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TITLE OF THE INVENTION (500 characters max)			
COMBINED USE OF CTLA-4 ANTIBODY AND PD-1 ANTIBODY FOR TREATMENT OF HYPERPROLIFERATIVE DISEASE			
Direct all correspondence to: CORRESPONDENCE ADDRESS			
☒ The address corresponding to Customer Number:		07278	
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ENCLOSED APPLICATION PARTS (check all that apply)			
☒ Application Data Sheet. See 37 CFR 1.76		☐ CD(s), Number of CDs _____	
☒ Specification Number of Pages 59		☒ Other (specify) Certificate of Express Mailing (1 page)	
☒ Drawing(s) Number of Sheets 54		Return Receipt Postcard	
Application Size Fee: Filing Fee of \$200 (\$100 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee due, which is \$250 (\$125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
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☐ Applicant claims small entity status. See 37 CFR 1.27.		TOTAL FEE AMOUNT (\$)	
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SIGNATURE



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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

[Number 1 of 1]

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**COMBINED USE OF CTLA-4 ANTIBODY AND PD-1 ANTIBODY
FOR TREATMENT OF HYPERPROLIFERATIVE DISEASE**

TECHNICAL FIELD

5 The present invention relates generally to immunotherapy in the treatment of human disease and reduction of adverse events related thereto. More specifically, the present invention relates to the use of a combination immunotherapy, including the combination of anti-CTLA-4 and anti-PD-1 antibodies, to treat cancer and/or to decrease the incidence or magnitude of
10 adverse events related to treatment with such antibodies individually.

BACKGROUND

15 The protein Programmed Death 1 (PD-1) is an inhibitory member of the CD28 family of receptors, which also includes CD28, CTLA-4, ICOS and BTLA. PD-1 is expressed on activated B cells, T cells, and myeloid cells (Agata *et al.*, *supra*; Okazaki *et al.* (2002) *Curr. Opin. Immunol.* 14: 391779-82; Bennett *et al.* (2003) *J Immunol* 170:711-8). One ligand for PD-1, PD-L1 is abundant in a variety of human cancers (Dong *et al.* (2002) *Nat. Med.* 8:787-9). The interaction between PD-1 and PD-L1 results in a decrease in tumor infiltrating lymphocytes,
20 a decrease in T-cell receptor mediated proliferation, and immune evasion by the cancerous cells (Dong *et al.* (2003) *J. Mol. Med.* 81:281-7; Blank *et al.* (2005) *Cancer Immunol. Immunother.* 54:307-314; Konishi *et al.* (2004) *Clin. Cancer Res.* 10:5094-100). Immune suppression can be reversed by inhibiting the local interaction of PD-1 with PD-L1, and the effect is additive when the interaction of PD-1 with PD-L2 is blocked as well (Iwai *et al.* (2002) *Proc. Nat'l. Acad. Sci.*
25 *USA* 99:12293-7; Brown *et al.* (2003) *J. Immunol.* 170:1257-66). Treatment with an anti-PD-1 antibody was shown to have a direct *in vivo* inhibitory effect on tumor growth, presumably PD-1 blockade raises the threshold of T cell activation and activates tumor responses in the host (*see*, *e.g.*, U.S. provisional application No. 60/679,466, filed May 9, 2005, the contents of which are hereby incorporated in their entirety).

30 Anti-CTLA-4 antibodies are known immunostimulatory agents (*see, e.g.*, PCT Application Publication Nos. WO 01/14424 and WO 00/37504, which describe human sequence anti-human CTLA-4 antibodies). Non-human CTLA-4 antibodies have been used in the various

studies. U.S. Patent No. 5,855,887 discloses a method of increasing the response of a mammalian T cell to antigenic stimulation by combining a T cell with a CTLA-4 blocking agent. U.S. Patent No. 5,811,097 discloses a method of decreasing the growth of non-T cell tumors by administering a CTLA-4 blocking agent. U.S. Patent Application Nos. 09/644,668 and 5 09/948,939 disclose human CTLA-4 antibodies. Each of these patents and applications is hereby incorporated by reference.

Therapy with an antibody can be associated with certain adverse events (*see, e.g.*, PCT Publication No. WO 01/1424). These adverse events, which can be severe and even life-threatening, include autoimmune responses, such as diarrhea, enterocolitis, dermatitis, 10 hypophysitis, panhypopituitarism, rash, pruritis, and vitiligo (*see, e.g.*, U.S. Patent Publication No. 2004/0241169 A1). For example, adverse events associated with anti-CTLA-4 antibody may be due to a loss of tolerance to some self-antigens or an exaggerated reaction to foreign antigens (*e.g.*, gut bacteria). In terms of organ system involvement, these anti-CTLA-4 associated adverse events have primarily involved the GI tract (diarrhea and colitis) or the skin 15 (rash and pruritis). Upper GI tract involvement including ileitis, duodenitis, and esophagitis has also been observed.

To date, immunostimulatory therapeutics for treating or preventing hyperproliferative disease has associated undesirable side-effect profiles. Therefore, there remains a need for improved methods of treating hyperproliferative disease. The instant invention fills such a need 20 and provides other related advantages.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A to 1D show the tumor volume over time in individual mice that were implanted with MC38 colon tumor cells (PD-L1⁺) and on the same day treated with one of the 25 following therapies: (A) mouse IgG (control), (B) anti-CTLA-4 antibody, (C) anti-PD-1 antibody, and (D) anti-CTLA-4 antibody and anti-PD-1 antibody. The mice received subsequent antibody treatments on days 3, 6 and 10 as described in Example 2 and tumor volume was monitored over 60 days.

Figure 2 shows the mean tumor volume of the mice shown in Figure 1.

30 Figure 3 shows the median tumor volume of the mice shown in Figure 1.

Figures 4A to 4D show the tumor volume over time in individual mice that were implanted with MC38 colon tumor cells (PD-L1⁺) and one week later treated with one of the following therapies: (A) mouse IgG (control), (B) anti-CTLA-4 antibody, (C) anti-PD-1 antibody, and (D) anti-CTLA-4 antibody and anti-PD-1 antibody. The tumor volume on the first day of treatment was about 315 mm³. The mice received subsequent antibody treatments on days 3, 6 and 10 as described in Example 3.

Figure 5 shows the mean tumor volume of the mice shown in Figure 4.

Figure 6 shows the median tumor volume of the mice shown in Figure 4.

Figure 7 shows the mean tumor volume over time in individual mice that were implanted with MC38 colon tumor cells (PD-L1⁺) (day -7) and then treated on days 0, 3, 6 and 10 post-implantation (as described in Example 4) with one of the following therapies: (A) mouse IgG as a control (20 mg/kg, X₂₀) (B) anti-PD-1 antibody (10 mg/kg) and mouse IgG (10 mg/kg) (P₁₀X₁₀), (C) anti-CTLA-4 antibody (10 mg/kg) and mouse IgG (10 mg/kg) (C₁₀X₁₀), (D) anti-CTLA-4 antibody and anti-PD-1 antibody (10 mg/kg each) (C₁₀P₁₀), (E) anti-CTLA-4 antibody and anti-PD-1 antibody (3 mg/kg each) (C₃P₃), and (F) anti-CTLA-4 antibody and anti-PD-1 antibody (1 mg/kg each) (C₁P₁). Two groups of mice were treated with each antibody sequentially as follows: (G) anti-CTLA-4 antibody (10 mg/kg, day 0), anti-CTLA-4 antibody (10 mg/kg, day 3), anti-PD-1 antibody (10 mg/kg, day 6), and anti-PD-1 antibody (10 mg/kg, day 10) (C₁₀C₁₀P₁₀P₁₀); and (H) anti-PD-1 antibody (10 mg/kg, day 0), anti-PD-1 antibody (10 mg/kg, day 3), anti-CTLA-4 antibody (10 mg/kg, day 6), and anti-CTLA-4 antibody (10 mg/kg, day 10) (P₁₀P₁₀C₁₀C₁₀).

Figure 8 shows the mean tumor volume of the mice shown in Figure 7.

Figure 9 shows the median tumor volume of the mice shown in Figure 7.

Figures 10A to 10F show the tumor volume over time in individual mice that were implanted with SA1/N fibrosarcoma cells (PD-L1⁺) and one day later treated with one of the following therapies: (A) PBS (vehicle control), (B) mouse IgG (antibody control, 10 mg/kg), (C) anti-PD-1 antibody (10 mg/kg), (D) anti-CTLA-4 antibody (10 mg/kg), (E) anti-CTLA-4 antibody (0.2 mg/kg), and (F) anti-PD-1 antibody (10 mg/kg) and anti-CTLA-4 antibody (0.2 mg/kg). The mice received subsequent antibody treatments on days 4, 7 and 11 as described in Example 5 and tumor volume was monitored over 41 days.

Figure 11 shows the mean tumor volume of the mice shown in Figure 9.

Figure 12 shows the median tumor volume of the mice shown in Figure 9.

Figures 13A to 13J show the tumor volume over time in individual mice that were implanted with SA1/N fibrosarcoma cells (PD-L1⁻) and then treated on days 7, 10, 13 and 17 post-implantation (as described in Example 6) with one of the following therapies: (A) PBS (vehicle control), (B) mouse IgG (antibody control, 10 mg/kg), (C) anti-CTLA-4 antibody (0.25 mg/kg), (D) anti-CTLA-4 antibody (0.5 mg/kg), (E) anti-CTLA-4 antibody (5 mg/kg), (F) anti-PD-1 antibody (3 mg/kg), (G) anti-PD-1 antibody (10 mg/kg), (H) anti-PD-1 antibody (10 mg/kg) and anti-CTLA-4 antibody (0.25 mg/kg), (I) anti-PD-1 antibody (10 mg/kg) and anti-CTLA-4 antibody (0.5 mg/kg), and (J) anti-PD-1 antibody (3 mg/kg) and anti-CTLA-4 antibody (0.5 mg/kg). The tumor volume on the first day of treatment was about 110 mm³.

Figure 14 shows the mean tumor volume of the mice shown in Figure 13.

Figure 15 shows the median tumor volume of the mice shown in Figure 13.

Figures 16A and 16B show the tumor volume over time in individual mice that were implanted with SA1/N fibrosarcoma cells (PD-L1⁻) and then treated on days 10, 13, 16 and 19 post-implantation (as described in Example 6) with one of the following therapies: (A) mouse IgG (antibody control, 10 mg/kg) or (B) anti-PD-1 antibody (10 mg/kg) and anti-CTLA-4 antibody (1 mg/kg). The tumor volume on the first day of treatment was about 250 mm³.

Figure 17 shows the mean tumor volume of the mice shown in Figure 16.

Figure 18 shows the median tumor volume of the mice shown in Figure 16.

Figure 19 shows the mean and median percent tumor inhibition calculated from the tumor volumes shown in Figures 13 and 16.

Figures 20A to 20D show the tumor volume in BALB/c mice that were implanted subcutaneously with RENCA renal adenocarcinoma cells (PD-L1⁺) (Murphy and Hrushesky (1973) *J. Nat'l. Cancer Res.* 50:1013-1025) (day -12) and then treated intraperitoneally on days 0, 3, 6 and 9 post-implantation with one of the following therapies: (A) mouse IgG (antibody control, 20 mg/kg), (B) anti-PD-1 antibody (10 mg/kg), (C) anti-CTLA-4 antibody (10 mg/kg), and (D) anti-PD-1 antibody (10 mg/kg) in combination with anti-CTLA-4 antibody (10 mg/kg). The tumor volume on the first day of treatment was about 115 mm³.

Figure 21 shows binding of mouse PD-L2-Fc fusion protein to mouse PD-1 (mPD-1) is blocked by anti-mPD-1 antibody 4H2 in a dose dependent manner. The binding is detected by

measuring fluorescence of FITC-labeled donkey-anti-rat IgG by ELISA. The greater the MFI (mean fluorescence intensity) the greater the binding.

Figure 22 shows binding curves of anti-mPD-1 antibodies to immobilized mPD-1-Fc fusion protein by ELISA.

5 Figure 23 shows the binding curve of rat anti-mPD-1 antibody 4H2.B3 to mPD-1-expressing CHO cells. Binding was detected with donkey-anti-rat IgG, FITC conjugated and measured by FACS (MFI).

Figure 24 shows the binding curve of mPD-L1-hFc fusion protein to mPD-1-expressing CHO cells in the presence of increasing concentrations of anti-mPD-1 antibody 4H2.B3.

10 Binding was detected with goat-anti-human IgG, FITC conjugated and measured by FACS (MFI).

Figure 25 shows the binding curves of rat anti-mPD-1 antibody 4H2.B3 to mPD-1-expressing CHO cells as compared to chimeric rat:mouse anti-mPD-1 antibody 4H2.

15 Figure 26 shows the binding curves of mPD-L1-hFc fusion protein to mPD-1-expressing CHO cells in the presence of increasing concentrations of either rat anti-mPD-1 antibody 4H2.B3 or chimeric rat:mouse anti-mPD-1 antibody 4H2.

Figure 27 shows the mean tumor volume of tumor-free mice previously treated with anti-PD1 antibody and re-challenged with SA1/N fibrosarcoma cells (PD-L1⁺). Also shown is the mean tumor volume of naïve mice (control, not previously challenged or treated) implanted
20 with SA1/N fibrosarcoma cells.

Figure 28 shows the tumor volume over time in individual mice, which survived tumor-free following implantation of MC38 colon tumor cells (PD-L1⁺) and treatment with anti-PD1 antibody or a combination of anti-PD1 antibody with anti-CTLA-4 antibody), re-challenged with 10x more MC38 colon tumor cells than the initial treatment. Also shown is
25 the mean tumor volume of naïve mice (control, not previously challenged or treated) implanted with MC38 colon tumor cells.

Figure 29 shows the mean tumor volume of the mice shown in Figure 28.

Figure 30A shows the nucleotide sequence (SEQ ID NO: 41) and amino acid sequence (SEQ ID NO: 1) of the heavy chain variable region of the 17D8 human monoclonal antibody.
30 The CDR1 (SEQ ID NO: 11), CDR2 (SEQ ID NO: 16) and CDR3 (SEQ ID NO: 21) regions are delineated and the V, D and J germline derivations are indicated.

Figure 30B shows the nucleotide sequence (SEQ ID NO: 46) and amino acid sequence (SEQ ID NO: 6) of the light chain variable region of the 17D8 human monoclonal antibody. The CDR1 (SEQ ID NO: 26), CDR2 (SEQ ID NO: 31) and CDR3 (SEQ ID NO: 36) regions are delineated and the V and J germline derivations are indicated.

5 Figure 31A shows the nucleotide sequence (SEQ ID NO: 42) and amino acid sequence (SEQ ID NO: 2) of the heavy chain variable region of the 2D3 human monoclonal antibody. The CDR1 (SEQ ID NO: 12), CDR2 (SEQ ID NO: 17) and CDR3 (SEQ ID NO: 22) regions are delineated and the V and J germline derivations are indicated.

10 Figure 31B shows the nucleotide sequence (SEQ ID NO: 47) and amino acid sequence (SEQ ID NO: 7) of the light chain variable region of the 2D3 human monoclonal antibody. The CDR1 (SEQ ID NO: 27), CDR2 (SEQ ID NO: 32) and CDR3 (SEQ ID NO: 37) regions are delineated and the V and J germline derivations are indicated.

15 Figure 32A shows the nucleotide sequence (SEQ ID NO: 43) and amino acid sequence (SEQ ID NO: 3) of the heavy chain variable region of the 4H1 human monoclonal antibody. The CDR1 (SEQ ID NO: 13), CDR2 (SEQ ID NO: 18) and CDR3 (SEQ ID NO: 23) regions are delineated and the V and J germline derivations are indicated.

20 Figure 32B shows the nucleotide sequence (SEQ ID NO: 48) and amino acid sequence (SEQ ID NO: 8) of the light chain variable region of the 4H1 human monoclonal antibody. The CDR1 (SEQ ID NO: 28), CDR2 (SEQ ID NO: 33) and CDR3 (SEQ ID NO: 38) regions are delineated and the V and J germline derivations are indicated.

Figure 33A shows the nucleotide sequence (SEQ ID NO: 44) and amino acid sequence (SEQ ID NO: 4) of the heavy chain variable region of the 5C4 human monoclonal antibody. The CDR1 (SEQ ID NO: 14), CDR2 (SEQ ID NO: 19) and CDR3 (SEQ ID NO: 24) regions are delineated and the V and J germline derivations are indicated.

25 Figure 33B shows the nucleotide sequence (SEQ ID NO: 49) and amino acid sequence (SEQ ID NO: 9) of the light chain variable region of the 5C4 human monoclonal antibody. The CDR1 (SEQ ID NO: 29), CDR2 (SEQ ID NO: 34) and CDR3 (SEQ ID NO: 39) regions are delineated and the V and J germline derivations are indicated.

30 Figure 34A shows the nucleotide sequence (SEQ ID NO: 45) and amino acid sequence (SEQ ID NO: 5) of the heavy chain variable region of the 4A11 human monoclonal antibody.

The CDR1 (SEQ ID NO: 15), CDR2 (SEQ ID NO: 20) and CDR3 (SEQ ID NO: 25) regions are delineated and the V and J germline derivations are indicated.

Figure 34B shows the nucleotide sequence (SEQ ID NO: 50) and amino acid sequence (SEQ ID NO: 10) of the light chain variable region of the 4A11 human monoclonal antibody.

5 The CDR1 (SEQ ID NO: 30), CDR2 (SEQ ID NO: 35) and CDR3 (SEQ ID NO: 40) regions are delineated and the V and J germline derivations are indicated.

Figure 35 shows the alignment of the amino acid sequence of the heavy chain variable region of 17D8, 2D3, 4H1, and 5C4 with the human germline V_H 3-33 amino acid sequence (SEQ ID NO: 51).

10 Figure 36 shows the alignment of the amino acid sequence of the light chain variable region of 17D8 with the human germline V_k L6 amino acid sequence (SEQ ID NO:52).

Figure 37 shows the alignment of the amino acid sequence of the light chain variable region of 2D3 with the human germline V_k L6 amino acid sequence (SEQ ID NO:52).

15 Figure 38 shows the alignment of the amino acid sequence of the light chain variable region of 4H1 with the human germline V_k L6 amino acid sequence (SEQ ID NO:52).

Figure 39 shows the alignment of the amino acid sequence of the light chain variable region of 5C4 with the human germline V_k L6 amino acid sequence (SEQ ID NO:52).

Figure 40 shows the alignment of the amino acid sequence of the heavy chain variable region of 4A11 with the human germline V_H 4-39 amino acid sequence (SEQ ID NO: 53).

20 Figure 41 shows the alignment of the amino acid sequence of the light chain variable region of 4A11 with the human germline V_k L15 amino acid sequence (SEQ ID NO:54).

Figures 42A and 42B show the results of flow cytometry experiments demonstrating that the human monoclonal antibodies 5C4 and 4H1, directed against human PD-1, binds the cell surface of CHO cells transfected with full-length human PD-1. Figure 42A shows to the flow
25 cytometry plot for 5C4. Figure 42B shows the flow cytometry plot for 4H1.

DETAILED DESCRIPTION

The present disclosure relates generally to the combination of two or more immunotherapeutic agents (such as anti-CTLA-4 and anti-PD-1 antibodies) against hyperproliferative disease (such as cancer). The instant disclosure also relates to methods for
30 reducing the incidence and/or severity of side effects of monotherapy by administering a

combination therapy against hyperproliferative disease. A representative immunotherapeutic combination to treat, for example, cancer is the combination of anti-CTLA-4 antibody with anti-PD-1 antibody. This antibody combination surprisingly showed an *in vivo* synergistic effect in a treatment against a cancerous tumor. The synergistic activity of the combination of an
5 anti-CTLA-4 antibody and an anti-PD-1 antibody allows for the use of a subtherapeutic dose of each or both antibodies in the combination therapy against hyperproliferative disease, and the subtherapeutic dose treatment results in reduced toxicity (*i.e.*, reduced adverse effects associated with a higher dose of antibody). In certain embodiments, the present invention provides a method for treating hyperproliferative disease in a subject, which method comprises
10 administering a PD-1 antibody and a CTLA-4 antibody to a subject in need thereof. In other embodiments, the present invention provides a method for altering adverse events associated with treatment with immunostimulatory agents, which method comprises administering a PD-1 antibody and a CTLA-4 antibody to a subject in need thereof.

Prior to setting forth the invention, it may be helpful to an understanding thereof to set
15 forth definitions of certain terms that are used herein. Additional definitions are set forth throughout the detailed description.

The term “treatment” or “therapy” refers to administering an active agent with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect a condition (*e.g.*, a disease), the symptoms of the condition, or to prevent or delay the onset of the symptoms,
20 complications, biochemical indicia of a disease, or otherwise arrest or inhibit further development of the disease, condition, or disorder in a statistically significant manner.

An “adverse event” (AE) as used herein is any unfavorable and generally unintended, even 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
25 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.

30 As used herein, “hyperproliferative disease” refers to conditions wherein cell growth is increased over normal levels. For example, hyperproliferative diseases or disorders include

malignant diseases (*e.g.*, esophageal cancer, colon cancer, biliary cancer) and non-malignant diseases (*e.g.*, atherosclerosis, benign hyperplasia, benign prostatic hypertrophy).

As used herein, "subtherapeutic dose" means a dose of a therapeutic compound (*e.g.*, an antibody) that is lower than the usual or typical dose of the therapeutic compound when administered alone for the treatment of a hyperproliferative disease (*e.g.*, cancer). For example, a subtherapeutic dose of CTLA-4 antibody is a single dose of the antibody at less than about 3 mg/kg, *i.e.*, the known dose of anti-CTLA-4 antibody.

The terms "cytotoxic T lymphocyte-associated antigen-4," "CTLA-4," "CTLA4," "CTLA-4 antigen" and "CD152" (*see, e.g.*, Murata, *Am. J. Pathol.* (1999) 155:453-460) are used interchangeably, and include variants, isoforms, species homologs of human CTLA-4, and analogs having at least one common epitope with CTLA-4 (*see, e.g.*, Balzano (1992) *Int. J. Cancer Suppl.* 7:28-32). The complete CTLA-4 nucleic acid sequence can be found under GenBank Accession No. L15006.

The terms "Programmed Death 1," "Programmed Cell Death 1," "Protein PD-1," "PD-1," "PD1," "PDCD1," "hPD-1" and "hPD-I" are used interchangeably, and include variants, isoforms, species homologs of human PD-1, and analogs having at least one common epitope with PD-1. The complete PD-1 sequence can be found under GenBank Accession No. U64863.

The term "immune response" refers to the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

Except when noted, the terms "patient" or "subject" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, and other animals. Animals include all vertebrates, *e.g.*, mammals and non-mammals, such as sheep, dogs, cows, chickens, amphibians, and reptiles.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is

comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1}, C_{H2} and C_{H3}. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of 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 antibodies 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 (C1q) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., PD-1). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody 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 V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

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

The term "human monoclonal antibody" refers to antibodies displaying a single binding
15 specificity which have variable regions in which both the framework and CDR regions are
derived from human germline immunoglobulin sequences. In one embodiment, the human
monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a
transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human
heavy chain transgene and a light chain transgene fused to an immortalized cell.

