biology. This hypothesis is supported by a recent study with a small cohort of colorectal carcinomas ($n=25$).[9] That study showed highly upregulated expression of multiple immune checkpoints, including PD-1, PD-L1, CTLA-4, LAG-3, and IDO in tumor-infiltrating inflammatory cells in tumors with activated CD8+ cytotoxic T cells as well as an activated Th1 pathway in the context of microsatellite instability, although the PD-L1 expression was identified in immune cells, not on the tumor cells.[9] Our study also confirms the link between microsatellite instability and PD-L1 expression in colorectal carcinoma, and supports the idea that immunogenic microsatellite-instability high tumors may acquire PD-L1 expression on the tumor cells under selective pressure to escape from the cytotoxic T-cell/Th1 immune response.

We also identified a positive association of PD-L1 expression with *BRAF* mutation and an inverse association with *KRAS* mutation in this colorectal carcinoma cohort. These findings are most likely explained by the frequent co-occurrence of *BRAF* mutation and microsatellite instability in the serrated pathway of colorectal carcinogenesis,[14] as well as the mutual exclusivity of *BRAF* and *KRAS* mutations in colorectal carcinoma.[20] Interestingly though, in lung adenocarcinoma *KRAS* mutations are positively correlated with PD-L1 expression.[21] The link between *KRAS* mutation and PD-L1 expression in non-small cell lung cancer is attributed in part to smoking, which increases the risk of developing *KRAS* mutations along with other passenger mutations.[21] As is hypothesized to occur in microsatellite-unstable colorectal carcinoma, the high mutational burden in such non-small cell lung cancer appears to lead to neo-antigen formation and increased immunogenicity.[22] In addition, as in microsatellite-unstable colorectal carcinoma, lung cancers with a high mutational burden and neo-antigen formation appear to be associated not only with induction of PD-L1 expression on tumor cells, but also with an improved objective response to anti-PD-1 agents with durable clinical benefit and better progression-free survival.[10,22] These findings suggest a common mechanism for induction of PD-L1 expression in these tumors despite different site of origin and driver mutations.

Interestingly, multivariate analysis revealed that only increased CD8-positive tumor-infiltrating lymphocytes, and medullary morphology, but not microsatellite instability, were significant independent predictors of PD-L1 expression. Given that only 14 (26%) of the 54 microsatellite-unstable tumors showed significant CD8-positive tumor-infiltrating

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lymphocytes in our cohort, it seems that the presence of a cytotoxic T-cell (and Th1)-rich tumor microenvironment is more closely associated with PD-L1 expression than the presence of microsatellite instability in general. Interestingly, *BRAF* mutation was also not an independent predictor of PD-L1 expression, although it did show a trend toward significance. These results suggest the possibility that distinct subtypes of colorectal carcinoma may arise within the classic microsatellite-instability pathway of colorectal carcinogenesis, including a PD-L1-positive immune activated subtype with increased tumor-infiltrating lymphocytes and subsequent, adaptive immune resistance, and a PD-L1-negative immune-tolerant subtype. Of note, the four hereditary nonpolyposis colorectal cancer cases did not show PD-L1 expression in this study. Additional studies with larger numbers of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal carcinomas may be helpful to clarify whether sporadic microsatellite instability is more prone to adaptive immune resistance than hereditary nonpolyposis colorectal cancer.

As for the prognostic role of clinicopathological variables, nodal status was an independent predictor of decreased disease-specific survival. In a dichotomized model, high PD-L1 expression did not show a convincing trend toward decreased survival ($P=0.1896$). However, when comparing those cases with very high PD-L1 expression (2+) to those with very low or no expression (0), high PD-L1 expression was more closely associated with a lower disease-specific survival (hazard ratio 16.9; $P=0.053$). The marginal statistical significance of these associations may be due to the small number of patients with PD-L1 expression and limited follow up in a number of PD-L1-positive cases, however, the magnitude of the difference (high hazard ratio) may make this finding clinically meaningful. Further studies are needed to elucidate these findings. When restricted to microsatellite-unstable cases, PD-L1 expression was correlated with significantly decreased survival ($P=0.0319$). This finding suggests that one of reasons microsatellite-unstable colorectal carcinoma has a more favorable prognosis in general may be due to immune surveillance. Negation of that immune surveillance (such as through the expression of PD-L1) may allow for the escape and spread of cancerous cells.

Our study has several important limitations. First, there is currently no gold standard in PD-L1 testing, and a number of different antibodies and scoring protocols have been applied.[23] To account for this, we used a commercially available, anti-PD-L1 antibody that has been independently validated.[24–26] We also evaluated PD-L1 expression using cut-offs that have been associated with beneficial response to PD-1/PD-L1 inhibitors in clinical studies.[7,27] Another issue associated with the analysis is the use of tissue microarrays, in which heterogeneous staining of tumors is difficult to evaluate. A recent study on non-small cell lung cancer demonstrated significant heterogeneity of stain with PD-L1 expression.[26] To overcome this issue, we constructed tissue microarrays of multiple 2-mm cores for each of the study cases, and the final score was made based on their collective assessment. Finally, molecular testing results were limited in some (6.1%) of the study cohort, although the vast majority was successfully evaluated for microsatellite-instability status.

In conclusion, we demonstrate that PD-L1 expression is characteristic of a subset of microsatellite-unstable colorectal carcinoma, with increased CD8-positive tumor-infiltrating lymphocytes and a medullary phenotype being independent predictors of high PD-L1 status. Our findings support the hypothesis that expression of PD-L1 may be induced by a cytotoxic T-cell/Th1-mediated immune reaction to tumor cells that arises in the setting of microsatellite instability and high neo-antigen load. Interestingly, a high level of PD-L1 expression showed a trend toward markedly reduced survival on multivariate analysis, and was associated with adverse prognosis within microsatellite-unstable tumors. This suggests that colorectal carcinoma with high PD-L1 expression may acquire a survival advantage though downregulation of the immune response. Our finding of PD-L1 expression in microsatellite instability also provides a histopathologic correlate to the mechanism underlying the known response of microsatellite-unstable colorectal carcinoma to PD-1 blockade.[10] This study calls attention to the importance of studying PD-1/PD-L1 axis blockade not only in colorectal carcinoma but in other tumors with microsatellite instability, frequent tumor-infiltrating lymphocytes, and/or a medullary phenotype. Furthermore, PD-L1 expression may be useful in prognostic stratification of microsatellite-instability high colorectal carcinoma, and potentially identifying colorectal carcinoma cases that might respond to PD-1/PD-L1 blockade.

Acknowledgments

The study was in part presented at the 104th annual meeting of the United States and Canadian Society of Pathology.

Disclosure/conflict of interest

MM-K has served as a consultant for Merrimack Pharmaceuticals. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)

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Science **314**, 268 (2006);
DOI: 10.1126/science.1133427

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The Consensus Coding Sequences of Human Breast and Colorectal Cancers

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The elucidation of the human genome sequence has made it possible to identify genetic alterations in cancers in unprecedented detail. To begin a systematic analysis of such alterations, we determined the sequence of well-annotated human protein-coding genes in two common tumor types. Analysis of 13,023 genes in 11 breast and 11 colorectal cancers revealed that individual tumors accumulate an average of ~90 mutant genes but that only a subset of these contribute to the neoplastic process. Using stringent criteria to delineate this subset, we identified 189 genes (average of 11 per tumor) that were mutated at significant frequency. The vast majority of these genes were not known to be genetically altered in tumors and are predicted to affect a wide range of cellular functions, including transcription, adhesion, and invasion. These data define the genetic landscape of two human cancer types, provide new targets for diagnostic and therapeutic intervention, and open fertile avenues for basic research in tumor biology.

It is widely accepted that human cancer is a genetic disease caused by sequential accumulation of mutations in oncogenes and tumor suppressor genes (*1*). These tumor-specific (that is, somatic) mutations provide clues to the cellular processes underlying tumorigenesis and have proven useful for diagnostic and therapeutic purposes. To date, however, only a small fraction of the genes has been analyzed, and the number and type of alterations responsible for the development of common tumor types are unknown (*2*). In the past, the selection of genes chosen for mutational analyses in cancer has been guided by information from linkage studies in cancer-prone families, identification of chromosomal abnormalities in tumors, or known functional attributes of individual genes or gene families (*2–4*). With the determination of the human genome sequence and recent improvements in sequencing and bioinformatic approaches, it is now possible in principle to examine the cancer cell genome in a comprehensive and unbiased manner. Such an approach not only provides the means to discover other genes that contribute to tumorigenesis, but also can lead to mechanistic insights that are only evident through a systems biological perspective. Comprehensive genetic analyses of human cancers could lead to discovery of a set of genes, linked together through a shared phenotype, that point to the importance of specific cellular processes or pathways.

To begin the systematic study of the cancer genome, we examined a major fraction of human genes in two common tumor types, breast and colorectal cancers. These cancers were chosen for study because of their substantial clinical importance worldwide; together they account for ~2.2 million cancer diagnoses (20% of the total) and ~940,000 cancer deaths each year (14% of the total) (*5*). For genetic evaluation of these tumors, we focused on a set of protein-coding genes, termed the consensus coding sequences (CCDS), that represent the most highly curated gene set currently available. The CCDS Database (*6*) contains full-length protein-coding genes that have been defined by extensive manual curation and computational processing and have gene annotations that are identical among reference databases.

The goals of this study were (i) to develop a methodological strategy for conducting genome-wide analyses of cancer genes in human tumors, (ii) to determine the spectrum and extent of somatic mutations in human tumors of similar and different histologic types, and (iii) to identify new cancer genes and molecular pathways that could lead to improvements in diagnosis or therapy.

Cancer mutation discovery screen. The initial step toward achieving these goals was the development of methods for high-throughput identification of somatic mutations in cancers. These methods included those for primer design, polymerase chain reaction (PCR), sequencing, and mutational analysis (Fig. 1). The first component involved extraction of all protein-coding sequences from the CCDS genes. A total of 120,839 nonredundant exons and adjacent intronic sequences were obtained from 14,661 different transcripts in CCDS. These sequences were used to design primers for PCR amplification and sequencing of exons and adjacent splice sites. Primers were designed using a number of criteria to ensure robust amplification and sequencing of template regions (*7*). Although most exons could be amplified in a single PCR reaction, we found that exons larger than 350 base pairs (bp) were more effectively amplified as multiple overlapping amplicons. One member of every pair of PCR primers was tailed with a universal primer sequence for subsequent sequencing reactions. A total of 135,483 primer pairs encompassing ~21 Mb of genomic sequence were designed in this manner (table S1).

Eleven cell lines or xenografts of each tumor type (breast and colorectal carcinomas) were used in the discovery screen (table S2, A and B). Two matching normal samples were used as controls to help identify normal sequence variations and amplicon-specific sequencing artifacts such as those associated with GC-rich regions. A total of ~3 million PCR products were generated and directly sequenced, resulting in 465 Mb of tumor sequence.

Sequence data were assembled for each amplicon and evaluated for quality within the target region with the use of software specifically designed for this purpose (*7*). The target region of each exon included all coding bases as well as the four intronic bases at both the 5′ and 3′ ends that serve as the major splice recognition sites. For an amplicon to be considered successfully analyzed, we required that ≥90% of bases in the target region have a Phred quality score—defined as $-10\{\log_{10}(\text{raw per-base error})\}$—of at least 20 in at least three-quarters of the tumor samples analyzed (*8*). This quality cutoff was chosen to provide high sensitivity for mutation detection while minimizing false positives. Using these criteria, 93% of the 135,483 amplicons and 90% of the total targeted bases in CCDS were successfully analyzed for potential alterations.

Examination of sequence traces from these amplicons revealed a total of 816,986 putative

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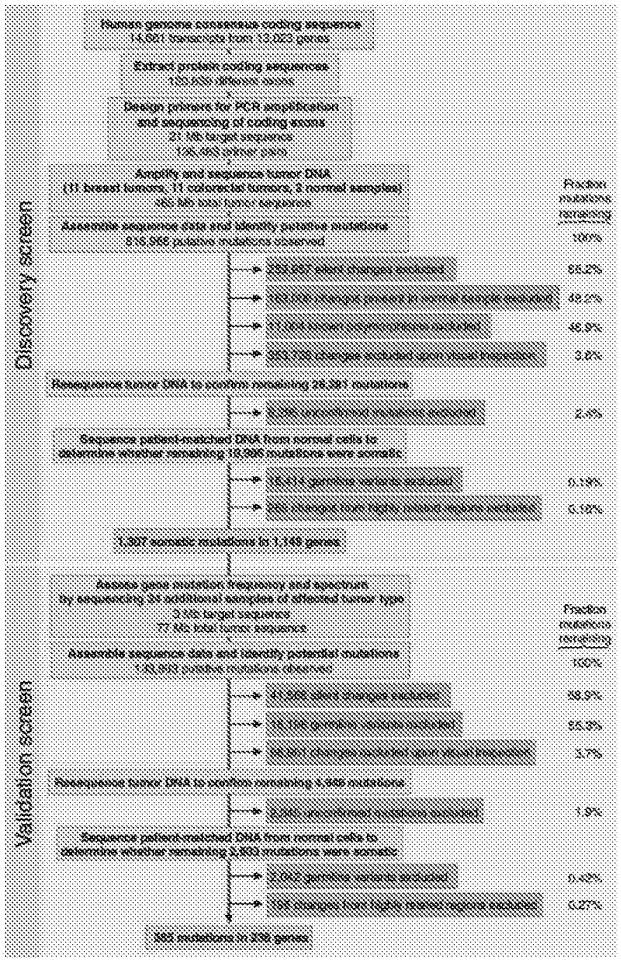
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nucleotide changes. Because the vast majority of changes that did not affect the amino acid sequence (i.e., synonymous or silent substitutions) were likely to be nonfunctional, these changes were not analyzed further. The remaining 537,029 changes could represent germline variants, artifacts of PCR or sequencing, or bona fide somatic mutations. Several bioinformatic and experimental steps were used to distinguish among these possibilities. First, any alterations that were also present in either of the two normal samples included in the discovery screen were removed, as these were likely to represent common germline polymorphisms or sequence artifacts. Second, as these two normal control samples would be expected to contain only a subset of known variants, any change corresponding to a germline polymorphism found in single-nucleotide polymorphism (SNP) databases was also removed (*7*). Finally, the sequence trace of each potential alteration was visually inspected so as to remove false positive calls in the automated analysis. The combination of these data analysis efforts was efficient, removing ~96% of the potential alterations and leaving 29,281 for further scrutiny (Fig. 1).

To ensure that the observed mutations did not arise artifactually during the PCR or sequencing steps, we independently reamplified and resequenced the regions containing them in the corresponding tumors. This step removed 9295 alterations. The regions containing the putative mutations were then sequenced in matched normal DNA samples to determine whether the mutations were truly somatic: 18,414 changes were observed to be present in the germ line of these patients, representing variants not currently annotated in SNP databases, and were excluded. As a final step, the remaining 1572 putative somatic mutations were carefully examined in silico to ensure that the alterations did not arise from mistargeted sequencing of highly related regions occurring elsewhere in the genome (*7*). Alterations in such duplicated regions may appear to be somatic when there is loss of one or both alleles of the target region in the tumor and when the selected primers closely match and therefore amplify similar areas of the genome. A total of 265 changes in closely related regions were excluded in this fashion, resulting in a total of 1307 confirmed somatic mutations in 1149 genes (Table 1).

Fig. 1. Schematic of mutation discovery and validation screens.



Validation screen. To evaluate the prevalence and spectrum of somatic mutations in these 1149 genes, we determined their sequence in additional tumors of the same histologic type (Fig. 1) (table S2, A and B). Genes mutated in at least one breast or colorectal tumor in the discovery screen were analyzed in 24 additional breast or colorectal tumors, respectively. This effort involved 453,024 additional PCR and sequencing reactions encompassing 77 Mb of tumor DNA. A total of 133,693 putative changes were identified in the validation screen. Methods similar to those used in the discovery screen were used to exclude silent changes, known and novel germline variants, false positives arising from PCR or sequencing artifacts, and apparent changes that were likely due to coamplification of highly related genes. Additionally, any changes corresponding to germline variants not found in SNP databases but identified in the discovery screen were excluded. The regions containing the remaining 4948 changes were reamplified and resequenced in the corresponding tumors (to ensure reproducibility) and in matched normal tissue to determine if they were somatic. An additional 365 somatic mutations in 236 genes were identified in this manner. In total, 921 and 751 somatic mutations were identified in breast and colorectal cancers, respectively (Fig. 1, Table 1, and table S4).

Mutation spectrum. The great majority of the 1672 mutations observed in the discovery or validation screens were single base substitutions: 81% of the mutations were missense, 7% were nonsense, and 4% were altered splice sites (Table 1). The remaining 8% were insertions, deletions, and duplications ranging from 1 to 110 nucleotides in length. Although the fraction of mutations that were single base substitutions was similar in breast and colorectal cancers, the spectrum and nucleotide contexts of the substitution mutations were very different between the two tumor types. The most striking of these differences occurred at C:G base pairs: 59% of the 696 colorectal cancer mutations were C:G to T:A transitions, whereas only 7% were C:G to G:C transversions (Table 2 and table S3). In contrast, only 35% of the mutations in breast cancers were C:G to T:A transitions, whereas 29% were C:G to G:C transversions. In addition, a large fraction (44%) of the mutations in colorectal cancers were at 5′-CpG-3′ dinucleotide sites, but only 17% of the mutations in breast cancers occurred at such sites. This 5′-CpG-3′ preference led to an excess of nonsynonymous mutations, resulting in changes of arginine residues in colorectal cancers but not in breast cancers (fig. S1). In contrast, 31% of mutations in breast cancers occurred at 5′-TpC-3′ sites (or complementary 5′-GpA-3′ sites), whereas only 11% of mutations in colorectal cancers occurred at these dinucleotide sites. The differences noted above were all highly significant ($P < 0.0001$) (*7*) and have substantial implications for the mechanisms underlying mutagenesis in the two tumor types.

Distinction between passenger and nonpassenger mutations. Somatic mutations in

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human tumors can arise either through selection of functionally important alterations (via their effect on net cell growth) or through accumulation of nonfunctional "passenger" alterations that arise during repeated rounds of cell division in the tumor or in its progenitor stem cell. In light of the relatively low rates of mutation in human cancer cells (*9*, *10*), a distinction between selected and passenger mutations is generally not required when the number of genes and tumors analyzed is small. In large-scale studies, however, such distinctions are of paramount importance (*11*, *12*). For example, it has been estimated that nonsynonymous passenger mutations are present at a frequency no higher than ~1.2 per Mb of DNA in cancers of the breast or colon (*13*–*15*). Because we assessed 542 Mb of tumor DNA, we would therefore have expected to observe ~650 passenger mutations. We actually observed 1672 mutations (Table 1), many more than what would have been predicted to occur by chance ($P < 1 \times 10^{-10}$) (*7*). Moreover, the frequency of mutations in the validation screen was significantly higher than in the discovery screen (5.8 versus 3.1 mutations per Mb, $P < 1 \times 10^{-10}$; Table 1). The mutations in the validation screen were also enriched for nonsense, insertion, deletion, duplication, and splice site changes relative to the discovery screen; each of these would be expected to have a functional effect on the encoded proteins.

To distinguish genes likely to contribute to tumorigenesis from those in which passenger mutations occurred by chance, we first excluded genes that were not mutated in the validation screen. We next developed statistical methods to estimate the probability that the number of mutations in a given gene was greater than expected from the background mutation rate. For each gene, this analysis incorporated the number of somatic alterations observed in either the discovery or validation screens, the number of tumors studied, and the number of nucleotides successfully analyzed (as indicated by the number of bases with Phred quality scores of ≥20). Because the mutation frequencies varied with nucleotide type and context and were different in breast versus colorectal cancers (Table 2), these factors were included in the calculations. The output of this analysis was a cancer mutation prevalence (CaMP) score for each gene analyzed. The CaMP score reflects the probability that the number of mutations observed in a gene reflects a mutation frequency that is higher than that expected to be observed by chance given the background mutation rate; its derivation is based on principles described in (*7*). The use of the CaMP score for analysis of somatic mutations is analogous to the use of the lod score for linkage analysis in familial genetic settings. For example, 90% of the genes with CaMP scores of >1.0 are predicted to have mutation frequencies higher than the background mutation frequency.

Candidate cancer genes. A complete list of the somatic mutations identified in this study is provided in table S4. Validated genes with CaMP scores greater than 1.0 were considered to be candidate cancer genes (*CAN* genes). The combination of experimental validation and statistical calculation thereby yielded four nested sets of genes: Of 13,023 genes evaluated, 1149 were mutated, 236 were validated, and 189 were *CAN* genes. Among these, the *CAN* genes were most likely to have been subjected to mutational selection during tumorigenesis. There were 122 and 69 *CAN* genes identified in breast and colorectal cancers, respectively (tables S5 and S6). Individual breast cancers examined in the discovery screen harbored an average of 12 (range 4 to 23) mutant *CAN* genes, whereas the average number of *CAN* genes in colorectal cancers was 9 (range 3 to 18) (table S3). Interestingly, each cancer specimen of a given tumor type carried its own distinct *CAN*-gene mutational signature, as no cancer had more than six mutant *CAN* genes in common with any other cancer (tables S4 to S6).

CAN genes could be divided into three classes: (i) genes previously observed to be mutationally altered in human cancers, (ii) genes in which no previous mutations in human cancers had been discovered but had been linked to cancer through functional studies, and (iii) genes with no previous strong connections to neoplasia.

The reidentification of genes that had been previously shown to be somatically mutated in cancers represented a critical validation of the approach used in this study. All of the CCDS genes previously shown to be mutated in >10% of either breast or colorectal cancers were found to be *CAN* genes in the current study. These included *TP53* (*2*), *APC* (*2*), *KRAS* (*2*), *SMAD4* (*2*), and *FBXW7* (*CDC4*) (*16*) (tables S4 to S6). In addition, we identified mutations in genes whose mutation prevalence in sporadic cancers was rather low. These genes included *EPHA3* (*17*), *MRE11A* (*18*), *NF1* (*2*), *SMAD2* (*19*, *20*), *SMAD3* (*21*), *TCF7L2* (*TCF4*) (*22*), *BRCA1* (*2*), and *TGFBRII* (*23*). We also detected mutations in genes that had been previously found to be altered in human tumors but not in the same tumor type identified in this study. These included *GNAS* (guanine nucleotide binding protein, α stimulating) (*24*), *KEAP1* (kelch-like ECH-associated protein) (*25*), *RET* (a proto-oncogene) (*2*), and *TCF1* (a transcription factor) (*26*). Finally, we found mutations in a number of genes that have been previously identified as targets of translocation or amplification in human cancers. These included *NUP214* (a nucleoporin) (*2*), *KIN1* (a kinesin receptor) (*27*), *DDX10* (DEAD box polypeptide 10) (*28*), *GLI1* (glioma-associated oncogene homolog 1) (*29*), and *MTG8* (the translocation target gene of runt-related transcription factor 1, *RUNX1T1*) (*2*). We conclude that if these genes had not already been shown to play a causative role in human tumors, they would have been discovered through the approach taken in this study. By analogy, the 167 other *CAN* genes in tables S5 and S6 are likely to

Table 1. Summary of somatic mutations. Numbers in parentheses refer to percentage of total mutations.