20 As used herein, an antibody that "specifically binds to" a particular antigen is intended to
refer to an antibody that binds to the antigen with a K_D of 1×10^{-7} M or less, more preferably
 5×10^{-8} M or less, more preferably 1×10^{-8} M or less, and more preferably 5×10^{-9} M or less.

The term " K_{assoc} " or " K_a ", as used herein, is intended to refer to the association rate of a
particular antibody-antigen interaction, whereas the term " K_{dis} " or " K_d ," as used herein, is
25 intended to refer to the dissociation rate of a particular antibody-antigen interaction. The term
" K_D ", as used herein, is intended to refer to the dissociation constant, which is obtained from the
ratio of K_d to K_a (*i.e.*, K_d/K_a) and is expressed as a molar concentration (M). K_D values for
antibodies can be determined using methods well established in the art. A preferred method for
determining the K_D of an antibody is use of surface plasmon resonance, preferably using a
30 biosensor system such as a Biacore® system.

As used herein, the term “high affinity” for an IgG antibody refers to an antibody having a K_D of 10^{-8} M or less, more preferably 10^{-9} M or less and even more preferably 10^{-10} M or less for a target antigen. However, “high affinity” binding can vary for other antibody isotypes. For example, “high affinity” binding for an IgM isotype refers to an antibody having a K_D of 10^{-7} M or less, more preferably 10^{-8} M or less, even more preferably 10^{-9} M or less.

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.

As used herein, “about” or “comprising essentially of” mean within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *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 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.

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.

IMMUNOSTIMULATORY THERAPEUTIC ANTIBODIES

The present invention is based, in part, on the following experimental data. Mouse tumor models (MC38 colon cancer and SA1/N fibrosarcoma) were used to examine the *in vivo* effect of treating a tumor by combining immunostimulatory therapeutic antibodies – anti-CTLA-4 and anti-PD-1. The immunotherapeutic combination was provided either simultaneous with the implant of tumor cells (Examples 2 and 5) or after the tumor cells were implanted for a time sufficient to become an established tumor (Examples 3, 4 and 6). Regardless of the timing of antibody treatment, it was found that anti-CTLA-4 antibody treatment alone and anti-PD-1

antibody (chimeric antibody in which a rat anti-mouse PD-1 was modified with a mouse immunoglobulin Fc region, *see* Example 1) treatment alone had a modest effect on reducing tumor growth in the MC38 tumor model (*see, e.g.,* Figures 1, 4 and 7). The anti-CTLA-4 antibody alone was quite effective in the SA1/N tumor model (*see* Figure 10D), which required a lower anti-CTLA-4 antibody dose for the combination studies in this model. Nonetheless, the combination treatment of anti-CTLA-4 antibody and anti-PD-1 antibody showed an unexpected, significantly greater effect on reducing tumor growth as compared to treatment with either antibody alone (*see, e.g.,* Figures 1D, 4D, 10F and 13H-J). In addition, the results of Examples 2, 4 and 6 show that the combination treatment of anti-CTLA-4 antibody and anti-PD-1 antibody had a significant (synergistic) effect on tumor growth even at sub-optimal therapeutic doses as compared to treatment with either antibody alone (*i.e.,* the combination therapy was surprisingly more effective at subtherapeutic doses than either monotherapy). Without wishing to be bound by theory, it is possible that by raising the threshold of T cell activation by PD-1 and CTLA-4 blockade, anti-tumor responses may be activated in a host.

In one embodiment, the present invention provides a method for treating a hyperproliferative disease, comprising administering a PD-1 antibody and a CTLA-4 antibody to a subject. In further embodiments, the anti-PD-1 antibody is administered at a subtherapeutic dose, the anti-CTLA-4 antibody is administered at a subtherapeutic dose, or both are administered at a subtherapeutic dose. In another embodiment, the present invention provides a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering an anti-PD-1 antibody and a subtherapeutic dose of anti-CTLA-4 antibody to a subject. In certain embodiments, the subject is human. In certain embodiments, the anti-CTLA-4 antibody is human sequence monoclonal antibody 10D1 and the anti-PD-1 antibody is human sequence monoclonal antibody, such as 17D8, 2D3, 4H1, 5C4 and 4A11. Human sequence monoclonal antibodies 17D8, 2D3, 4H1, 5C4 and 4A11 have been isolated and structurally characterized, as described in U.S. Provisional Patent No. 60/679,466.

The anti-CTLA-4 antibody and anti-PD-1 monoclonal antibodies (mAbs) and the human sequence antibodies of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology, *e.g.,* the standard somatic cell hybridization technique of Kohler and Milstein (1975) *Nature* 256:495. Any technique for producing

monoclonal antibody can be employed, *e.g.*, viral or oncogenic transformation of B lymphocytes. One animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known (*see, e.g.*, Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York).

For example, human monoclonal antibodies and human sequence antibodies directed against human CTLA-4 or PD-1 can be generated using transgenic mice carrying a human immune system rather than the mouse system. These transgenic mice, also referred to herein as "HuMAb-Mouse™", contain a human immunoglobulin gene miniloci that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg *et al.* (1994) *Nature* 368(6474): 856-859 and U.S. Patent No. 5,770,429). Accordingly, the mice exhibit reduced expression of mouse IgM or κ , and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal (Lonberg *et al.* (1994), *supra*; reviewed in Lonberg (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg and Huszar (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93, and Harding and Lonberg (1995) *Ann. N.Y. Acad. Sci.* 764:536-546). The preparation of transgenic mice is described in Taylor *et al.* (1992) *Nucleic Acids Research* 20:6287-6295; Chen *et al.* (1993) *International Immunology* 5: 647-656; Tuaillon *et al.* (1993) *Proc. Natl. Acad. Sci USA* 90:3720-3724; Choi *et al.* (1993) *Nature Genetics* 4:117-123; Chen, J. *et al.* (1993) *EMBO J.* 12:821-830; Tuaillon *et al.* (1994) *J. Immunol.* 152:2912-2920; Lonberg *et al.*, (1994) *Nature* 368(6474): 856-859; Lonberg (1994) *Handbook of Experimental Pharmacology* 113:49-101; Taylo *et al.* (1994) *International Immunology* 6: 579-591; Lonberg and Huszar (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93; Harding and Lonberg (1995) *Ann. N.Y. Acad. Sci.* 764:536-546; Fishwild *et al.* (1996) *Nature Biotechnology* 14: 845-851. *See further*, U.S. Patent Nos. 5,625,126 and 5,770,429, both to Lonberg and Kay, and GenPharm International; U.S. Patent No. 5,545,807 to Surani *et al.*; International Publication Nos. WO 98/24884, published on June 11, 1998; WO 94/25585, published November 10, 1994; WO 93/1227, published June 24, 1993; WO 92/22645, published December 23, 1992; WO 92/03918,

published March 19, 1992. For example, human anti-CTLA-4 antibodies can be generated as described in U.S. Patent Publication No. 2005/0201994, and human anti-PD-1 antibodies can be generated as described in U.S. Provisional Patent No. 60/679,466 (and Examples 8 and 9).

In another embodiment, human antibodies of the instant disclosure can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchromosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. Such mice, referred to herein as "KM miceTM", are described in detail in PCT Publication WO 02/43478 to Ishida *et al.*

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CTLA-4 or anti-PD-1 antibodies of the instant disclosure. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CTLA-4 or anti-PD-1 antibodies of the instant disclosure. For example, mice carrying both a human heavy chain transchromosome and a human light chain transchromosome, referred to as "TC mice" can be used; such mice are described in Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art (Kuroiwa *et al.* (2002) *Nature Biotechnology* 20:889-894) and can be used to raise anti-CTLA-4 or anti-PD-1 antibodies of the instant disclosure.

Human monoclonal antibodies of the instant disclosure can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage display methods for isolating human antibodies are established in the art. See, for example, U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*

Human monoclonal antibodies of the invention can also be prepared using SCID mice into which human immune cells have been reconstituted such that a human antibody response

can be generated upon immunization. Such mice are described in, for example, U.S. Patent Nos. 5,476,996 and 5,698,767 to Wilson *et al.*

Anti-CTLA-4 antibodies of the instant invention can bind to an epitope on human CTLA-4 so as to inhibit CTLA-4 from interacting with a human B7 counterreceptor. Because interaction of human CTLA-4 with human B7 transduces a signal leading to inactivation of T-cells bearing the human CTLA-4 receptor, antagonism of the interaction effectively induces, augments or prolongs the activation of T cells bearing the human CTLA-4 receptor, thereby prolonging or augmenting an immune response. Anti-CTLA-4 antibodies are described in U.S. Patent Nos. 5,811,097; 5,855,887; 6,051,227; in PCT Application Publication Nos.

WO 01/14424 and WO 00/37504; and in U.S. Patent Publication No. 2002/0039581. Each of these references is specifically incorporated herein by reference for purposes of description of anti-CTLA-4 antibodies. An exemplary clinical anti-CTLA-4 antibody is human monoclonal antibody 10D1 as disclosed in WO 01/14424 and U.S. Patent Application No. 09/644,668. Antibody 10D1 has been administered in single and multiple doses, alone or in combination with a vaccine, chemotherapy, or interleukin-2 to more than 500 patients diagnosed with metastatic melanoma, prostate cancer, lymphoma, renal cell cancer, breast cancer, ovarian cancer, and HIV. Other anti-CTLA-4 antibodies encompassed by the methods of the present invention include, for example, those disclosed in: WO 98/42752; WO 00/37504; U.S. Patent No. 6,207,156; Hurwitz *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95(17):10067-10071; Camacho *et al.* (2004) *J. Clin. Oncology* 22(145): Abstract No. 2505 (antibody CP-675206); and Mokyr *et al.* (1998) *Cancer Res.* 58:5301-5304. In certain embodiments, the methods of the instant invention comprise use of an anti-CTLA-4 antibody that is a human sequence antibody, preferably a monoclonal antibody and in another embodiment is monoclonal antibody 10D1.

In certain embodiments, the anti-CTLA-4 antibody binds to human CTLA-4 with a K_D of 5×10^{-8} M or less, binds to human CTLA-4 with a K_D of 1×10^{-8} M or less, binds to human CTLA-4 with a K_D of 5×10^{-9} M or less, or binds to human CTLA-4 with a K_D of between 1×10^{-8} M and 1×10^{-10} M or less.

Anti-PD-1 antibodies useful in the instant invention may be characterized by particular functional features or properties. For example, the antibodies may bind specifically to PD-1 (*e.g.*, bind to human PD-1 or may cross-react with PD-1 from other species). In certain embodiments, an antibody that binds to PD-1 may be by high affinity, for example, with a K_D of

1 x 10⁻⁷ M or less. In certain embodiments, the anti-PD-1 antibodies of the invention exhibit one or more of the following characteristics: (a) binds to human PD-1 with a K_D of 1 x 10⁻⁷ M or less; (b) does not substantially bind to human CD28, CTLA-4 or ICOS; (c) increases T-cell proliferation in an Mixed Lymphocyte Reaction (MLR) assay; (d) increases interferon-gamma 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-L1 and/or PD-L2 to PD-1; (h) stimulates antigen-specific memory responses; (i) stimulates antibody responses; or (j) inhibits tumor cell growth *in vivo*. In certain embodiments, anti-PD-1 antibodies for use in the methods of the instant invention are human sequence antibodies, preferably human sequence monoclonal antibodies, such as 17D8, 2D3, 4H1, 5C4 and 4A11.

In certain embodiments; the PD-1 antibody binds to human PD-1 with a K_D of 5 x 10⁻⁸ M or less, binds to human PD-1 with a K_D of 1 x 10⁻⁸ M or less, binds to human PD-1 with a K_D of 5 x 10⁻⁹ M or less, or binds to human PD-1 with a K_D of between 1 x 10⁻⁸ M and 1 x 10⁻¹⁰ M or less.

In another aspect, the present invention features an anti-PD-1 antibody or anti-CTLA-4 antibody, or antigen binding fragment thereof, conjugated to a therapeutic moiety, such as a cytotoxin, a drug (*e.g.*, an immunosuppressant) or a radiotoxin. Such conjugates are referred to herein as “immunoconjugates”. Immunoconjugates that include one or more cytotoxins are referred to as “immunotoxins.” A cytotoxin or cytotoxic agent includes any agent that is detrimental to (*e.g.*, kills or injures) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents also include, for example, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

Other preferred examples of therapeutic cytotoxins that can be conjugated to an antibody of the invention include duocarmycins, calicheamicins, maytansines and auristatins, and derivatives thereof. An example of a calicheamicin antibody conjugate is commercially available (Mylotarg™; Wyeth-Ayerst).

5 Cytotoxins can be conjugated to antibodies of the invention using linker technology available in the art. Examples of linker types that have been used to conjugate a cytotoxin to an antibody include, but are not limited to, hydrazones, thioethers, esters, disulfides and peptide-containing linkers. A linker can be chosen that is, for example, susceptible to cleavage by low pH within the lysosomal compartment or susceptible to cleavage by proteases, including
10 proteases preferentially expressed in tumor tissue, such as cathepsins (*e.g.*, cathepsins B, C, D) or susceptible to cleavage in a reducing tumor microenvironment.

For further discussion of types of cytotoxins, linkers and methods for conjugating therapeutic agents to antibodies, see also Saito *et al.* (2003) *Adv. Drug Deliv. Rev.* 55:199-215; Trail *et al.* (2003) *Cancer Immunol. Immunother.* 52:328-337; Payne (2003) *Cancer Cell* 3:207-
15 212; Allen (2002) *Nat. Rev. Cancer* 2:750-763; Pastan and Kreitman (2002) *Curr. Opin. Investig. Drugs* 3:1089-1091; Senter and Springer (2001) *Adv. Drug Deliv. Rev.* 53:247-264.

Antibodies of the present invention also can be conjugated to a radioactive isotope to generate cytotoxic radiopharmaceuticals, also referred to as radioimmunoconjugates. Examples of radioactive isotopes that can be conjugated to antibodies for use diagnostically or
20 therapeutically include, but are not limited to, Iodine¹³¹, Indium¹¹¹, Yttrium⁹⁰ and Lutetium¹⁷⁷. Methods for preparing radioimmunoconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including Zevalin™ (IDEC Pharmaceuticals) and Bexxar™ (GlaxoSmithKline), and similar methods can be used to prepare radioimmunoconjugates using the antibodies of the invention.

25 The antibody conjugates of the invention can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a
30 protein such as tumor necrosis factor or interferon-γ; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"),

granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.* (1982) "The Preparation and Cytotoxic Properties of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58.

15 PHARMACEUTICAL COMPOSITIONS

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing a combination of two or more antibodies, or antigen-binding portion(s) thereof, formulated with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of (*e.g.*, two or more different) antibodies or immunoconjugates of the invention. For example, a pharmaceutical composition of the invention can comprise a combination of antibodies (or immunoconjugates) that bind to different epitopes on the target antigen or that have complementary activities.

Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include an anti-PD-1 antibody and an anti-CTLA-4 antibody of the present invention combined with at least one other anti-inflammatory or immunosuppressant agent. Examples of therapeutic agents that can be used in combination therapy are described in greater detail below in the section on therapeutic uses of the combined antibodies of the invention.

As used herein, "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). Depending on the route of administration, the active compounds, *i.e.*, antibodies or immunoconjugates may be coated in a material to protect the compounds from the action of acids and other natural conditions that may inactivate the compounds.

5 In certain embodiments, the methods of the instant disclosure provide pharmaceutical compositions, which may be administered concurrently or sequentially, comprising an anti-PD-1 antibody and a pharmaceutically acceptable carrier, and comprising an anti-CTLA-4 antibody and a pharmaceutically acceptable carrier. The anti-PD-1 and anti-CTLA-4 antibodies may be formulated in separate pharmaceutical compositions or admixed as a single pharmaceutical
10 composition comprising both antibodies.

The pharmaceutical compounds of the invention may include one or more pharmaceutically acceptable salts. A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of
15 such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts
20 include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chlorprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the invention also may include a pharmaceutically
25 acceptable anti-oxidant. Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid,
30 ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, *supra*, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion

5 medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

10 The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, 15 preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent of active ingredient in combination with a pharmaceutically acceptable carrier.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as 20 indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required 25 pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

30 Alternatively, antibody 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 antibody in the patient. In general, human antibodies show the longest half life,

followed by humanized antibodies, chimeric antibodies, and nonhuman antibodies. 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 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 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, or the ester, salt or amide thereof, 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.

For administration of the anti-PD-1 antibody or anti-CTLA-4 antibody, the dosage can range from about 0.0001 to 100 mg/kg, or more usually 0.01 to 5 mg/kg, of the host body weight. For example, a dosage can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or any dose within the range of 1-10 mg/kg. Representative treatment regimes entail 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. In certain embodiments, dosage regimens for an anti-PD-1 antibody or anti-CTLA-4 antibody or both include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the antibodies given using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks. In other

embodiments, a composition containing the combination of anti-PD-1 and anti-CTLA-4 antibodies at a subtherapeutic dose is administered as needed.

5 A "therapeutically effective dosage" of an anti-PD-1 or an anti-CTLA-4 antibody of the invention preferably results in 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. For example, for the treatment of tumors, a "therapeutically effective dosage" 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. The ability of a
10 compound to inhibit tumor growth can be evaluated in an animal model system predictive of efficacy in human tumors, as described herein. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit *in vitro*, such inhibition *in vitro* can be measured by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms
15 in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

In another aspect, the instant disclosure provides a pharmaceutical kit of parts comprising an anti-PD-1 antibody and an anti-CTLA-4 antibody, as described herein. The kit may also
20 further comprise instructions for use in the treatment of a hyperproliferative disease (such as cancer as described herein). In another embodiment, the anti-PD-1 and anti-CTLA-4 antibodies may be co-packaged in unit dosage form.

In certain embodiments, two or more monoclonal antibodies with different binding specificities (*e.g.*, anti-PD-1 and anti-CTLA-4) are administered simultaneously, in which case
25 the dosage of each antibody administered falls within the ranges indicated. Antibody can be administered as a single dose or more commonly can be administered on multiple occasions. Intervals between single dosages can be, for example, weekly, monthly, every three months or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to the target antigen in the patient. In some methods, dosage is adjusted to achieve a plasma antibody
30 concentration of about 1-1000 µg /ml and in some methods about 25-300 µg /ml.

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 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. Preferred routes of administration for antibodies of the invention include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, 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, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Alternatively, an antibody composition of the instant disclosure 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.

The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicaments through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for

continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

5 In certain embodiments, the human monoclonal antibodies of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Patent Nos. 4,522,811; 5,374,548; and
10 5,399,331. The liposomes may comprise one or more moieties that are selectively transported into specific cells or organs and, thus, enhance targeted drug delivery (*see, e.g.*, Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (*see, e.g.*, U.S. Patent 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.* (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134); p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); *see also* Keinanen and Laukkanen (1994) *FEBS Lett.* 346:123; Killion and Fidler (1994) *Immunomethods* 4:273.

THERAPEUTIC METHODS

20 The combination of antibodies is useful for enhancement of an immune response against a hyperproliferative disease by blockade of PD-1 and CTLA-4. In a preferred embodiment, the antibodies of the present invention are human antibodies. For example, these molecules can be administered to cells in culture, *in vitro* or *ex vivo*, or to human subjects, *e.g., in vivo*, to enhance immunity in a variety of situations. Accordingly, in one aspect, the invention provides a method
25 of modifying an immune response in a subject comprising administering to the subject an antibody combination, or a combination of antigen-binding portions thereof, of the invention such that the immune response in the subject is modified. Preferably, the response is enhanced, stimulated or up-regulated. In another embodiment, the instant disclosure provides a method of altering adverse events associated with treatment of a hyperproliferative disease with an

immunostimulatory therapeutic agent, comprising administering an anti-PD-1 antibody and a subtherapeutic dose of anti-CTLA-4 antibody to a subject.

Blockade of PD-1 and CTLA-4 by antibodies can enhance the immune response to cancerous cells in the patient. Cancers whose growth may be inhibited using the antibodies of the instant disclosure include cancers typically responsive to immunotherapy. Representative examples of cancers for treatment with the combination therapy of the instant disclosure include melanoma (*e.g.*, metastatic malignant melanoma), renal cancer, prostate cancer, breast cancer, colon cancer and lung cancer. Examples of other cancers that may be treated using the methods of the instant disclosure include bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian 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, 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, environmentally induced cancers including those induced by asbestos, and combinations of said cancers. The present invention is also useful for treatment of metastatic cancers.

In certain embodiments, the combination of therapeutic antibodies discussed herein may be administered concurrently as a single composition in a pharmaceutically acceptable carrier, or concurrently as separate compositions with each antibody in a pharmaceutically acceptable carrier. In another embodiment, the combination of therapeutic antibodies can be administered sequentially. For example, an anti-CTLA-4 antibody and an anti-PD-1 antibody can be administered sequentially, such as anti-CTLA-4 being administered first and anti-PD-1 second, or anti-PD-1 being administered first and anti-CTLA-4 second. Furthermore, if more than one dose of the combination therapy is administered sequentially, the order of the sequential

administration can be reversed or kept in the same order at each time point of administration, sequential administrations may be combined with concurrent administrations, or any combination thereof. For example, the first administration of a combination anti-CTLA-4 antibody and anti-PD-1 antibody may be concurrent, the second administration may be sequential with anti-CTLA-4 first and anti-PD-1 second, and the third administration may be sequential with anti-PD-1 first and anti-CTLA-4 second, etc. Another representative dosing scheme may involve a first administration that is sequential with anti-PD-1 first and anti-CTLA-4 second, and subsequent administrations may be concurrent.

Optionally, the combination of anti-PD-1 and anti-CTLA-4 antibodies can be further combined with an immunogenic agent, such as cancerous cells, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells, and cells transfected with genes encoding immune stimulating cytokines (He *et al.* (2004) *J. Immunol.* 173:4919-28). Non-limiting examples of tumor vaccines that can be used include peptides of melanoma antigens, such as peptides of gp100, MAGE antigens, Trp-2, MART1 and/or tyrosinase, or tumor cells transfected to express the cytokine GM-CSF (discussed further below).

A combined PD-1 and CTLA-4 blockade can be further combined with a vaccination protocol. Many experimental strategies for vaccination against tumors have been devised (*see* Rosenberg, S. (2000) Development of Cancer Vaccines, ASCO Educational Book Spring: 60-62; Logothetis, C., 2000, ASCO Educational Book Spring: 300-302; Khayat, D. (2000) ASCO Educational Book Spring: 414-428; Foon, K. (2000) ASCO Educational Book Spring: 730-738; see also Restifo and Sznol, Cancer Vaccines, Ch. 61, pp. 3023-3043 in DeVita *et al.* (eds.), 1997, Cancer: Principles and Practice of Oncology. Fifth Edition). In one of these strategies, a vaccine is prepared using autologous or allogeneic tumor cells. These cellular vaccines have been shown to be most effective when the tumor cells are transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff *et al.* (1993) *Proc. Natl. Acad. Sci U.S.A.* 90: 3539-43).

The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg (1999) *Immunity* 10:281-7). In many cases, these tumor specific antigens are differentiation antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE antigens, and Trp-2. More importantly, many of these antigens can be shown to be the

targets of tumor specific T cells found in the host. In certain embodiments, a combined PD-1 and CTLA-4 blockade using the antibody compositions described herein may be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. These proteins are normally viewed by the immune system as self-antigens and are, therefore, tolerant to them. The tumor antigen may also include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim *et al.* (1994) *Science* 266: 2011-2013). (These somatic tissues may be protected from immune attack by various means). Tumor antigen may also be "neo-antigens" expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (*i.e.*, bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

Other tumor vaccines may include the proteins from viruses implicated in human cancers such a Human Papilloma Viruses (HPV), Hepatitis Viruses (HBV and HCV) and Kaposi's Herpes Sarcoma Virus (KHSV). Another form of tumor specific antigen which may be used in conjunction with PD-1 blockade is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat shock proteins contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity (Suot & Srivastava (1995) *Science* 269:1585-1588; Tamura *et al.* (1997) *Science* 278:117-120).

Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC's can be produced *ex vivo* and loaded with various protein and peptide antigens as well as tumor cell extracts (Nestle *et al.* (1998) *Nature Medicine* 4: 328-332). DCs may also be transduced by genetic means to express these tumor antigens as well. DCs have also been fused directly to tumor cells for the purposes of immunization (Kugler *et al.* (2000) *Nature Medicine* 6:332-336). As a method of vaccination, DC immunization may be effectively further combined with a combined PD-1 and CTLA-4 blockade to activate more potent anti-tumor responses.

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. In these instances, as is observed with the

combination of anti-PD-1 and anti-CTLA-4 antibodies, it may be possible to reduce the dose of other chemotherapeutic reagent administered with the combination of the instant disclosure (Mokyr *et al.* (1998) *Cancer Research* 58: 5301-5304). An example of such a combination is a combination of anti-PD-1 and anti-CTLA-4 antibodies further in combination with decarbazine for the treatment of melanoma. Another example is a combination of anti-PD-1 and anti-CTLA-4 antibodies further in combination with interleukin-2 (IL-2) for the treatment of melanoma. 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.

A combination of PD-1 and CTLA-4 blocking antibodies can also be used in combination with bispecific antibodies that target Fc α or Fc γ receptor-expressing effector cells to tumor cells (see, *e.g.*, U.S. Pat. Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. For example anti-Fc receptor/anti tumor antigen (*e.g.*, Her-2/neu) bispecific antibodies have been used to target macrophages to sites of tumor. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the use of a combined PD-1 and CTLA-4 blockade. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies which bind to tumor antigen and a dendritic cell specific cell surface marker.

In another example, a combination of anti-PD-1 and anti-CTLA-4 antibodies can be used in conjunction with anti-neoplastic antibodies, such as Rituxan® (rituximab), Herceptin® (trastuzumab), Bexxar® (tositumomab), Zevalin® (ibritumomab), Campath® (alemtuzumab), Lymphocide® (epratuzumab), Avastin® (bevacizumab), and Tarceva® (erlotinib), and the like. By way of example and not wishing to be bound by theory, treatment with an anti-cancer antibody or an anti-cancer antibody conjugated to a toxin can lead to cancer cell death (*e.g.*, tumor cells) which would potentiate an immune response mediated by CTLA-4 or PD-1. In an

exemplary embodiment, a treatment of a hyperproliferative disease (*e.g.*, a cancer tumor) may include an anti-cancer antibody in combination with anti-PD-1 and anti-CTLA-4 antibodies, concurrently or sequentially or any combination thereof, which may potentiate an anti-tumor immune responses by the host.

5 Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of proteins, which are expressed by the tumors and which are immunosuppressive. These include, among others, TGF- β (Kehrl, J. *et al.* (1986) *J. Exp. Med.* 163: 1037-1050), IL-10 (Howard, M. & O'Garra, A. (1992) *Immunology Today* 13: 198-200), and Fas ligand (Hahne, M. *et al.* (1996) *Science* 274: 1363-1365). In
10 another example, antibodies to each of these entities may be further combined with an anti-PD-1 and anti-CTLA-4 combination to counteract the effects of immunosuppressive agents and favor anti-tumor immune responses by the host.

Other antibodies that may be used to activate host immune responsiveness can be further used in combination with an anti-PD-1 and anti-CTLA-4 combination. These include molecules
15 on the surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity (Ridge, J. *et al.* (1998) *Nature* 393: 474-478) and can be used in conjunction with an anti-PD-1 and anti-CTLA-4 combination (Ito, N. *et al.* (2000) *Immunobiology* 201 (5) 527-40). Activating antibodies to T cell costimulatory molecules, such as OX-40 (Weinberg, A. *et al.* (2000) *Immunol* 164: 2160-
20 2169), 4-1BB (Melero, I. *et al.* (1997) *Nature Medicine* 3: 682-685 (1997), and ICOS (Hutloff, A. *et al.* (1999) *Nature* 397: 262-266) may also provide for increased levels of T cell activation.

Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, therapeutic benefit may be obtained from graft vs. tumor responses. A combined PD-1 and
25 CTLA-4 blockade can be used to increase the effectiveness of the donor engrafted tumor specific T cells.

There are also several experimental treatment protocols that involve *ex vivo* activation and expansion of antigen specific T cells and adoptive transfer of these cells into recipients in order to antigen-specific T cells against tumor (Greenberg, R. & Riddell, S. (1999) *Science* 285:
30 546-51). These methods may also be used to activate T cell responses to infectious agents such

as CMV. *Ex vivo* activation in the presence of anti-PD-1 and anti-CTLA-4 antibodies may be expected to increase the frequency and activity of the adoptively transferred T cells.

As set forth herein, organs can exhibit immune-related adverse events following immunostimulatory therapeutic antibody therapy, such as the GI tract (diarrhea and colitis) and the skin (rash and pruritis) after treatment with anti-CTLA-4 antibody. For example, non-colonic gastrointestinal immune-related adverse events have also been observed in the esophagus (esophagitis), duodenum (duodenitis), and ileum (ileitis) after anti-CTLA-4 antibody treatment.

In certain embodiments, the present invention provides a method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, comprising administering a anti-PD-1 antibody and a subtherapeutic dose of anti-CTLA-4 antibody to a subject. For example, the methods of the present invention provide for a method of reducing the incidence of immunostimulatory therapeutic antibody-induced colitis or diarrhea by administering a non-absorbable steroid to the patient. Because any patient who will receive an immunostimulatory therapeutic antibody is at risk for developing colitis or diarrhea induced by such an antibody, this entire patient population is suitable for therapy according to the methods of the present invention. Although steroids have been administered to treat inflammatory bowel disease (IBD) and prevent exacerbations of IBD, they have not been used to prevent (decrease the incidence of) IBD in patients who have not been diagnosed with IBD. The significant side effects associated with steroids, even non-absorbable steroids, have discouraged prophylactic use.

In further embodiments, a combination PD-1 and CTLA-4 blockade (*i.e.*, immunostimulatory therapeutic antibodies anti-PD-1 and anti-CTLA-4) can be further combined with the use of any non-absorbable steroid. As used herein, a "non-absorbable steroid" is a glucocorticoid that exhibits extensive first pass metabolism such that, following metabolism in the liver, the bioavailability of the steroid is low, *i.e.*, less than about 20%. In one embodiment of the invention, the non-absorbable steroid is budesonide. Budesonide is a locally-acting glucocorticosteroid, which is extensively metabolized, primarily by the liver, following oral administration. ENTOCORT EC® (Astra-Zeneca) is a pH- and time-dependent oral formulation of budesonide developed to optimize drug delivery to the ileum and throughout the colon. ENTOCORT EC® is approved in the U.S. for the treatment of mild to moderate Crohn's disease

involving the ileum and/or ascending colon. The usual oral dosage of ENTOCORT EC® for the treatment of Crohn's disease is 6 to 9 mg/day. ENTOCORT EC® is released in the intestines before being absorbed and retained in the gut mucosa. Once it passes through the gut mucosa target tissue, ENTOCORT EC® is extensively metabolized by the cytochrome P450 system in the liver to metabolites with negligible glucocorticoid activity. Therefore, the bioavailability is low (about 10%). The low bioavailability of budesonide results in an improved therapeutic ratio compared to other glucocorticoids with less extensive first-pass metabolism. Budesonide results in fewer adverse effects, including less hypothalamic-pituitary suppression, than systemically-acting corticosteroids. However, chronic administration of ENTOCORT EC® can result in systemic glucocorticoid effects such as hypercorticism and adrenal suppression. *See PDR 58th ed. 2004; 608-610.*

In still further embodiments, a combination PD-1 and CTLA-4 blockade (*i.e.*, immunostimulatory therapeutic antibodies anti-PD-1 and anti-CTLA-4) in conjunction with a non-absorbable steroid can be further combined with a salicylate. Salicylates include 5-ASA agents such as, for example: sulfasalazine (AZULFIDINE®, Pharmacia & UpJohn); olsalazine (DIPENTUM®, Pharmacia & UpJohn); balsalazide (COLAZAL®, Salix Pharmaceuticals, Inc.); and mesalamine (ASACOL®, Procter & Gamble Pharmaceuticals; PENTASA®, Shire US; CANASA®, Axcan Scandipharm, Inc.; ROWASA®, Solvay).

In accordance with the methods of the present invention, a salicylate administered in combination with anti-PD-1 and anti-CTLA-4 antibodies and a non-absorbable steroid can include any overlapping or sequential administration of the salicylate and the non-absorbable steroid for the purpose of decreasing the incidence of colitis induced by the immunostimulatory antibodies. Thus, for example, methods for reducing the incidence of colitis induced by the immunostimulatory antibodies according to the present invention encompass administering a salicylate and a non-absorbable concurrently or sequentially (*e.g.*, a salicylate is administered 6 hours after a non-absorbable steroid), or any combination thereof. Further, according to the present invention, a salicylate and a non-absorbable steroid can be administered by the same route (*e.g.*, both are administered orally) or by different routes (*e.g.*, a salicylate is administered orally and a non-absorbable steroid is administered rectally), which may differ from the route(s) used to administer the anti-PD-1 and anti-CTLA-4 antibodies.

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

EXAMPLES

Example 1 Generation of Chimeric (Rat-Mouse) anti-PD-1 Antibody 4H2

Rat monoclonal antibody against mouse PD-1 antibodies (rat anti-mPD-1) were generated from rats immunized with mPD-1-hFc fusion protein using standard hybridoma production methods (see Kohler and Milstein (1975) *Nature* 256:495; and Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York). Eight hybridomas were subcloned, and antibodies were isolated and screened for their ability to block mouse PD-L2 (mPD-L2) binding to mPD-1. Several anti-mPD-1 antibodies capable of blocking mPD-L2 binding to mPD-1 were identified (*see*, e.g., activity of 4H2, Figure 21) and the binding affinity of several of these antibodies to mPD-1-Fc fusion protein was determined by ELISA (Figure 22).

Antibody 4H2.B3 was further characterized, which is referred to interchangeably herein as "4H2." CHO cells expressing mouse PD-1 were constructed and incubated with 4H2 anti-mPD-1 antibody at a concentration ranging from 200 µg/ml to 0.012 µg/ml to determine the binding affinity of 4H2 to PD-1. Binding of anti-mPD-1 antibody to the PD-1 expressing CHO cells was detected by incubating with donkey-anti-rat IgG, FITC conjugated and measured by FACS. The anti-mPD-1 antibody had an EC₅₀ (50% effective concentration) of about 0.38 µg (Figure 23) and a K_D of 4.7 x 10⁻⁹ M. To examine the inhibition of PD-L1 binding to PD-1, the same assay was performed except that the cells were also incubated with 0.16 µg mPD-L1-hFc fusion protein, then binding of PD-L1 to the PD-1 expressing CHO cells was detected by incubating with goat-anti-human IgG (Fc specific), FITC conjugated and measuring binding signal by FACS (MFI, mean fluorescence intensity). The anti-mPD-1 antibody had an EC₅₀ of about 0.72 µg (Figure 24).

For use in the mouse tumor models, the 4H2 rat anti-mPD1 needed to be modified so the mouse immune system would not neutralize the immunotherapeutic antibody (*i.e.*, so the antibody would have better pharmacokinetics) and to avoid antibody-dependent cellular cytotoxicity (ADCC) by reducing Fc receptor interactions (*i.e.*, so blockade by anti-PD-1 could be evaluated with being compromised by ADCC effects). The original rat anti-mPD-1 antibody, 4H2, was determined to be a rat IgG2a isotype. Hence, the Fc-portion of the 4H2 antibody was replaced with an Fc-portion from a mouse IgG1 isotype. Using the assay described above, the

binding affinity of the rat-mouse chimeric 4H2 to mPD-1 was found to be comparable to the rat 4H2.B3 anti-mPD-1 antibody (Figure 25). Similarly, inhibition of PD-L1 binding to PD-1 was comparable for both antibodies (Figure 26). Thus, the rat-mouse chimeric 4H2 anti-mPD-1 antibody was used to examine the therapeutic efficacy of anti-PD-1 in combination with anti-CTLA-4.

Example 2 *In Vivo* Efficacy of Combination Therapy (anti-CTLA-4 and anti-PD-1 Antibodies) on Tumor Establishment and Growth

MC38 colorectal cancer cells (PD-L1⁺) (available from Dr. N. Restifo, National Cancer Institute, Bethesda, MD; or Jeffrey Schlom, National Institutes of Health, Bethesda, MD) were implanted in C57BL/6 mice (2 x 10⁶ cells/mouse). On day 0 (*i.e.*, the day the MC38 cells were implanted in the mice), each of four groups of 10 mice each was injected intraperitoneally (IP) with one of the following: (1) mouse IgG (control), (2) anti-CTLA-4 monoclonal antibody 9D9 (mouse anti-mouse CTLA-4, obtained from J. Allison, Memorial Sloan-Kettering Cancer Center, New York, NY), (3) anti-PD-1 monoclonal antibody 4H2 (chimeric antibody in which a rat anti-mouse PD-1 was modified with a mouse Fc region, as described in Example 6), or (4) anti-CTLA-4 antibody 9D9 and anti-PD-1 antibody 4H2. Antibody injections were then further administered on days 3, 6 and 10. The single antibody treatments were dosed at 10 mg/kg, and the combination of anti-CTLA-4 antibody and anti-PD-1 antibody was dosed at 5 mg/kg of each antibody (*i.e.*, 10 mg/kg of total antibody). Using an electronic caliper, the tumors were measured three dimensionally (height x width x length) and tumor volume was calculated. Mice were euthanized when the tumors reached a designated tumor end-point. The results are shown in Table 1 and Figure 1.

Table 1. Percentage of Tumor-Free Mice Following Anti-PD-1 and/or Anti-CTLA-4 Treatment

Treatment	Total mice studied	Tumor-free mice (%)
mIgG1	10	0
anti-CTLA-4	10	1 (10)
anti-PD-1	10	3 (30)
anti-CTLA-4 + anti-PD-1	10	6 (60)

Eight mice in the IgG group reached the tumor end-point by about day 30 and two mice (86066 and 87260) in the IgG group had ulcerated tumors (Figure 1A). In the anti-CTLA-4 antibody alone group, seven mice reached the tumor end-point by about day 60, one mouse had an ulcerated tumor (84952), one mouse had a tumor with a volume of less than 1500 mm³ (85246), and one mouse was tumor-free (86057) (Figure 1B). In the anti-PD-1 antibody alone group, six mice reached the tumor end-point by about day 60, one mouse had an ulcerated tumor (86055), and three mice were tumor-free (84955, 85239 and 86750) (Figure 1C). In the anti-CTLA-4 antibody and anti-PD-1 antibody combination group, four mice reached the tumor end-point by about Day 40, and six mice were tumor-free (84596, 85240, 86056, 86071, 86082 and 86761) (Figure 1D).

Figure 2 shows that the mean tumor volume measured at day 21 was about 2955 mm³ for the IgG control group; about 655 mm³ for the CTLA-4 antibody alone group, about 510 mm³ for the PD-1 antibody alone group, and about 280 mm³ for the anti-CTLA-4 antibody and anti-PD-1 antibody combination group. Figure 3 shows that the median tumor volume measured at day 21 was about 2715 mm³ for the IgG group; about 625 mm³ for the CTLA-4 antibody alone group; about 525 mm³ for the PD-1 antibody alone group; and about 10 mm³ for the CTLA-4 antibody and PD-1 antibody combination group (and down to 0 mm³ by day 32).

This study indicates that, in a murine tumor model, CTLA-4 antibody treatment alone and PD-1 antibody treatment alone have a modest effect on tumor growth, and that the combination treatment of CTLA-4 antibody and PD-1 antibody has a significantly greater effect on tumor growth. It is interesting to note that the combination treatment with CTLA-4 antibody and PD-1 antibody had a more significant effect on tumor growth at a dose of 5 mg/kg of each antibody as compared to the effect of either antibody alone when each is administered at a higher dose of 10 mg/kg.

Example 3 *In Vivo* Efficacy of Combination Therapy (anti-CTLA-4 and anti-PD-1 Antibodies) on Established Tumor Growth

MC38 colorectal cancer cells (PD-L1⁺) were implanted in C57BL/6 mice (2 x 10⁶ cells/mouse) for a time sufficient (about 6 to 7 days) to permit the formation of tumors. On day 6 post-implantation (day -1), tumor measurements were taken and mice were randomized based on mean tumor volume (about 250 mm³) into 11 groups for subsequent antibody therapy. At day

0 (*i.e.*, one week after the MC38 cells were implanted), mice were injected IP with (1) mouse IgG (control), (2) anti-CTLA-4 monoclonal antibody 9D9, (3) anti-PD-1 monoclonal antibody 4H2, or (4) anti-CTLA-4 monoclonal antibody 9D9 and anti-PD-1 antibody monoclonal antibody 4H2, at a concentration of 10 mg/kg per mouse. Antibody injections were also
 5 administered on days 3, 6 and 10. The monoclonal antibody compositions used had low levels of endotoxin and did not significantly aggregate. Using an electronic caliper, the tumors were measured three dimensionally (height x width x length) and tumor volume was calculated. Tumor measurements were taken on day 0 (tumors at the beginning of treatment had a volume of about 125 mm³), and on days 3, 6, 10, 13, 17 and 20 post-antibody injection. Mice were
 10 euthanized when the tumors reached a designated tumor end-point (a particular tumor volume such as 1500 mm³ and/or when the mice showed greater than about 15% weight loss).

All eleven mice in the IgG group reached the tumor end-point by about day 17 (Figure 4A). In the anti-CTLA-4 antibody alone group, seven of eleven mice reached the tumor end-point by about day 12 (Figure 4B). In the anti-PD-1 antibody alone group, four mice
 15 reached the tumor end-point by about day 13 and two mice were tumor-free (Figure 4C). In the anti-CTLA-4 antibody and anti-PD-1 antibody combination group, one mouse reached the tumor end-point by about day 17, one mouse reached the tumor end-point by about day 45 and nine mice were tumor-free on day 45 (Figure 4D).

Figure 5 shows that the mean tumor volume measured at day 10 was about 1485 mm³ for the IgG control group; about 1010 mm³ for the CTLA-4 antibody alone group; about 695 mm³
 20 for the PD-1 antibody alone group; and about 80 mm³ for the anti-CTLA-4 antibody and anti-PD-1 antibody combination group. Figure 6 shows that the median tumor volume measured at day 10 was about 1365 mm³ for the IgG group; about 1060 mm³ for the anti-CTLA-4 antibody alone group; about 480 mm³ for the anti-PD-1 antibody alone group; and about 15 mm³
 25 for the anti-CTLA-4 antibody and anti-PD-1 antibody combination group (which was down to 0 mm³ by day 17).

This study indicates that, in a murine tumor model, treatment with the combination of CTLA-4 antibody and PD-1 antibody has a significantly greater effect on tumor growth than either antibody alone, even when a tumor is already well established.

Example 4 Dose Titration of Combination Therapy (anti-CTLA-4 and anti-PD-1 Antibodies) on Established Tumor Growth

MC38 colorectal cancer cells (PD-L1⁺) were implanted in C57BL/6 mice (2 x 10⁶ cells/mouse) for a time sufficient (about 6 to 7 days) to permit the formation of tumors as described in Example 3. Groups of 10 mice were injected IP at days 0, 3, 6 and 10 as follows: Group (A) mouse IgG (control, 20 mg/kg), Group (B) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg) and mouse IgG (10 mg/kg), Group (C) anti-CTLA-4 monoclonal antibody 9D9 (10 mg/kg) and mouse IgG (10 mg/kg), Group (D) anti-CTLA-4 monoclonal antibody 9D9 (10 mg/kg) and anti-PD-1 antibody monoclonal antibody 4H2 (10 mg/kg), Group (E) anti-CTLA-4 monoclonal antibody 9D9 (3 mg/kg) and anti-PD-1 antibody monoclonal antibody 4H2 (3 mg/kg), or Group (F) anti-CTLA-4 monoclonal antibody 9D9 (1 mg/kg) and anti-PD-1 antibody monoclonal antibody 4H2 (1 mg/kg). Using an electronic caliper, the tumors were measured three dimensionally (height x width x length) and tumor volume was calculated. Tumor measurements were taken at the beginning of treatment (*i.e.*, on day 0 tumors had an average volume of about 90 mm³), and on days 3, 6, 10, 13, 17 and 20 post-antibody treatment. Mice were euthanized when the tumors reached a designated tumor end-point (a particular tumor volume such as 1500 mm³ and/or when the mice showed greater than about 15% weight loss).

Figure 7A shows that all 10 control mice had reached a tumor end-point. Figure 7B shows that the group treated with 10 mg/kg anti-PD-1 antibody (Group B) had 6 mice that reached the tumor end-point and 4 mice with tumors having a volume of about 750 mm³ or less. Figure 7C shows that the group treated with 10 mg/kg anti-CTLA-4 antibody (Group C) had 3 mice that reached the tumor end-point and 7 mice with tumors having a volume of about 1000 mm³ or less. Figure 7D shows that the group treated with a combination of 10 mg/kg anti-PD-1 antibody with 10 mg/kg anti-CTLA-4 antibody (Group D) had 2 mice with tumors having a volume of about 1000 mm³ or less, and 8 mice that were tumor free. Figure 7E shows that the group treated with a combination of 3 mg/kg anti-PD-1 antibody with 3 mg/kg anti-CTLA-4 antibody (Group E) had one mouse that had reached the tumor end-point, 7 mice with tumors having a volume of about 500 mm³ or less, and 2 mice that were tumor free. Figure 7F shows that the group treated with a combination of 1 mg/kg anti-PD-1 antibody with 1 mg/kg anti-CTLA-4 antibody (Group F) had 4 mice that had reached the tumor end-point,

5 mice with tumors having a volume of about 1100 mm³ or less, and one mouse that was tumor free.