Tumor	Discovery screen*			Validation screen†			Both screens combined		
	Colorectal	Breast	Total	Colorectal	Breast	Total	Colorectal	Breast	Total
Number of mutated genes	519	673	1149	105	137	236	519	673	1149
Number of mutations	574	733	1307	177	188	365	751	921	1672
Nonsynonymous mutations in coding sequences									
Missense	482 (84.0)	600 (81.9)	1082 (82.8)	126 (71.2)	145 (77.1)	271 (74.2)	608 (81.0)	745 (80.9)	1353 (80.9)
Nonsense	35 (6.1)	39 (5.3)	74 (5.7)	26 (14.7)	8 (4.3)	34 (9.3)	61 (8.1)	47 (5.1)	108 (6.5)
Insertion	3 (0.5)	3 (0.4)	6 (0.5)	2 (1.1)	2 (1.1)	4 (1.1)	5 (0.7)	5 (0.5)	10 (0.6)
Deletion	18 (3.1)	48 (6.5)	66 (5.0)	10 (5.6)	13 (6.9)	23 (6.3)	28 (3.7)	61 (6.6)	89 (5.3)
Duplication	17 (3.0)	2 (0.3)	19 (1.5)	3 (1.7)	12 (6.4)	15 (4.1)	20 (2.7)	14 (1.5)	34 (2.0)
Mutations in noncoding sequences									
Splice site‡	17 (3.0)	37 (5.0)	54 (4.1)	9 (5.1)	8 (4.3)	17 (4.7)	26 (3.5)	45 (4.9)	71 (4.2)
UTR§	2 (0.3)	4 (0.5)	6 (0.5)	1 (0.6)	0 (0.0)	1 (0.3)	3 (0.4)	4 (0.4)	7 (0.4)
Nucleotides successfully analyzed (Mb)‖	208.5	209.2	417.7	28.7	34.3	63.0	237.2	243.5	480.7
Mutation frequency (mutations/Mb)	2.8	3.5	3.1	6.2	5.5	5.8	3.2	3.8	3.5

*Coding and adjacent noncoding regions of 13,023 CCDS genes were sequenced in 11 colorectal and 11 breast cancers. †Genes mutated in the discovery screen were sequenced in 24 additional tumor samples of the affected tumor type. ‡Intronic mutations within 4 bp of exon/intron boundary. §Mutations in untranslated regions (UTR) within 4 bp 5′ of initiation codon or 4 bp 3′ of termination codon. ‖Nucleotides with Phred quality score of at least 20.

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play important roles in breast, colorectal, and perhaps other types of cancers.

Although genetic alterations currently provide the most reliable indicator of a gene's importance in human neoplasia (*1*, *30*), many other genes are thought to play key roles on the basis of functional or expression studies. Our study provides genetic evidence supporting the importance of several of these genes in neoplasia. For example, we discovered intragenic mutations in *EPHB6* (an ephrin receptor) (*31*), *MLL3* (mixed-lineage leukemia 3) (*32*), *GSN* (gelsolin) (*33*), *CDH10* and *CDH20* (cadherins), *FLNB* (actin and SMAD binding protein filamin B) (*34*), *PTPRD* (protein tyrosine phosphatase receptor) (*35*), and *AMFR* (autocrine motility factor receptor) (*36*).

In addition to these two classes of genes, our study revealed a large number of genes that had not been strongly suspected to be involved in cancer. This third class of genes included *PKHD1* (polycystic kidney and hepatic disease 1), *GUCY1A2* (guanylate cyclase 1), *TBX22* (a transcription factor), *SEC8L1* (an exocyst complex component), *TTLL3* (a tubulin tyrosine ligase), *ATP8B1* (an ATP-dependent transporter), *CUBN* (an intrinsic factor-cobalamin receptor), *DBN1* (an actin binding protein), and *TECTA* (tectorin α). In addition, seven *CAN* genes corresponded to genes for which no biologic role has yet been established.

We examined the distribution of mutations within *CAN*-gene products to see whether clustering occurred in specific regions or functional domains. In addition to the well-documented hotspots in *TP53* (*37*) and *KRAS* (*38*), we identified three mutations in *GNAS* in colorectal cancers that affected a single amino acid residue (Arg^{201}). Alterations of this residue have previously been shown to lead to constitutive activation of the encoded heterotrimeric guanine nucleotide–binding protein (G protein) α_s through inhibition of guanosine triphosphatase (GTPase) activity (*24*). Two mutations in the EGF-like gene *EGFL6* in breast tumors affected the same nucleotide position and resulted in a $Leu^{918} \rightarrow Phe$ change in the MAM adhesion domain. A total of seven genes had alterations located within five amino acid residues of each other, and an additional 12 genes had clustering of multiple mutations within a specific protein domain (13 to 78 amino acids apart). Thirty-one of 40 of these changes affected residues that were evolutionarily conserved. Although the effects of these alterations are unknown, their clustering suggests specific roles for the mutated regions in the neoplastic process.

***CAN*-gene groups.** An unbiased screen of a large set of genes can provide insights into pathogenesis that would not be apparent through single-gene mutational analysis. This has been exemplified by large-scale mutagenesis screens in experimental organisms (*39*–*41*). We therefore attempted to assign each *CAN* gene to a functional group based on Gene Ontology (GO) molecular function or biochemical process groups, the presence of specific INTERPRO sequence domains, or previously published literature (Table 3 and Fig. 2). Several of the groups identified in this way were of special interest. For example, 22 of the 122 (18%) breast *CAN* genes and 13 of the 69 (19%) colorectal *CAN* genes were transcriptional regulators. At least one of these genes was mutated in more than 80% of the tumors of each type. Zinc-finger transcription factors were particularly highly represented (eight genes mutated collectively in 43% of breast cancer samples). Similarly, genes involved in cell adhesion represented ~22% of *CAN* genes and affected more than two-thirds of tumors of either type. Genes involved in signal transduction represented ~23% of *CAN* genes, and at least one such gene was mutated in 77% and 94% of the breast and colorectal cancer samples, respectively. Subsets of these groups were also of interest and included metalloproteinases (part of the cell adhesion and motility group and mutated in 37% of colorectal cancers) and G proteins and their regulators (part of the signal transduction group and altered in 43% of breast cancers). These data suggest that dysregulation of specific cellular processes is genetically selected during neoplasia and that distinct members of each group may serve similar roles in different tumors.

Discussion. Four important points have emerged from this comprehensive mutational analysis of human cancer. First, a relatively large number of previously uncharacterized *CAN* genes exist in breast and colorectal cancers, and these genes can be discovered by unbiased approaches such as that used in our study. These results support the notion that large-scale mutational analyses of other tumor types will prove useful for identifying genes not previously known to be linked to human cancer.

Second, our results suggest that the number of mutational events occurring during the evolution of human tumors from a benign to a metastatic state is much larger than previously thought. We found that breast and colorectal cancers harbor an average of 52 and 67 nonsynonymous somatic mutations in CCDS genes, of which an average of 9 and 12, respectively, were in *CAN* genes (table S3). These data can be used to estimate the total number of nonsynonymous mutations in coding genes that arise in a "typical" cancer through sequential rounds of mutation and selection. If we assume that the mutation prevalence in genes that have not yet been sequenced is similar to that of the genes so far analyzed, we estimate that there are 81 and 105 mutant genes (average 93) in the typical colorectal or breast cancer, respectively (*7*). Of these, an average of 14 and 20, respectively, would be expected to be *CAN* genes. In addition to the *CAN* genes, there were other mutated CCDS genes that were likely to have been selected for during tumorigenesis but were not altered at a frequency high enough to warrant confidence in their interpretation.

A third point emerging from our study is that breast and colorectal cancers show substantial differences in their mutation spectra. In colorectal cancers, a bias toward C:G to T:A transitions at 5′-CpG-3′ sites was previously noted in *TP53* (*42*). Our results suggest that this bias is genome-wide

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Table 2. Spectrum of single base substitutions. Base substitutions in coding sequences resulting in nonsynonymous changes as well as substitutions in noncoding sequences are included (see Table 1). Numbers in parentheses indicate percentage of total mutations.

Tumor	Discovery screen			Validation screen			Both screens combined		
	Colorectal	Breast	Total	Colorectal	Breast	Total	Colorectal	Breast	Total
Total number of substitutions	535	678	1213	161	160	321	696	838	1534
Substitutions at C:G base pairs									
C:G → T:A	325 (60.7)	230 (33.9)	555 (45.6)	88 (54.7)	59 (36.9)	147 (45.8)	413* (59.3)	289* (34.5)	702 (45.8)
C:G → G:C	36 (6.7)	207 (30.5)	243 (20.0)	12 (7.5)	32 (20.0)	44 (13.7)	46* (6.9)	239* (28.5)	287 (18.7)
C:G → A:T	70 (13.1)	110 (16.2)	180 (14.8)	23 (14.3)	38 (23.8)	61 (19.0)	93 (13.4)	148 (17.7)	241 (15.7)
Substitutions at T:A base pairs									
T:A → C:G	42 (7.9)	54 (8.0)	96 (7.9)	14 (8.7)	18 (11.3)	32 (10.0)	56 (8.0)	72 (8.6)	128 (8.3)
T:A → G:C	38 (7.1)	30 (4.4)	68 (5.6)	13 (8.1)	5 (3.1)	18 (5.6)	51 (7.3)	35 (4.2)	86 (5.6)
T:A → A:T	24 (4.5)	47 (6.9)	71 (5.9)	11 (6.8)	8 (5.0)	19 (5.9)	35 (5.0)	55 (6.6)	90 (5.9)
Substitutions at specific dinucleotides†									
5′-CpG-3′	254 (47.5)	115 (17.0)	369 (30.4)	55 (34.2)	24 (15.0)	79 (24.6)	309* (44.4)	139* (16.6)	448 (29.2)
5′-TpC-3′	54 (10.1)	235 (34.7)	289 (23.8)	25 (15.5)	22 (13.8)	47 (14.6)	79* (11.4)	257* (30.7)	336 (21.9)

*Values in this category were significantly different between breast and colorectal cancers ($P < 0.0001$). †Includes substitutions at the C or G of the 5′-CpG-3′ dinucleotide, the C of the 5′-TpC-3′ dinucleotide, or the G of the 5′-GpA-3′ dinucleotide.

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rather than representing a selection for certain nucleotides within *TP53*. This bias may reflect a more extensive methylation of 5′-CpG-3′ dinucleotides in colorectal cancers than in breast cancers, or it may be an effect of dietary carcinogens (*43*, *44*). In breast cancers, the fraction of mutations at 5′-TpC-3′ sites was far higher in the CCDS genes examined in this study than previously reported for *TP53* (*37*). It has been noted that a small fraction of breast tumors may have a defective repair system, resulting in 5′-TpC-3′ mutations (*15*). Our studies confirm that some breast

Table 3. Functional classification of *CAN* genes, with CaMP score to the right of each gene name. *CAN* genes were assigned to functional classes using Gene Ontology (GO) groups, INTERPRO domains, and available literature. Representative GO groups and INTERPRO domains are listed for each class.

Breast cancers						Colorectal cancers			
Cellular adhesion and motility (examples: cytoskeletal protein binding GO:0008092, cell adhesion GO:0007155, metallopeptidase activity GO:0008237)									
FLNB	3.4	*TMPRSS6*	2.0	*RAPH1*	1.4	*PKHD1*	3.5	*CNTN4*	1.6
MYH1	2.7	*COL11A1*	1.8	*PCDHB15*	1.4	*ADAMTSL3*	3.3	*CHL1*	1.3
SPTAN1	2.6	*DNAH9*	1.7	*CMYA1*	1.4	*OBSCN*	3.0	*HAPLN1*	1.2
DBN1	2.5	*OBSCN*	1.7	*MACF1*	1.3	*ADAMTS18*	2.7	*MGC33407*	1.2
TECTA	2.4	*COL7A1*	1.5	*SYNE2*	1.3	*MMP2*	2.3	*MAP2*	1.0
ADAM12	2.3	*MAGEE1*	1.5	*NRCAM*	1.1	*TTLL3*	2.2		
GSN	2.2	*CDH10*	1.5	*COL19A1*	1.1	*EVL*	2.0		
CDH20	2.2	*SULF2*	1.5	*SEMA5B*	1.1	*ADAM29*	2.0		
BGN	2.1	*CNTN6*	1.4	*ITGA9*	1.1	*CSMD3*	1.9		
ICAM5	2.1	*THBS3*	1.4			*ADAMTS15*	1.8		
Signal transduction (examples: intracellular signaling cascade GO:0007242, receptor activity GO:0004872, GTPase regulator GO:0030695)									
VEPH1	2.1	*PFC*	1.5	*PRPF4B*	1.3	*APC*	>10	*PTPRD*	2.2
SBNO1	2.1	*GAB1*	1.5	*CENTG1*	1.3	*KRAS*	>10	*MCP*	2.1
DNASE1L3	1.9	*ARHGEF4*	1.4	*MAP3K6*	1.3	*EPHA3*	4.2	*NF1*	1.9
RAP1GA1	1.8	*NALP8*	1.4	*APC2*	1.3	*GUCY1A2*	3.5	*PTPRU*	1.4
EGFL6	1.8	*RGL1*	1.4	*STARD8*	1.2	*EPHB6*	3.5	*CD109*	1.3
AMFR	1.7	*PPM1E*	1.4	*PTPN14*	1.1	*TGFBR2*	2.9	*PHIP*	1.2
CENTB1	1.7	*PKDREJ*	1.4	*IRTA2*	1.1	*GNAS*	2.6		
GPNMB	1.7	*CNNM4*	1.3	*RASGRF2*	1.1	*RET*	2.3		
INHBE	1.7	*ALS2CL*	1.3	*MTMR3*	1.1	*P2RY14*	2.2		
FLJ10458	1.6	*RASAL2*	1.3			*LGR6*	2.2		
Transcriptional regulation (examples: regulation of transcription GO:0045449, zinc finger C2H2-subtype IPR007066)									
TP53	>10	*CHD5*	1.8	*ZFP64*	1.4	*TP53*	>10	*ZNF442*	1.9
FLJ13479	3.4	*CIC*	1.7	*ZNF569*	1.4	*SMAD4*	4.6	*SMAD3*	1.9
SIX4	2.5	*KEAP1*	1.6	*EHMT1*	1.3	*MLL3*	3.7	*EYA4*	1.5
KIAA0934	2.5	*HOXA3*	1.6	*ZFYVE26*	1.2	*TBX22*	3.3	*PKNOX1*	1.4
LRRFIP1	2.4	*TCF1*	1.6	*BCL11A*	1.1	*SMAD2*	3.1	*MKRN3*	1.3
GLI1	2.3	*HDAC4*	1.6	*ZNF318*	1.1	*TCF7L2*	2.8		
RFX2	2.1	*MYOD1*	1.5			*HIST1H1B*	2.5		
ZCSL3	1.8	*NCOA6*	1.5			*RUNX1T1*	2.4		
Transport (examples: ion transporter activity GO:0015075, ligand-gated ion channel activity GO:0015276, carrier activity GO:0005386)									
ATP8B1	3.1	*ABCB8*	1.7	*ABCB10*	1.4	*ABCA1*	2.8	*C6orf29*	1.1
CUBN	2.5	*KPNA5*	1.7	*SCNN1B*	1.3	*SLC29A1*	1.9		
GRIN2D	2.4	*ABCA3*	1.7	*NUP133*	1.1	*SCN3B*	1.9		
HDLBP	2.2	*SLC9A2*	1.6			*P2RX7*	1.3		
NUP214	1.8	*SLC6A3*	1.5			*KCNQ5*	1.2		
Cellular metabolism (examples: aromatic compound metabolism GO:0006725, generation of precursor metabolites GO:0016445, biosynthesis GO:0009058)									
ACADM	2.0	*NCB5OR*	1.7	*PHACS*	1.4	*UQCRC2*	1.9		
PRPS1	1.8	*ASL*	1.6	*XDH*	1.3	*ACSL5*	1.6		
CYP1A1	1.7	*GALNT5*	1.4			*GALNS*	1.2		
Intracellular trafficking (examples: endoplasmic reticulum targeting sequence IPR000866, membrane fusion GO:0006944)									
OTOF	2.2	*PLEKHA8*	1.8	*KTN1*	1.5	*SYNE1*	2.3	*PRKD1*	1.9
LRBA	2.1	*LOC283849*	1.7	*GGA1*	1.4	*SEC8L1*	2.2	*LRP2*	1.2
AEGP	1.8	*SORL1*	1.7			*SDBCAG84*	2.2		
RNA metabolism (examples: RNA processing GO:0008353, RNA splice site selection GO:0006376)									
C14orf155	3.3	*RNU31P2*	1.7	*KIAA0427*	1.5	*SFRS6*	1.3		
SP110	1.8	*C22orf9*	1.5	*DDX10*	1.3				
Other (examples: response to DNA damage stimulus GO:0006974, protein ubiquitination GO:0016567)									
FLJ40869	2.1	*SERPINB1*	1.4			*FBXW7*	5.1	*K6IRS3*	1.2
BRCA1	2.0					*UHRF2*	1.5	*CD248*	1.2
MRE11A	1.6					*LMO7*	1.3	*ERCC6*	1.0
Unknown									
KIAA1632	2.4	*KIAA0999*	1.3			*C10orf137*	2.7	*KIAA1409*	1.6
MGC24047	2.1					*LOC157697*	2.0	*C15orf2*	1.0

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cancers have higher fractions of 5′-TpC-3′ mutations than others, but also show that mutations at this dinucleotide are generally more frequent than in colorectal cancers (Table 2 and table S3).

Finally, our results reveal that there are substantial differences in the panel of *CAN* genes mutated in the two tumor types (Table 3). For example, metalloproteinase genes were mutated in a large fraction of colorectal but only in a small fraction of breast cancers (tables S5 and S6). Transcriptional regulator genes were mutated in a high fraction of both breast and colorectal tumors, but the specific genes affected varied according to tumor type (Table 3). There was also considerable heterogeneity among the *CAN* genes mutated in different tumor specimens derived from the same tissue type (tables S4 to S6). It has been documented that virtually all biochemical, biological, and clinical attributes are heterogeneous within human cancers of the same histologic subtype (*45*). Our data suggest that differences in the genes mutated in various tumors could account for a major part of this heterogeneity. This might explain why it has been so difficult to correlate the behavior, prognosis, or response to therapy of common solid tumors with the presence or absence of a single gene alteration; such alterations reflect only a small component of each tumor's mutational composition. On the other hand, disparate genes contributing to cancer are often functionally equivalent, affecting net cell growth through the same molecular pathway (*1*). Thus, *TP53* and *MDM2* mutations exert comparable effects on cells, as do mutations in *RB1*, *CDKN2A* (*p16*), *CCND1*, and *CDK4*. It will be of interest to determine whether a limited number of pathways include most *CAN* genes, a possibility consistent with the groupings in Fig. 2 and Table 3.

Like a draft version of any genome project, our study has limitations. First, only genes present in the current version of CCDS were analyzed; of the genes not yet included, there are ~5000 genes for which excellent supporting evidence exists (*46*). Second, we were not able to successfully sequence ~10% of the bases within the coding sequences of the 13,023 CCDS genes (equivalent to 1302 unsequenced genes). Third, our identification of genes mutated at significant frequencies assumed that the background mutation frequency was constant throughout the genome. Although it cannot currently be determined whether certain genomic regions have higher background mutation frequencies, we have included the number of mutations observed per Mb sequenced in tables S5 and S6 to facilitate such analyses in the future. Fourth, although our screen would be expected to identify the most common types of mutations found in cancers, some genetic alterations—including mutations in noncoding genes, mutations in noncoding regions of coding genes, relatively large deletions or insertions, amplifications, and translocations—would not be detectable by the methods we used. Future studies using a combination of different technologies, such as those envisioned by the Cancer Genome Atlas Project (*47*), will be able to address these issues.

The results of this study inform future cancer genome sequencing efforts in several important ways:

1) A major technical challenge of such studies will be discerning somatic mutations from the large number of sequence alterations identified. In our study, 557,029 nonsynonymous sequence alterations were detected in the discovery screen, but after subsequent analyses only 0.23% of these were identified as legitimate somatic mutations (Fig. 1). Fewer than 10% of the putative nonsynonymous alterations were known polymorphisms; many of the rest were uncommon germline variants or sequence artifacts that were not reproducible. Inclusion of matched normal samples and sequencing both strands of each PCR product would reduce false positives in the discovery screen but would increase the cost of sequencing by a factor of 4. Although recently developed sequencing methods could reduce the cost of such studies in the future (*48*), the higher error rates of these approaches may result in an even lower ratio of bona fide somatic mutations to putative alterations.

2) Another technical issue is that careful design of primers is important to eliminate sequence artifacts due to the inadvertent amplification and sequencing of related genes. The primer pairs that resulted in successful amplification and sequencing represent a valuable resource in this regard. Even with well-designed primers, it is essential to examine any observed mutation to ensure that it is not found as a normal variant in a related gene.

3) Although it is likely that studies of other solid tumor types will also identify a large number of somatic mutations, it will be important to apply rigorous approaches to identify those mutations that have been selected for during tumorigenesis. Statistical techniques, such as those used in this study or described by Greenman *et al.* (*11*), can provide strong evidence for selection of mutated genes. These approaches are likely to improve as more cancer genomic sequencing data are accumulated through the Cancer Genome Atlas Project (*47*) and other projects now under way.

4) There has been much discussion about which genes should be the focus of future sequencing efforts. Our results suggest that many genes not previously implicated in cancer are mutated at significant levels and may provide novel clues to pathogenesis. From these data, it would seem that large-scale unbiased screens of coding genes may be more informative than screens based on previously defined criteria.

5) The results also raise questions about the optimum number of tumors of any given type that should be assessed in a cancer genome study. Our study was designed to determine the nature and types of alterations present in an "average" breast or colorectal cancer and to discover genes mutated at reasonably high frequencies. With this design, our power to detect genes mutated in more than 20% of tumors of a given type was 90%, but only 50% of genes mutated in 6% of tumors would have been discovered. Detection of genes mutated in 6% or 1% of tumors with >99% probability in a discovery screen would require sequence determination of at least 75 or 459 tumors, respectively. Although it will be impossible to detect all mutations that may occur in tumors, strategies that would identify the most important ones at an affordable cost can be envisioned on the basis of the data and analysis reported herein.

6) Ultimately, the sequences of entire cancer genomes, including intergenic regions, will be obtainable. Our studies demonstrate the inherent difficulties in determining the consequences of somatic mutations, even those that alter the amino acid sequence of highly annotated and well-studied genes. Establishing the consequences of mutations in noncoding regions of the genome will likely be much more difficult. Until new tools for solving this problem become available, it is likely that gene-centric sequencing analyses of cancer will be more useful than whole-genome sequencing.

Our results provide a large number of future research opportunities in human cancer. For genetics, it will be of interest to elucidate the timing

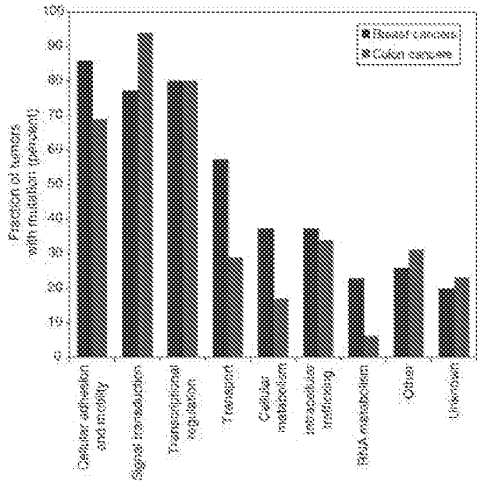


Fig. 2. Mutation frequency of *CAN*-gene groups. *CAN* genes were grouped by function with the use of Gene Ontology groups, INTERPRO domains, and available literature. Bars indicate the fraction of tumors (35 breast or 35 colorectal) with at least one mutated gene in the functional group.