Figures 7G and 7H show the tumor volumes in mice treated sequentially with anti-PD-1 antibody first and anti-CTLA-4 antibody second, and vice versa. The mice of Figure 7G first received 10 mg/kg anti-CTLA-4 on each of days 0 and 3, and then received 10 mg/kg anti-PD-1 antibody on each of days 6 and 10. The mice of Figure 7H first received 10 mg/kg anti-PD-1 antibody on each of days 0 and 3, and then received 10 mg/kg anti-CTLA-4 antibody on each of days 6 and 10. For group G at day 27, 8 mice reached the tumor end-point, one mouse had a very small tumor (which, after a significant delay, eventually grew out) and one mouse was tumor free. For group H at day 27, 8 mice reached the tumor end-point and 2 were tumor free.

Figure 8 shows that the mean tumor volume measured at day 10 was about 1250 mm³ for the IgG control group; about 470 mm³ for the PD-1 antibody with the IgG control; about 290 mm³ for the CTLA-4 antibody with the IgG control (measured at day 6); about 40 mm³ for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 165 mm³ for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 400 mm³ for the anti-CTLA-4 antibody (1 mg/kg) and anti-PD-1 antibody (1 mg/kg) combination group. Figure 9 shows that the median tumor volume measured at day 13 was about 1680 mm³ for the IgG control group; about 400 mm³ for the PD-1 antibody with the IgG control; about 660 mm³ for the CTLA-4 antibody with the IgG control; 0 mm³ for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 90 mm³ for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 650 mm³ for the anti-CTLA-4 antibody (1 mg/kg) and anti-PD-1 antibody (1 mg/kg) combination group. For the combination treatment of the anti-PD-1 antibody with the anti-CTLA-4 antibody, the number of mice per group that were tumor free at day 27 of the study was 8/10 (10 mg/kg), 2/10 (3 mg/kg) and 1/10 (1 mg/kg) (data not shown).

This study indicates that, in a murine tumor model, treatment with the combination of CTLA-4 antibody and PD-1 antibody functions in a dose dependent manner and has a significantly greater effect on tumor growth than both antibodies alone, even at a lower dose and even when a tumor is already well established. Moreover, the antibodies may be administered sequentially (anti-CTLA-4 antibody first and anti-PD-1 antibody second, or vice versa) and the combination is still superior to the antibody monotherapies.

Example 5 *In Vivo* Efficacy of Combination Therapy (anti-CTLA-4 and anti-PD-1 Antibodies) on Fibrosarcoma Establishment and Growth

SA1/N fibrosarcoma cells (PD-L1⁺) (Leach *et al.* (1996) *Science* 271:1734-1736) were implanted subcutaneously in A/J mice (2×10^6 cells/mouse) on day 0. On days 1, 4, 7 and 11 post-implantation, mice were injected IP as follows: Group (A) PBS alone (referred to as the “vehicle”); Group (B) mouse IgG (control, 10 mg/kg per mouse), Group (C) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse), Group (D) anti-CTLA-4 monoclonal antibody 9D9 (10 mg/kg or 0.2 mg/kg per mouse), and Group (E) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse) in combination with anti-CTLA-4 monoclonal antibody 9D9 (0.2 mg/kg per mouse). The study lasted 41 days and tumor measurements were taken on various days throughout the course of the study (*see* Figure 9). Tumor volume was calculated by measuring tumors in three dimensions (height x width x length) using an electronic caliper. Mice were euthanized when the tumors reached a designated tumor end-point – a volume of 1500 mm³ and/or an ulcerated tumor.

Figures 10A and 10B show that 19 out of the 20 control (9/10 in Group A and 10/10 in Group B) mice had either reached a tumor end-point or had developed ulcerated tumors. Figure 10C shows that the group treated with 10 mg/kg anti-PD-1 antibody (Group C) had 6 mice that reached a tumor end-point (2 with a volume greater than 1500 mm³ and 4 with an ulcerated tumor) and 4 mice that were tumor free. Figure 10D shows that the group treated with 10 mg/kg anti-CTLA-4 antibody (Group D) had 5 mice that reached a tumor end-point (2 with a volume greater than 1500 mm³ and 3 with an ulcerated tumor), one mouse with a small tumor (volume of about 70 mm³) and 4 mice that were tumor free. Figure 10E shows that the group treated with 0.2 mg/kg anti-CTLA-4 antibody (Group E) had 10 mice that reached a tumor end-point (6 with a volume greater than 1500 mm³ and 4 with an ulcerated tumor). Figure 10F shows that the group treated with a combination of 10 mg/kg anti-PD-1 antibody with 0.2 mg/kg anti-CTLA-4 antibody (Group F) had 2 mice that reached a tumor end-point (one with a volume greater than 1500 mm³ and one with an ulcerated tumor) and 8 mice that were tumor free.

Figures 11 and 12 show the mean and median tumor volume, respectively, that developed in treated and untreated mice over the course of this study. The tumor growth inhibition in mice treated with these antibodies, as compared to mice treated with the control antibody mouse IgG, is summarized in Table 2.

Table 2. Tumor Growth Inhibition and Tumor Free Mice Following Anti-PD-1 and/or Anti-CTLA-4 Treatment

Group [†]	Median Tumor Volume – mm ³ (Day 15)	TGI* (%) (Day 15)	Median Tumor Volume – mm ³ (Day 19)	TGI (%) (Day 19)	No. of Tumor Free Mice (Day 41)
A	985	–	1140	–	0/10
B	635	–	1060	–	0/10
C	465	27	310	71	4/10
D	235	63	90	91	4/10
E	600	6	805	24	0/10
F	330	48	90	92	8/10

* TGI = tumor growth inhibition; the median could be calculated only when fewer than 50% of the mice reached the tumor end point.

5 † Groups are as defined in Figure 10. A = vehicle (PBS); B = mouse IgG; C = anti-PD-1, 10 mg/kg; D = anti-CTLA-4, 10 mg/kg; E = anti-CTLA-4, 0.2 mg/kg; and F = anti-PD-1, 10 mg/kg with anti-CTLA-4, 0.2 mg/kg.

These data further indicate that the combination therapy comprising anti-PD-1 and anti-CTLA-4 antibodies is substantially more effective than treatment with either antibody alone.

10 Indeed, the combination is still more effective than single antibody treatments even when the combination therapy contains a subtherapeutic dose of anti-CTLA-4 antibody. These data also indicate that surprisingly the presence or absence of PD-L1 on the tumor may have no effect on the efficacy of treatment with this antibody combination, although the presence of PD-L1 may influence the effect of the antibody monotherapies in that expression of PD-L1 on the tumor
15 may also lead to inhibition of anti-tumor T cell responses (see Figure 20).

Example 6 *In Vivo* Efficacy and Dose Titration of Combination Therapy (anti-CTLA-4 and anti-PD-1 Antibodies) on PD-L1⁺ Fibrosarcoma Growth

SA1/N fibrosarcoma cells (PD-L1⁺) were implanted subcutaneously in A/J mice (2 x 10⁶ cells/mouse) on day 0 for a time sufficient (about 7 days) to permit the establishment of a tumor.
20 On days 7, 10, 13 and 16 post-implantation, ten groups of 8 mice having an average tumor volume of 110 mm³ were injected IP as follows: Group (A) PBS alone (referred to as the “vehicle”); Group (B) mouse IgG (control, 10 mg/kg per mouse); Group (C) anti-CTLA-4

monoclonal antibody 9D9 (0.25 mg/kg); Group (D) anti-CTLA-4 monoclonal antibody 9D9 (0.5 mg/kg per mouse); Group (E) anti-CTLA-4 monoclonal antibody 9D9 (5 mg/kg); Group (F) anti-PD-1 monoclonal antibody 4H2 (3 mg/kg per mouse); Group (G) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse); Group (H) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse) in combination with anti-CTLA-4 monoclonal antibody 9D9 (0.25 mg/kg per mouse); Group (I) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse) in combination with anti-CTLA-4 monoclonal antibody 9D9 (0.5 mg/kg per mouse); and Group (J) anti-PD-1 monoclonal antibody 4H2 (3 mg/kg per mouse) in combination with anti-CTLA-4 monoclonal antibody 9D9 (0.5 mg/kg per mouse).

On days 10, 13, 16 and 19 post-implantation, two groups of 6 mice having an average tumor volume of 255 mm³ were injected IP as follows: Group (K) mouse IgG (control, 10 mg/kg per mouse); and Group (L) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse) in combination with anti-CTLA-4 monoclonal antibody 9D9 (1 mg/kg per mouse). The study lasted 51 days and tumor measurements were taken on various days throughout the course of the study (*see* Figures 13-18). Tumor volume was calculated by measuring tumors in three dimensions (height x width x length) using an electronic caliper. Mice were euthanized when the tumors reached a designated tumor end-point – a volume of 1500 mm³ and/or an ulcerated tumor.

Figure 13 shows the response to immunostimulatory antibody treatment in mice with tumors having an initial volume of about 110 mm³ (*i.e.*, at the time of the first antibody treatment).

Figures 13A and 13B show that all 16 control mice (Groups A and B) reached a tumor end-point (15 with a tumor volume greater than 1500 mm³ and 1 with an ulcerated tumor). Figures 13C-13E show that tumor bearing mice respond to treatment with anti-CTLA-4 antibody in a dose-dependent manner (*e.g.*, Group C receiving 0.25 mg/kg had 7/8 mice reach the tumor end-point and one mouse with a tumor volume less than 200 mm³, whereas Group E receiving 5 mg/kg had 6/8 mice reach the tumor end-point and two mice were tumor free). Figures 13F and 13G show that mice responded about the same regardless of the anti-PD-1 antibody dose (Group F received 3 mg/kg and Group G received 10 mg/kg). In contrast, the mice receiving a combination treatment of 10 or 3 mg/kg anti-PD-1 antibody with 0.25 or 0.5 mg/kg anti-CTLA-4 antibody (Groups H, I and J) showed a significant reduction in tumor growth. For example, Figure 13J shows that the group treated with a combination of 3 mg/kg anti-PD-1 antibody with 0.5 mg/kg anti-CTLA-4 antibody (Group J) had 2 mice that had ulcerated tumors,

2 mice with a tumor volume less than 500 mm³, and 4 mice that were tumor free. The unexpected synergistic effect of an anti-PD-1 antibody combined with an anti-CTLA-4 antibody, along with the surprising effectiveness of subtherapeutic levels of anti-CTLA-4 antibody in the combination, are shown in Figures 14 (mean tumor volume) and 15 (median tumor volume).

5 Figure 16 shows the response to immunostimulatory antibody treatment in mice with larger tumors, those having an initial volume of about 250 mm³ (*i.e.*, at the time of the first antibody treatment). Figure 16A shows that all 6 control mice (Group K) reached a tumor end-point (4 with a tumor volume greater than 1500 mm³ and 2 with an ulcerated tumor). Figure 16B shows that the group treated with a combination of 10 mg/kg anti-PD-1 antibody with 1
10 mg/kg anti-CTLA-4 antibody (Group L) had one mouse with an ulcerated tumor, 4 mice with a tumor volume greater than 1500 mm³, and one mouse that was tumor free. The mean and median tumor volumes are shown in Figures 17 and 18.

The tumor growth inhibition in mice treated with these antibodies, as compared to mice treated with the control antibody mouse IgG, is summarized in Table 3 and Figure 19.

15 **Table 3. Tumor Growth Inhibition Following Anti-PD-1 and/or Anti-CTLA-4 Treatment**

Group	Mean Tumor Volume – mm ³ (Day 23)	TGI* (Mean)	Median Tumor Volume – mm ³ (Day 23)	TGI (Median)	Tumor Free Mice (Day 51)	No. Mice at Tumor End Point
A	700	–	1,380	–	–	–
B	1,710	–	1,360	–	–	–
C	1,050	39%	925	32	–	–
D	770	55%	505	63	–	–
E	155	91%	100	93	2/8	6/8
F	1,050	39%	675	50	–	7/8
G	1,070	37%	1,145	16	–	6/8
H	85	95%	25	98	4/8	3/8
I	75	96%	60	95	4/8	1/8
J	80	95%	5	99	4/8	0/8
K	1,900	–	2,125	–	–	–
L	1,115	41	1,090	49	1/6	–

* TGI = tumor growth inhibition; the median could only be calculated when fewer than 50% of the mice reached the tumor end point.

[†] Groups are as defined in Figures 13 and 16. For smaller initial tumor: A = vehicle (PBS); B = mouse IgG, 10 mg/kg; C = anti-CTLA-4, 0.25 mg/kg; D = anti-CTLA-4, 0.5 mg/kg; E = anti-CTLA-4, 5 mg/kg; F = anti-PD-1, 3 mg/kg; G = anti-PD-1, 10 mg/kg; H = anti-PD-1, 10 mg/kg with anti-CTLA-4, 0.25 mg/kg; I = anti-PD-1, 10 mg/kg with anti-CTLA-4, 0.5 mg/kg; and J = anti-PD-1, 3 mg/kg with anti-CTLA-4, 0.5 mg/kg. For larger initial tumor: K = mouse IgG, 10 mg/kg; and L = anti-PD-1, 10 mg/kg with anti-CTLA-4, 0.25 mg/kg.

Together these data indicate that the combination therapy comprising anti-PD-1 and anti-CTLA-4 antibodies is substantially more effective than treatment with either antibody alone. In addition, surprisingly the dose of each antibody can be reduced without affecting the synergistic efficacy of this combination of immunostimulatory therapeutic antibodies. The combination therapy still seems to be effective even when the tumor mass is more mature (*i.e.*, larger).

Example 7 Tumor Immunity in Mice Following anti-PD-1 Antibody Treatment and Re-Challenge with PD-L1⁺ Fibrosarcoma Cells

Mice that survived tumor-free from a challenge with tumor cells and treatment with anti-PD-1 antibody (*i.e.*, treatment similar to the efficacy studies described in Examples 5 and 6) were then re-challenged with tumor cells to investigate immunity to tumor formation after such a treatment. Briefly, in the initial challenge, SA1/N fibrosarcoma cells (PD-L1⁺) were implanted subcutaneously in A/J mice (1×10^6 cells/mouse) on day 0. On days 1, 4, 7, 10, 14, 17 and 20 post-implantation, groups of mice were injected IP with either mouse IgG (control, 10 mg/kg per mouse) or with one of various doses of anti-PD-1 monoclonal antibody 4H2 (30, 10, 3, 1 and 0.3 mg/kg per mouse). Tumor formation and volume was monitored with a precision electronic caliper twice a week until the study was complete. A group of 8 mice were tumor-free after the anti-PD1 antibody treatment (4 that were treated with 30 mg/kg, 2 with 3 mg/kg, one with 1 mg/kg, and one with 0.3 mg/kg).

The eight treated, tumor-free A/J mice were re-challenged by subcutaneously implanting 1×10^6 SA1/N fibrosarcoma cells/mouse. As a control, nine naïve mice were subcutaneously implanted with 1×10^6 SA1/N fibrosarcoma cells/mouse. Tumor formation and volume was monitored with a precision electronic caliper twice a week until day 62 post-implantation. All nine naïve (control) mice reached the tumor end-point by day 22 post-implantation of the fibrosarcoma cells. In contrast, the eight tumor-free mice re-challenged with fibrosarcoma cells

did not develop tumors up to 62 days post-implantation. Figure 27 shows the mean tumor volume for the naïve and re-challenged mice. These results demonstrate that treatment with an immunostimulatory antibody, such as anti-PD-1, provides the treated subject with immunity to further tumor formation, even in the presence of cells capable of forming a tumor.

Example 8 Tumor Immunity in Mice Following Single Antibody Therapy (anti-PD-1) or Combination Antibody Therapy (anti-CTLA-4 and anti-PD-1) Re-Challenged with PD-L1⁺ Colorectal Cancer Cells

Mice that survived tumor-free from a challenge with tumor cells and treatment with either anti-PD-1 antibody alone or anti-PD-1 antibody combined with anti-CTLA-4 antibody (*i.e.*, treatment similar to the efficacy studies described in Examples 2-4) were then re-challenged with tumor cells to investigate immunity to tumor formation after such treatments. Briefly, in the initial challenge, MC38 colorectal cancer cells (PD-L1⁺) were implanted in C57BL/6 mice (2 x 10⁶ cells/mouse) on day 0. On days 0, 3, 6 and 10 post-implantation, groups of mice were injected IP with one of the following treatments: (1) mouse IgG (control, 10 mg/kg per mouse), anti-PD-1 monoclonal antibody 4H2, or (3) anti-PD-1 monoclonal antibody 4H2 in combination with anti-CTLA-4 monoclonal antibody 9D9. Tumor growth was monitored with a precision electronic caliper as described in Example 4. A group of 11 mice were tumor-free after the anti-PD1 antibody treatment (2 total) or the combination anti-PD-1 / anti-CTLA-4 antibody treatment (9 total).

The 11 treated, tumor-free C57BL/6 mice were re-challenged by implantation of 2 x 10⁷ MC38 colorectal cancer cells/mouse (*i.e.*, a dose of cells 10 x greater than the initial challenge). As a control, seven naïve mice were implanted with 2 x 10⁷ MC38 colorectal cancer cells/mouse. Tumor formation and volume was monitored with a precision electronic caliper for the duration of the re-challenge experiment (at least 20 days). Figure 28 shows that all seven naïve (control) mice developed a tumor and reached the tumor end-point by day 18 post-implantation of the colorectal cancer cells. In contrast, all 11 tumor-free mice re-challenged with colorectal cancer cells did not develop tumors up to 18 days post-implantation. Figure 29 shows the mean tumor volume for the naïve and re-challenged mice. These data indicate that, similar to the antibody monotherapy, the combination antibody therapy resulting in PD-1 and CTLA-4 blockade produces a persistent immunity to tumor relapse.

Example 9 Generation of Human Monoclonal Antibodies Against PD-1

Antigen

Immunization protocols utilized as antigen both (i) a recombinant fusion protein comprising the extracellular portion of PD-1 and (ii) membrane bound full-length PD-1. Both
5 antigens were generated by recombinant transfection methods in a CHO cell line.

Transgenic HuMab and KM miceTM

Fully human monoclonal antibodies to PD-1 were prepared using the HCo7 strain of HuMab transgenic mice and the KM strain of transgenic transchromosomal mice, each of which express human antibody genes. In each of these mouse strains, the endogenous mouse kappa
10 light chain gene has been homozygously disrupted as described in Chen *et al.* (1993) *EMBO J.* 12:811-820 and the endogenous mouse heavy chain gene has been homozygously disrupted as described in Example 1 of PCT Publication WO 01/09187. Each of these mouse strains carries a human kappa light chain transgene, KCo5, as described in Fishwild *et al.* (1996) *Nature Biotechnology* 14:845-851. The HCo7 strain carries the HCo7 human heavy chain transgene as
15 described in U.S. Patent Nos. 5,545,806; 5,625,825; and 5,545,807. The KM strain contains the SC20 transchromosome as described in PCT Publication WO 02/43478.

HuMab and KM Immunizations:

To generate fully human monoclonal antibodies to PD-1, HuMab mice and KM miceTM were immunized with purified recombinant PD-1 fusion protein and PD-1-transfected CHO cells
20 as antigen. General immunization schemes for HuMab mice are described in Lonberg, N. *et al.* (1994) *Nature* 368(6474): 856-859; Fishwild, D. *et al.* (1996) *Nature Biotechnology* 14: 845-851 and PCT Publication WO 98/24884. The mice were 6-16 weeks of age upon the first infusion of antigen. A purified recombinant preparation (5-50 µg) of PD-1 fusion protein antigen and 5 - 10x10⁶ cells were used to immunize the HuMab mice and KM miceTM intraperitoneally,
25 subcutaneously (Sc) or via footpad injection.

Transgenic mice were immunized twice with antigen in complete Freund's adjuvant or Ribi adjuvant IP, followed by 3-21 days IP (up to a total of 11 immunizations) with the antigen in incomplete Freund's or Ribi adjuvant. The immune response was monitored by retroorbital bleeds. The plasma was screened by ELISA (as described below), and mice with sufficient titers
30 of anti-PD-1 human immunoglobulin were used for fusions. Mice were boosted intravenously

with antigen 3 days before sacrifice and removal of the spleen. Typically, 10-35 fusions for each antigen were performed. Several dozen mice were immunized for each antigen.

Selection of HuMab or KM MiceTM Producing Anti-PD-1 Antibodies:

To select HuMab or KM miceTM producing antibodies that bound PD-1, sera from
 5 immunized mice were tested by ELISA as described by Fishwild, D. *et al.* (1996). Briefly, microtiter plates were coated with purified recombinant PD-1 fusion protein from transfected CHO cells at 1-2 µg /ml in PBS, 100 µl/wells incubated 4°C overnight then blocked with 200 µl/well of 5% fetal bovine serum in PBS/Tween (0.05%). Dilutions of sera from PD-1 immunized mice were added to each well and incubated for 1-2 hours at ambient temperature.
 10 The plates were washed with PBS/Tween and then incubated with a goat-anti-human IgG polyclonal antibody conjugated with horseradish peroxidase (HRP) for 1 hour at room temperature. After washing, the plates were developed with ABTS substrate (Sigma, A-1888, 0.22 mg/ml) and analyzed by spectrophotometer at OD 415-495. Mice that developed the highest titers of anti-PD-1 antibodies were used for fusions. Fusions were performed as
 15 described below and hybridoma supernatants were tested for anti-PD-1 activity by ELISA.

Generation of Hybridomas Producing Human Monoclonal Antibodies to PD-1:

The mouse splenocytes, isolated from the HuMab mice, were fused with PEG to a mouse myeloma cell line based upon standard protocols. The resulting hybridomas were then screened for the production of antigen-specific antibodies. Single cell suspensions of splenocytes from
 20 immunized mice were fused to one-fourth the number of SP2/0 nonsecreting mouse myeloma cells (ATCC, CRL 1581) with 50% PEG (Sigma). Cells were plated at approximately 1x10⁵/well in flat bottom microtiter plate, followed by about two week incubation in selective medium containing 10% fetal bovine serum, 10% P388D1 (ATCC, CRL TIB-63) conditioned medium, 3-5% origen (IGEN) in DMEM (Mediatech, CRL 10013, with high glucose, L-
 25 glutamine and sodium pyruvate) plus 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 mg/ml gentamycin and 1x HAT (Sigma, CRL P-7185). After 1-2 weeks, cells were cultured in medium in which the HAT was replaced with HT. Individual wells were then screened by ELISA (described above) for human anti-PD-1 monoclonal IgG antibodies. Once extensive hybridoma growth occurred, medium was monitored usually after 10-14 days. The antibody-secreting
 30 hybridomas were replated, screened again and, if still positive for human IgG, anti-PD-1

monoclonal antibodies were subcloned at least twice by limiting dilution. The stable subclones were then cultured *in vitro* to generate small amounts of antibody in tissue culture medium for further characterization.

Hybridoma clones 17D8, 2D3, 4H1, 5C4, and 4A11 were selected for further analysis.

5 **Example 10** Structural Characterization of Human Monoclonal Antibodies 17D8, 2D3, 4H1, 5C4, and 4A11

10 The cDNA sequences encoding the heavy and light chain variable regions of the 17D8, 2D3, 4H1, 5C4, and 4A11 monoclonal antibodies were obtained from the 17D8, 2D3, 4H1, 5C4, and 4A11 hybridomas, respectively, using standard PCR techniques and were sequenced using standard DNA sequencing techniques.

 The nucleotide and amino acid sequences of the heavy chain variable region of 17D8 are shown in Figure 30A and in SEQ ID NO: 41 and 1, respectively.

 The nucleotide and amino acid sequences of the light chain variable region of 17D8 are shown in Figure 30B and in SEQ ID NO: 46 and 6, respectively.

15 Comparison of the 17D8 heavy chain immunoglobulin sequence to the known human germline immunoglobulin heavy chain sequences demonstrated that the 17D8 heavy chain utilizes a VH segment from human germline VH 3-33, an undetermined D segment, and a JH segment from human germline JH 4b. The alignment of the 17D8 VH sequence to the germline VH 3-33 sequence is shown in Figure 35. Further analysis of the 17D8 VH sequence using the
20 Kabat system of CDR region determination led to the delineation of the heavy chain CDR1, CDR2 and CD3 regions as shown in Figures 30A and 35, and in SEQ ID NOs: 11, 16 and 21, respectively.

 Comparison of the 17D8 light chain immunoglobulin sequence to the known human germline immunoglobulin light chain sequences demonstrated that the 17D8 light chain utilizes a
25 VL segment from human germline VK L6 and a JK segment from human germline JK 4. The alignment of the 17D8 VL sequence to the germline VK L6 sequence is shown in Figure 7. Further analysis of the 17D8 VL sequence using the Kabat system of CDR region determination led to the delineation of the light chain CDR1, CDR2 and CD3 regions as shown in Figures 30B and 36, and in SEQ ID NOs: 26, 31 and 36, respectively.

The nucleotide and amino acid sequences of the heavy chain variable region of 2D3 are shown in Figure 31A and in SEQ ID NO: 42 and 2, respectively.

The nucleotide and amino acid sequences of the light chain variable region of 2D3 are shown in Figure 31B and in SEQ ID NO: 47 and 7, respectively.

5 Comparison of the 2D3 heavy chain immunoglobulin sequence to the known human
germline immunoglobulin heavy chain sequences demonstrated that the 2D3 heavy chain utilizes
a VH segment from human germline VH 3-33, a D segment from human germline 7-27, and a
JH segment from human germline JH 4b. The alignment of the 2D3 VH sequence to the
germline VH 3-33 sequence is shown in Figure 35. Further analysis of the 2D3 VH sequence
10 using the Kabat system of CDR region determination led to the delineation of the heavy chain
CDR1, CDR2 and CD3 regions as shown in Figures 31A and 35, and in SEQ ID NOs: 12, 17 and
22, respectively.

 Comparison of the 2D3 light chain immunoglobulin sequence to the known human
germline immunoglobulin light chain sequences demonstrated that the 2D3 light chain utilizes a
15 VL segment from human germline VK L6 and a JK segment from human germline JK 4. The
alignment of the 2D3 VL sequence to the germline VK L6 sequence is shown in Figure 8.
Further analysis of the 2D3 VL sequence using the Kabat system of CDR region determination
led to the delineation of the light chain CDR1, CDR2 and CD3 regions as shown in Figures 31B
and 37, and in SEQ ID NOs: 27, 32 and 37, respectively.

20 The nucleotide and amino acid sequences of the heavy chain variable region of 4H1 are
shown in Figure 32A and in SEQ ID NO: 43 and 3, respectively.

 The nucleotide and amino acid sequences of the light chain variable region of 4H1 are
shown in Figure 32B and in SEQ ID NO: 48 and 8, respectively.

 Comparison of the 4H1 heavy chain immunoglobulin sequence to the known human
25 germline immunoglobulin heavy chain sequences demonstrated that the 4H1 heavy chain utilizes
a VH segment from human germline VH 3-33, an undetermined D segment, and a JH segment
from human germline JH 4b. The alignment of the 4H1 VH sequence to the germline VH 3-33
sequence is shown in Figure 35. Further analysis of the 4H1 VH sequence using the Kabat
system of CDR region determination led to the delineation of the heavy chain CDR1, CDR2 and
30 CD3 regions as shown in Figures 32A and 35, and in SEQ ID NOs: 13, 18 and 23, respectively.

Comparison of the 4H1 light chain immunoglobulin sequence to the known human germline immunoglobulin light chain sequences demonstrated that the 4H1 light chain utilizes a VL segment from human germline VK L6 and a JK segment from human germline JK 1. The alignment of the 4H1 VL sequence to the germline VK L6 sequence is shown in Figure 38.

5 Further analysis of the 4H1 VL sequence using the Kabat system of CDR region determination led to the delineation of the light chain CDR1, CDR2 and CD3 regions as shown in Figures 32B and 38, and in SEQ ID NOs: 28, 33 and 38, respectively.

The nucleotide and amino acid sequences of the heavy chain variable region of 5C4 are shown in Figure 33A and in SEQ ID NO: 44 and 4, respectively.

10 The nucleotide and amino acid sequences of the light chain variable region of 5C4 are shown in Figure 33B and in SEQ ID NO: 49 and 9, respectively.

Comparison of the 5C4 heavy chain immunoglobulin sequence to the known human germline immunoglobulin heavy chain sequences demonstrated that the 5C4 heavy chain utilizes a VH segment from human germline VH 3-33, an undetermined D segment, and a JH segment
15 from human germline JH 4b. The alignment of the 5C4 VH sequence to the germline VH 3-33 sequence is shown in Figure 35. Further analysis of the 5C4 VH sequence using the Kabat system of CDR region determination led to the delineation of the heavy chain CDR1, CDR2 and CD3 regions as shown in Figures 33A and 35, and in SEQ ID NOs: 14, 19 and 24, respectively.

Comparison of the 5C4 light chain immunoglobulin sequence to the known human
20 germline immunoglobulin light chain sequences demonstrated that the 5C4 light chain utilizes a VL segment from human germline VK L6 and a JK segment from human germline JK 1. The alignment of the 5C4 VL sequence to the germline VK L6 sequence is shown in Figure 39. Further analysis of the 5C4 VL sequence using the Kabat system of CDR region determination led to the delineation of the light chain CDR1, CDR2 and CD3 regions as shown in Figures 33B
25 and 39, and in SEQ ID NOs: 29, 34 and 39, respectively.

The nucleotide and amino acid sequences of the heavy chain variable region of 4A11 are shown in Figure 34A and in SEQ ID NO: 45 and 5, respectively.

The nucleotide and amino acid sequences of the light chain variable region of 4A11 are shown in Figure 34B and in SEQ ID NO: 50 and 10, respectively.

30 Comparison of the 4A11 heavy chain immunoglobulin sequence to the known human germline immunoglobulin heavy chain sequences demonstrated that the 4A11 heavy chain

utilizes a VH segment from human germline VH 4-39, a D segment from human germline 3-9, and a JH segment from human germline JH 4b. The alignment of the 4A11 VH sequence to the germline VH 4-39 sequence is shown in Figure 40. Further analysis of the 4A11 VH sequence using the Kabat system of CDR region determination led to the delineation of the heavy chain CDR1, CDR2 and CD3 regions as shown in Figures 5A and 40, and in SEQ ID NOs: 15, 20 and 25, respectively.

Comparison of the 4A11 light chain immunoglobulin sequence to the known human germline immunoglobulin light chain sequences demonstrated that the 4A11 light chain utilizes a VL segment from human germline VK L15 and a JK segment from human germline JK 1. The alignment of the 4A11 VL sequence to the germline VK L6 sequence is shown in Figure 41. Further analysis of the 4A11 VL sequence using the Kabat system of CDR region determination led to the delineation of the light chain CDR1, CDR2 and CD3 regions as shown in Figures 34B and 41, and in SEQ ID NOs: 30, 35 and 40, respectively.

Example 11 Characterization of Binding Specificity and Binding Kinetics of Anti-PD-1 Human Monoclonal Antibodies

In this example, binding affinity and binding kinetics of anti-PD-1 antibodies were examined by Biacore analysis. Binding specificity and cross-competition were examined by flow cytometry.

Binding Affinity and Kinetics

Anti-PD-1 antibodies were characterized for affinities and binding kinetics by Biacore analysis (Biacore AB, Uppsala, Sweden). Purified recombinant human PD-1 fusion protein was covalently linked to a CM5 chip (carboxy methyl dextran coated chip) via primary amines, using standard amine coupling chemistry and kit provided by Biacore. Binding was measured by flowing the antibodies in HBS EP buffer (provided by Biacore AB) at a concentration of 267 nM at a flow rate of 50 µl/min. The antigen-antibody association kinetics was followed for 3 minutes and the dissociation kinetics was followed for 7 minutes. The association and dissociation curves were fit to a 1:1 Langmuir binding model using BIAevaluation software (Biacore AB). To minimize the effects of avidity in the estimation of the binding constants, only

the initial segment of data corresponding to association and dissociation phases were used for fitting. The K_D , k_{on} and k_{off} values that were determined are shown in Table 4.

Table 4. Biacore binding data for PD-1 human monoclonal antibodies.

Sample #	Sample ID	Affinity $K_D \times 10^{-9}$ (M)	On rate $k_{on} \times 10^5$ (1/Ms)	Off rate $k_{off} \times 10^{-4}$ 1/s
1	17D8	0.16	2.56	0.45
2	2D3	1.20	3.77	4.52
3	4H1	5.46	3.15	1.72
4	5C4	0.73	4.32	3.15
5	4A11	0.13	0.76	0.099

Binding Specificity by Flow Cytometry

Chinese hamster ovary (CHO) cell lines that express recombinant human PD-1 at the cell surface were developed and used to determine the specificity of PD-1 human monoclonal antibodies by flow cytometry. CHO cells were transfected with expression plasmids containing full length cDNA encoding transmembrane forms of PD-1. Binding of the 5C4 and 4H1 anti-PD-1 human monoclonal antibodies was assessed by incubating the transfected cells with the anti-PD-1 human monoclonal antibodies at a concentration of 20 μ g/ml. The cells were washed and binding was detected with a FITC-labeled anti-human IgG Ab. Flow cytometric analyses were performed using a FACScan flow cytometry (Becton Dickinson, San Jose, CA). The results are depicted in Figures 42A (5C4) and 42B (4H1). The anti-PD-1 human monoclonal antibodies bound to the CHO cells transfected with PD-1 but not to CHO cells that were not transfected with human PD-1. These data demonstrate the specificity of anti-PD-1 human monoclonal antibodies for PD-1.

Binding specificity by ELISA against other CD28 family members

A comparison of the binding of anti-PD-1 antibodies to CD28 family members was performed by standard ELISA using four different CD28 family members to examine the specificity of binding for PD-1.

Fusion proteins of CD28 family members, ICOS, CTLA-4 and CD28 (R&D Biosystems) were tested for binding against the anti-PD-1 human monoclonal antibodies 17D8, 2D3, 4H1, 5C4, and 4A11. Standard ELISA procedures were performed. The anti-PD-1 human monoclonal antibodies were added at a concentration of 20 μ g/ml. Goat-anti-human IgG (κ)

chain-specific) polyclonal antibody conjugated with horseradish peroxidase (HRP) was used as secondary antibody. Each of the anti-PD-1 human monoclonal antibodies 17D8, 2D3, 4H1, 5C4, and 4A11 bound with high specificity to PD-1, but not to the other CD28 family members (data not shown).

5 The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims. The invention is, therefore, to be limited only by the terms of the appended claims along with the full
10 scope of equivalents to which the claims are entitled.

Patents, patent applications, publications, product descriptions, and protocols cited in this application are hereby incorporated by reference in their entireties for all purposes.

CLAIMS

We claim:

1. A method for treating a hyperproliferative disease, which method comprises:
 - (a) administering a anti-PD-1 antibody to a subject, and
 - (b) administering a anti-CTLA-4 antibody to the subject.
2. The method of claim 1, wherein the anti-PD-1 antibody is administered at a subtherapeutic dose.
3. The method of claim 1, wherein the anti-CTLA-4 antibody is administered at a subtherapeutic dose.
4. The method of claim 1, wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are each administered at a subtherapeutic dose.
5. The method according to claim 1 wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are administered sequentially.
6. The method according to claim 5 wherein the anti-PD-1 antibody is administered before the anti-CTLA-4 antibody.
7. The method according to claim 5 wherein the anti-CTLA-4 antibody is administered before the anti-PD-1 antibody.
8. The method according to claim 1 wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are administered concurrently.
9. The method according to claim 1 wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are admixed as a single composition and administered concurrently.
10. The method according to claim 1 comprising administering to a subject (a) a composition comprising the anti-PD-1 antibody and a pharmaceutically acceptable carrier and (b) a composition comprising the anti-CTLA-4 antibody and a pharmaceutically acceptable carrier.

11. The method of any one of claims 1 to 10 wherein the hyperproliferative disease is cancer.
12. The method of claim 11 wherein the cancer is colon cancer.
13. The method of any one of claims 1 to 10 wherein the subject is a human.
14. The method of any one of claims 1 to 10 wherein the anti-PD-1 antibody is a human sequence antibody.
15. The method of claim 14 wherein the anti-PD-1 human sequence antibody is a monoclonal antibody.
16. The method of claim 15 wherein the anti-PD-1 human sequence monoclonal antibody is 17D8, 2D3, 4H1, 5C4 or 4A11.
17. The method of any one of claims 1 to 10 wherein the anti-CTLA-4 antibody is a human sequence antibody.
18. The method of claim 17 wherein the anti-CTLA-4 human sequence antibody is a monoclonal antibody.
19. The method of claim 18 wherein the anti-CTLA-4 human sequence monoclonal antibody is 10D1 (MDX-010).
20. A method for altering an adverse event associated with treatment of a hyperproliferative disease with an immunostimulatory agent, which method comprises:
 - (a) administering an anti-PD-1 antibody to a subject, and
 - (b) administering a subtherapeutic dose of anti-CTLA-4 antibody to the subject.
21. The method of claim 18 wherein the anti-PD-1 antibody is administered in a subtherapeutic dose.
22. The method according to claim 20 wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are administered sequentially.

23. The method according to claim 22 wherein the anti-PD-1 antibody is administered before the anti-CTLA-4 antibody.
24. The method according to claim 22 wherein the anti-CTLA-4 antibody is administered before the anti-PD-1 antibody.
25. The method according to claim 20 wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are administered concurrently.
26. The method according to claim 20 wherein the anti-PD-1 antibody and the anti-CTLA-4 antibody are admixed as a single composition and administered concurrently.
27. The method according to claim 20 comprising administering to a subject (a) a composition comprising the anti-PD-1 antibody and a pharmaceutically acceptable carrier and (b) a composition comprising the anti-CTLA-4 antibody and a pharmaceutically acceptable carrier.
28. The method of any one of claims 20 to 27 wherein the hyperproliferative disease is cancer.
29. The method of claim 28 wherein the cancer is colon cancer.
30. The method of any one of claims 20 to 27 wherein the subject is a human.
31. The method of any one of claims 20 to 27 wherein the anti-PD-1 antibody is a human sequence antibody.
32. The method of claim 31 wherein the anti-PD-1 human sequence antibody is a monoclonal antibody.
33. The method of claim 32 wherein the anti-PD-1 human sequence monoclonal antibody is 17D8, 2D3, 4H1, 5C4 or 4A11.
34. The method of any one of claims 20 to 27 wherein the anti-CTLA-4 antibody is a human sequence antibody.

35. The method of claim 34 wherein the anti-CTLA-4 human sequence antibody is a monoclonal antibody.
36. The method of claim 35 wherein the anti-CTLA-4 human sequence monoclonal antibody is 10D1 (MDX-010).

ABSTRACT

The present invention provides methods for using a combination immunotherapy, such as the combination of anti-CTLA-4 and anti-PD-1 antibodies, to treat hyperproliferative disease, such as cancer. The invention also provides methods for altering adverse events related to treatment with such antibodies individually.

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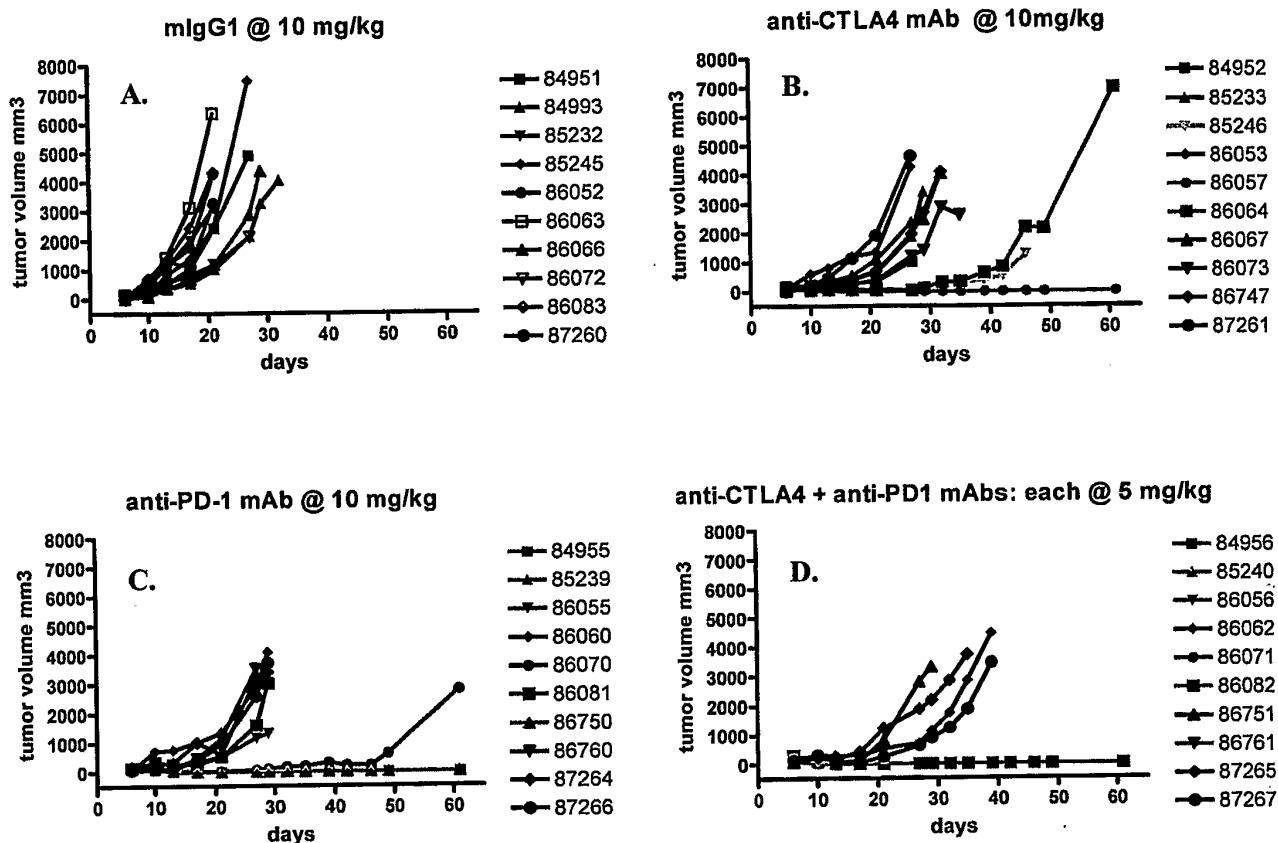