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and extent of *CAN*-gene mutations in breast and colorectal cancers, whether these genes are mutated in other tumor types, and whether germline variants in *CAN* genes are associated with cancer predisposition. For immunology, the finding that tumors contain an average of ~90 different amino acid substitutions not present in any normal cell can provide novel approaches to engender antitumor immunity. For epidemiology, the remarkable difference in mutation spectra of breast and colorectal cancers suggests the existence of organ-specific carcinogens. For cancer biology, it is clear that no current animal or in vitro model of cancer recapitulates the genetic landscape of an actual human tumor. Understanding and capturing this landscape and its heterogeneity may provide models that more successfully mimic the human disease. For epigenetics, it is possible that a subset of *CAN* genes can also be dysregulated in tumors through changes in chromatin or DNA methylation rather than through mutation. For diagnostics, the *CAN* genes define a relatively small subset of genes that could prove useful as markers for neoplasia. Finally, some of these genes, particularly those on the cell surface or those with enzymatic activity, may prove to be good targets for therapeutic development.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1133427/DC1
Materials and Methods
Figs. S1 and S2
Tables S1 to S5
References

3 August 2006; accepted 30 August 2006
Published online 7 September 2006;
10.1126/science.1133427
Include this information when citing this paper.

REPORTS

Self-Assembly of CdTe Nanocrystals into Free-Floating Sheets

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In their physical dimensions, surface chemistry, and degree of anisotropic interactions in solution, CdTe nanoparticles are similar to proteins. We experimentally observed their spontaneous, template-free organization into free-floating particulate sheets, which resemble the assembly of surface layer (S-layer) proteins. Computer simulation and concurrent experiments demonstrated that the dipole moment, small positive charge, and directional hydrophobic attraction are the driving forces for the self-organization process. The data presented here highlight the analogy of the solution behavior of the two vastly different classes of chemical structures.

Understanding the ability of nanoparticles (NPs) to self-assemble will influence both the fundamental picture of the properties of matter on the nanoscale and the practical realization of bottom-up fabrication technologies (*1*–*3*). Self-organization processes in solution have been well established for proteins and other biomacromolecules (*4*–*6*), which have physical dimensions of several nanometers, i.e., the same scale as that of many inorganic nanocolloids. Recent studies demonstrate nanoparticle self-organization into one-dimensional (1D) structures driven by anisotropic dipolar interparticle forces (*7*–*9*). Highly uniform nanocolloids, with presumably isotropic interactions, can form 3D arrays or crystals (*10*–*14*). Two-dimensional arrays of NPs produced at two-phase interfaces—such as gas-solid (*10*), liquid-solid (*15*), gas-liquid (*16*), or liquid-liquid (*17*)—where the interfaces act as a template, are well known. We report that the combination of the electrostatic interaction and anisotropic hydrophobic attraction between NPs with tetrahedral shape result in the spontaneous formation of 2D free-floating sheets. The sheets display considerable mechanical robustness and retain size-quantized properties of the semiconductor NP with characteristic luminescence. Notably, they do not require any interface that may give dimension-restrictive cues

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Downloaded from www.sciencemag.org on April 2, 2010

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Annals of Oncology 27 (Supplement 6): vi149–vi206, 2016
doi:10.1093/annonc/mdw370.27

gastrointestinal tumours, colorectal

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Nivolumab ± ipilimumab treatment (Tx) efficacy, safety, and biomarkers in patients (Pts) with metastatic colorectal cancer (mCRC) with and without high microsatellite instability (MSI-H): results from the CheckMate-142 study

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Background: Globally, about 10% of new cancers each year are CRC and ~15% of these are MSI-H. Nivolumab (N), a fully human anti-PD-1 mAb, and ipilimumab (I), a humanized anti-CTLA-4 mAb, are immune checkpoint inhibitors with favorable safety and efficacy profiles in multiple tumor types. This phase 2 study evaluates N ± I in MSI-H and non-MSI-H pts with mCRC.

Methods: MSI-H pts received N 3 mg/kg q2 wk (N3) or N 3 mg/kg + I 1 mg/kg q3 wk (N3 + I1) x 4 doses followed by N3 until disease progression (PD) or other discontinuation. Initial evaluation of N + I was also completed in non-MSI-H pts. Primary endpoint was investigator-reported ORR by RECIST 1.1. Other endpoints included safety, OS, PFS, and clinical activity in biomarker-defined subpopulations (KRAS, BRAF status, and PD-L1).

Results: 70 (N3) and 30 (N3 + I1) MSI-H pts and 3 (N1 + I1), 10 (N1 + I3), and 10 (N3 + I1) non-MSI-H pts were enrolled. All non-MSI-H pts and 87% (N3) and 93% (N3 + I1) of MSI-H pts had ≥2 prior regimens. 47 (67%; N3) and 18 (60%; N3 + I1) MSI-H pts remain on tx. Efficacy data for MSI-H pts are shown in the Table. Responses were also seen in non-MSI-H pts. Median (95% CI) PFS across all non-MSI-H pts was 1.4 mo (1.2, 1.9). Responses were observed regardless of tumor PD-L1 expression. Treatment-related adverse events (TRAEs) occurred in 41 (59%; N3) and 25 (83%; N3 + I1) MSI-H pts; 10 (14%; N3) and 8 (27%; N3 + I1) pts had Grade 3–4 TRAEs. One pt on N3 had a Grade 5 TRAE (sudden death). Additional biomarker data including MSI assessment and influence of BRAF/KRAS mutations will be presented.

Conclusions: N ± I demonstrated promising clinical activity with a favorable overall safety profile in pts with mCRC, regardless of tumor PD-L1 expression. Additional biomarker analyses are ongoing.

Legal entity responsible for the study: Sponsored by Bristol-Myers Squibb

Funding: Sponsored by Bristol-Myers Squibb

Disclosure: H-J. Lenz: Has served as a consultant/advisory board member and received travel expenses from Bayer, Merck Serono, and Roche. He has received honoraria from Bayer, Boehringer Ingelheim, Celgene, Merck Serono, and Roche. P. Garcia-Alfonso: Has served on advisory boards for Roche, Merck, Sanofi, Amgen, Bayer, and Lilly. A. Hill: Is employed by, owns stock in, and has received research funding from Tasman Oncology Research. He has received travel accommodations/expenses from BMS. R.A. Moss: Is employed by and owns stock in Bristol-Myers Squibb. M.V. Goldberg, C-S. Lin, H. Tang: Is an employee of Bristol-Myers Squibb. T. André: Has received honoraria from BMS and consultant fees from Roche. All other authors have declared no conflicts of interest.

Table: 4758P MSI-H[a] efficacy

	N3 (n = 70)	N3 + I1 (n = 30)
ORR, n (%)[b]	12 (25.5)	9 (33.3)
CR PR SD PD Not determined/not reported	0 12 (25.5) 14 (29.8) 17 (36.2) 4 (8.5)	0 9 (33.3) 14 (51.9) 3 (11.1) 1 (3.7)
Median DOR (95% CI), mo	NR (4.2, NE)	NR (NE, NE)
PFS rates, % (95% CI) 6 mo 9 mo 12 mo	45.9 (29.8, 60.7) 45.9 (29.8, 60.7) 45.9 (29.8, 60.7)	66.6 (45.5, 81.1) NR NR
OS rates, % (95% CI) 6 mo 9 mo 12 mo	75.0 (58.5, 85.7) 65.6 (48.0, 78.6) 65.6 (48.0, 78.6)	85.1 (65.0, 94.2) 85.1 (65.0, 94.2) NR
Baseline PD-L1 expression, n (%) ≥1% <1% Indeterminate/not evaluable/missing	17 (24.3) 29 (41.4) 24 (34.3)	7 (23.3) 20 (66.7) 3 (10.0)
NR, not reached; NE, not estimable		

[a]By local screen
[b]Reported for patients with 12 weeks of follow-up, N3 (n = 47) and N3 + I1 (n = 27)

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NIH Public Access
Author Manuscript
N Engl J Med. Author manuscript; available in PMC 2013 January 14.

Published in final edited form as:
N Engl J Med. 2012 June 28; 366(26): 2443–2454. doi:10.1056/NEJMoa1200690.

Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer

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No other potential conflict of interest relevant to this article was reported.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Abstract

Background—Blockade of programmed death 1 (PD-1), an inhibitory receptor expressed by T cells, can overcome immune resistance. We assessed the antitumor activity and safety of BMS-936558, an antibody that specifically blocks PD-1.

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Methods—We enrolled patients with advanced melanoma, non–small-cell lung cancer, castration-resistant prostate cancer, or renal-cell or colorectal cancer to receive anti–PD-1 antibody at a dose of 0.1 to 10.0 mg per kilogram of body weight every 2 weeks. Response was assessed after each 8-week treatment cycle. Patients received up to 12 cycles until disease progression or a complete response occurred.

Results—A total of 296 patients received treatment through February 24, 2012. Grade 3 or 4 drug-related adverse events occurred in 14% of patients; there were three deaths from pulmonary toxicity. No maximum tolerated dose was defined. Adverse events consistent with immune-related causes were observed. Among 236 patients in whom response could be evaluated, objective responses (complete or partial responses) were observed in those with non–small-cell lung cancer, melanoma, or renal-cell cancer. Cumulative response rates (all doses) were 18% among patients with non–small-cell lung cancer (14 of 76 patients), 28% among patients with melanoma (26 of 94 patients), and 27% among patients with renal-cell cancer (9 of 33 patients). Responses were durable; 20 of 31 responses lasted 1 year or more in patients with 1 year or more of follow-up. To assess the role of intratumoral PD-1 ligand (PD-L1) expression in the modulation of the PD-1–PD-L1 pathway, immunohistochemical analysis was performed on pretreatment tumor specimens obtained from 42 patients. Of 17 patients with PD-L1–negative tumors, none had an objective response; 9 of 25 patients (36%) with PD-L1–positive tumors had an objective response ($P = 0.006$).

Conclusions—Anti–PD-1 antibody produced objective responses in approximately one in four to one in five patients with non–small-cell lung cancer, melanoma, or renal-cell cancer; the adverse-event profile does not appear to preclude its use. Preliminary data suggest a relationship between PD-L1 expression on tumor cells and objective response. (Funded by Bristol-Myers Squibb and others; ClinicalTrials.gov number, NCT00730639.)

Human cancers harbor numerous genetic and epigenetic alterations, generating neoantigens that are potentially recognizable by the immune system.[1] Although an endogenous immune response to cancer is observed in preclinical models and patients, this response is ineffective, because tumors develop multiple resistance mechanisms, including local immune suppression, induction of tolerance, and systemic dysfunction in T-cell signaling.[2-5] Moreover, tumors may exploit several distinct pathways to actively evade immune destruction, including endogenous "immune checkpoints" that normally terminate immune responses after antigen activation. These observations have resulted in intensive efforts to develop immunotherapeutic approaches for cancer, including immune-checkpoint-pathway inhibitors such as anti–CTLA-4 antibody (ipilimumab) for the treatment of patients with advanced melanoma.[6-8]

Programmed death 1 (PD-1) is a key immune-checkpoint receptor expressed by activated T cells, and it mediates immunosuppression. PD-1 functions primarily in peripheral tissues, where T cells may encounter the immunosuppressive PD-1 ligands PD-L1 (B7-H1) and PD-L2 (B7-DC), which are expressed by tumor cells, stromal cells, or both.[9-12] Inhibition of the interaction between PD-1 and PD-L1 can enhance T-cell responses in vitro and mediate preclinical antitumor activity.[11,13] In a dose-escalation study, the anti–PD-1 monoclonal antibody BMS-936558 (also known as MDX-1106 and ONO-4538) was administered as a single dose in 39 patients with advanced solid tumors.[14] A favorable safety profile and preliminary evidence of clinical activity were shown in this pilot study, establishing the basis for the current multiple-dose trial involving patients with diverse cancers. We report clinical results for 296 patients in this trial.

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Methods

Study Design

This study was sponsored by Bristol-Myers Squibb, which provided the study drug and worked jointly with the senior academic authors to design, collect, analyze, and interpret the study results. All the authors signed a confidentiality agreement with the sponsor. The protocol, including a detailed statistical analysis plan, is available with the full text of this article at NEJM.org. All drafts of the manuscript were prepared by the authors with editorial assistance from a professional medical writer paid by the sponsor. All the authors vouch for the accuracy and completeness of the reported data and for the fidelity of this report to the trial protocol, and all the authors made the decision to submit the manuscript for publication.

This phase 1 study assessed the safety, anti-tumor activity, and pharmacokinetics of BMS-936558, a fully human IgG4-blocking monoclonal antibody directed against PD-1, in patients with selected advanced solid tumors. All patients (or their legal representatives) gave written informed consent before enrollment. The antibody was administered as an intravenous infusion every 2 weeks of each 8-week treatment cycle. Response was assessed after each treatment cycle. Patients received treatment for up to 2 years (12 cycles), unless they had a complete response, unacceptable adverse effects, or progressive disease or they withdrew consent. In clinically stable patients, study treatment could be continued beyond apparent initial disease progression until progression was confirmed, as outlined by proposed immune-response criteria.[15] Patients with stable disease or an ongoing objective response (complete or partial response) at the end of treatment were followed for up to 1 year and were offered retreatment for 1 additional year in the event of disease progression.

Safety evaluations (clinical examination and laboratory assessments) were conducted for all treated patients at baseline and regular intervals. The severity of adverse events was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.[16]

Dose Escalation

Patients with advanced melanoma, non–small-cell lung cancer, renal-cell cancer, castration-resistant prostate cancer, or colorectal cancer were enrolled. Cohorts of three to six patients per dose level were enrolled sequentially at doses of 1.0, 3.0, or 10.0 mg per kilogram of body weight. Dose escalation proceeded when a minimum of three patients had completed the safety-evaluation period (56 days) at a given dose level, with dose-limiting toxicity in less than one third of patients. Intrapatient dose escalation was not permitted.

Cohort Expansion

A maximum tolerated dose was not reached. Initially, five expansion cohorts of approximately 16 patients each were enrolled at doses of 10.0 mg per kilogram for melanoma, non–small-cell lung cancer, renal-cell cancer, castration-resistant prostate cancer, and colorectal cancer. On the basis of initial signals of activity, additional expansion cohorts of approximately 16 patients each were enrolled for melanoma (at a dose of 1.0 or 3.0 mg per kilogram, followed by cohorts randomly assigned to 0.1, 0.3, or 1.0 mg per kilogram), lung cancer (patients with the squamous or nonsquamous subtype, randomly assigned to a dose of 1.0, 3.0, or 10.0 mg per kilogram), and renal-cell cancer (at a dose of 1.0 mg per kilogram).

Patients

Eligible patients had documented advanced solid tumors; an age of 18 years or older; a life expectancy of 12 weeks or more; an Eastern Cooperative Oncology Group performance

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status of 0, 1, or 2 (on a scale from 0 to 5, with 0 indicating that the patient is asymptomatic, 1 that the patient is restricted in strenuous activity, and 2 that the patient is ambulatory but unable to work)[17]; measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0,[18] with modification (see Methods S1 in the Supplementary Appendix, available at NEJM.org; and the protocol); adequate hematologic, hepatic, and renal function; and a history of one to five systemic treatment regimens. Patients with radiographically stable treated brain metastases were enrolled. Patients with a history of chronic autoimmune disease, prior therapy with antibodies that modulate T-cell function (e.g., anti–CTLA-4, anti–PD-1, and anti–PD-L1), conditions requiring immunosuppressive medications, or chronic infection (e.g., human immunodeficiency virus infection and hepatitis B or C) were excluded.

Immunohistochemical Analysis for PD-L1

Immunohistochemical analysis for PD-L1 was performed on archival or newly obtained pretreatment formalin-fixed, paraffin-embedded tumor specimens with the use of the murine antihuman PD-L1 monoclonal antibody 5H1.[11,19] The percentage of tumor cells exhibiting cell-surface staining for PD-L1 was scored by two independent pathologists who were unaware of outcomes. PD-L1 positivity was defined per specimen by a 5% expression threshold[19,20]; patients with multiple specimens were considered PD-L1–positive if any specimen met this criterion.

Pharmacokinetics and Pharmacodynamics

For pharmacokinetic analysis, serum concentrations of anti–PD-1 antibody were quantified with the use of an enzyme-linked immunosorbent assay. For pharmacodynamic analysis, peripheral-blood mononuclear cells (PBMCs) were isolated from patients at baseline and after the first treatment cycle to estimate PD-1–receptor occupancy by the antibody on circulating CD3+ T cells by means of flow cytometry.[14]

Statistical Analysis

Data on all 296 patients treated as of the date of analysis for this report (February 24, 2012) were used for summaries of baseline characteristics and adverse events. Pharmacokinetic and molecular-marker analyses included treated patients with available data as of February 24, 2012. The efficacy analysis included the 236 patients who could be evaluated for a response and who began treatment by July 1, 2011. Adverse events were coded with the use of the Medical Dictionary for Regulatory Activities (MedDRA), version 14.1. Adverse events of special interest, with a potential immune-related cause, were identified with the use of a predefined list of MedDRA terms. The best responses in individual patients were derived from investigator-reported data per modified RECIST, version 1.0. Objective responses were confirmed by at least one sequential tumor assessment, and objective response rates were calculated as [(complete responses + partial responses) ÷ number of patients] × 100. Fisher's exact test was used to assess the association between PD-L1 expression and objective response.

Results

Baseline Patient Characteristics

A total of 296 patients with advanced solid tumors, including melanoma (104 patients), non–small-cell lung cancer (122), renal-cell cancer (34), castration-resistant prostate cancer (17), and colorectal cancer (19), began treatment with anti–PD-1 antibody between October 2008 and February 24, 2012. The majority of patients were heavily pretreated; 47% had received at least three prior regimens (Table S1-A in the Supplementary Appendix). Notable prior

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therapies included immunotherapy and BRAF inhibitors in patients with melanoma (64% and 8% of patients, respectively); platinum-based chemotherapy and tyrosine kinase inhibitors in patients with lung cancer (94% and 34%, respectively); and nephrectomy, immunotherapy, and antiangiogenic therapy in patients with renal-cell cancer (94%, 59%, and 74%, respectively) (Tables S1-B, S1-C, and S1-D in the Supplementary Appendix). Baseline characteristics of the total treated population (296 patients) were similar to those of the efficacy population (236 patients).

Safety

A maximum tolerated dose was not defined at the doses tested in this study. A relative dose intensity (the proportion of administered doses relative to planned doses) of 90% or more was achieved in 86% of patients (Table S2-A in the Supplementary Appendix). Fifteen of 296 patients (5%) discontinued treatment owing to treatment-related adverse events (Tables S2-B and S3-A in the Supplementary Appendix). As of the date of analysis, 62 patients (21%) had died; disease progression was the most common cause of death (Table S2-C in the Supplementary Appendix).

The most common adverse events, regardless of causality, were fatigue, decreased appetite, diarrhea, nausea, cough, dyspnea, constipation, vomiting, rash, pyrexia, and headache (Table S3-A in the Supplementary Appendix). Common treatment-related adverse events included fatigue, rash, diarrhea, pruritus, decreased appetite, and nausea (Tables S3-A and S3-B in the Supplementary Appendix). Grade 3 or 4 treatment-related adverse events were observed in 41 of 296 patients (14%). Drug-related serious adverse events (as defined in Table S4 in the Supplementary Appendix) occurred in 32 of 296 patients (11%). The spectrum, frequency, and severity of treatment-related adverse events were generally similar across the dose levels tested. Drug-related adverse events of special interest (e.g., those with potential immune-related causes) included pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis (Table 1 and Fig. 1C).

Hepatic or gastrointestinal adverse events were managed with treatment interruption and, as necessary, with the administration of glucocorticoids. These events (e.g., diarrhea in 33 patients, including three grade 3 or 4 events and elevated alanine aminotransferase levels in 11 patients, including two grade 3 or 4 events) were reversible in all cases. Endocrine disorders were managed with replacement therapy. At the discretion of the treating physician, treatment with anti–PD-1 antibody was reinitiated once the adverse event had been successfully managed. Drug-related pneumonitis occurred in 9 of the 296 patients (3%). Grade 3 or 4 pneumonitis developed in 3 patients (1%). No clear relationship between the occurrence of pneumonitis and tumor type, dose level, or the number of doses received was noted. Early-grade pneumonitis in 6 patients was reversible with treatment discontinuation, glucocorticoid administration, or both. In 3 patients with pneumonitis, infliximab, mycophenolate, or both were used for additional immunosuppression; however, given the small number of patients and variable outcomes, the effectiveness of such treatment was unclear. There were three drug-related deaths (1%) due to pneumonitis (two in patients with non–small-cell lung cancer and one in a patient with colorectal cancer).

Clinical Activity

Antitumor activity was observed at all doses tested. Objective responses were observed in a substantial proportion of patients with non–small-cell lung cancer, melanoma, or renal-cell cancer (Table 2 and Fig. 1) and in various sites of metastasis, including the liver, lung, lymph nodes, and bone. At the time of data analysis, two patients with lung cancer who received 10 mg per kilogram had unconfirmed responses, and eight additional patients (with melanoma, lung cancer, or renal-cell cancer) had a persistent reduction in baseline target

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lesions in the presence of new lesions (a finding consistent with an immune-related response pattern[15]). None of these patients were categorized as having had a response for the purpose of calculating objective-response rates. Objective responses, prolonged disease stabilization, or both were observed in patients who had received a variety of prior therapies. No objective responses were observed in patients with colorectal or prostate cancer.

In patients with lung cancer, 14 objective responses were observed at doses of 1.0, 3.0, or 10.0 mg per kilogram, with response rates of 6%, 32%, and 18%, respectively. Objective responses were observed across non–small-cell histologic types: in 6 of 18 patients (33%) with squamous tumors, 7 of 56 (12%) with nonsquamous tumors, and 1 of 2 with tumors of unknown type. All 14 patients with objective responses started treatment 24 weeks or more before data analysis, and of these, 8 had a response that lasted 24 weeks or more (Table 2). Five of 14 patients with objective responses started treatment 1 year or more before data analysis, and of these, 2 had a response that lasted 1 year or more. Stable disease lasting 24 weeks or more was observed in 5 patients (7%) with lung cancer, all of whom had nonsquamous tumors.

In patients with melanoma, 26 objective responses were observed at doses ranging from 0.1 to 10.0 mg per kilogram, with response rates ranging from 19 to 41% per dose level. At a dose of 3.0 mg per kilogram, objective responses were noted in 7 of 17 patients (41%). Of 26 patients with melanoma who had an objective response, 18 started treatment 1 year or more before February 24, 2012, and of these, 13 had a response that lasted 1 year or more. The remaining 8 patients with objective responses received study medication for less than 1 year, and 6 had responses ranging from 1.9 to 5.6 months. Stable disease lasting 24 weeks or more was observed in 6 patients (6%).

Among patients with renal-cell cancer, objective responses occurred in 4 of 17 patients (24%) treated with a dose of 1.0 mg per kilogram and 5 of 16 (31%) treated with 10.0 mg per kilogram. Of 8 patients with objective responses who started treatment 1 year or more before data analysis, 5 had a response that lasted 1 year or more. Stable disease lasting 24 weeks or more was observed in an additional 9 patients (27%).

Pharmacokinetics and Pharmacodynamics

The median time to the peak concentration of anti PD-1 antibody was 1 to 4 hours after the start of infusion. The pharmacokinetics of the antibody were linear, with a dose-proportional increase in the peak concentration and area under the curve calculated from day 1 to day 14 in the dose range of 0.1 to 10.0 mg per kilogram (35 patients). The pharmacodynamics of anti–PD-1 antibody were assessed according to PD-1–receptor occupancy on circulating CD3+ T cells. In PBMCs from 65 patients with melanoma who were treated with one cycle of anti–PD-1 antibody at a dose of 0.1 to 10.0 mg per kilogram every 2 weeks, the median PD-1–receptor occupancy by anti–PD-1 antibody was 64 to 70% according to dose level (Fig. 2A).

PD-L1 Expression in Tumors

Sixty-one pretreatment tumor specimens from 42 patients (18 with melanoma, 10 with non–small-cell lung cancer, 7 with colorectal cancer, 5 with renal-cell cancer, and 2 with prostate cancer) (Table S5 in the Supplementary Appendix) were analyzed for PD-L1 expression on the surface of tumor cells (Fig. 2B). Biopsy specimens from 25 of the 42 patients were positive for PD-L1 expression by immunohistochemical analysis. Of these 25 patients, 9 (36%) had an objective response. None of the 17 patients with PD-L1– negative tumors had an objective response. This analysis is based on optional biopsies in a non-random subset of

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the population, and testing of a statistical hypothesis was not prespecified. These preliminary results must therefore be interpreted with caution.