Figure 1

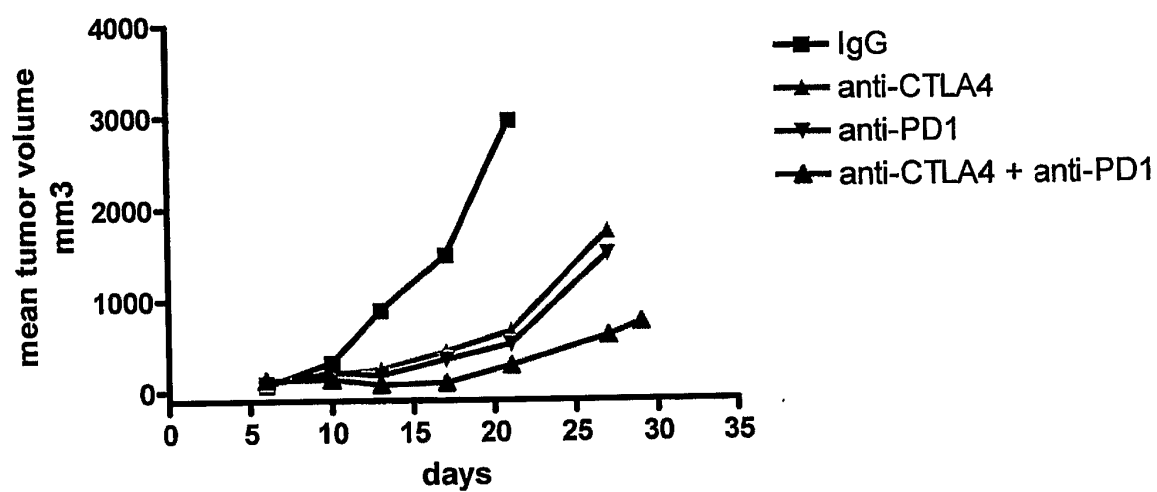


Figure 2

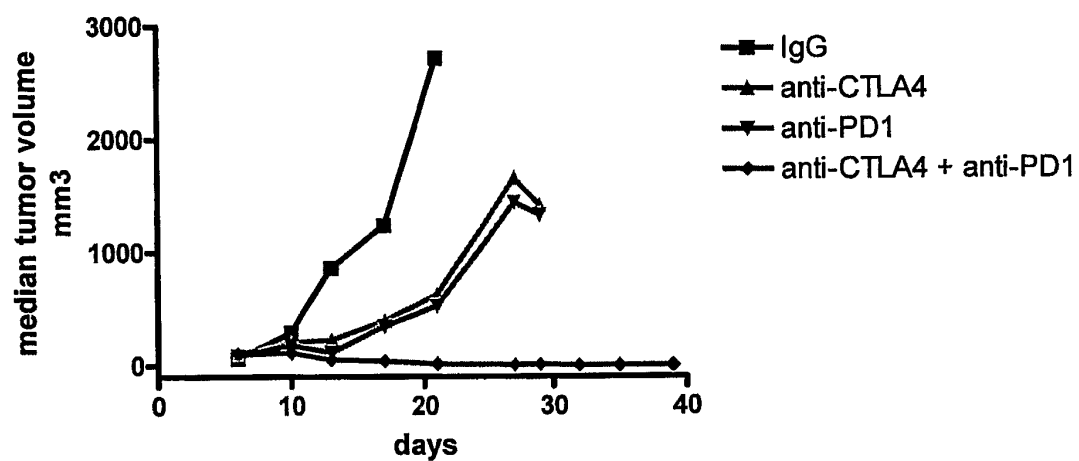


Figure 3

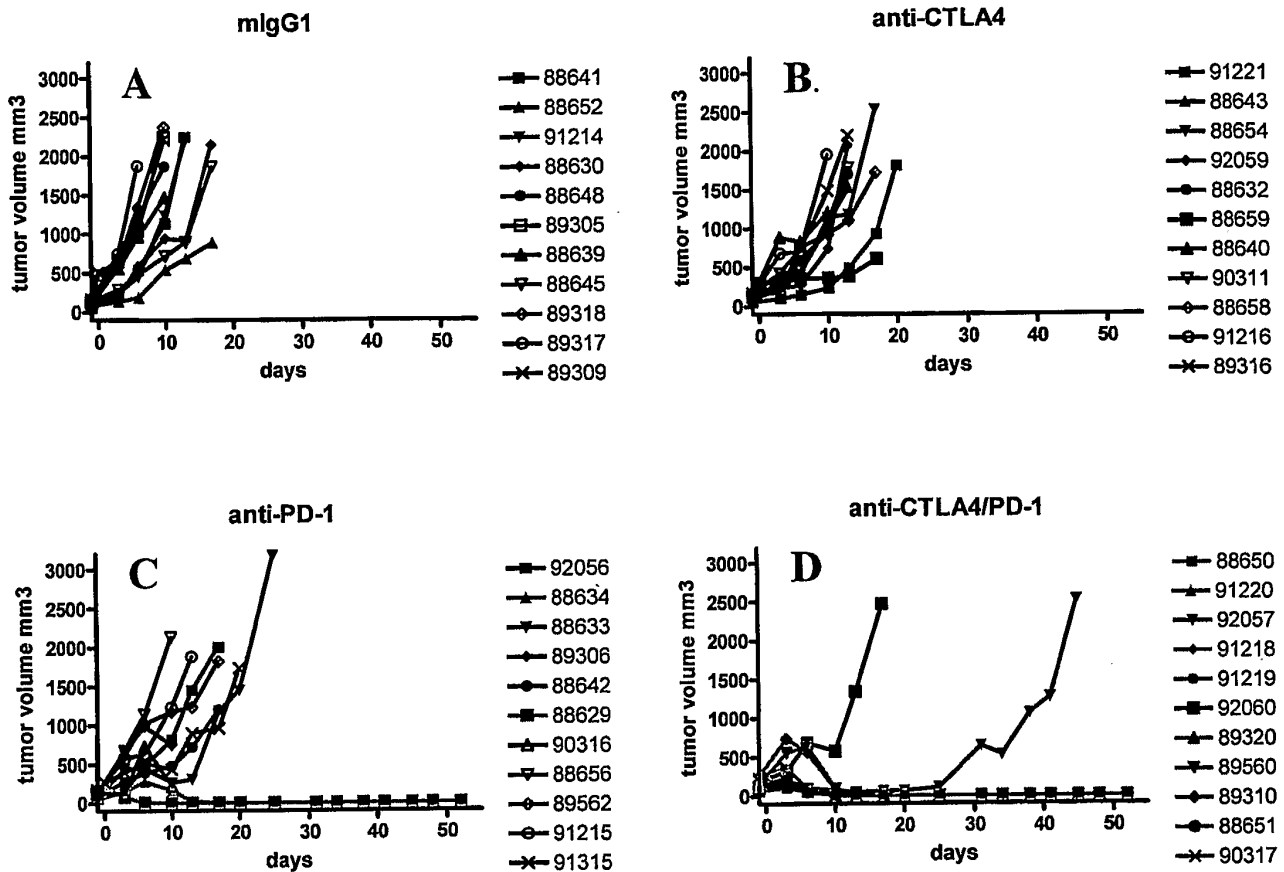


Figure 4

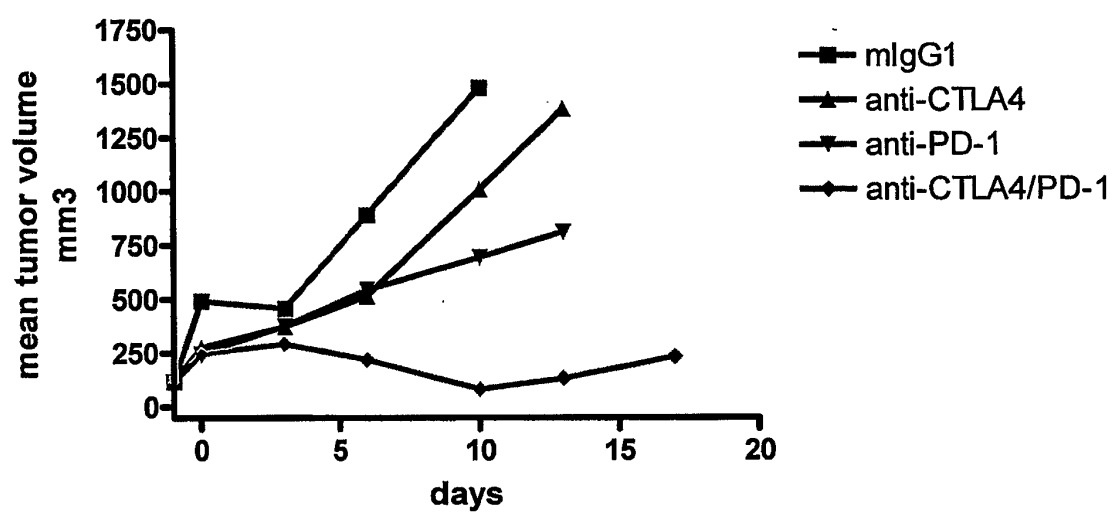


Figure 5

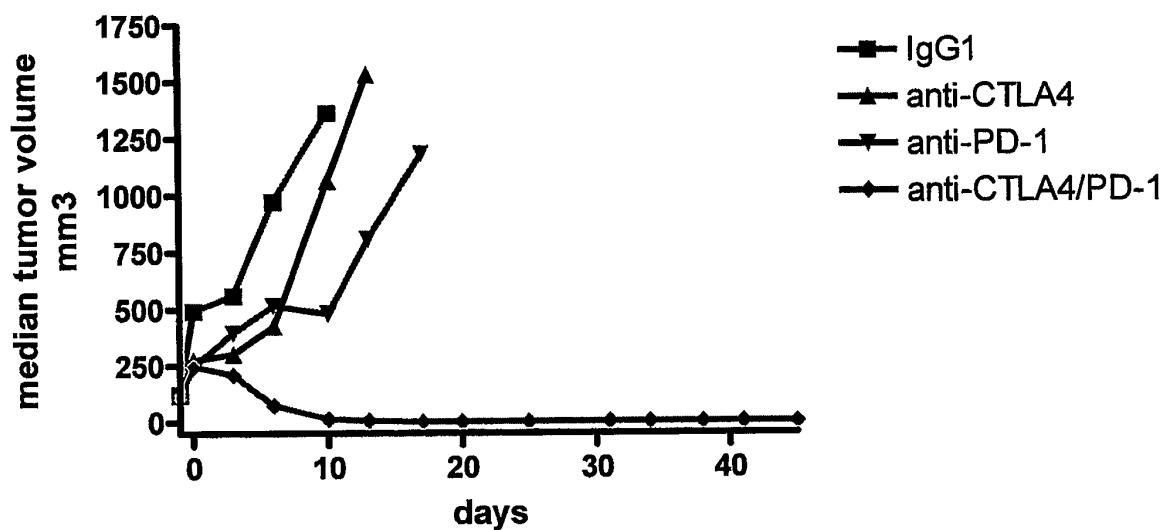


Figure 6

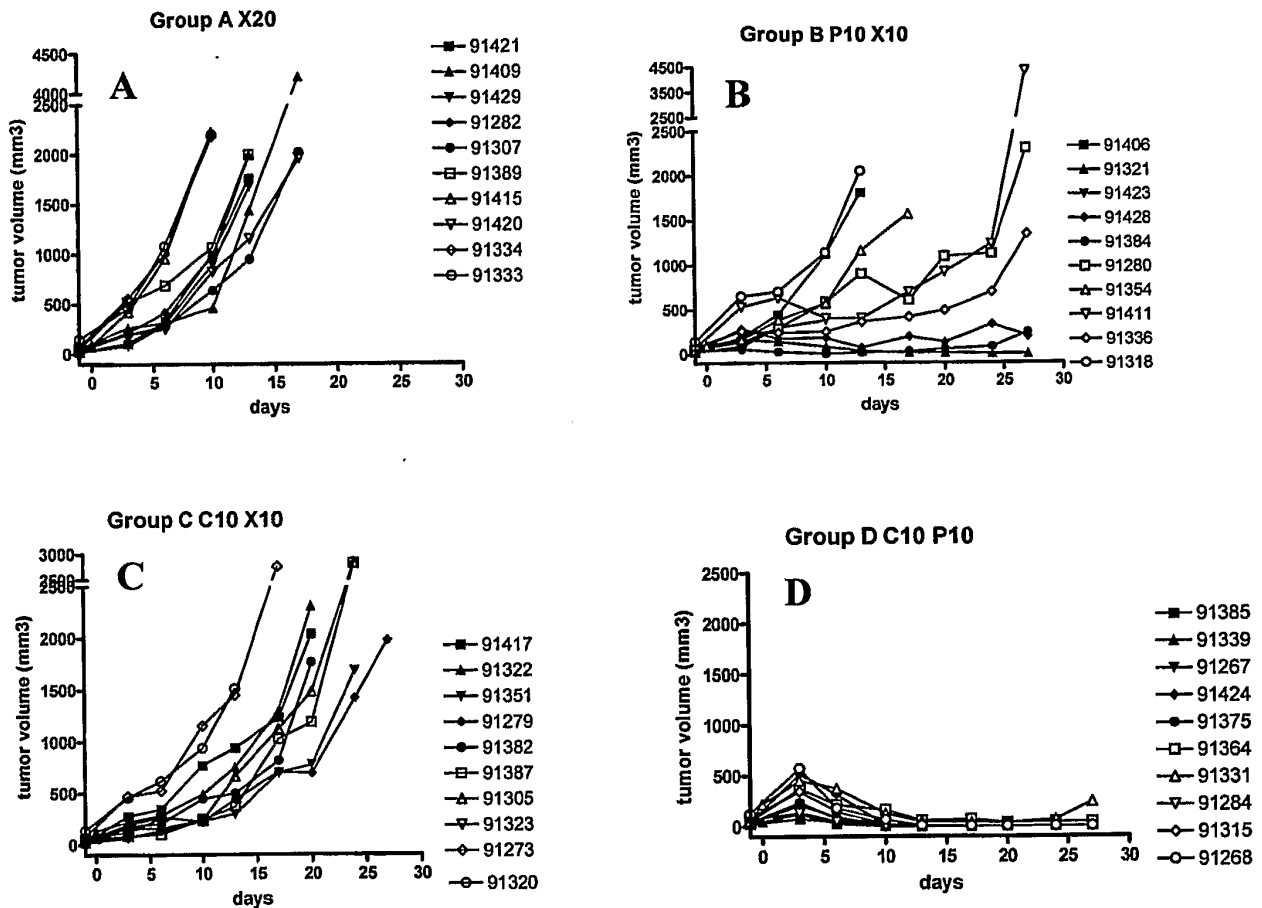


Figure 7

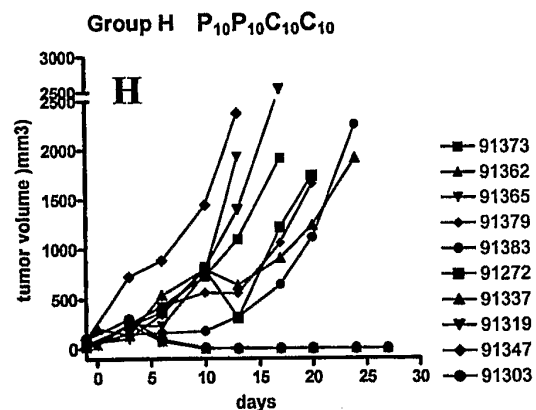
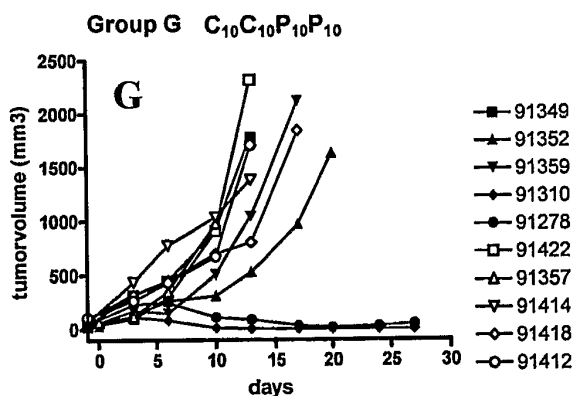
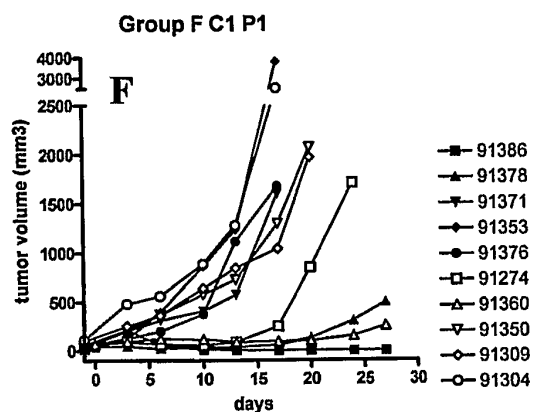
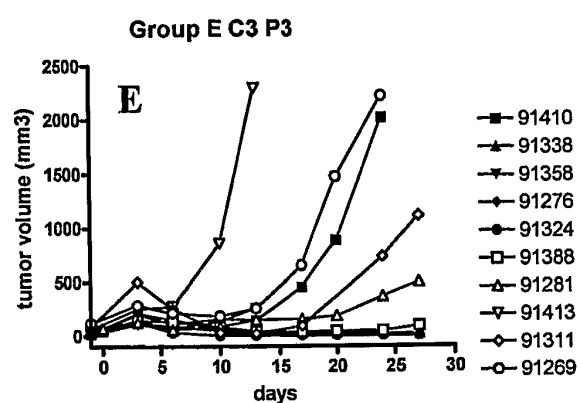


Figure 7 (cont.)