Discussion

Our data show that approximately one in four to one in five patients treated with anti–PD-1 antibody had objective responses with durability; these occurred in heavily pretreated patients with diverse tumor types. PD-1 blockade extends the spectrum of clinical activity by immunotherapy beyond immunogenic tumor types, such as melanoma and renal-cell cancer, to treatment-refractory, meta-static non–small-cell lung cancer, a tumor type that is generally not considered to be responsive to immunotherapy. The level of activity seen with anti–PD-1 antibody in patients with lung cancer who had received substantial amounts of prior therapy (55% with at least three lines of previous therapy) (Table S1-B in the Supplementary Appendix) and across histologic types is of interest, particularly in the patients with squamous tumors.[22,23] These unexpected findings underscore the possibility that any neoplasm could be immunogenic with proper immune activation; however, the reason why only a minority of patients had a response (i.e., tumor or patient 'host' factors or both) is not known. The full therapeutic potential of PD-1 blockade across other tumor types remains to be defined.

The durability of objective responses across multiple cancer types in patients treated with anti–PD-1 antibody was also notable. Although anti–PD-1 antibody was not compared with other therapies in this study, this durability contrasts with the relatively modest durability of objective responses observed in many patients with non–small-cell lung cancer, melanoma, or renal-cell cancer who are treated with conventional chemotherapy, tyrosine kinase inhibitors, or both. The response durability in heavily pretreated patients with lung cancer who received anti–PD-1 antibody is particularly interesting, because standard salvage therapies historically have shown modest benefit in these patients.[24] As measured by standard RECIST criteria in this study, objective responses were long-lasting, with response durations of 1 year or more in 20 of 31 patients who had a response with 1 year or more of follow-up. In addition, patterns of tumor regression consistent with immune-related patterns of response[15,21,25] were observed. That is, index lesions often responded as previously undetected lesions became detectable, a finding that is possibly related to lymphocyte infiltration of previously unknown small nests of tumor cells. Although the full effect of these unconventional response patterns remains to be defined in randomized trials with survival end points, these observations are reminiscent of findings with ipilimumab in which a significant extension of overall survival was observed in treated patients.[7,8]

Drug-related grade 3 or 4 toxic effects occurred in 14% of patients who received anti–PD-1 antibody, suggesting that therapy can be delivered in an outpatient setting with minimal supportive care. Among adverse events of special interest, pneumonitis was observed, with findings ranging from isolated radiographic abnormalities to progressive, diffuse infiltrates associated with clinical symptoms in a small number of patients. Although three deaths occurred, mild-to-moderate pneumonitis was managed successfully with either observation or glucocorticoids.

A particular challenge in cancer immunotherapy has been the identification of mechanism-based predictive biomarkers that could be used to identify candidates for such treatment and guide disease-management decisions. Our findings suggest that PD-L1 expression in tumors is a candidate molecular marker that warrants further exploration for use in selecting patients for immunotherapy with anti–PD-1 antibody. Our observation of an objective response in 36% of the patients with PD-L1–positive tumors and in none of those with PD-L1–negative tumors suggests that PD-L1 expression on the surface of tumor cells in pretreatment tumor

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specimens may be associated with an objective response. Although tumor-cell expression of PD-L1 may be driven by constitutive oncogenic pathways, recent research suggests that it may also reflect adaptive immune resistance in response to an endogenous antitumor immune response, which may remain in check unless it is unleashed through blockade of the PD-1–PD-L1 pathway.[19] Although our data on PD-L1 expression are consistent with current knowledge of the role of PD-L1 in tumor immune resistance, additional studies will be needed to define the role of PD-L1 as a potential predictive marker of response to anti–PD-1 antibody therapy.

This study and a companion study of anti–PD-L1 antibody, now reported in the *Journal*,[26] describe clinical activity with these agents that validates the importance of the PD-1–PD-L1 pathway for the treatment of some cancers. The signals of clinical activity in patients with non–small-cell lung cancer, melanoma, and renal-cell cancer treated with anti–PD-1 antibody and the possibility of basing patient selection for such treatment on PD-L1 expression in tumors need to be prospectively assessed. Phase 2 trials involving immunologic and molecular-marker correlates (ClinicalTrials.gov numbers, NCT01354431 and NCT01358721) are under way, and phase 3 studies of anti–PD-1 antibody for the treatment of non–small-cell lung cancer, melanoma, and renal-cell cancer are being planned. An assessment of such treatment for other tumor types is also of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by Bristol-Myers Squibb, Ono Pharmaceutical, and grants from the National Institutes of Health (5R01 CA142779, to Drs. Topalian and Pardoll) and the Melanoma Research Alliance (to Drs. Topalian and Pardoll).

Dr. Topalian reports receiving grant support and reimbursement for travel expenses from Bristol-Myers Squibb. Dr. Hodi reports receiving reimbursement for travel expenses from Bristol-Myers Squibb and funding for clinical trials from Bristol-Myers Squibb, Genentech, Pfizer, Novartis, and Synta Pharmaceuticals on behalf of his institution. Dr. Brahmer reports receiving grant support from MedImmune, Regeneron, Merck, and Synta Pharmaceuticals, consulting fees and reimbursement for travel expenses from Genentech, consulting fees from Eli Lilly, reimbursement for travel expenses from Bristol-Myers Squibb, and payment for the development of educational presentations from Quintiles and the Network for Continuing Medical Education. Dr. Smith reports receiving grant support from Bristol-Myers Squibb. Dr. McDermott reports receiving grant support and consulting fees from Prometheus Laboratories and consulting fees from Bristol-Myers Squibb and Roche/Genentech. Dr. Powderly reports receiving grant support (including for the development of educational presentations) and lecture fees from Bristol-Myers Squibb; receiving grant support, consulting fees, and lecture fees from Genentech; receiving grant support and consulting fees from Celldex; receiving grant support and consulting fees from Amplimmune; receiving grant support from ImClone; receiving consulting fees and lecture fees from Genoptix; receiving consulting fees from Veridex; receiving lecture fees from Dendreon; and being the founder of and holding stock in BioCytics, which has received contract work from Genentech and Millennium. Dr. Carvajal reports receiving consulting fees from Morphotek and Novartis and lecture fees from the Florida Society of Dermatology and Dermatologic Surgery and Imedex. Dr. Atkins reports receiving consulting fees from Bristol-Myers Squibb, Genentech, CureTech, Merck, Prometheus Laboratories, Nektar, and AstraZeneca. Dr. Spigel reports receiving consulting fees from Bristol-Myers Squibb on behalf of his institution. Dr. Drake reports receiving consulting fees and payment for the development of educational presentations from Bristol-Myers Squibb, receiving consulting fees from Dendreon and Pfizer, holding stock or stock options in Amplimmune, holding patents regarding the blockade of lymphocyte checkpoint molecules, and receiving patent royalties from Bristol-Myers Squibb and Amplimmune on behalf of his institution. Dr. Pardoll reports receiving consulting fees from Aduro Biotech, Immune Design, NexImmune, ImmuneXcite, Amplimmune, GlaxoSmithKline, Abbott, Jennerex, Pfizer, and Anza Therapeutics; receiving reimbursement for travel expenses from Bristol-Myers Squibb; holding patents regarding immunotherapy with the B7-DC gene and its products, combinatorial therapy of cancer with vaccines plus anti–B7-H1 antibodies, vaccine strategies, and the blockade of lymphocyte checkpoint molecules; and receiving patent royalties from Bristol-Myers Squibb, Amplimmune, GlaxoSmithKline, Aduro Biotech, and BioSante on behalf of his institution. Dr.Chen reports serving as a board observer of, receiving consulting fees from, and holding stock options in Amplimmune; receiving patent licensing payment and research funding from Amplimmune on behalf of Johns Hopkins University and Yale University; receiving patent licensing payment regarding B7-H1– related

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technologies from Bristol-Myers Squibb on behalf of the Mayo Clinic; receiving funding for research on B7-H1 immunohistochemistry from Bristol-Myers Squibb; and receiving patent licensing payment from ImmuNext on behalf of the Mayo Clinic. Dr. Sharfman reports receiving consulting fees from Genentech and Merck and lecture fees from Prometheus Laboratories. Dr. Anders reports receiving grant support and reimbursement for travel expenses from Bristol-Myers Squibb. Dr. Taube reports receiving grant support from Bristol-Myers Squibb on behalf of her institution. Ms. McMiller reports receiving compensation for sponsored laboratory research from Bristol-Myers Squibb on behalf of her institution. Dr. Korman reports being an employee of, holding stock or stock options in, and receiving reimbursement for travel expenses from Bristol-Myers Squibb. Drs. Jure-Kunkel, Agrawal, Kollia, Gupta, and Wigginton report being employees of and holding stock or stock options in Bristol-Myers Squibb. Mr. McDonald reports being an employee of, holding stock or stock options in, and receiving reimbursement for travel expenses from Bristol-Myers Squibb. Dr. Sznol reports receiving consulting fees from and owning stock options in Genesis Biopharma; receiving consulting fees from Bristol-Myers Squibb, Anaeropharma Science, Prometheus Laboratories, Nektar, and Abbott; and receiving lecture fees from the Center for Biomedical Continuing Education and Institute for Medical Education and Research, both of which are sponsored in part by Bristol-Myers Squibb.

We thank the patients who participated in this study; clinical faculty and personnel, including Ashley Bagheri, Cherylann Carr, Tianna Dauses, Robert Gray, Hans Hammers, Marina Laiko, Dung Le, Evan Lipson, Christian Meyer, Alice Pons, Pritish John, Joanne Riemer, and Theresa M. Salay, of the Johns Hopkins University School of Medicine and the Sidney Kimmel Comprehensive Cancer Center; Donald Lawrence of Massachusetts General Hospital; Toni Choueiri, Leena Gandhi, and David Jackman of Dana–Farber Cancer Institute; Daniel Cho, Natasha Coleman, Rose Marujo, and James Mier of Beth Israel Deaconess Medical Center; Miriam Akita, Marianne Davies, Emily Duffield, Christina Lakomski, Daniel Morgensztern, Isabel Oliva, Von Potter, Elin Rowen, Rebecca Sipples, and Danielle Wanik of Yale Cancer Center; Suzanne Burke, Kim Feldhaus, Elaine Granch, Nabeela Iqbal, and Prathima Koppolu of the University of Michigan; Jahleen Byers, Bryan Greene, Jill Hinson, Eric Keller, Lori Lipocky, and Vanica Pharoah of Carolina BioOncology Institute; and David Carbone, Ken Hande, William Pao, Igor Puzanov, and Serena Rucker of Vanderbilt University Medical Center; Qiankun Sun, statistician, and Mubing Li, lead statistical programmer, of Bristol-Myers Squibb; and Susan Leinbach, medical writer, of Clinical Solutions Group.

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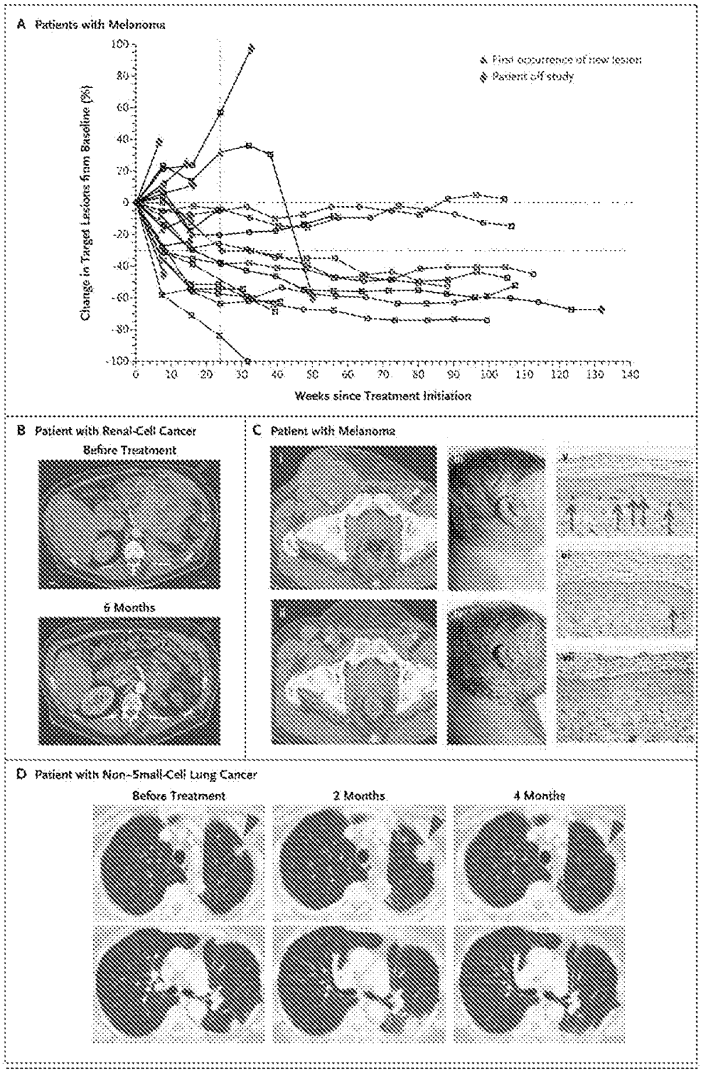


Figure 1. Activity of Anti–Programmed Death 1 (PD-1) Antibody in Patients with Treatment-Refractory Melanoma, Non–Small-Cell Lung Cancer, or Renal-Cell Cancer

In Panel A, a representative plot shows changes from baseline in the tumor burden, measured as the sum of the longest diameters of target lesions, in 27 patients with melanoma who received anti–PD-1 antibody at a dose of 1.0 mg per kilogram of body weight every 2 weeks. In the majority of patients who had an objective response, responses were durable and evident by the end of cycle 2 (16 weeks) of treatment. The vertical dashed line marks the 24-week time point at which the progression-free survival rate was calculated, and the horizontal dashed line marks the threshold for objective response (partial tumor regression) according to modified Response Evaluation Criteria in Solid Tumors. Tumor regression followed conventional as well as immune-related patterns of response, such as prolonged reduction in tumor burden in the presence of new lesions.[15,21] Panel B shows partial regression of metastatic renal-cell cancer in a 57-year-old patient who received anti–PD-1 antibody at a dose of 1.0 mg per kilogram. This patient had previously undergone radical surgery, and progressive disease had developed after treatment with sunitinib, temsirolimus,

sorafenib, and pazopanib. The arrowheads show regression of recurrent tumor in the operative field. Panel C shows a complete response in a 62-year-old patient with metastatic melanoma who received anti–PD-1 antibody at a dose of 3.0 mg per kilogram. Pretreatment computed tomographic scanning (i) revealed inguinal-lymph-node metastasis (arrowhead), which regressed completely after 13 months of treatment (ii). Numerous metastases in the subcutaneous tissue and retroperitoneum also regressed completely (not shown). Vitiligo, which developed after 6 months of treatment, is evident in photographs taken at 9 months under visible light (iii) and ultraviolet light (iv). Skin-biopsy specimens with immunohistochemical staining for micro-ophthalmia–associated transcription factor show that melanocytes (arrows) are abundant at the epidermal–dermal junction in normal skin (v), scarce in skin partially affected by vitiligo (vi), and absent in skin fully affected by vitiligo (vii). Panel D shows a partial response in a patient with metastatic non–small-cell lung cancer (nonsquamous histologic type) who received anti–PD-1 antibody at a dose of 10.0 mg per kilogram. The arrowheads show initial progression in pulmonary lesions, followed by regression (an immune-related pattern of response)

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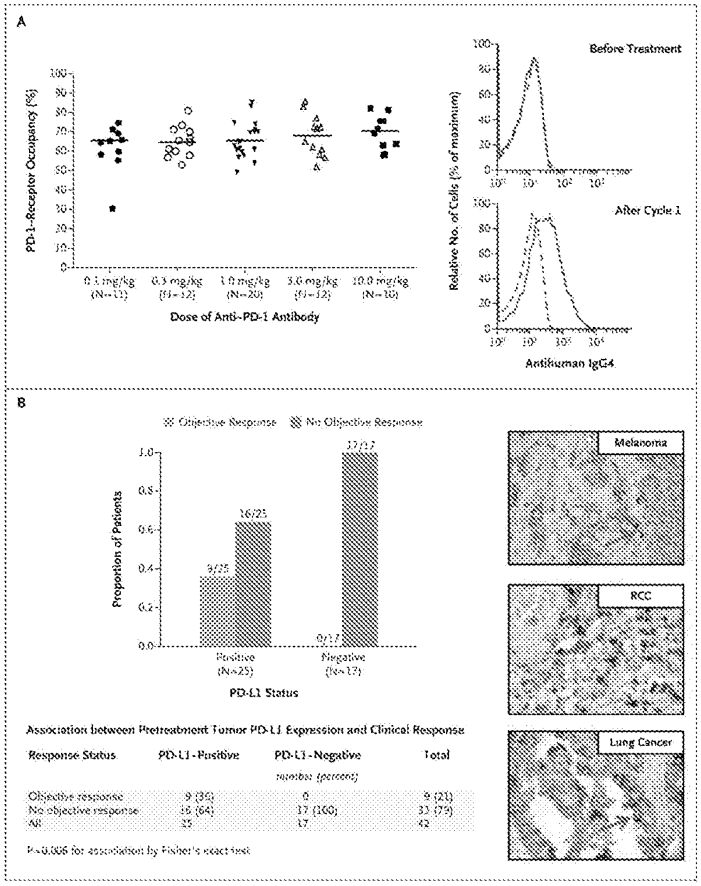


Figure 2. Pharmacodynamic and Molecular-Marker Assessments

Panel A shows PD-1–receptor occupancy by anti–PD-1 antibody. The graph at the left shows PD-1–receptor occupancy on circulating T cells in 65 patients with melanoma after one cycle (8 weeks) of treatment at a dose of 0.1 to 10.0 mg per kilogram every 2 weeks. Bars indicate median values. The graphs at the right show PD-1–receptor occupancy on CD3-gated peripheral-blood mononuclear cells from a patient with melanoma who received 0.1 mg per kilogram, before treatment (top) and after one treatment cycle (bottom). Cells were stained with biotinylated antihuman IgG4 to detect infused anti–PD-1 antibody bound to PD-1 molecules on the cell surface. Detection was accomplished with the use of streptavidin–phycoerythrin, followed by flow-cytometric analysis. Dashed lines indicate isotype staining controls, and solid lines antihuman IgG4. Panel B shows the correlation of pretreatment tumor cell-surface expression of PD-1 ligand (PD-L1), as determined with immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens, with an objective response to PD-1 blockade in 42 patients with advanced cancers: 18 with melanoma, 10 with non–small-cell lung cancer, 7 with colorectal cancer, 5 with renal-cell cancer, and 2 with castration-resistant prostate cancer. Tumor cell-surface expression of PD-L1 was significantly correlated with an objective clinical response (graph at the left). No patients with PD-L1–negative tumors had an objective response. Of the 25 patients with PD-L1–positive tumors, 2 who were categorized as not having had a response at the time of data analysis are still under evaluation. Shown at the right are immunohistochemical analysis with the anti–PD-L1 monoclonal antibody 5H1 in a specimen of a lymph-node metastasis

from a patient with melanoma (top), a nephrectomy specimen from a patient with renal-cell cancer (RCC) (middle), and a specimen of a brain metastasis from a patient with lung adenocarcinoma (bottom). The arrow in each specimen indicates one of many tumor cells with surface-membrane staining for PD-L1. The asterisk indicates a normal glomerulus in the nephrectomy specimen, which was negative for PD-L1 staining.

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Table 1

Treatment-Related Adverse Events of Special Interest That Occurred in at Least 1% of All Treated Patients.

Event	Anti-PD-1 Antibody, 0.1 mg/kg N = 18)		Anti-PD-1 Antibody, 0.3 mg/kg (N = 19)		Anti-PD-1 Antibody, 1.0mg/ kg(N = 79)		Anti-PD-1 Antibody, 3.0 mg/kg (N = 50)		Anti-PD-1 Antibody, 10.0 mg/kg (N = 130)		Anti-PD-1 Antibody, Total (N = 296)	
	All Events	Grade 3 or 4 Events	All Events	Grade 3 or 4 Events	All Events	Grade 3 or 4 Events	All Events	Grade 3 or 4 Events	All Events	Grade 3 or 4 Events	All Events	Grade 3 or 4 Events
	number of patients (percent)											
Any adverse event of special interest *	8 (44)	0	6 (32)	0	39 (49)	5 (6)	19 (38)	2 (4)	50 (38)	11 (8)	122 (41)	18 (6)
Pulmonary disorders												
Pneumonitis	0	0	0	0	3 (4)	2 (3)	1 (2)	0	5 (4)	1 (1)	9 (3)	3 (1)
Allergic rhinitis	1 (6)	0	0	0	2 (3)	0	0	0	1 (1)	0	4 (1)	0
Diarrhea	1 (6)	0	2 (11)	0	15 (19)	0	3 (6)	0	12 (9)	3 (2)	33 (11)	3 (1)
Skin events												
Rash	3 (17)	0	2 (11)	0	16 (20)	0	4 (8)	0	11 (8)	0	36 (12)	0
Pruritus	0	0	2 (11)	0	13 (16)	0	4 (8)	0	9 (7)	1 (1)	28 (9)	1 (<1)
Vitiligo	3 (17)	0	0	0	3 (4)	0	2 (4)	0	0	0	8 (3)	0
Pruritic rash	0	0	0	0	1 (1)	0	1 (2)	0	4 (3)	0	6 (2)	0
Urticaria	0	0	0	0	0	0	3 (6)	0	2 (2)	0	5 (2)	0
Macular rash	0	0	0	0	0	0	1 (2)	0	3 (2)	1 (1)	4 (1)	1 (<1)
Alopecia	0	0	0	0	2 (3)	0	0	0	1 (1)	0	3 (1)	0
Hypopigmentation	0	0	0	0	2 (3)	0	0	0	1 (1)	0	3 (1)	0
Laboratory investigations †												
Alanine aminotransferase increased	0	0	1 (5)	0	4 (5)	0	2 (4)	0	4 (3)	2 (2)	11 (4)	2 (1)
Thyroid-stimulating hormone increased	2 (11)	0	0	0	2 (3)	0	2 (4)	0	3 (2)	1 (1)	9 (3)	1 (<1)
Aspartate aminotransferase increasec	0	0	1 (5)	0	2 (3)	0	2 (4)	1 (2)	3 (2)	1 (1)	8 (3)	2 (1)
Endocrine disorders												
Hypothyroidism	0	0	1 (5)	0	2 (3)	0	1 (2)	0	3 (2)	1 (1)	7 (2)	1 (<1)
Hyperthyroidism	1 (6)	0	0	0	0	0	1 (2)	0	1 (1)	1 (1)	3 (1)	1 (<1)
Infusion-related reaction or hypersensitivty	0	0	0	0	2 (3)	0	3 (6)	0	4 (3)	1 (1)	9 (3)	1 (<1)

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[*] The numbers reported within a column may not add up to the total number reported for "any adverse event of special interest" because patients who had more than one adverse event were counted for each event but were counted only once for "any adverse event of special interest" and because data for only those events that were reported in at least 1% of all treated patients are shown. Colitis, hepatitis, hypophysitis, and thyroiditis were among the adverse events of special interest that were reported in less than 1% of patients. PD-1 denotes programmed death 1.

[†] Levels of alanine aminotransferase, thyroid-stimulating hormone, and aspartate aminotransferase were considered to be increased if they exceeded the upper limit of the normal range for the local laboratory.