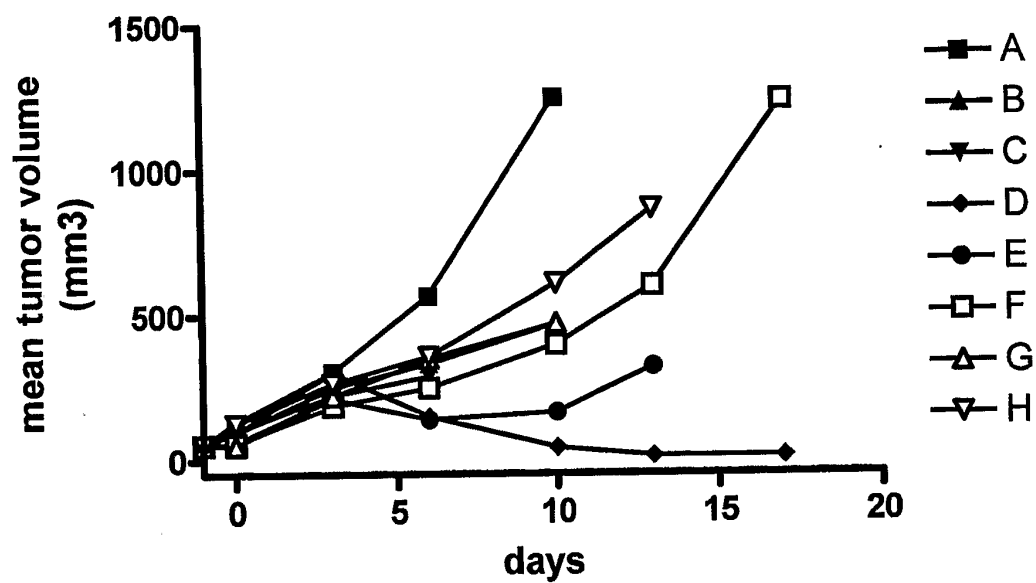


Figure 8

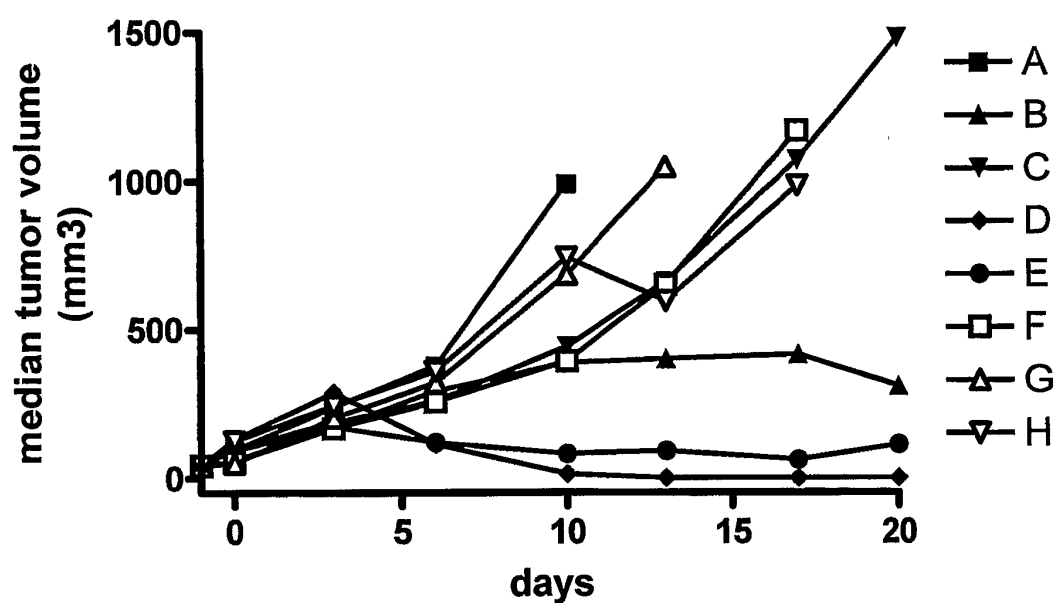


Figure 9

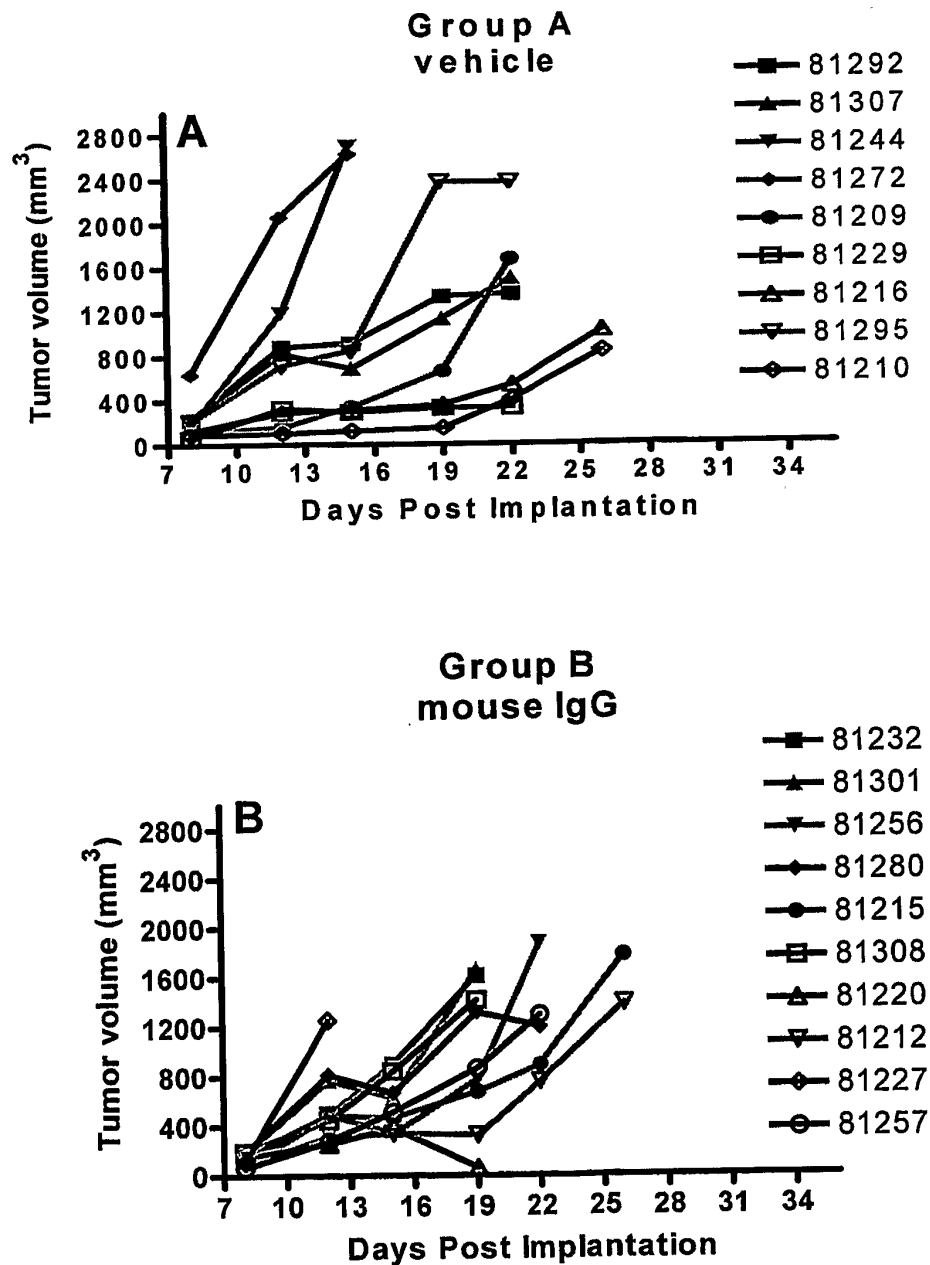
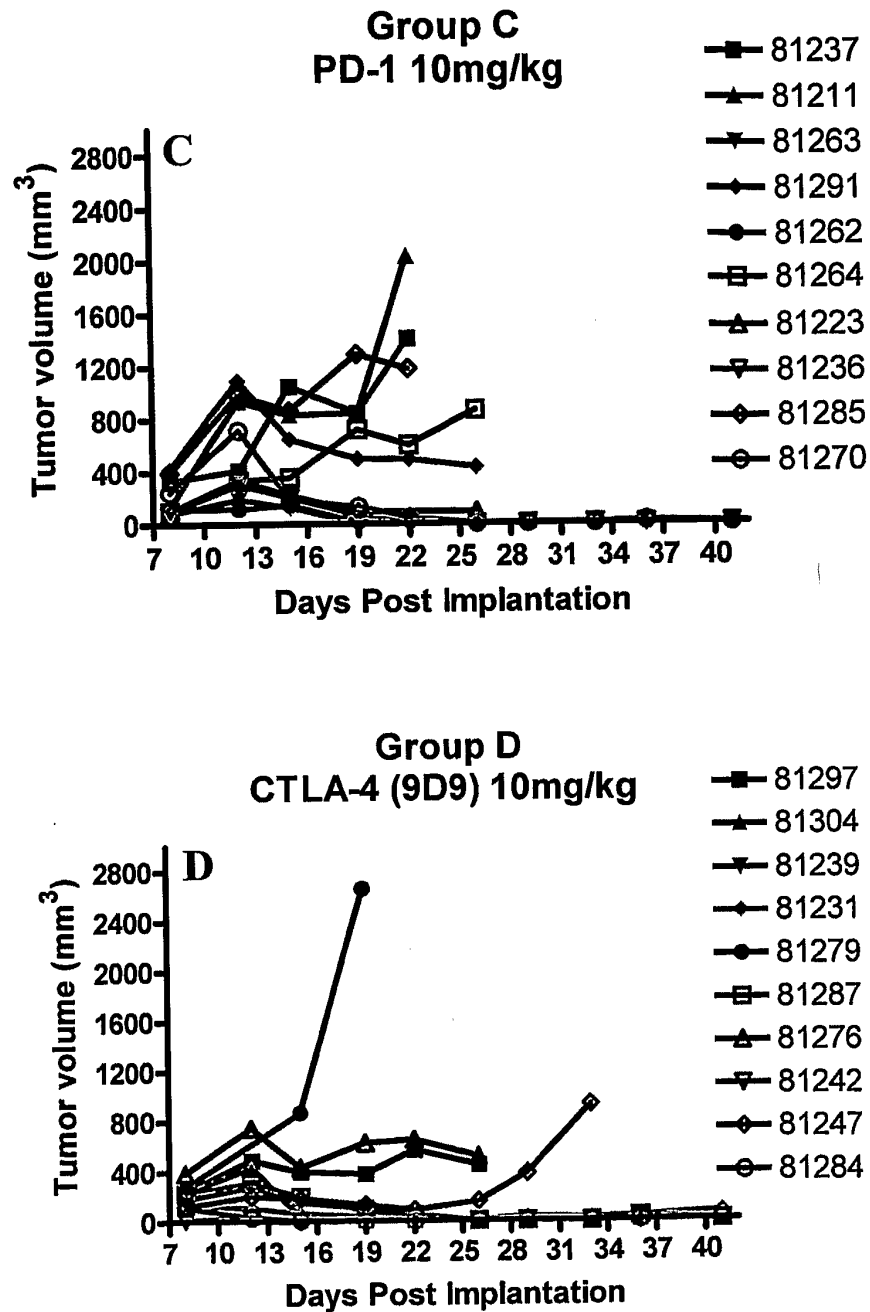
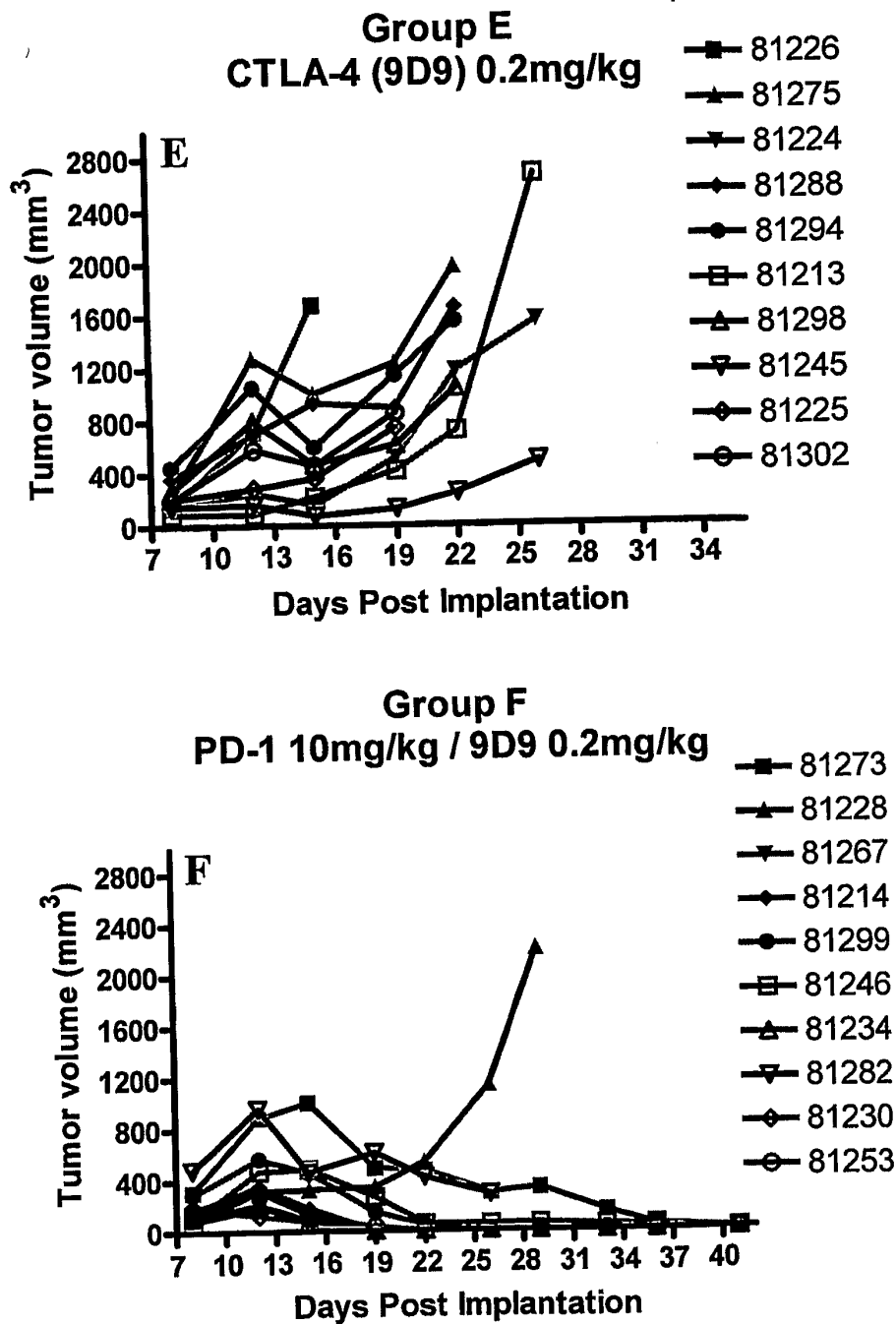


Figure 10





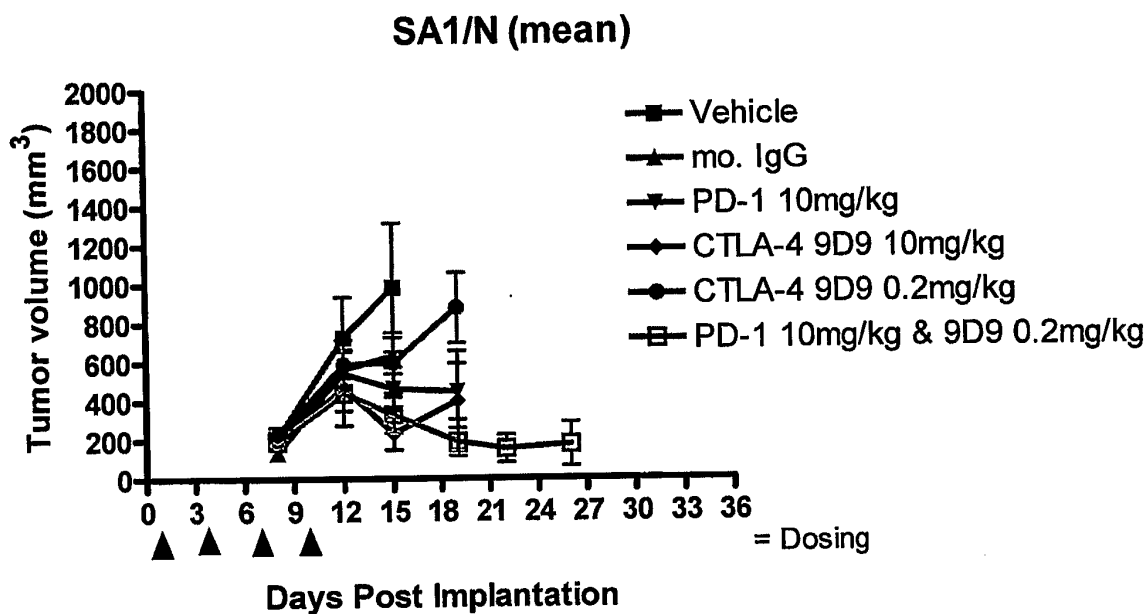


Figure 11

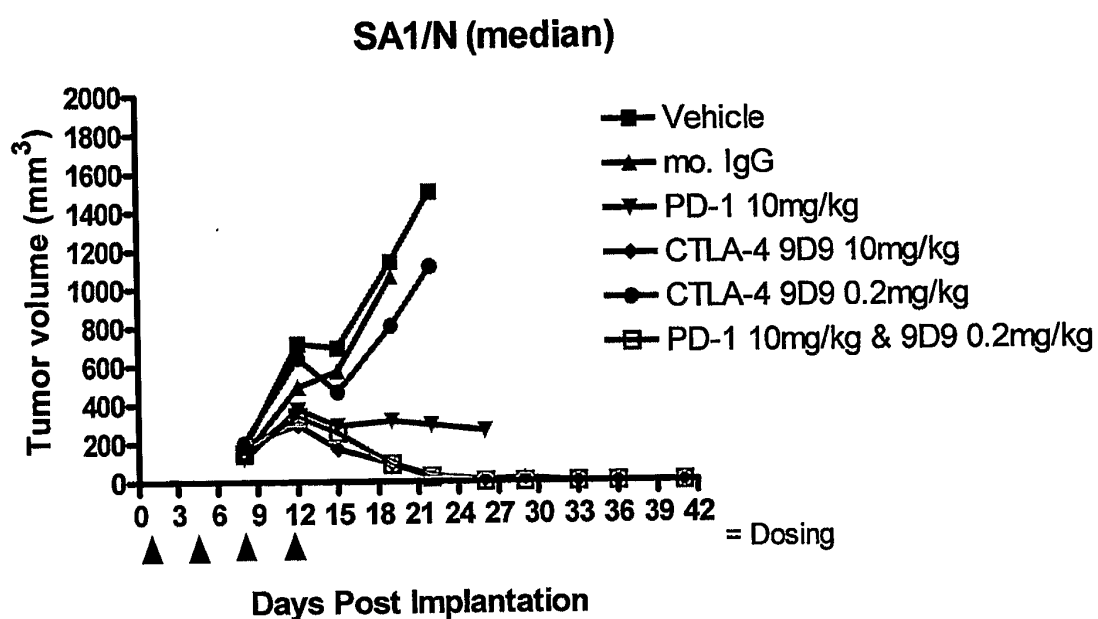


Figure 12

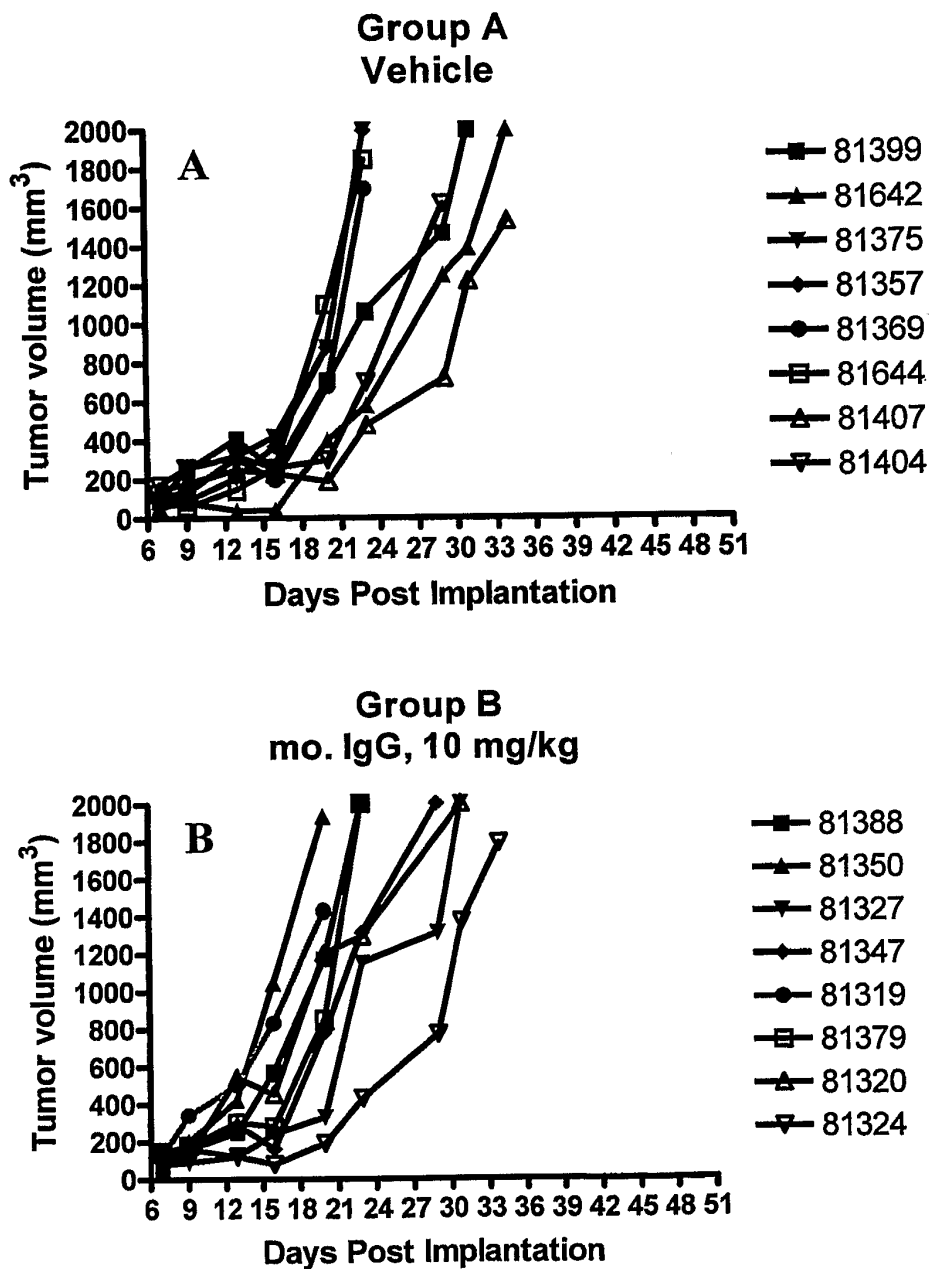


Figure 13

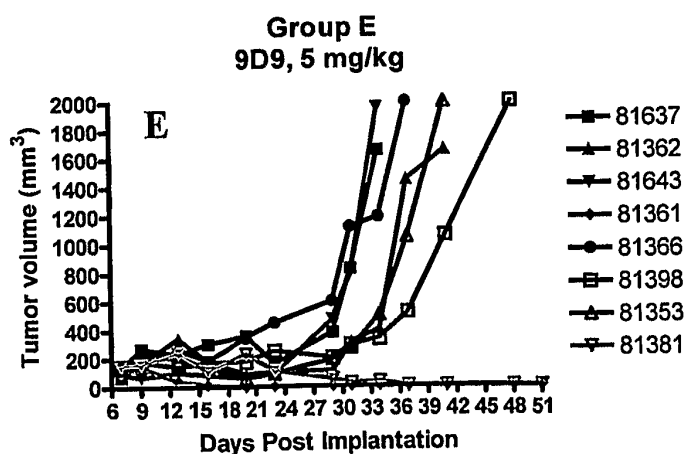
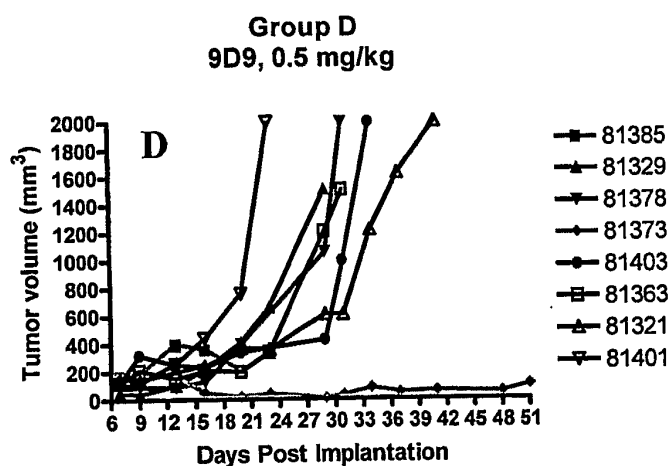
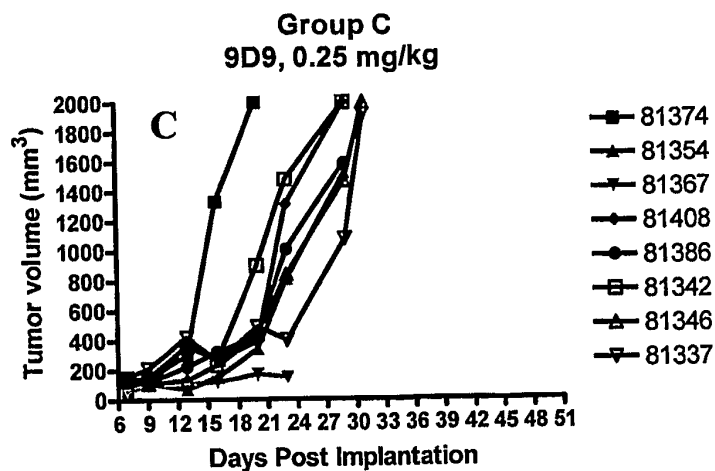


Figure 13 (cont.)

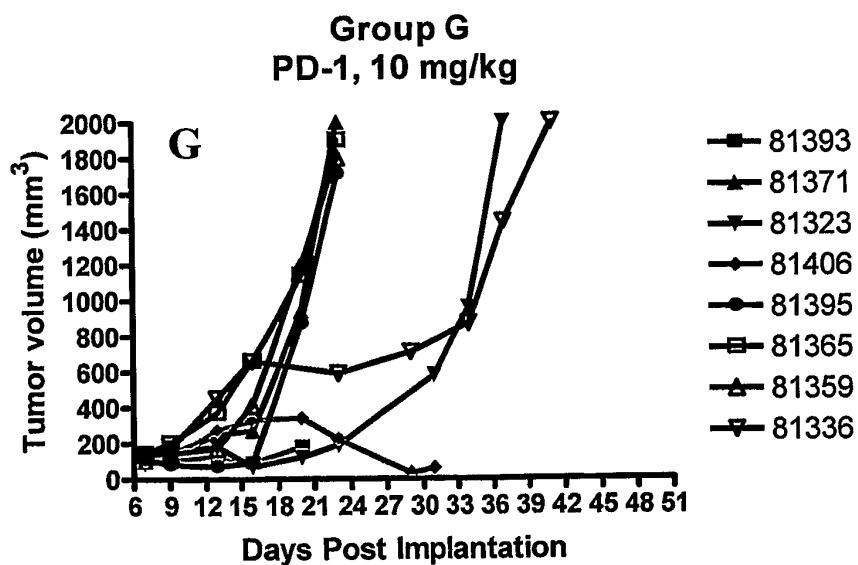
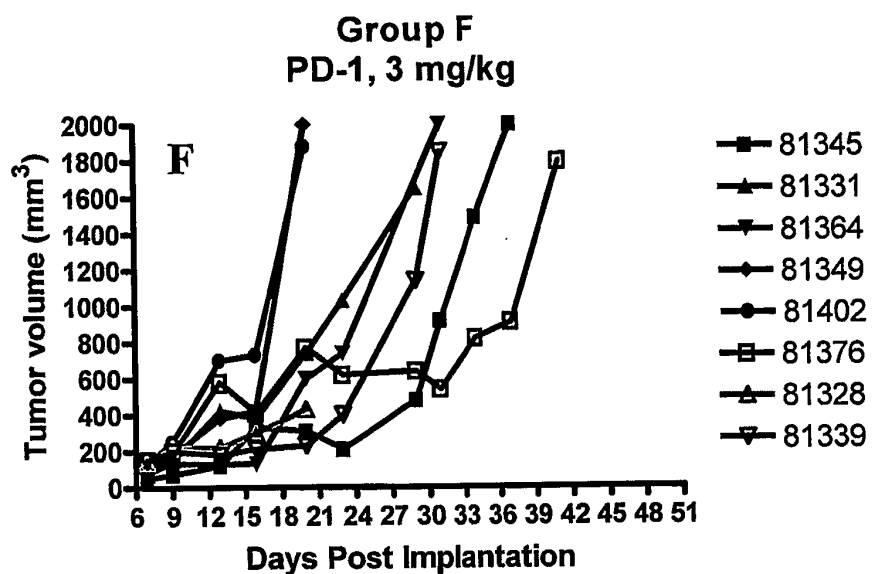