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Table 2

Clinical Activity of Anti–PD-1 Antibody in the Efficacy Population.*

Dose of Anti–PD-1 Antibody	Objective Response†	Objective-Response Rate‡	Duration of Response§	Stable Disease ≥24 wk		Progression-free Survival Rate at 24 wk¶
	no. of patients/total no. of patients	*% (95% CI)*	*mo*	*no. of patients/total no. of patients*	*% (95% CI)*	*% (95% CI)*
Melanoma						
0.1 mg/kg	4/14	29 (8–58)	7.5+, 5.6+, 5.6, 5.6	1/14	7 (0.2–34)	40 (13–66)
0.3 mg/kg	3/16	19 (4–46)	3.8+, 2.1+, 1.9+	1/16	6 (0.2–30)	31 (9–54)
1.0 mg/kg	8/27	30 (14–50)	24.9+, 22.9, 20.3+, 19.3+, 18.4+, 7.6+, 5.6+, 5.3+	3/27	11 (2–29)	45 (26–65)
3.0 mg/kg	7/17	41 (18–67) //	22.4+, 18.3+, 15.2+, 12.9, 11.1, 9.3, 9.2+	1/17	6 (0.1–29)	55 (30–80)
10.0 mg/kg	4/20	20 (6–44)	24.6+, 23.9+, 18.0+, 17.0	0/20	0	30 (9–51)
All doses	26/94	28 (19–38)		6/94	6 (2–13)	41 (30–51)
Non–small-cell lung cancer						
Squamous						
1.0 mg/kg	0/5	0		0/5	0	0
3.0 mg/kg	3/6	50 (12–88)	ND	0/6	0	50 (10–90)
10.0 mg/kg	3/7	43 (10–82)	ND	0/7	0	43 (6–80)
All doses	6/18	33 (13–59)	ND	0/18	0	33 (12–55)
Nonsquamous						
1.0 mg/kg	0/12	0		1/12	8 (0.2–39)	14 (0–37)
3.0 mg/kg	3/13	23 (5–54)	ND	2/13	15 (2–45)	37 (10–64)
10.0 mg/kg	4/31	13 (4–30)	ND	2/31	6 (0.8–21)	21 (6–36)
All doses	7/56	12 (5–24)	ND	5/56	9 (3–20)	22 (11–34)
Unknown type						
1.0 mg/kg	1/1	NA	ND	0/1	0	NA
10.0 mg/kg	0/1	0		0/1	0	0
All types						
1.0 mg/kg	1/18	6 (0.1–27)	9.2+	1/18	6 (0.1–27)	16 (0–34)

Dose of Anti–PD-1 Antibody	Objective Response[†]	Objective-Response Rate[‡]	Duration of Response[§]	Stable Disease ≥24 wk		Progression-free Survival Rate at 24 wk[¶]
	no. of patients/total no. of patients	*% (95% CI)*	*mo*	*no. of patients/total no. of patients*	*% (95% CI)*	*% (95% CI)*
3.0 mg/kg	6/19	32 (13–57)	30.8+, 7.6+, 5.5+, 3.7+, 1.9+, NA[**]	2/19	11 (1–33)	41 (18–64)
10.0 mg/kg	7/39	18 (8–34)	14.8+, 7.6+, 7.3+, 6.7, 4.2, 3.7+, 3.7	2/39	5 (0.6–17)	24 (11–38)
All doses	14/76	18 (11–29)		5/76	7 (2–15)	26 (16–36)
Renal-cell cancer						
1.0 mg/kg	4/17	24 (7–50)	17.5+, 9.2+, 9.2, 5.6+	4/17	24 (7–50)	47 (23–71)
10.0 mg/kg	5/16	31 (11-59) //	22.3+, 21.7+, 12.9, 12.0, 8.4	5/16	31 (11–59)	67 (43–91)
All doses	9/33	27 (13–46)		9/33	27 (13–46)	56 (39–73)

[*] The efficacy population consisted of patients in whom the response could be evaluated, whose treatment was initiated by July 1, 2011, and who had measurable disease at baseline with one of the following: at least one scan obtained during treatment, clinical evidence of disease progression, or death. NA denotes not applicable, and ND not determined.

[†] Responses were adjudicated according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0, with modification (see Methods S1 in the Supplementary Appendix and the protocol).

[‡] Objective response rate = [(complete responses + partial responses) ÷ total no. of patients] × 100. Rates were calculated on the basis of confirmed responses, with confidence intervals calculated with the use of the Clopper–Pearson method.

[§] Duration of response was defined as the time from the first response to the time of documented progression, death, or, for censored data (denoted by a plus sign), the most recent tumor assessment.

[¶] The progression-free survival rate is the proportion of patients without disease progression who were alive at 24 weeks; the rate was calculated according to the Kaplan–Meier method, with confidence intervals calculated with the use of the Greenwood method.

// Two patients (one with melanoma who received 3.0 mg of anti–PD-1 antibody per kilogram and one with renal-cell cancer who received 10.0 mg per kilogram) had a complete response.

[**] One patient continued to receive treatment after an initial detection of progressive disease in preexisting lesions and subsequently had a partial response; this patient was classified as having had a response for the purpose of calculating response rates according to RECIST, version 1.0, but was not included in the calculation of duration of response.

N Engl J Med. Author manuscript; available in PMC 2013 January 14.

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Curr Opin Immunol. Author manuscript; available in PMC 2013 April 1.

Published in final edited form as:
Curr Opin Immunol. 2012 April ; 24(2): 207–212. doi:10.1016/j.coi.2011.12.009.

Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity

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Abstract

Genetic alterations and epigenetic dysregulation in cancer cells create a vast array of neoepitopes potentially recognizable by the immune system. Immune checkpoint blockade has the capacity to enhance and sustain endogenous immunity against non-mutated tumor-associated antigens as well as uniquely mutant antigens, establishing durable tumor control. Recent evidence from preclinical models highlights the pivotal role of the Programmed Death-1 (PD-1) T cell co-receptor and its ligands, B7-H1/PD-L1 and B7-DC/PD-L2, in maintaining an immunosuppressive tumor microenvironment. Encouraging early clinical results using blocking agents against components of the PD-1 pathway have validated its importance as a target for cancer immunotherapy.

INTRODUCTION

Ever since it became clear that all cancer cells express tumor-specific and tumor-selective antigens derived from genetic alterations and epigenetic dysregulation, the immunology community has embraced the potential for immune-based therapies to induce targeted anti-tumor responses. However, skepticism about the clinical value of immunotherapies designed to specifically enhance T cell-mediated immunity against preselected commonly expressed tumor antigens, including cancer vaccines and adoptive T cell transfer strategies, has escalated [1]. A game changer for cancer immunotherapy has emerged in the form of monoclonal antibodies (mAbs) that block inhibitory receptors on immune effector cells or their ligands on tumor cells and antigen presenting cells (APCs) – so-called "immune checkpoints". Immune checkpoint blockade has the potential to enhance and sustain endogenous immunity against non-mutated as well as uniquely mutant antigens, establishing

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DISCLOSURE STATEMENT

Consulting or advisory role: S.L.T., Bristol-Myers Squibb (uncompensated) and Amplimmune Inc. (spouse); C.G.D., Bristol-Myers Squibb and Amplimmune Inc.; D.M.P., Bristol-Myers Squibb (uncompensated) and Amplimmune Inc.. *Stock ownership*: C.G.D., Amplimmune Inc.. *Research funding*: S.L.T., Bristol-Myers Squibb.

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durable tumor control. Recent evidence highlights the pivotal role of the Programmed Death-1 (PD-1) T cell co-receptor and its ligands B7-H1/PD-L1 and B7-DC/PD-L2 in maintaining an immunosuppressive tumor microenvironment. Encouraging early results from clinical blockade of this pathway have validated its potential as a target for cancer immunotherapy.

RECEPTOR/LIGAND BIOLOGY

The two checkpoint receptors that have been most actively studied in the context of clinical cancer immunotherapy, CTLA-4 and PD-1 [2–8], play distinct roles in regulating immunity (Figure 1). CTLA-4, which regulates the amplitude of early activation of naïve and memory T cells, is transported to the T cell surface at levels that depend on the strength of the TCR signal. It acts physiologically as a signal dampener. Its importance in modulating T cell responses is demonstrated by the rapidly lethal autoimmune/hyperimmune phenotype of CTLA-4 knockout (KO) mice, which predicted the significant immune toxicity associated with blockade of this receptor.

In contrast to CTLA-4, the major role of PD-1 is to limit the activity of T cells in the periphery during an inflammatory response to infection and to limit autoimmunity [5–9]. The basis for this physiology is that the ligands for PD-1, B7-H1/PD-L1 [10,11] and B7-DC/PD-L2 [12,13], are up-regulated in response to inflammation. B7-H1/PD-L1 is up-regulated on many cell types – hematopoietic, endothelial and epithelial - in response to proinflammatory cytokines, notably interferon-gamma, while B7-DC/PD-L2 is upregulated on dendritic cells and macrophages in response to different proinflammatory cytokines such as IL-4 [14,15]. PD-1 is expressed to various degrees on activated T cells; thus, the co-expression of ligand and receptor in inflamed tissues mitigates against collateral tissue destruction by T cells at these sites. In keeping with this biologic role, PD-1 and B7-H1/PD-L1 KO mice do not develop spontaneous autoimmune responses in the first year of age, but do demonstrate exacerbated tissue responses to infection or accelerated disease in autoimmune-prone strains.

Further complexity to the PD-1/PD-1ligand system was appreciated with the surprising discovery that B7-H1/PD-L1 binds B7.1 in addition to PD-1 [16]. The binding sites appear to be adjacent but not overlapping, allowing for anti-B7-H1/PD-L1 mAbs that block one or both of these interactions. Application of these mAbs in various selective KO models suggests that interactions between B7-H1/PD-L1 and B7.1 on T cells inhibit T cell activity independent of interactions with PD-1 [17,18]. These findings suggest that there are three distinct inhibitory interactions within this pathway and that anti-PD-1 and anti-B7-H1/PD-L1 antibodies might behave differently owing to their ability to block distinct sets of interactions (Figure 2).

The finding that B7-H1/PD-L1 is commonly up-regulated on many different tumor types, where it inhibits local anti-tumor T cell responses [19,20], and that PD-1 is expressed on the majority of tumor infiltrating lymphocytes [21,22] creates an important rationale for mAb blockade of this pathway for cancer immunotherapy, as validated by multiple murine tumor studies [23,24].

The clinical development of PD-1 pathway blockers requires an understanding of the signals that induce expression of its ligands within the tumor. In the case of the PD-1 ligands, constitutive oncogene-driven expression of B7-H1/PD-L1 has been suggested. However, recent findings support an alternative model, that B7-H1/PD-L1 up-regulation on tumor cells reflects their adaptation to endogenous immune responses directed at tumor antigens – a process we term adaptive resistance (J. Taube et al., unpublished). In adaptive resistance, the tumor co-opts the natural physiology of the PD-1 pathway for tissue protection in the

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face of inflammation, to protect itself from an anti-tumor response. Expression of B7-H1/PD-L1 as an adaptive response to anti-tumor immunity likely occurs because this ligand is induced on most epithelial cancers in response to interferon-gamma, similarly to epithelial and stromal cells in normal tissues [25, 26]. In lymphoid malignancies, B7-DC/ PD-L2 is more commonly up-regulated [27], likely in response to different set of proinflammatory cytokine signals. The adaptive resistance mechanism directly implies that any treatment that induces anti-tumor immunity (e.g., vaccination) will provide therapeutic synergy with PD-1 pathway blockade.

PRECLINICAL MODELS

Preclinical evidence for PD-1's inhibitory role comes from several sources. Knockout mice develop late-onset, strain specific autoimmunity; on a C57/Bl6 background this manifests as sporadic glomerulonephritis [28], on a Balb/c background as an antibody-mediated cardiomyopathy [29]. These findings extend to models of organ-specific autoimmunity; on the non-obese diabetic (NOD) background, PD-1 KO mice develop accelerated insulitis as well as increased T cell production of effector cytokines [30]. Taken together, these data suggest that the PD-1/B7-H1(PD-L1) axis maintains T cell tolerance to persistently expressed antigens. A similar immunological picture emerges in models of chronic infection, where persistent pathogen exposure results in a population of "exhausted" antigen-specific CD8 T cells that express PD-1. In a chronic infection setting, the importance of this axis was elegantly demonstrated by studies in which blockade of PD-1/B7-H1(PD-L1) interactions restored the function of non-functional virus-specific CD8 T cells [31].

While much of this work has focused on CD8+ T cells, recent studies have highlighted a role for PD-1 on other cell types as well. One interesting set of experiments examined the acute B cell depletion that occurs during HIV infection, using a primate model. Here, PD-1 was shown to mediate the depletion of activated memory B cells and PD-1 blockade ameliorated this effect, restoring antibody titers [32]. While expression of PD-1 on B cells has been previously described, these recent data support a potential role for PD-1 in B cell immunity - the relative importance of this mechanism in cancer patients has yet to be explored. Expression of PD-1 on CD4+ T cells may also play a role in their dysfunction; a recent study used MHC class II tetramers to study HCV-specific CD4 T cell function in patients with either chronic or resolved infection. Similar to the case for CD8+ T cells, PD-1 blockade partially restored specific CD4 function in chronically infected patients, but T_H1 cytokine production was most effectively restored when PD-1 blockade was combined with TGF-β and/or IL-10 blockade. Finally, PD-1 is highly expressed on induced regulatory T cells (iTregs), and PD-1: PD-L1 interactions appear to promote the induction/conversion and maintenance of Tregs, suggesting an additional mechanism for immunosuppression in a tumor microenvironment rich in PD-1 ligands [33, 34]. These studies reinforce the notion that PD-1 blockade may affect multiple cell types, a critical observation in understanding the mechanism of action of antibody-mediated blockade.

Perhaps even more interesting are accumulating data that non-functional CD8+ cells coordinately express multiple immune checkpoint molecules with PD-1, and that blockade of several checkpoints may augment anti-tumor (as well as anti-viral) immunity. This notion was first demonstrated in the chronic LCMV model, where PD-1 was co-expressed with 2B4 and lymphocyte activating gene-3 (LAG-3) [35]. Recent studies extended this concept to cancer; PD-1/LAG-3 co-expression was documented on tumor antigen-specific CD8+ T cells in women with ovarian cancer, and dual (but not single) blockade was shown to restore CD8+ T cell function in vitro [36]. Indeed, PD-1 and LAG-3 may mediate non-overlapping tolerance-maintaining pathways; an important recent study showed that PD-1/LAG-3 double

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KO mice developed an accelerated autoimmune phenotype not present in either single KO strain [37]. Another cell surface molecule that may work with PD-1 to maintain tolerance is Tim-3, which is co-expressed with PD-1 in multiple preclinical models. Indeed, in an animal model of acute myelogenous leukemia (AML), blockade of PD-1 or Tim-3 alone was insufficient to rescue mice from cancer progression, while blockade of both pathways resulted in additive disease protection [38]. Similarly, PD-1/Tim-3 co-expression has been reported on NY-ESO-1 specific CD8+ T cells from melanoma patients [39], reinforcing the notion that other checkpoints likely cooperate with PD-1 to control anti-tumor immunity. Determining the relative expression of relevant checkpoint molecules in the tumor microenvironment, or in tumor draining lymph nodes, should provide key insights into combinatorial treatment strategies for clinical exploration.

CLINICAL TRANSLATION

An understanding of PD-1 pathway biology and the preclinical demonstration of its pivotal role in immunosuppression have fueled the clinical development of PD-1 pathway blockade for cancer therapy. There are currently four anti-PD-1 agents in the clinic: MDX-1106/BMS-936558/ONO-4538, CT-011, MK-3475, and AMP-224. The first three are reported to be PD-1 blocking mAbs, while the last is a B7-DC/IgG1 fusion protein (Table 1).

To date, most clinical experience with PD-1 blockade has been gained with MDX-1106. The first-in-human phase I trial of this fully human IgG4 mAb employed intermittent dosing in patients with treatment-refractory metastatic solid tumors, allowing detailed analysis of pharmacokinetics (PK) and pharmacodynamics over a wide dose range [40]. Clinical activity was observed in patients with melanoma, renal cell carcinoma, colorectal cancer and non-small cell lung cancer (NSCLC). Tumor cell surface expression of B7-H1/PD-L1 in pretreatment biopsies emerged as a potential biomarker of response, consistent with pathway biology. Unexpectedly, the pharmacodynamic effects of PD-1 receptor occupancy by MDX-1106 were prolonged well beyond the measured half-life of 12–20 days, indicating the biological durability of this high-affinity mAb. An ongoing follow-up trial of biweekly MDX-1106 administration has already shown durable complete or partial tumor regressions in approximately one-third of patients with advanced melanoma and kidney cancer, with confirmed activity against NSCLC (M. Sznol et al., abstract 2506 in *J Clin Oncol* 2010, 28: suppl 15s). The activity of anti-PD-1 against NSCLC, long thought to be a "non-immunogenic" tumor, suggests the potential for PD-1 blockade to reactivate endogenous anti-tumor immunity in a wide spectrum of malignancies. Grade ≥3 adverse clinical events occurred in approximately 12% of patients, and included immune-related phenomena similar to those encountered with anti-CTLA-4 therapy (DF McDermott et al., abstract 331 in *J Clin Oncol* 2011, 29: suppl 7).

Another PD-1 blocking mAb, MK-3475, recently entered phase I clinical evaluation for cancer therapy. In contrast to the fully human MDX-1106, MK-3475 is a humanized mAb. However, similar to MDX-1106, its engineered IgG4 isotype is designed to obviate antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CMC).

Two anti-PD-1 agents with IgG1 isotypes may coordinately activate T cell-mediated and innate anti-tumor immunity. CT-011, a humanized mAb, was first tested in a single-dose regimen in 17 patients with advanced hematologic malignancies [41], and was generally well-tolerated. A complete response in a previously untreated patient with follicular B cell lymphoma and a minor response in a patient with refractory AML were reported. CT-011 is currently undergoing further testing in next-generation clinical trials for patients with advanced hematologic malignancies and a variety of solid tumors. In vitro studies have

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demonstrated its capacity to activate human NK cells against multiple myeloma, suggesting engagement of the innate immune system [42]. Finally, AMP-224, a recombinant protein fusing the extracellular domain of human B7-DC/PD-L2 to IgG1, also commenced phase I clinical testing in 2011 for patients with treatment-refractory metastatic cancers. Based on its composition, AMP-224 has the potential to block the inhibitory B7-DC:PD-1 interaction while engaging NK cells.

A blocking antibody against the ligand B7-H1/PD-L1 (MDX-1105/BMS-936559) is also currently in phase I clinical testing in patients with advanced solid tumors, and it has shown preliminary evidence of activity against melanoma, kidney cancer and NSCLC. These complementary results further validate the PD-1 pathway as a target for immunotherapy.

CONCLUSIONS

Despite early successes with monotherapies blocking PD-1 pathways, preclinical models indicate that combinatorial therapies will deliver maximum clinical impact. Several clinical trials are already planned or in progress, combining anti-PD-1 mAbs with cancer vaccines (melanoma, prostate cancer, renal cell carcinoma, AML), anti-tumor mAbs (lymphoma), or chemotherapies (pancreatic cancer, NSCLC). These synergistic treatment strategies will provide a foundation for the next generation of clinical investigations.

Highlights

1. PD-1/B7-H1 are pivotal in maintaining an immunosuppressive tumor microenvironment.
2. PD-1 and CTLA-4 play distinct roles in regulating immunity.
3. B7-H1 up-regulation on tumor cells likely reflects "adaptive resistance".
4. PD-1 blockade is active against NSCLC, thought to be a "non-immunogenic" tumor.
5. Monotherapies blocking PD-1 may be more effective in combinatorial regimens.

Acknowledgments

Supported by NIH grants R01 CA142779 (S.L.T., D.M.P.), R01 CA127153 (C.G.D), and 1P50CA58236-15 (C.G.D); a grant from the Melanoma Research Alliance (S.L.T., C.G.D, D.M.P.); the Laverna Hahn Charitable Trust and the Barney Family Foundation (S.L.T.); the Patrick C. Walsh Fund, the OneInSix Foundation, and the Prostate Cancer Foundation (C.G.D).

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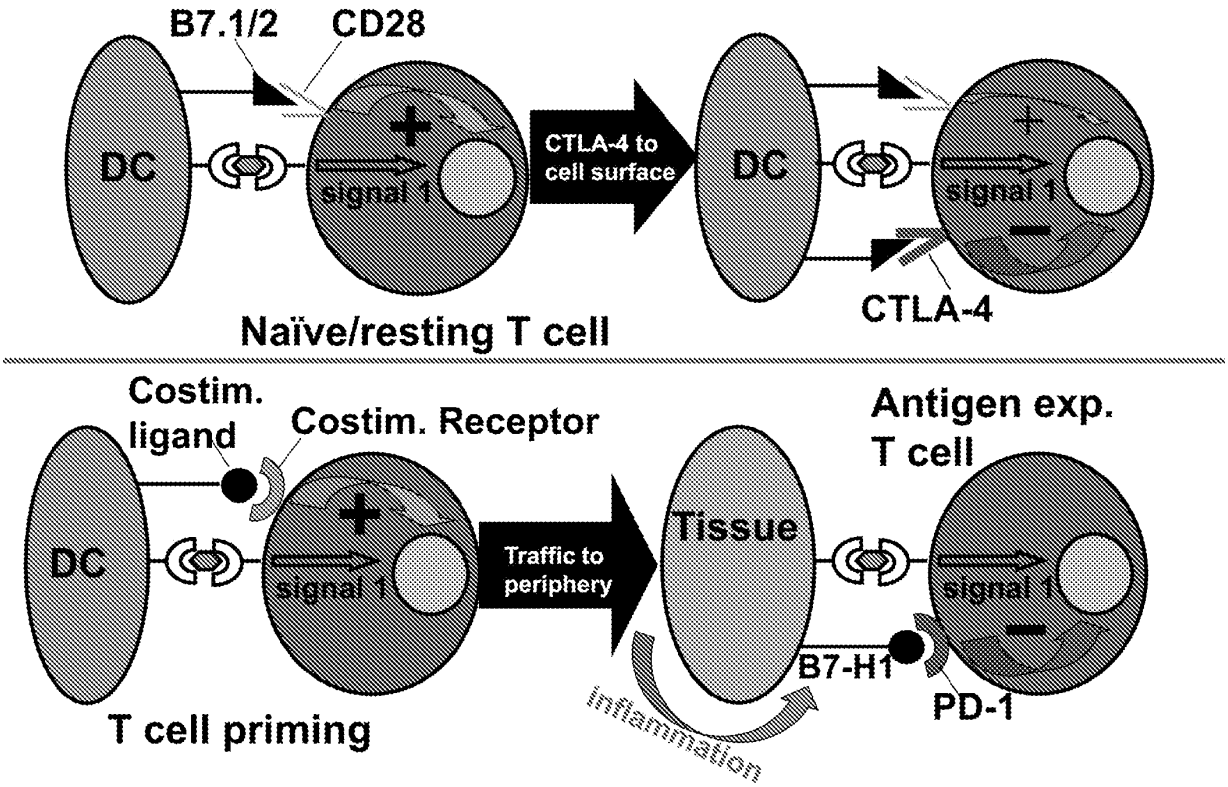


Figure 1.

PD-1 and CTLA-4 play distinct roles in regulating T cell immunity. CTLA-4 modulates the early phases of activation of naïve or memory T cells in response to TCR stimulation by MHC-peptide complexes displayed by antigen presenting cells ("signal 1"). In contrast, PD-1 is expressed on antigen-experienced T cells in the periphery, and serves to limit the activity of T cells at the time of an inflammatory response, thereby protecting normal tissues from collateral destruction. DC, dendritic cell.

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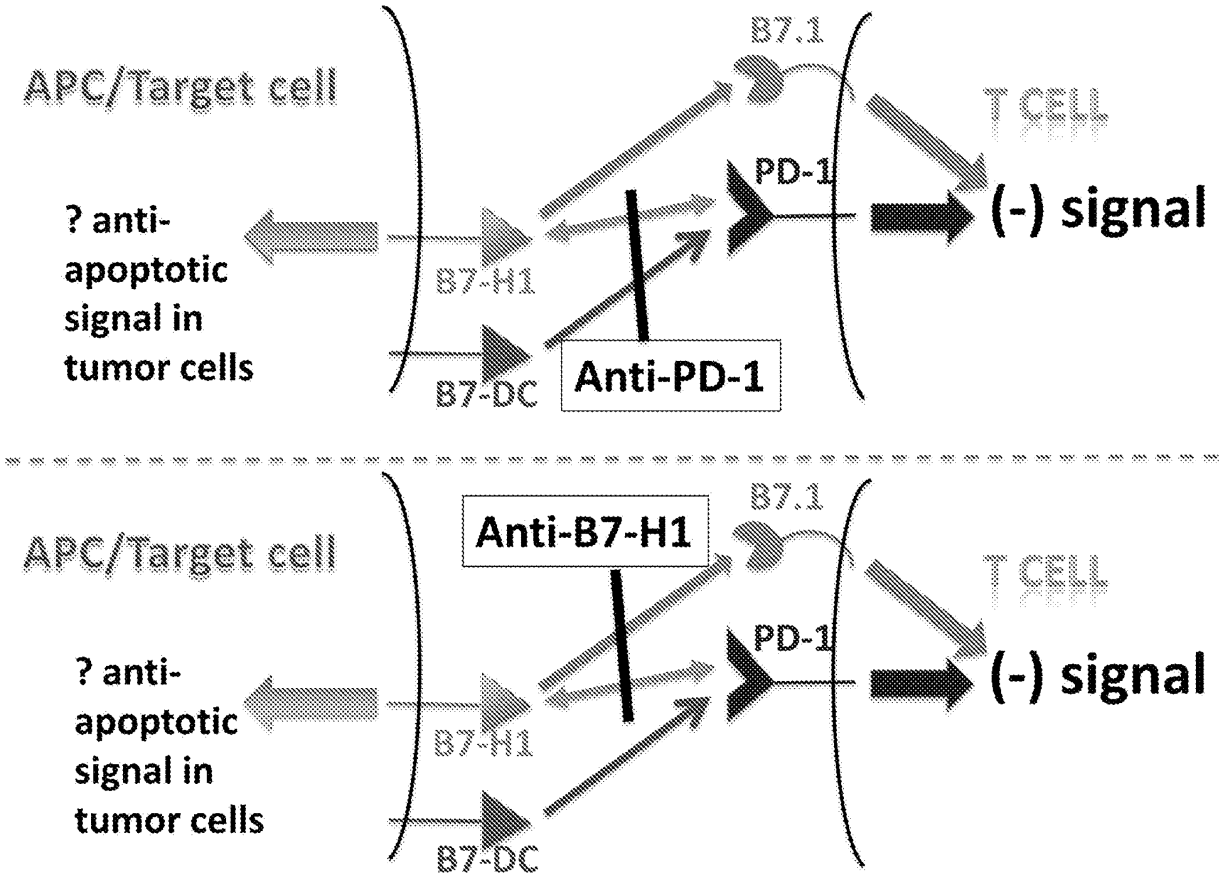


Figure 2.

Anti-PD-1 and anti-B7-H1/PD-L1 antibodies (mAbs) might behave differently owing to their ability to block distinct sets of inhibitory interactions. Anti-PD-1 mAbs can block its binding to both B7-H1/PD-L1 and B7-DC/PD-L2, abrogating an inhibitory PD-1-mediated signal in T cells; however, the inhibitory interaction of B7-H1/PD-L1 with B7.1 on T cells is not affected. Conversely, anti-B7-H1/PD-L1 mAbs can block its interactions with both B7.1 and PD-1, but will not block the inhibitory interaction of B7-DC/PD-L2 with PD-1. Both anti-PD-1 and anti-B7-H1/PD-L1 mAbs could potentially block transmission of a retrograde pro-survival signal through B7-H1/PD-L1 into tumor cells [43]. APC, antigen presenting cell.

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Table 1

PD-1 and B7-H1/PD-L1 blocking agents currently in clinical testing[a]

	Target Molecule	
Source	**PD-1**	**B7-H1/PD-L1**
Amplimmune Inc./GlaxoSmithKline	AMP-224 (B7-DC/IgG1 fusion protein)	N/A
Bristol-Myers Squibb	MDX-1106/BMS-936558/ONO-4538 (fully human IgG4 mAb)	MDX-1105/BMS-936559 (fully human IgG4 mAb)
CureTech/Teva	CT-011 (humanized IgG1 mAb)	N/A
Merck	MK-3475 (humanized IgG4 mAb)	N/A

[a] Information about clinical trials can be found at ClinicalTrials.gov, http://www.clinicaltrials.gov.

VOLUME 32 · NUMBER 10 · APRIL 1 2014

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Survival, Durable Tumor Remission, and Long-Term Safety in Patients With Advanced Melanoma Receiving Nivolumab

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See accompanying editorial on page 986; listen to the podcast by Dr Gulley at www.jco.org.podcasts

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Published online ahead of print at www.jco.org on March 3, 2014.

Supported by Bristol-Myers Squibb and Ono Pharmaceutical Company.

S.L.T. and M.S. contributed equally to this work.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Presented in part at the 49th Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, May 31-June 4, 2013.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical trial information: NCT00730639.

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0732-183X/14/3210w-1020w/$20.00

DOI: 10.1200/JCO.2013.53.0105

ABSTRACT

Purpose

Programmed cell death 1 (PD-1) is an inhibitory receptor expressed by activated T cells that downmodulates effector functions and limits the generation of immune memory. PD-1 blockade can mediate tumor regression in a substantial proportion of patients with melanoma, but it is not known whether this is associated with extended survival or maintenance of response after treatment is discontinued.

Patients and Methods

Patients with advanced melanoma (N = 107) enrolled between 2008 and 2012 received intravenous nivolumab in an outpatient setting every 2 weeks for up to 96 weeks and were observed for overall survival, long-term safety, and response duration after treatment discontinuation.

Results

Median overall survival in nivolumab-treated patients (62% with two to five prior systemic therapies) was 16.8 months, and 1- and 2-year survival rates were 62% and 43%, respectively. Among 33 patients with objective tumor regressions (31%), the Kaplan-Meier estimated median response duration was 2 years. Seventeen patients discontinued therapy for reasons other than disease progression, and 12 (71%) of 17 maintained responses off-therapy for at least 16 weeks (range, 16 to 56+ weeks). Objective response and toxicity rates were similar to those reported previously; in an extended analysis of all 306 patients treated on this trial (including those with other cancer types), exposure-adjusted toxicity rates were not cumulative.

Conclusion

Overall survival following nivolumab treatment in patients with advanced treatment–refractory melanoma compares favorably with that in literature studies of similar patient populations. Responses were durable and persisted after drug discontinuation. Long-term safety was acceptable. Ongoing randomized clinical trials will further assess the impact of nivolumab therapy on overall survival in patients with metastatic melanoma.

J Clin Oncol 32:1020-1030. © 2014 by American Society of Clinical Oncology

INTRODUCTION

Melanoma harbors one of the highest somatic mutation frequencies among human cancers.[1] Although the diversity of genetic alterations in melanoma creates challenges for targeted therapies, it provides a common denominator for immunotherapy, namely, the creation of tumor-specific antigens recognizable by the immune system. The adaptive immune system has powerful anticancer potential, with a broad capacity and exquisite specificity to respond to diverse tumor antigens. It also demonstrates considerable plasticity and a memory component, making immunotherapy unique among all cancer treatment modalities. Evidence suggests that a properly educated immune system can provide a self-perpetuating mechanism to eliminate or durably control melanoma and other cancers.[2] The clinical translation of cancer immunotherapy has recently accelerated as advances in molecular immunology have elucidated mechanistic pathways that subvert antitumor immunity. These include dysfunctional T-cell signaling,[3] suppressive regulatory cells,[4] and key "immune checkpoints" that regulate the outcome of lymphocyte engagement with antigen-presenting cells and tumor cells.[5,6] In

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particular, immune checkpoints, which serve to downmodulate the intensity of adaptive immune responses and protect normal tissues from collateral damage, can be co-opted by cancer cells to evade immune attack, which provides a spectrum of potential new targets for cancer immunotherapy.

The recent clinical success of anti-CTLA-4 (CD152) (ipilimumab) in improving survival in patients with advanced melanoma was achieved by blocking a prototypical T-cell checkpoint. This innovation established a therapeutic role for targeting immune inhibitory receptors and ligands and fueled efforts to explore the clinical effects of inhibiting other molecules in the CD28 and B7 families.[7,8] Programmed cell death 1 (PD-1) is a key inhibitory receptor expressed by activated T and B cells. Its binding with programmed cell death ligand 1 (PD-L1 [B7-H1]) and PD-L2 (B7-DC), expressed on antigen-presenting cells and human cancers, delivers a negative signal to lymphocytes.[9-12] In the first-in-human study of the PD-1 immune checkpoint inhibitor nivolumab (BMS-936558, MDX-1106, ONO-4538), an acceptable safety profile and durable objective tumor regressions were observed in patients with advanced solid tumors, including melanoma.[13,14] On the basis of these findings, this study of a multidose nivolumab regimen was undertaken. We have reported preliminary findings showing that approximately 20% to 30% of patients with advanced treatment–refractory melanoma, non–small-cell lung cancer, or kidney cancer experienced objective tumor regressions.[15] We now report overall survival outcomes in patients with melanoma who received nivolumab. Response characteristics, including durability and persistence after treatment discontinuation, and the long-term safety profile are presented in patients with a minimum of 14 months and up to 4.3 years since treatment initiation.

Patients and Methods

Study Design

This dose-escalation, cohort expansion study evaluated the antitumor activity and safety of nivolumab, a fully human immunoglobulin G4 monoclonal antibody blocking PD-1 in patients with advanced cancers, including melanoma and non–small-cell lung, kidney, colorectal, and castration-resistant prostate cancer. Study design and methods, including the protocol, amendments, and detailed statistical analysis plan, were previously published.[15] The study was approved by local institutional review boards, and all patients or their legal representatives gave written informed consent before enrollment. Nivolumab was administered intravenously once every 2 weeks in an outpatient setting in 8-week treatment cycles at 1, 3, or 10 mg/kg during dose escalation. After completion of dose escalation, each dose cohort was expanded to accrue approximately 16 patients with advanced melanoma. Following a 6.5-month hiatus for protocol amendment, additional melanoma cohorts randomly assigned to 0.1, 0.3, and 1.0 mg/kg were enrolled. In patients with melanoma receiving 0.1 or 0.3 mg/kg who had progressive disease, intrapatient dose escalation to 1.0 mg/kg was permitted. On the basis of observed objective responses, the protocol was further amended to evaluate overall survival.

Tumors were reassessed radiographically following each treatment cycle. Treatment continued up to 96 weeks (12 cycles), until patients experienced confirmed complete response, unacceptable toxicity, or progressive disease or until they withdrew consent. In clinically stable patients, treatment could be continued beyond initial disease progression pending subsequent confirmation of progression, consistent with proposed immune response criteria.[16] Patients with stable disease or an ongoing objective response (complete or partial) at the end of treatment were observed for up to 1 year and were offered re-treatment for 1 additional year if disease progressed.

Clinical and laboratory safety assessments were conducted on all treated patients at regular intervals during therapy and were reported up to 70 days following the last drug administration. Adverse event severity was graded on the basis of the National Cancer Institute's Common Terminology Criteria for Adverse Events, v3.0.[17]

Patients

Eligibility criteria have been previously described.[15] Patients with melanoma arising from any primary site, including ocular, were required to have measurable disease by RECIST (Response Evaluation Criteria in Solid Tumors) v1.0[18] with modification. Those with brain metastases were eligible if lesions had been treated and were clinically stable for at least 8 weeks. Patients must have received at least one but not more than five prior systemic cancer therapies. Those with a history of autoimmune disease, prior therapy with T-cell modulating antibodies (eg, anti–PD-1, anti–PD-L1, anti–CTLA-4), conditions requiring immunosuppression, chronic infections, or history of other invasive cancers within the previous 2 years were excluded.

Statistical Analysis

Baseline characteristics, response rates, adverse events, and survival results for all patients with melanoma (N = 107) are reported here as of March 5, 2013. Interim response rates for 94 patients and adverse events for 104 patients were previously reported as of February 2012.[15] Tumor measurements were collected by investigators, and individual best responses were centrally assessed by the sponsor per modified RECIST v1.0. Objective response and stable disease rates with CIs were estimated by using the Clopper-Pearson method. Time-to-event end points, including progression-free survival, overall survival, survival rates, and response duration, were estimated by using the Kaplan-Meier method, with CIs based on Greenwood's formula. Survival data were collected retrospectively. Progression-free survival estimates take into account all deaths, including four that occurred during the follow-up for survival. Adverse events were coded by using the Medical Dictionary for Regulatory Activities (MedDRA), version 15.1. Categories of select adverse events with potential immunologic etiologies, defined as adverse events that require more frequent monitoring or intervention with immune suppression or hormone replacement, were based on a prespecified list of MedDRA Terms (Data Supplement). An exposure-adjusted analysis of select adverse events that was based on the event rate per 100 person-years of nivolumab exposure was conducted for all 306 treated patients, including those with melanoma and non–small-cell lung, kidney, colorectal, and castration-resistant prostate cancer.[15]

Results

Patient Characteristics

In all, 107 patients with melanoma initiated treatment with nivolumab from November 2008 through January 2012. Baseline characteristics are presented in the Data Supplement. Of note, 62% had received at least two prior systemic treatments for melanoma, 78% had a visceral metastatic lesion, and 36% had an increased lactate dehydrogenase level in the blood, a factor associated with adverse prognosis in patients with advanced melanoma.

Overall and Progression-Free Survival

We undertook a retrospective analysis of overall survival in patients with advanced melanoma receiving nivolumab that was based on preliminary findings of durable tumor regression in these patients.[15] All patients initiated treatment at least 14 months before this analysis. The estimated median overall survival was 16.8 months (95% CI, 12.5 to 31.6 months; Table 1 and Fig 1A). One- and 2-year survival

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Table 1. Clinical Activity of Nivolumab in Melanoma by Dose Level

	ORR*			Duration of Response (weeks)†		Stable Disease ≥24 Weeks			PFS (months)		OS (months)	
Dose (mg/kg)	n/N	%	95% CI	Median	Individual Responses	n/N	%	95% CI	Median	95% CI	Median	95% CI
All doses	33/107	30.8	22.3 to 40.5	104	—	7/107	6.5	2.7 to 13.0	3.7	1.9 to 9.1	16.8	12.8 to 31.6
0.1‡	6/17	35.3	14.2 to 61.7	NR§	24.1, 24.1, 34.3, 44.1+, 48.1+, 48.7+	0	0	0	3.6	1.7 to 9.1	16.2	8.6 to NE
0.3‡	5/18	27.8	9.7 to 53.5	NR§	18.4, 44.4+, 64.8+, 65.1+, 66.3+	1/18	5.6	0.1 to 27.3	1.9	1.8 to 9.3	12.5	7.7 to NE
1	11/35	31.4	16.9 to 49.3	104	32.4, 32.4, 43.0+, 64.1+, 74.1+, 80.1+, 82.1+, 99.4, 100.9+, 104.1, 108.1+	5/35	14.3	4.8 to 30.3	9.1	1.8 to 24.7	25.3	14.6 to NE
3	7/17	41.2	18.4 to 67.1	75.9	40.1+, 40.4, 48.1, 56.1, 95.7, 106.7+, 115.4+	1/17	5.9	0.1 to 28.7	9.7	1.9 to 16.4	20.3	8.2 to NE
10	4/20	20.0	5.7 to 43.7	112	73.9, 78.3+, 111.7, 117.0+	0	0	0	3.7	1.7 to 20.5	11.7	7.2 to 37.9

Abbreviations: NE, not estimable; n/N, No. of patients/total No. of patients; NR, not reached; ORR, objective response rate; OS, overall survival; PFS, progression-free survival.
*Objective response rates {[(CR + PR) ÷ N] × 100} have been calculated on the basis of confirmed responses with CIs calculated by using the Clopper-Pearson method. Individual patient responses were adjudicated per Response Evaluation Criteria in Solid Tumors (RECIST) v1.0 with modification. One complete response was noted.
†Kaplan-Meier estimate, time from first response to time of documented progression, death, or for censored data (denoted by "+"), time to last tumor assessment.
‡Five patients with tumor progression received dose-escalation from 0.1 to 1.0 mg/kg, and six patients received dose-escalation from 0.3 to 1.0 mg/kg. None of these patients responded to therapy.
§The time point at which the probability that responder's progress drops below 50% has not been reached because of insufficient number of events and/or follow-up.

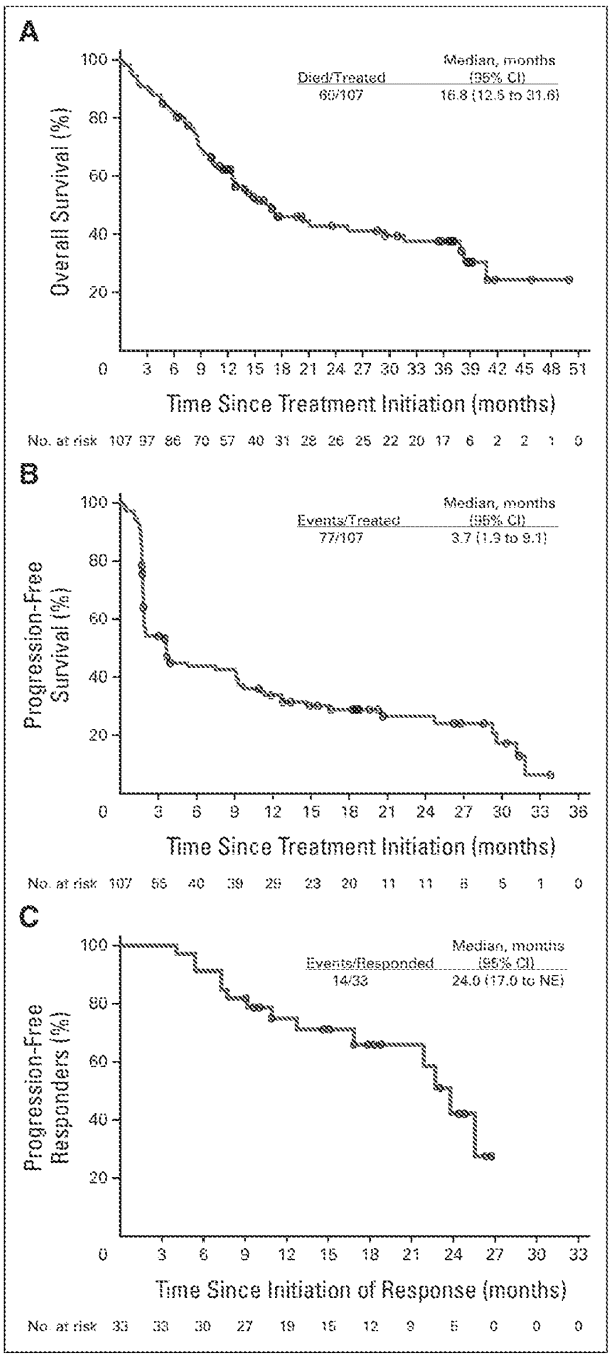


Fig 1. Kaplan-Meier curves of (A) overall survival and (B) progression-free survival in 107 nivolumab-treated patients with melanoma and (C) response duration in 33 objective responders. Analysis includes patients from all dose cohorts. (A) Patients with melanoma had 1- and 2-year overall survival rates of 62% and 43% and a median overall survival of 16.8 months. (B) Progression-free survival rates were 36% and 27% at 1 and 2 years, and the median was 3.7 months. (C) The median duration of response in 33 responding patients was 24 months. Open circles indicate censored events defined for progression-free survival as the time to the last tumor assessment before the date of data analysis for patients without disease progression or death, and for overall survival as the time to the last known alive date before the date of data analysis for patients without a death. NE, not estimable

rates were 62% (95% CI, 53% to 72%) and 43% (95% CI, 32% to 53%), respectively (Fig 1A). Median progression-free survival was 3.7 months (95% CI, 1.9 to 9.1 months), with 1- and 2-year progression-free survival rates of 36% (95% CI, 27% to 46%) and 27% (95% CI, 17% to 36%), respectively (Table 1 and Fig 1B). Both the overall and progression-free survival curves appeared to flatten beyond the median, although verification of this observation will require longer follow-up.

Response Rate and Duration

Objective responses were observed in 31% of patients (33 of 107) with melanoma, and an additional 7% of patients (seven of 107) experienced stable disease lasting for 24 weeks or more (Table 1). Durable responses were observed across all nivolumab doses tested within a 2-log range (0.1 to 10 mg/kg). Changes in the sum of target lesion dimensions compared with baseline are shown in Figure 2A. Unconventional response patterns that did not meet RECIST criteria (eg, persistent reduction in target lesions in the presence of new lesions or regression following initial progression)[16] were observed in four patients (4%); three of them received nivolumab at 1 mg/kg (Fig 2B), and a fourth received 10 mg/kg. Among 11 patients who experienced disease progression following treatment with nivolumab at 0.1 or 0.3 mg/kg, none responded following dose escalation to 1.0 mg/kg.

In 33 patients with objective responses, the Kaplan-Meier estimated median duration of response was 2 years (Fig 1C). Nineteen of 33 responses (58%) were ongoing at the time of data analysis (Table 1 and Fig 2C). Fifteen responses (45%) occurred rapidly and were documented at the first tumor assessment 8 weeks after starting treatment (Fig 2B-C). Tumor regression was observed at various anatomic sites and in primary and metastatic lesions, as exemplified in Figures 3 and 4. Seventeen of 33 patients stopped therapy for reasons other than disease progression during response and were observed; 12 (71%) of 17 maintained their responses for at least 16 weeks off-drug (16 to 56+ weeks), and eight of the 12 had responses ongoing at the time of analysis. Figure 4 shows an example of continued regression in multiple metastatic lesions after nivolumab discontinuation.

Safety

The maximum-tolerated dose of nivolumab was not reached within the tested dose range. With extended observation since our initial report (median time on treatment, 22 weeks; range, 2 to 122 weeks), the spectrum and severity of treatment-related adverse events remained stable (Table 2 and Data Supplement).[15] The most common events of any grade that occurred in patients with melanoma were fatigue (32%), rash (23%), and diarrhea (18%). Twenty-four (22%) of 107 patients with melanoma experienced grade 3 to 4 treatment-related adverse events. Select adverse events with potential immune-related causality, previously termed "immune-related adverse events" or "adverse events of special interest,"[15] were also analyzed (categorized in the Data Supplement). Treatment-related select adverse events of any grade were observed in 58 (54%) of 107 patients with melanoma, the most common of which included skin disorders (36%), GI events (18%), and endocrinopathies (13%; Data Supplement). Grade 3 to 4 treatment-related select events were seen in five patients (5%). There were no drug-related deaths in the population of patients

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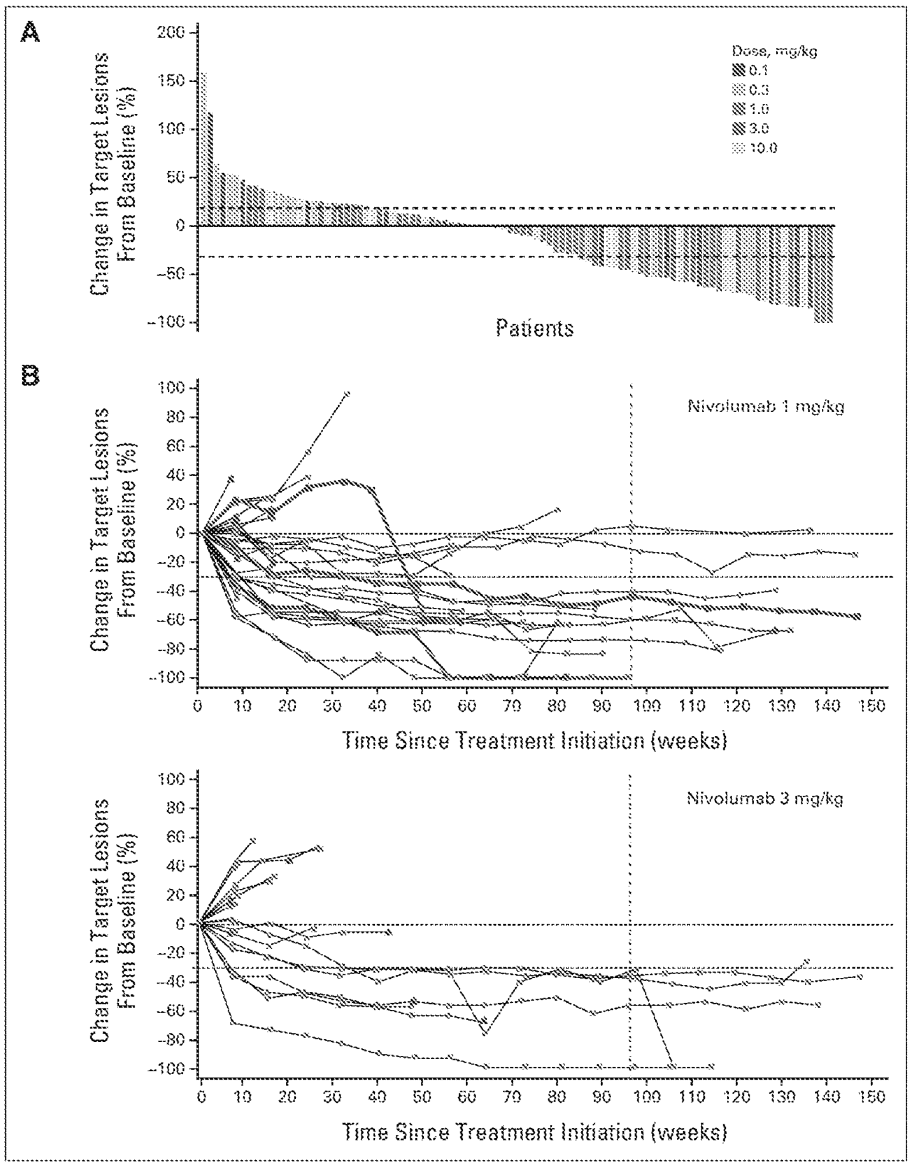


Fig 2. Characteristics of tumor regression in patients with melanoma receiving nivolumab therapy. (A) Maximum reduction or minimum increase in the sum of target lesion measurements compared with baseline in all treated patients with tumor measurements during treatment (n = 97). Responses were observed at all dose levels. Horizontal line at +20% indicates the threshold for defining progressive disease according to RECIST. Horizontal line at −30% indicates threshold for defining objective response (partial tumor regression) in the absence of new lesions or nontarget disease progression according to RECIST. (B) Response kinetics in patients receiving nivolumab at 1 mg/kg (n = 31) or 3 mg/kg (n = 17). Baseline tumor measurements are standardized to zero. Tumor burden is measured as the sum of the longest diameters of target lesions. Triangles indicate first occurrence of a new lesion. Vertical line at 96 weeks indicates the protocol-defined maximum duration of continuous nivolumab therapy. Horizontal line at −30% marks the threshold for defining objective response (partial tumor regression) according to RECIST. Blue curves indicate three unconventional immune-related response patterns in the 1 mg/kg dose cohort that did not meet RECIST criteria (eg, persistent reduction in target lesions in the presence of new lesions or regression following initial progression). Objective responses, unconventional responses, and stable disease persisted following treatment discontinuation in some patients. (Continued on next page.)

with melanoma, although there were three mortalities following treatment-related adverse events in the overall patient population (1%; two patients with non–small-cell lung cancer and one with colorectal cancer) associated with pneumonitis. Taking into account multiple adverse events occurring in individual patients, we analyzed the select adverse event rate as adjusted for person-years of nivolumab treatment in the total patient population, including those with melanoma and those with other solid tumors (N = 306; Data Supplement). Notably, with up to 2 years of safety monitoring for some patients, most adverse events occurred within the first 6 months of therapy, and cumulative toxicities were not observed with prolonged drug exposure.

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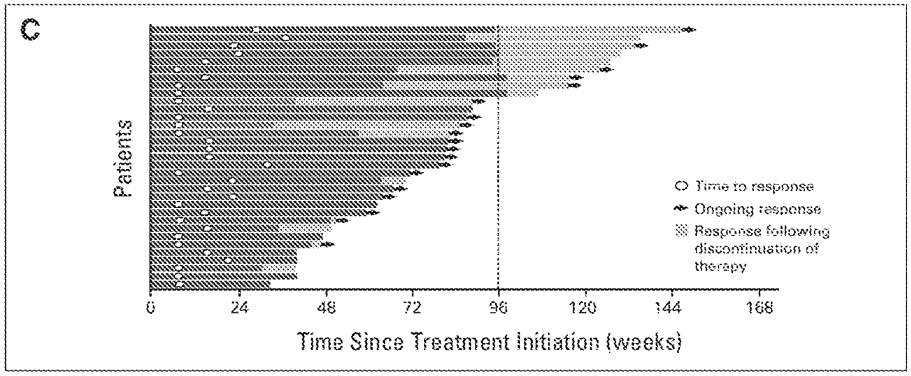


Fig 2. Continued. (C) Durability of tumor regressions in patients with melanoma who had objective responses to nivolumab therapy according to conventional RECIST criteria. Among 107 patients, 33 (31%) responded, including six (35%) of 17 who received nivolumab at 0.1 mg/kg, five (28%) of 18 at 0.3 mg/kg, 11 (31%) of 35 at 1 mg/kg, seven (41%) of 17 at 3 mg/kg, and four (20%) of 20 at 10 mg/kg. Blue bars indicate the time to and duration of response while on treatment; gold bars indicate response duration after treatment discontinuation; open circles indicate first evidence of objective response; arrows indicate ongoing response at time of analysis. Vertical line at 96 weeks indicates maximum planned duration of continuous nivolumab therapy. Reasons for treatment discontinuation with ongoing response included investigator-assessed complete response (n = 2), attainment of maximum treatment duration (n = 5), adverse events (n = 6), and other (eg, withdrew consent or investigator decision [n = 4]).

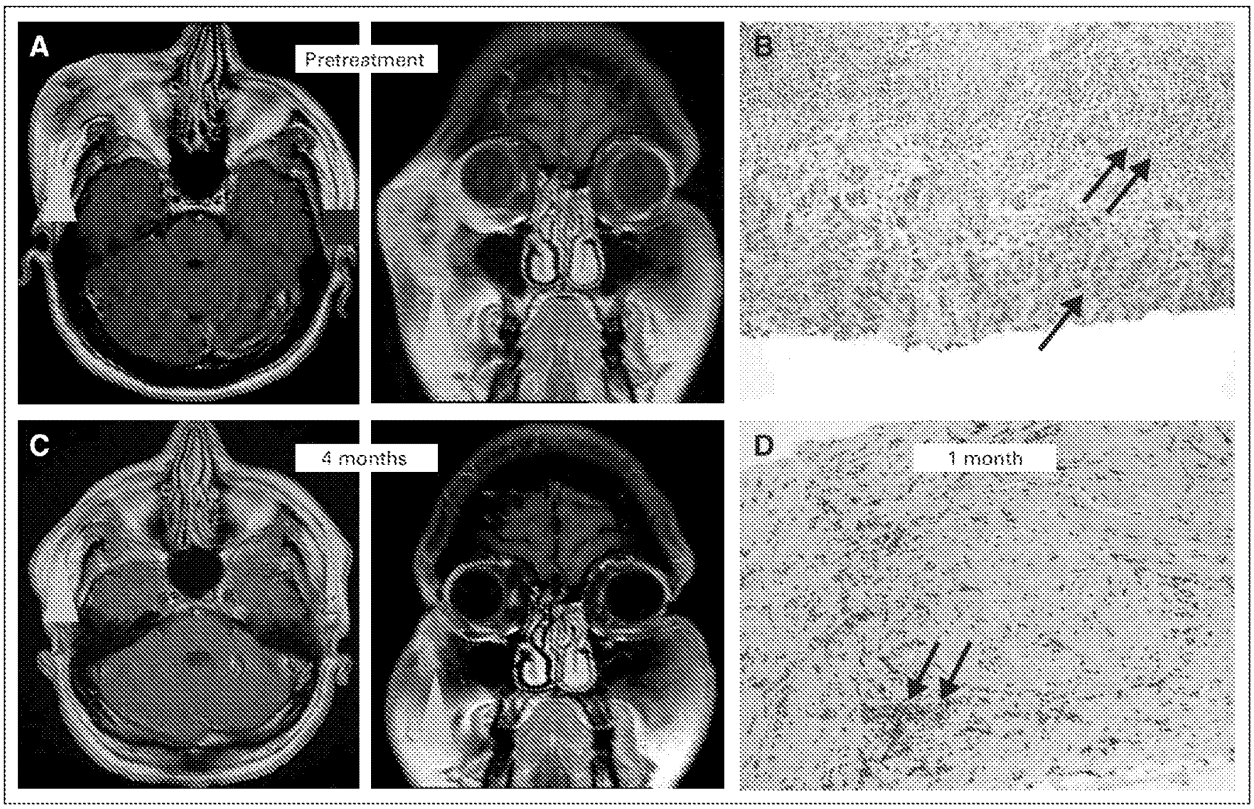


Fig 3. Partial response of locally advanced unresectable primary melanoma to nivolumab therapy in a 34-year-old man with xeroderma pigmentosum. Tumor had progressed through prior treatment with high-dose interleukin-2. (A) Pretreatment magnetic resonance imaging scans show right facial tumor eroding the zygomatic bone and extending into the orbit (gold arrows). (B) Immediate pretreatment core-needle tumor biopsy of the facial mass shows melanoma cells (single arrow) adjacent to infiltrating lymphocytes (double arrows). (C) A partial response was observed after 4 months (two cycles) of nivolumab therapy at 0.3 mg/kg every 2 weeks. This patient remains in partial response 2 years after treatment initiation. (D) Post-treatment core-needle biopsy shows fibrosis and infiltrating lymphocytes (double arrows); no tumor was present in this specimen. Hematoxylin and eosin stain; original magnification, ×200.

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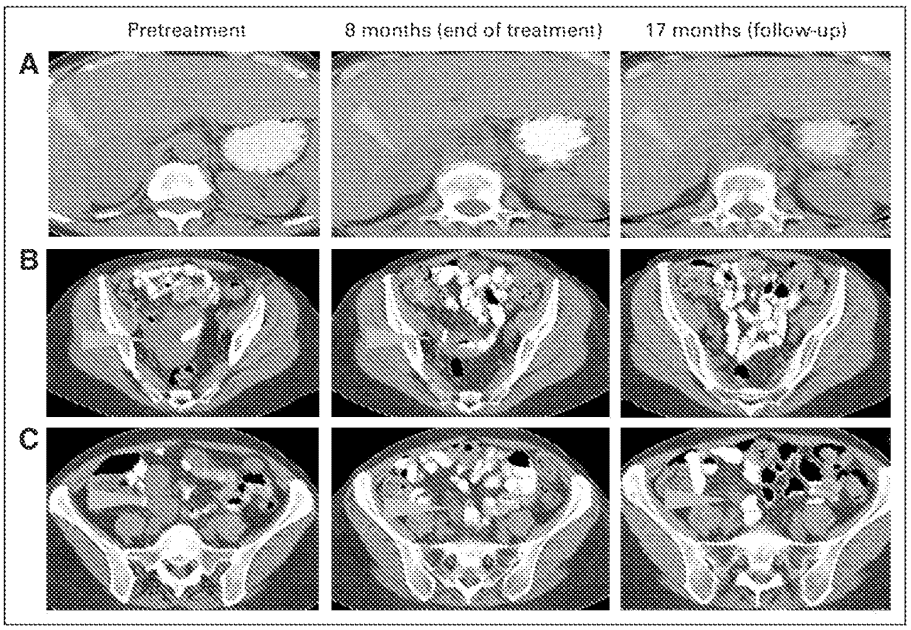


Fig 4. Partial response of metastatic melanoma to nivolumab, with continued tumor regression after drug discontinuation. This 59-year-old woman previously had experienced disease progression following high-dose interleukin-2, temozolomide, and sorafenib therapies. She received nivolumab 10 mg/kg every 2 weeks and achieved a partial tumor regression at 2 months. Treatment was discontinued at 8 months (four treatment cycles) as a result of exacerbation of an upper extremity neuropathy. Tumor regression continued after drug discontinuation. Computed tomographic scanning was performed with oral contrast but without intravenous contrast dye. Gold arrows indicate melanoma metastases involving (A) the right adrenal gland, (B) small bowel, and (C) mesenteric lymph nodes.

DISCUSSION

The critical role of the PD-1 pathway in suppressing antitumorimmunity, first revealed in laboratory models, has now been validated in clinical studies. Monotherapy with drugs blocking PD-1 (nivolumab, MK-3475 [lambrolizumab])[15,19] or its major ligand PD-L1 (BMS-936559, MPDL3280A)[20,21] can mediate rapid and durable regressions in patients with advanced treatment–refractory solid tumors, including epithelial cancers not traditionally viewed as immune responsive. These findings have established the PD-1 pathway as a new therapeutic focus in oncology.[22] This study presents the longest follow-up to date in patients with melanoma treated with a PD-1 pathway inhibitor, nivolumab, and allows us to assess for the first time survival outcomes and the durability of clinical activity mediated by this therapeutic approach. In addition to persistence of conventional responses and unconventional (immune-related) responses in patients receiving this therapy, the follow-up presented here assesses response maintenance after treatment discontinuation and treatment-associated toxicity as a function of time on therapy.

In the context of published clinical experience with similar patient populations, the survival outcomes associated with nivolumab therapy in melanoma in this early-phase study are particularly important. Overall survival rates of 62% at 1 year and 43% at 2 years were achieved, with a median overall survival of 16.8 months. In a recent phase III trial enrolling patients with melanoma who had at least one prior treatment for metastatic disease, ipilimumab increased median overall survival from 6.4 to 10.1 months compared with a gp100 peptide vaccine.[8] In phase II trials of ipilimumab in previously treated patients, median overall survivals of 8.7 to 11.4 months were observed in patients receiving 3 or 10 mg/kg, with 1-year and 2-year survival rates of 39% to 49% and 24% to 33%, respectively.[23] Treatment with selective BRAF and MEK inhibitors is restricted to patients with mutation-positive melanomas, which are found almost exclusively in tumors of cutaneous origin. In contrast, the trial of nivolumab described here enrolled patients regardless of anatomic site of melanoma origin or oncogene mutational status. The median overall survival in previously treated patients with *BRAF*-mutant melanoma enrolled onto a large phase II trial of vemurafenib was 15.9 months, and the 1-year survival rate was 58%; among 53% of patients with objective tumor regressions, the median response duration was 6.7 months.[24] Treatment of a similar patient population with the MEK inhibitor trametinib in those who had not previously received a BRAF inhibitor resulted in a median overall survival of 14.2 months and estimated 1-year survival of 59%; among the 25% of patients with objective tumor regressions, the median response duration was 5.7 months.[25] Of interest, PD-1 blockade has been reported to be effective in melanoma regardless of *BRAF* mutational status.[19,26] Despite the limitations of cross-study comparisons, this information suggests that nivolumab therapy may have a favorable impact on the survival of patients with advanced melanoma.

Notably, overall survival in patients with melanoma who received nivolumab was considerably longer than progression-free survival. Both survival curves appear to flatten after 1 year of follow-up. This mirrors observations reported for ipilimumab,[8] suggesting that early disease progression in some patients receiving immune checkpoint blockade can evolve to durable disease stabilization or regression. These findings suggest that progression-free survival may underestimate the efficacy of immunomodulatory agents such as nivolumab.[27]

We report here that 31% of patients with melanoma experienced confirmed objective tumor regressions when they were given nivolumab therapy, and 7% had disease stabilization lasting at least 6 months. In addition, 4% of patients manifested unconventional immune-related response patterns.[28] The apparent durability of clinical activity in nivolumab-treated patients is remarkable, because this has generally not been observed with chemotherapy or small molecule kinase inhibitors to date but has been seen to a

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Table 2. Treatment-Related Adverse Events That Occurred in at Least 3% of the Total Population of Patients With Melanoma

Treatment-Related Adverse Event	Patients (N = 107)			
	All Grades		Grades 3 to 4	
	No	%	No.	%
Any adverse event*†	90	84.1	24	22.4
General disorders				
Fatigue	34	31.8	2	1.9
Pyrexia	5	4.7	0	0
Pain	4	3.7	0	0
Skin and subcutaneous tissue disorders				
Rash	25	23.4	0	0
Pruritus	14	13.1	0	0
Vitiligo	10	9.3	0	0
Dermatitis acneiform	6	5.6	0	0
Photosensitivity reaction	4	3.7	0	0
GI disorders				
Diarrhea	19	17.8	2	1.9
Nausea	9	8.4	1	0.9
Abdominal pain	8	7.5	2	1.9
Dry mouth	7	6.5	1	0.9
Vomiting	5	4.7	1	0.9
Musculoskeletal disorders				
Arthralgia	7	6.5	0	0
Myalgia	4	3.7	0	0
Metabolism and nutrition disorders				
Decreased appetite	7	6.5	0	0
Hyperuricemia	4	3.7	1	0.9
Hypophosphatemia	4	3.7	1	0.9
Blood and lymphatic system disorders				
Lymphopenia	7	6.5	3	2.8
Investigations				
Blood thyroid-stimulating hormone increased	6	5.6	1	0.9
Weight decreased	6	5.6	0	0
Alanine aminotransferase increased	5	4.7	0	0
Hemoglobin decreased	5	4.7	1	0.9
Platelet count decreased	5	4.7	1	0.9
Aspartate aminotransferase increased	4	3.7	0	0
WBC count decreased	4	3.7	0	0
Endocrine disorders				
Hypothyroidism	6	5.6	1	0.9
Procedural complications				
Infusion-related reaction	6	5.6	0	0
Respiratory disorders				
Cough	4	3.7	0	0
Vascular disorders				
Flushing	4	3.7	0	0
Hypotension	4	3.7	0	0

NOTE. Treatment-related adverse events are reported according to the nivolumab dose cohort in the Data Supplement.

*Treatment-related adverse events that were reported in less than 3% of the total melanoma population included pneumonitis, colitis, and renal failure (two each, 2%) and hepatitis, hypophysitis, thyroiditis, uveitis, and tubulointerstitial nephritis (one each; 1%).

†The numbers reported within a column may not add up to the total number reported under any adverse event, because patients who had more than one adverse event were counted for each event but were counted only once for any adverse event. Data for only those events that were reported in at least 3% of the treated patient population are presented.

lesser degree in some patients with advanced melanoma receiving immunotherapies including ipilimumab and high-dose interleukin 2.[5,8] Among 33 patients with objective responses to nivolumab, the median response duration was 2 years. Unconventional responses also appeared to be long-lasting and may contribute to overall survival outcomes.

Beyond durability, a distinguishing feature of response to nivolumab therapy is the maintenance of response status after treatment discontinuation. The persistence of partial tumor regressions and stable disease following nivolumab discontinuation in the absence of any other systemic cancer therapy is not commonly seen with chemotherapy or kinase inhibitors and suggests that PD-1 blockade may

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reset the immune equilibrium between tumor and host. If PD-1 blockade served only to reverse inhibition of effector T-cell functions in the tumor microenvironment, tumors might be expected to re-engage this pathway and progress after drug discontinuation. Instead, our findings are consistent with a mechanism by which an effective tumor-selective immune memory response may have been established in some patients, similar to immune memory against specific infectious organisms after antigen exposure.[29] In other responding patients, persistent radiographic abnormalities may represent residual scarring rather than viable tumor (Fig 3), leading to an underestimation of true complete response rates. Further research will be needed to define mechanisms underlying the persistence of nivolumab-mediated tumor regressions after drug discontinuation and to explain disease recurrence following prolonged regression. Another implication worthy of further study is that the immune response unleashed by PD-1 blockade may adapt dynamically as the tumor evolves to mitigate the development of treatment resistance.

The acceptable long-term safety profile of nivolumab supports its continued development in the outpatient setting. Importantly, the exposure-adjusted toxicity rate in the total study population, including 306 patients with melanoma or other solid tumors who initiated therapy at least 14 months before data analysis, was not cumulative. Although the optimal duration of continuous nivolumab treatment is not yet known, this finding reduces potential concerns about the extended administration of anti–PD-1 therapy. In contrast, toxicities associated with chemotherapy typically accumulate with ongoing treatment. Select adverse events with potential immune-related causation are consistent with nivolumab's immunologic mechanism of action and include disorders of the lung, GI tract, skin, endocrine system, and kidney. The occurrence rate of grade 3 to 4 pneumonitis in 1% of all 306 patients receiving nivolumab on this study is lower than rates typically reported for some chemotherapies, tyrosine kinase inhibitors, and lung irradiation.[30-32] High-grade pneumonitis was not observed in any of the 107 patients with melanoma treated on this trial. Nivolumab-associated pneumonitis can be managed by drug discontinuation, corticosteroids, and other immune suppressive agents; however, vigilance and a multidisciplinary approach are needed.

Although nivolumab monotherapy may have an impact on the survival of patients with melanoma and other cancers,[33,34] preclinical evidence suggests that synergistic treatment combinations based on PD-1 pathway blockade could have even more potent effects. The apparently favorable therapeutic index of nivolumab as monotherapy supports its testing in treatment combinations. The clinical activity of nivolumab combined with ipilimumab, whose mechanism of action is distinct from that of nivolumab,[35] has been reported in melanoma,[36] and the potential advantage of this combination over monotherapy with either agent is being explored in a phase III trial (NCT01844505). In addition, combination trials with chemotherapy (NCT01454102), kinase inhibitors (NCT01472081), cancer vaccines (NCT01176461, NCT01176474), and other immune-modulating therapies (anti-killer inhibitory receptor, NCT01714739; interleukin-21, NCT01629758) are ongoing in patients with a variety of solid tumor types. Tumor cell expression of PD-L1 has been hypothesized as a molecular marker that may be associated with response to PD-1 or PD-L1 blockade.[15,21] Clinical trials incorporating molecular analyses to further explore the association of PD-L1 expression with clinical outcome and to profile the complex tumor immune microenvironment in patients treated with nivolumab may reveal additional therapeutic cotargets.[37,38] Innovative, rational treatment combinations based on preclinical evidence may be needed to realize the full therapeutic potential of PD-1 blockade.

In summary, these results suggest that nivolumab may have an impact on overall survival in patients with advanced melanoma with an acceptable long-term safety profile. Controlled phase III trials in melanoma with prospective survival end points (NCT01673867, NCT01721772, NCT01642004, NCT01668784, and NCT01721746) are currently underway.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Manuscript writing: All authors
Final approval of manuscript: All authors

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GLOSSARY TERMS

antigen: a substance that promotes, or is the target of, an immune response.

CTLA4 (CD152): receptor on activated T cells that binds B7 molecules with a higher affinity than CD28, downregulating T-cell responses by inhibiting CD28 signaling.

immunotherapy: a therapeutic approach that uses cellular and/or humoral elements of the immune system to fight a disease.

interleukin-2 (IL-2): a cytokine that stimulates proliferation of activated T cells and, at high doses, is used as antitumor therapy in metastatic renal cell carcinoma.

monoclonal antibody: an antibody that is secreted from a single clone of an antibody-forming cell. Large quantities of monoclonal antibodies are produced from hybridomas, which are produced by fusing single antibody-forming cells to tumor cells. The process is initiated with initial immunization against a particular antigen, stimulating the production of antibodies targeted to different epitopes of the antigen. Antibody-forming cells are subsequently isolated from the spleen. By fusing each antibody-forming cell to tumor cells, hybridomas can each be generated with a different specificity and targeted against a different epitope of the antigen.

RECIST (Response Evaluation Criteria in Solid Tumors): a model proposed by the Response Evaluation Criteria Group by which a combined assessment of all existing lesions, characterized by target lesions (to be measured) and nontarget lesions, is used to extrapolate an overall response to treatment.

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Acknowledgment

We thank the patients who participated in this study; clinical faculty, and personnel, including David Spigel of Sarah Cannon Research Institute/Tennessee Oncology; Cherylann Carr, Tianna Dauses, Robert Gray, Marina Laiko, and Alice Pons, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins; Miriam Akita, Marianne Davies, Emily Duffield, Christina Lakomski, Daniel Morgensztern, Isabel Oliva, Von Potter, Elin Rowen, Rebecca Sipples, and Danielle Wanik, Yale University School of Medicine and Smilow Cancer Center, Yale-New Haven Hospital; Daniel Cho, Jessica Jordan, Rose Marujo, and James Mier, Beth Israel Deaconess Medical Center; Jennifer L. Winkelmann, Nana S. Akom, and Jedd D. Wolchok, Memorial Sloan-Kettering Cancer Center; Jahleen Byers, Bryan Greene, Jill Hinson, Eric Keller, Lori Lipocky, and Vanica Pharoah, Carolina BioOncology Institute; Serena Rucker, Vanderbilt University Medical Center; Suzanne Burke, Kim Feldhaus, Elaine Granch, Nabeela Iqbal, Prathima Koppolu, and Terri O'Neill, University of Michigan; Ryan Sullivan, Massachusetts General Hospital; Philip Friedlander, Dana-Farber Cancer Institute; John Gerks, Susan Vigen, Ye Zhou, Mark Salvati, and Nils Lonberg, Bristol-Myers Squibb; and Susan Leinbach, medical writer, Clinical Solutions Group (funded by Bristol-Myers Squibb).

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Published OnlineFirst May 28, 2014; DOI: 10.1158/2326-6066.CIR-14-0040

Research Article

Cancer Immunology Research

In Vitro Characterization of the Anti-PD-1 Antibody Nivolumab, BMS-936558, and *In Vivo* Toxicology in Non-Human Primates

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Abstract

The programmed death-1 (PD-1) receptor serves as an immunologic checkpoint, limiting bystander tissue damage and preventing the development of autoimmunity during inflammatory responses. PD-1 is expressed by activated T cells and downmodulates T-cell effector functions upon binding to its ligands, PD-L1 and PD-L2, on antigen-presenting cells. In patients with cancer, the expression of PD-1 on tumor-infiltrating lymphocytes and its interaction with the ligands on tumor and immune cells in the tumor microenvironment undermine antitumor immunity and support its rationale for PD-1 blockade in cancer immunotherapy. This report details the development and characterization of nivolumab, a fully human IgG4 (S228P) anti-PD-1 receptor-blocking monoclonal antibody. Nivolumab binds to PD-1 with high affinity and specificity, and effectively inhibits the interaction between PD-1 and its ligands. *In vitro* assays demonstrated the ability of nivolumab to potently enhance T-cell responses and cytokine production in the mixed lymphocyte reaction and superantigen or cytomegalovirus stimulation assays. No *in vitro* antibody-dependent cell-mediated or complement-dependent cytotoxicity was observed with the use of nivolumab and activated T cells as targets. Nivolumab treatment did not induce adverse immune-related events when given to cynomolgus macaques at high concentrations, independent of circulating anti-nivolumab antibodies where observed. These data provide a comprehensive preclinical characterization of nivolumab, for which antitumor activity and safety have been demonstrated in human clinical trials in various solid tumors. *Cancer Immunol Res; 2(9); 846–56. ©2014 AACR.*

Introduction

Cancer can be considered as an inability of the host to eliminate transformed cells. Although the immune system is the principal mechanism of cancer prevention, transformed cells counteract immunosurveillance. Natural control mechanisms that limit T-cell activation, thereby preventing collateral damage from unrestrained T-cell activity, may be exploited by tumors to evade immune responses (1). Restoring the capacity of immune effector cells—especially T cells—to recognize and eliminate cancer is the goal of immunotherapy. The concept of inhibitory receptor blockade, also known as checkpoint blockade, has been validated in humans with the approval of the anti-CTLA-4 antibody ipilimumab for metastatic melanoma (2, 3).

Programmed death-1 (PD-1) is an additional inhibitory receptor expressed by T cells. Engagement of PD-1 by its ligands, PD-L1 and PD-L2, induces an inhibitory signal resulting in reduced T-cell proliferation, cytokine production, and cytotoxic activity (4, 5). PD-1 deletion in mice can lead to autoimmunity (6, 7), most notably when bred onto backgrounds of autoimmune-susceptible mouse strains (8). Elevated PD-1 expression on T cells, observed during chronic viral infections in humans and mice, is associated with reduced T-cell functionality or "exhaustion." T cells become progressively more nonresponsive as they express additional inhibitory receptors (9). Tumor-infiltrating T cells may also be functionally inert, due in part to the expression of PD-1 along with other inhibitory receptors (10, 11). In multiple syngeneic mouse tumor models, blockade of PD-1 or its ligands promotes antitumor activity (12–14); anti-PD-1 activity *in vivo* can be enhanced by combination with antibodies to other T-cell negative regulators, such as CTLA-4 and LAG-3 (15–17).

PD-L1 is expressed by many human tumors, including melanoma, lung, and kidney (10, 18, 19). PD-L1 engagement of PD-1 may be one mechanism whereby tumors evade immunosurveillance by directly limiting effector T-cell activity. Several studies support the notion that PD-L1 expression and, in some cases, PD-L2 expression is associated with tumor aggressiveness and adverse patient outcome (14, 20–22).

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunres.aacrjournals.org/).

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doi: 10.1158/2326-6066.CIR-14-0040

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Published OnlineFirst May 28, 2014; DOI: 10.1158/2326-6066.CIR-14-0040

Alternatively, PD-L1 expression in metastatic melanoma, upregulated by the expression of IFNγ through locally activated T cells, may indicate preexisting antitumor activity. Accordingly, patients with PD-L1$^+$ tumors had improved survival relative to those with PD-L1$^-$ tumors (23). Although greater responses to PD-1 blockade in humans are associated with PD-L1 expression on 5% or more of melanoma tumor cells, responses have also been seen in PD-L1$^-$ patients (24).

Here, we describe the selection and characterization of the anti-PD-1 antibody nivolumab (BMS-936558, MDX-1106, and ONO-4538). Nivolumab was generated in transgenic mice, which contain a human immunoglobulin minilocus for the heavy chain together with the human immunoglobulin light chain kappa locus along with mutations that prevent the production of murine antibodies. Antibodies arising from immunization of these mice are fully human and have low immunogenicity in human patients. Nivolumab has shown promising early results in patients with advanced malignancies, including melanoma, lung, and renal cancer, with generally manageable side effects (25–27).

Materials and Methods

Antibody generation

Transgenic mice comprising germline configuration human immunoglobulin minilloci in an endogenous IgH and Igκ knockout background (28, 29) were used to generate human anti-PD-1 monoclonal antibodies (mAb). The transgenic mice were immunized with recombinant human PD-1-Fc protein consisting of the extracellular domain of PD-1 (amino acids 1–167) and the Fc portion of human IgG1, and Chinese hamster ovary (CHO) cells expressing human PD-1 (CHO-PD-1 cells). Spleen cells from immunized mice were fused with SP2/0 myeloma cells and screened for hybridomas producing human mAbs reactive to PD-1-Fc by enzyme-linked immunosorbent assay (ELISA). The CHO cell line was provided by Dr. Lawrence Chasin (Columbia University, New York, NY). The SP2/0 and SK-MEL-3 cell lines were purchased from the ATCC. All cell lines were confirmed to be *Mycoplasma*-free by RT-PCR analysis. No other authentication assays were performed.

Nivolumab binding to human and cynomolgus PD-1

CD4$^+$ T cells purified from human peripheral blood mononuclear cells (PBMC) using a CD4$^+$ T-cell positive selection kit (Dynal) were activated with plate-coated anti-CD3 antibody (clone UCHT-1; BD Biosciences) for 4 days and tested for nivolumab binding in a fluorescence-activated cell sorting (FACS)–based assay using a fluorescein isothiocyanate (FITC)–conjugated anti-human kappa antibody (Jackson ImmunoResearch). Binding kinetics of nivolumab to PD-1 were determined using recombinant human PD-1-Fc (R&D Systems) or FLAG-tagged cynomolgus PD-1 protein (containing amino acids 1–169 of the cynomolgus PD-1 extracellular domain) coated on a CM5 (Biacore) sensor chip or captured on a CM5 sensor chip precoated with anti-FLAG mAb M2 (Sigma-Aldrich) with low antigen density, respectively. Nivolumab was flowed over the antigen-coated chip, and avidity was determined using surface plasmon resonance (Biacore). Alternatively, affinity was determined by capture of nivolumab on an anti-CH1 antibody precoated CM5 chip over which human PD-1-Fc protein was applied.

Immunohistochemistry

The nivolumab tissue-binding profile was assessed in a small panel of normal human tissues, including tonsil (hyperplasia, three samples), spleen, cerebellum, heart, kidney, liver, lung, and pituitary (five samples). Snap-frozen, optimal cutting temperature compound–embedded, unfixed tissues were purchased from Analytical Biological Services Inc., Asterand Inc., Cooperative Human Tissue Network, and National Disease Research Interchange (Philadelphia, PA). FITC-conjugated nivolumab (0.2–10 μg/mL) was applied to acetone-fixed sections, followed by anti-FITC as a bridging antibody, and visualized using the EnVision+ System (Dako).

In vitro functional assays

Mixed lymphocyte reaction. Dendritic cells (DC) were generated by culturing monocytes isolated from PBMCs using a monocyte purification kit (Miltenyi Biotec) *in vitro* for 7 days with 500 U/mL interleukin-4 (IL-4) and 250 U/mL GM-CSF (R&D Systems). CD4$^+$ T cells (1×10^5) and allogeneic DCs (1×10^4) were cocultured with or without dose titrations of nivolumab added at the initiation of the assay. After 5 days, IFNγ secretion in culture supernatants was analyzed by ELISA (BD Biosciences), and cells were labeled with ^{3}H-thymidine for an additional 18 hours to measure T-cell proliferation.

Staphylococcal enterotoxin B stimulation of PBMCs. PBMCs from healthy human donors ($N = 18$) were cultured for 3 days with nivolumab or an isotype control antibody (20 μg/mL) at the initiation of the assay together with serial dilutions of staphylococcal enterotoxin B (SEB; Toxin Technology). IL-2 levels in culture supernatants were measured by ELISA analysis (BD Biosciences).

***Antigen-specific recall response* in vitro.** In a cytomegalovirus (CMV)-restimulation assay, 2×10^5 PBMCs from a CMV-positive donor (Astarte) were stimulated using lysate of CMV-infected cells (Astarte), with serial dilutions of nivolumab added at the initiation of the assay. After 4 days, supernatants were assayed for IFNγ.

Suppression assay with regulatory T cells. CD4$^+$CD25$^+$ regulatory T cells (Tregs) and CD4$^+$CD25$^-$ responder T cells were purified from PBMCs (CD4$^+$CD25$^+$ Treg isolation kit; Miltenyi Biotec). In an allogeneic mixed lymphocyte reaction (MLR) assay, Tregs (5×10^4) were cocultured with 1×10^5 responder T cells and 2×10^3 monocyte-derived DCs, with 30 μg/mL nivolumab. After 5 days, IFNγ production was assessed in supernatants, and cells were labeled with ^{3}H-thymidine for an additional 18 hours for proliferation analysis.

Antibody-dependent cell-mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) was assayed using the DELFIA Cell Cytotoxicity Kit (PerkinElmer). PBMCs were incubated overnight with 50 U/mL IL-2 (R&D Systems) and used as effector cells. Activated CD4$^+$ T cells labeled with BATDA reagent were used as target cells at an effector-to-target cell ratio of 50:1. Serial dilutions of nivolumab or positive control [anti–major histocompatibility

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Published OnlineFirst May 28, 2014; DOI: 10.1158/2326-6066.CIR-14-0040

complex (MHC) class I antibody; Bristol-Myers Squibb] were added; the cells were incubated for 3 hours at 37°C. To measure cytotoxicity, supernatant was mixed with Europium solution and read using a RUBYstar Model 460 microplate reader (BMG LABTECH).

Pharmacokinetics, toxicity, and immunogenicity of nivolumab in cynomolgus macaques

In a single-dose pharmacokinetic (PK) study, cynomolgus monkeys (*Macaca fascicularis*) received i.v. nivolumab, 1 mg/kg (3 males and 3 females) or 10 mg/kg (3 males). The optical densities (OD) of a set of nivolumab concentration standards were determined and used to plot an OD versus concentration standard curve that was analyzed by four-parameter curve fit. Nivolumab serum concentrations were determined from the standard curve using SOFTmax Pro version 4.3 software. Anti-nivolumab antibodies were measured using a bridge ELISA and were detected with biotinylated nivolumab. Post-dose samples with mean OD > 1.5 × predose mean OD were reported as positive for anti-nivolumab antibody response. Each positive sample at day 28 was further characterized by dilutional titration and recovery of spiked nivolumab.

In a 3-month toxicity study, cynomolgus monkeys (6 males and 6 females per dose group) were injected i.v. with 0 (vehicle), 10, or 50 mg/kg nivolumab twice weekly for a total of 27 doses. Dosing levels were based on results from a 1-month toxicity study, in which doses up to 50 mg/kg weekly were well tolerated (results not shown). Twenty-four monkeys (4/gender/group) were euthanatized 1 day following the last dose for primary necropsy. The remaining 12 monkeys (2/gender/group) were euthanatized 28 days after the last dose for recovery necropsy. Analyses included body weight, cardiovascular, neurologic, and respiratory assessments; urinalysis; clinical pathology [hematologic assessments and analysis of plasma hormones: triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), growth hormone, ACTH, and α-MSH]; organ weights; and macroscopic and microscopic pathology.

Immunization of SK-MEL-3 melanoma cells and hepatitis B virus surface antigen in cynomolgus macaques

Vaccination studies were undertaken to examine the effects of nivolumab on activation of immune responses. Groups of 6 cynomolgus monkeys were dosed monthly with 10 mg/kg i.v. nivolumab, ipilimumab, or saline control (three doses total). In addition, all groups simultaneously received mitomycin C–inactivated SK-MEL-3 melanoma cells (5×10^6 cells) and hepatitis B virus surface antigen (Engerix-B; GlaxoSmithKline) injected subcutaneously at three independent sites. Peripheral blood samples from all animals were drawn immediately before and 2 weeks following each immunization. Antibodies to HBsAg were quantified using a commercially available kit (DiaSorin).

Antibody responses to the SK-MEL-3 cellular vaccine were measured with SK-MEL-3 cells incubated with monkey plasma samples at 4°C for 30 minutes. After washing, bound antibodies were detected with a phycoerythrin (PE)-conjugated F(ab′)2 goat anti-human IgG, Fcγ-specific antibody (Jackson Immunoresearch), and analyzed by FACS. Anti-human leukocyte antigen (HLA)-A2404 titer was measured in a 96-well plate coated with A2404 monomer (Baylor College of Medicine, Houston, TX) at 2 μg/mL.

Results

Binding analysis of nivolumab and inhibition of ligand binding

One clone (PD1.5) was selected from a panel of human antibodies generated by immunization of human immunoglobulin transgenic mice based on its ability to bind PD-1 with high affinity and specificity, to inhibit PD-1 ligand binding to PD-1, and to promote T-cell function. The variable regions of this antibody were sequenced and grafted onto human kappa and IgG4 constant region sequences containing an S228P mutation, and the resulting antibody (nivolumab) was expressed and purified from a transfected CHO cell line. The complete characterization of nivolumab is described below; preliminary data have been reported previously (25, 30).

Nivolumab bound to CHO cells expressing PD-1 with an EC_{50} of 1.66 nmol/L, but did not bind to the parental CHO cell line (data not shown). To confirm that nivolumab recognized native PD-1, binding of nivolumab to activated human $CD4^+$ T cells was assessed (Supplementary Fig. S1A). Nivolumab bound to PD-1 on activated T cells with an EC_{50} of 0.64 nmol/L. Additional flow cytometric analysis of human T-cell subsets revealed that nivolumab stained memory and effector, but not naïve $CD4^+$ or $CD8^+$ T cells from human peripheral blood (Supplementary Fig. S1B). $CD4^+CD25^{hi}$ Tregs were also bound by nivolumab (Supplementary Fig. S1C). By Scatchard analysis, nivolumab bound to PD-1 on activated human $CD4^+$ T cells, which expressed approximately 10,000 PD-1 receptors per activated T cell, with an affinity of 2.6 nmol/L (data not shown). Nivolumab demonstrated a similar affinity for cynomolgus PD-1 (1.7 nmol/L) by assessing binding to activated T cells by Scatchard analysis (data not shown). Cynomolgus PD-1 has a 96% identity with human PD-1 in the extracellular domain (Genbank NP_001271065.1). Using surface plasmon resonance, the affinity of nivolumab for recombinant human PD-1 protein was 3.06 nmol/L when the chip was coated with low antigen density, and 2.64 nmol/L when antibody was captured on the chip using anti-IgG, in good agreement with the Scatchard analysis. The affinity for cynomolgus PD-1 was 3.92 nmol/L. Using Bio-layer Interferometry (ForteBio), the affinity of nivolumab for PD-1-Fc protein was substantially higher, at 2.7 pmol/L (data not shown). The reason for this difference is unclear.

The molecular epitope of nivolumab on human PD-1 was determined using mass spectrometry. Two peptides from protease-treated human PD-1, ^{29}SFVLNWYR-MSPSNQTDKLAAFPEDR^{53} (putative glycosylation site underlined) and ^{85}SGTYLCGAISLAPKAQIKE^{103}, bound to nivolumab (Supplementary Fig. S2A). Previous studies identified several human PD-1 residues as critical for PD-L1 and PD-L2 binding (31–34), and these residues are contained within the two sequences (Supplementary Fig. S2B). The amino acid sequence of the nivolumab epitope is identical between cynomolgus and human species.

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Published OnlineFirst May 28, 2014; DOI: 10.1158/2326-6066.CIR-14-0040

Nivolumab inhibits the interaction between PD-1 and its ligands, PD-L1 and PD-L2, with IC_{50} values of 2.52 and 2.59 nmol/L, respectively, as shown by surface plasmon resonance (Supplementary Fig. S3). In a previous study using FACS to evaluate ligand binding to PD-1 expressed on CHO cells, the IC_{50} values for nivolumab-mediated inhibition of PD-1 binding to PD-L1 or PD-L2 were similar (1.04 and 0.97 nmol/L, respectively; ref. 35).

Binding specificity and immunohistochemistry of nivolumab in normal human tissues

Nivolumab binds specifically to PD-1 and not to other immunoglobulin superfamily proteins, such as CD28, CTLA-4, ICOS, and BTLA (35). The specificity and tissue-binding properties of nivolumab were assessed by immunohistochemistry using a panel of normal human tissues. In tonsil, there was strong, specific staining by nivolumab in a subset of small- to medium-sized lymphocytes (Fig. 1A and B). These PD-1–positive cells were primarily in the periphery of reactive germinal centers (centrocytes), with a few scattered PD-1–positive cells in the mantle zone and the interfollicular region. When follicle zonation was observed, positive cells were primarily in the light zone. These results are consistent with PD-1$^+$ expression on T_{FH} cells (35, 36).

In four of five pituitary samples, immunoreactivity was detectable in a very small number of scattered endocrine cells (Fig. 1G–I); staining was primarily cytoplasmic and observed at higher antibody concentrations (5 or 10 μg/mL). In two samples, staining was localized mainly in large cytoplasmic spherical organelles, likely enigmatic bodies (large lysosomes), which are characteristic structures of corticotroph cells. Pituitary immunoreactivity required a 20-fold increase in antibody concentration, suggesting that PD-1 expression in the pituitary is very low or that nivolumab binds a cross-reactive pituitary tissue antigen with low affinity. However, PD-1 expression has not been reported in this cell type, and RT-PCR analysis for PD-1 mRNA in pituitary cells was also negative (data not shown). There was no specific staining in the other tissues examined (Fig. 1C–F), including cerebellum, heart, liver, lung, kidney, and spleen.

In vitro activity of nivolumab

The ability of nivolumab to promote T-cell responses was evaluated *in vitro* using human T cells; these assays include an allogeneic MLR, stimulation of human PBMCs by the superantigen SEB, and antigen-specific stimulation of T cells from CMV-responsive donors. In an allogeneic MLR, PD-1 blockade with nivolumab systematically resulted in a titratable

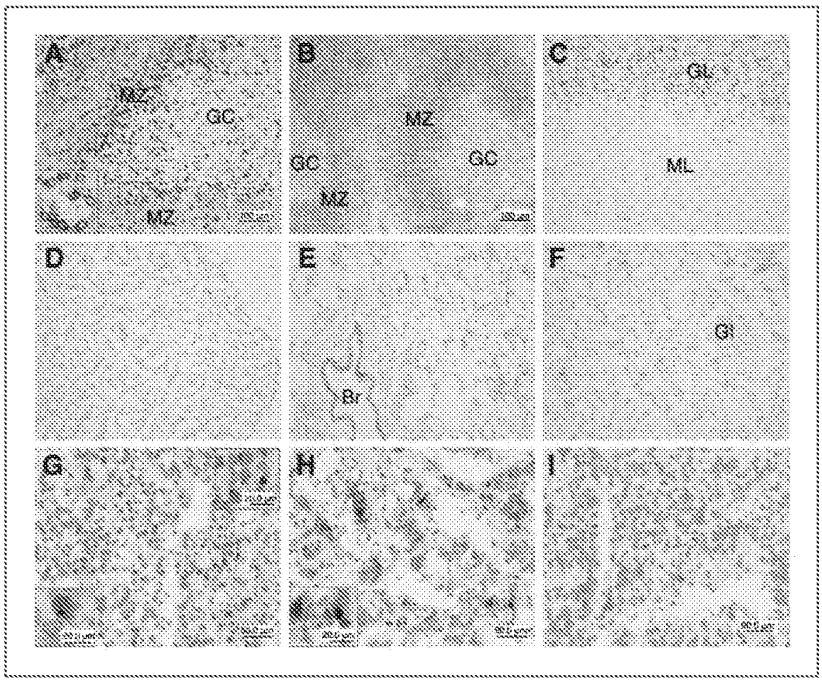


Figure 1. Limited PD-1 expression in normal human tissues. Immunohistochemistry in positive control tissue, hyperplastic tonsil (A), using FITC-conjugated nivolumab. Strong immunoreactivity was distributed in subsets of lymphocytes primarily in germinal center of the tonsil; FITC-conjugated human IgG4 was used as an isotype control in tonsil (B). No specific staining was observed in cerebellum (C), heart (D), lung (E), or kidney (F). Positive staining was revealed in a very small number of scattered endocrine cells (G–I) in four of five pituitary samples. G and H represent two positive samples, and I represents negative pituitary tissue. Insets in G and H are high-power views showing strong immunoreactivity in large cytoplasmic spherical organelles, enigmatic body-like structures, with weak cytoplasmic staining. Br, bronchiole of the lung; GC, germinal center of the tonsil; Gl, glomerulus of the kidney; GL, granular layer of the cerebellum cortex; ML, molecular layer of the cerebellum cortex; MZ, mantle zone of the tonsil.

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