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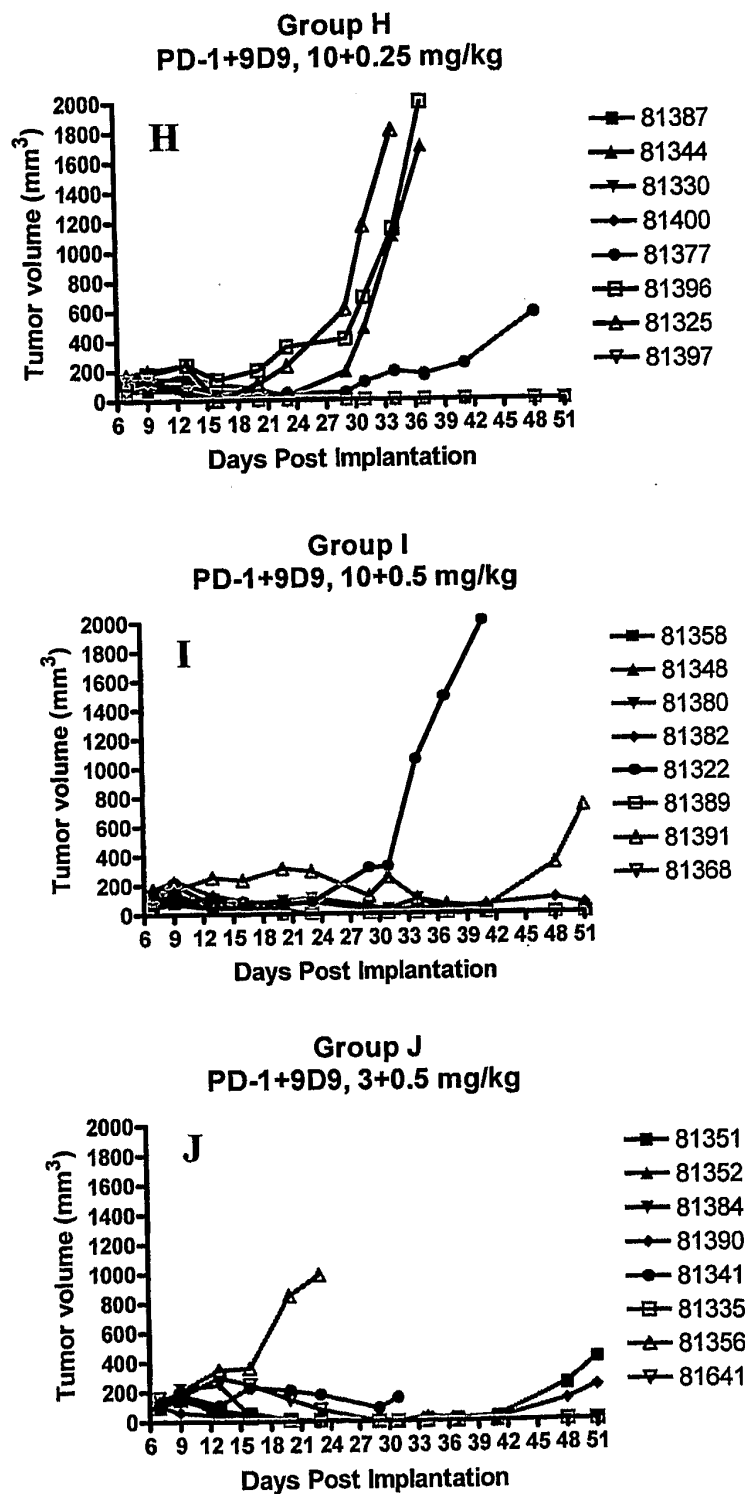


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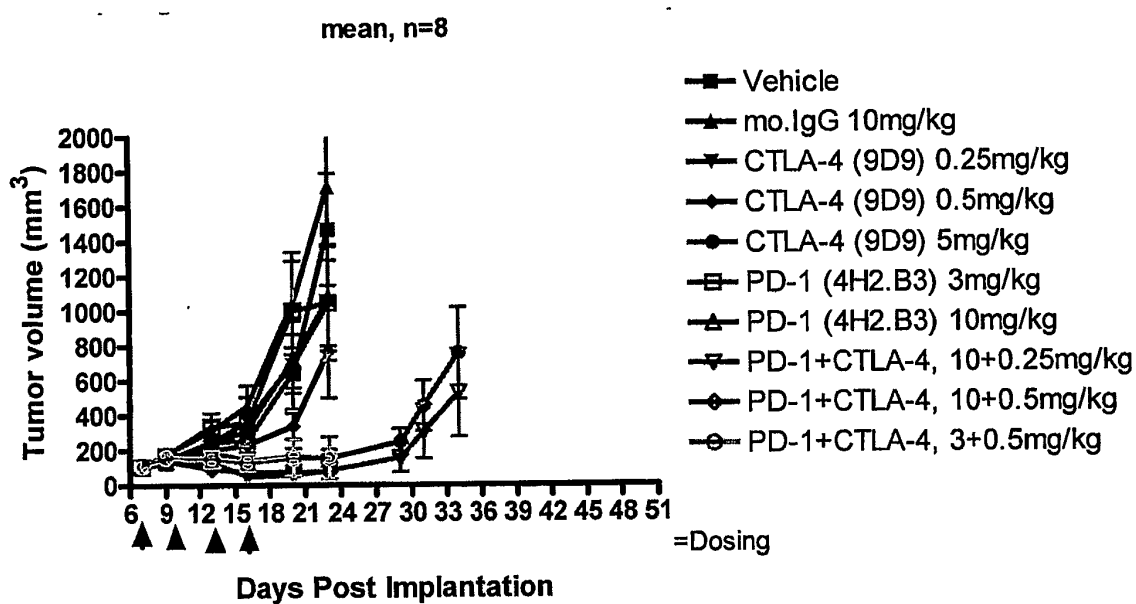


Figure 14

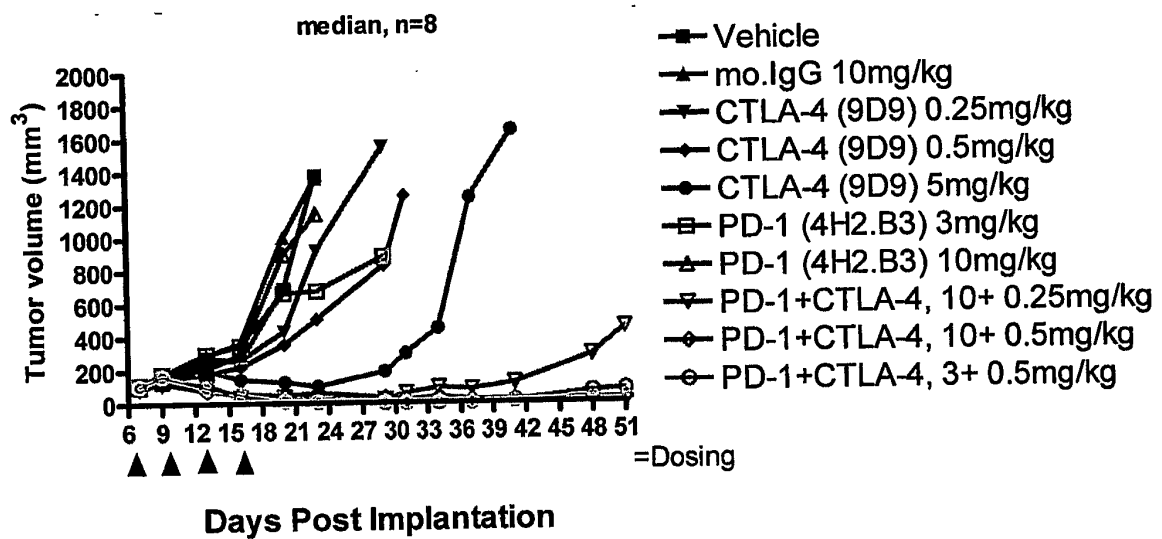


Figure 15

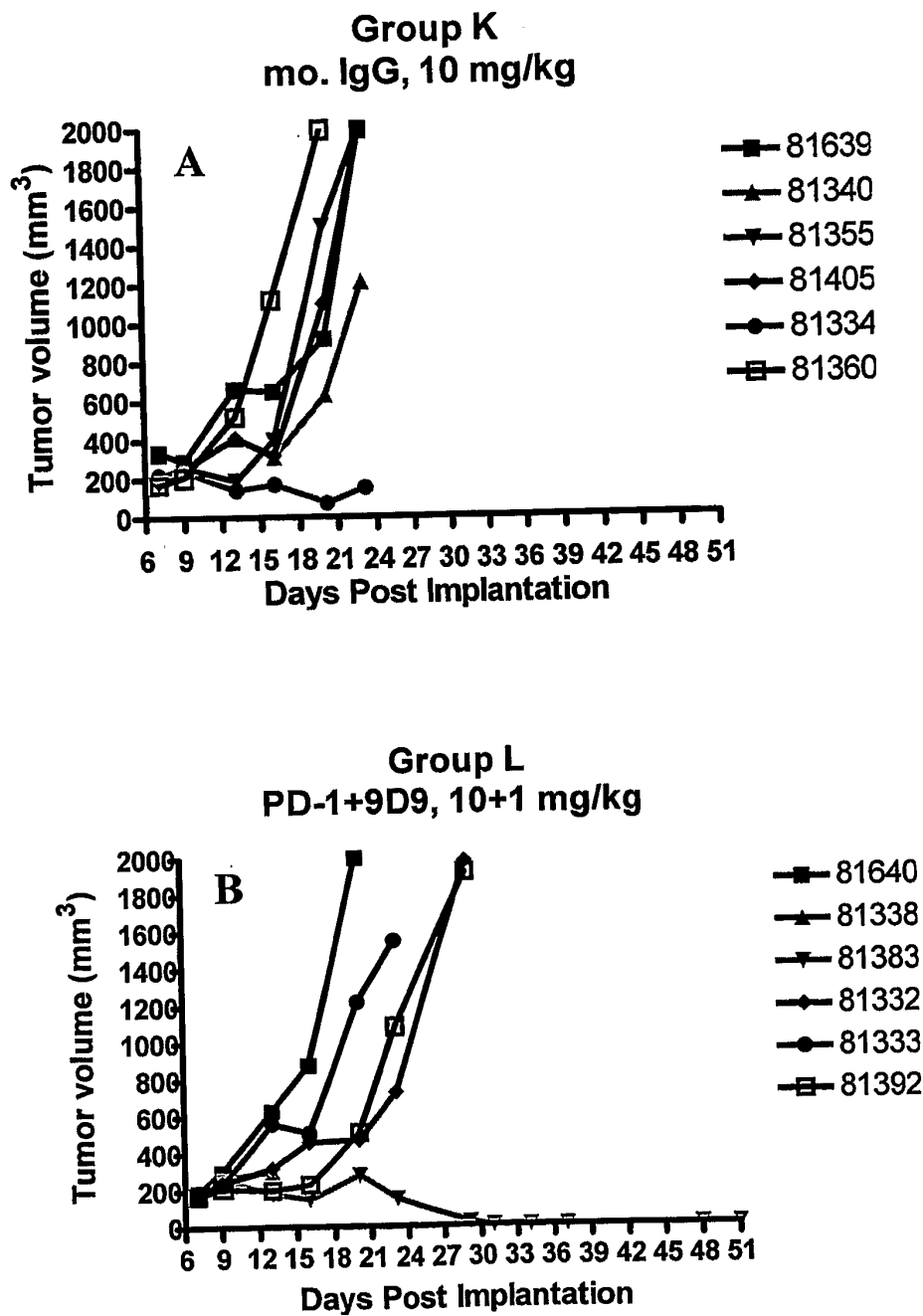


Figure 16

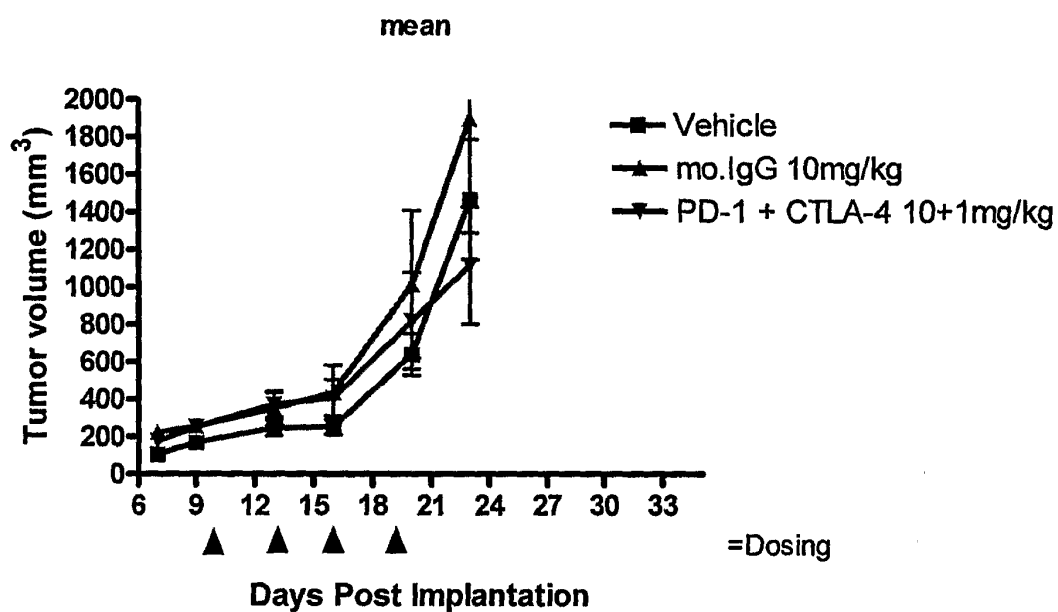


Figure 17

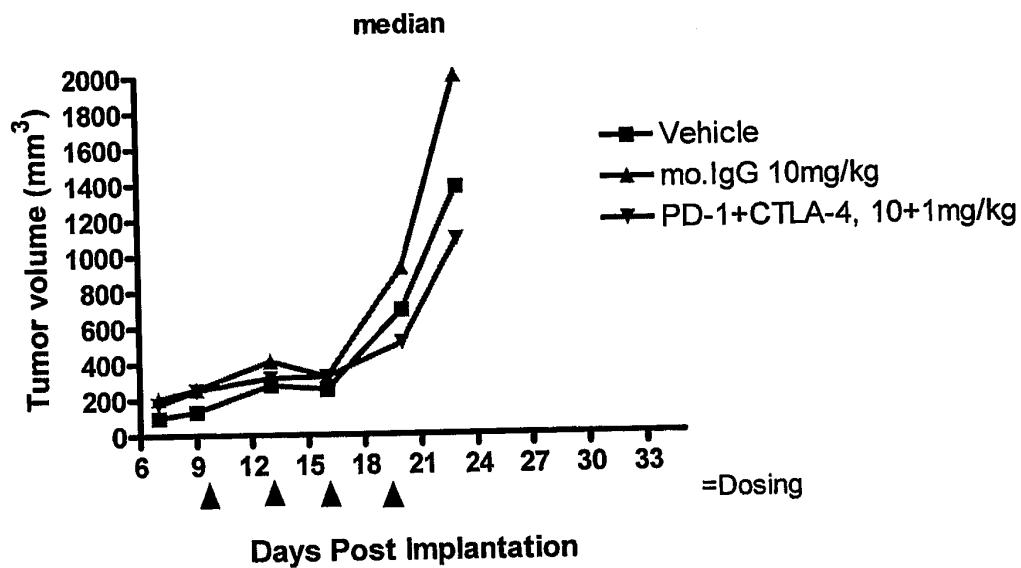


Figure 18

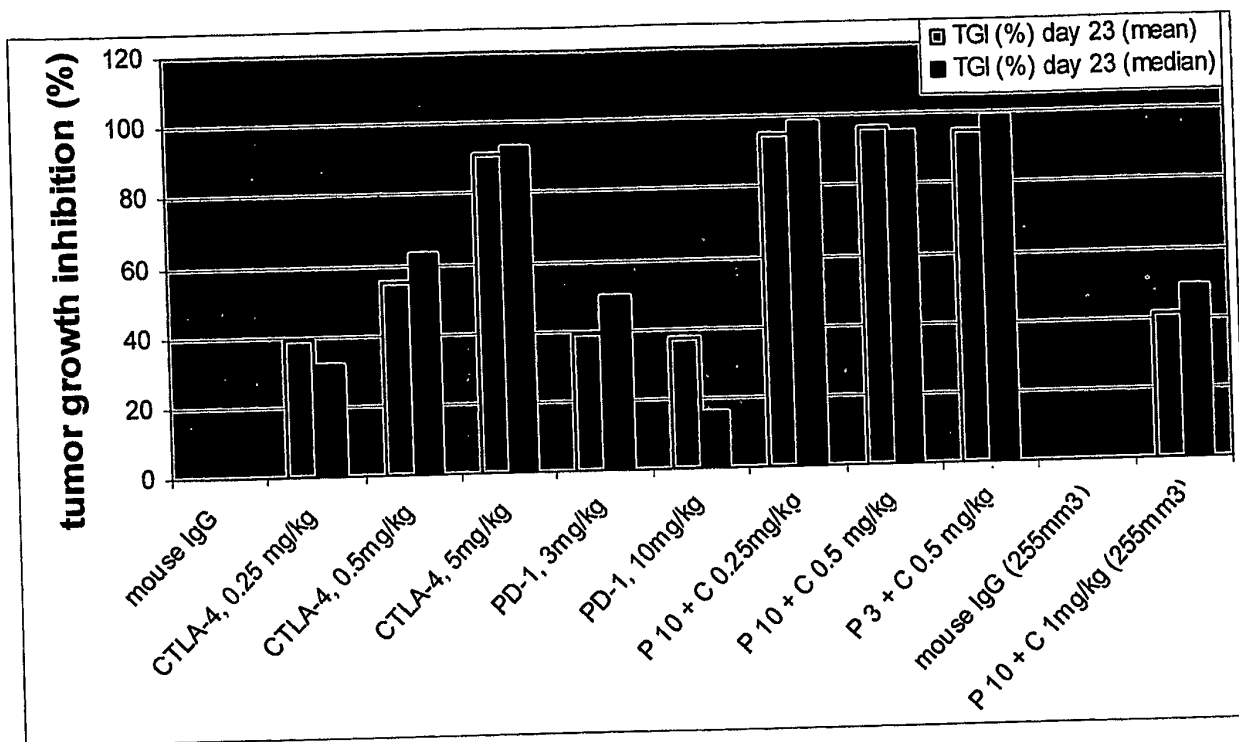


Figure 19

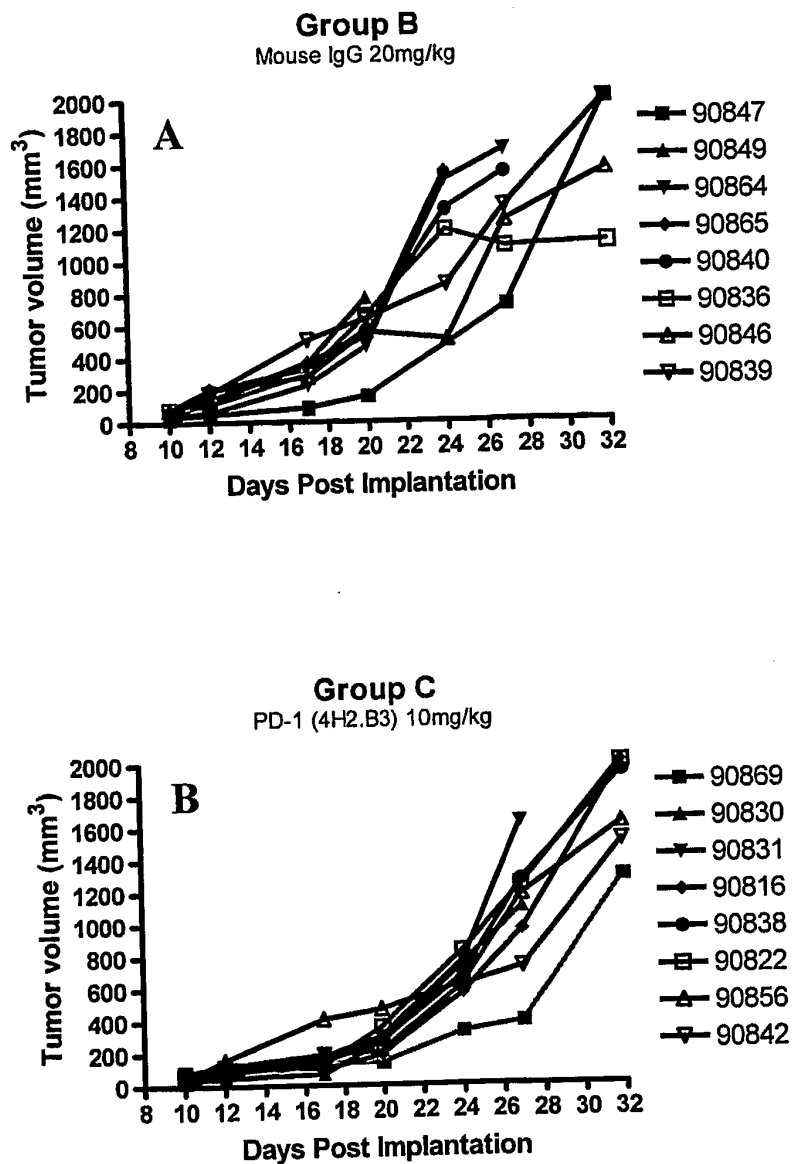


Figure 20

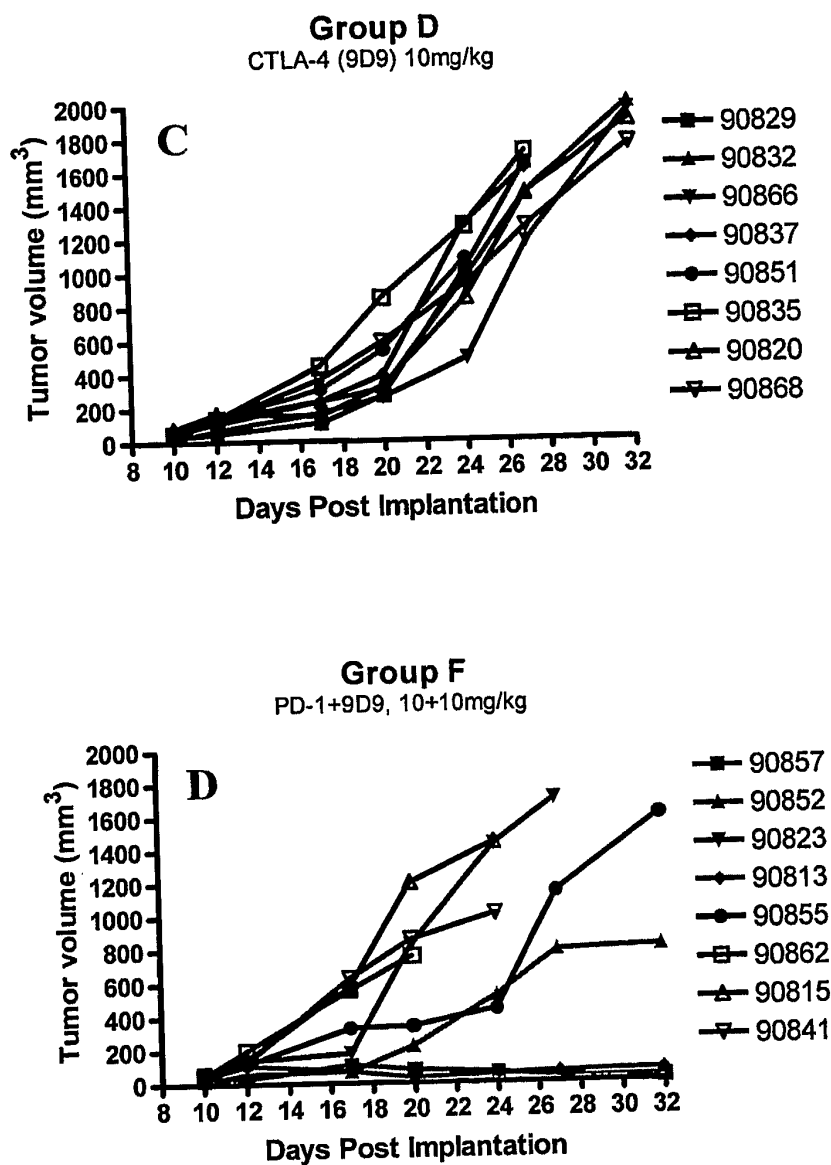


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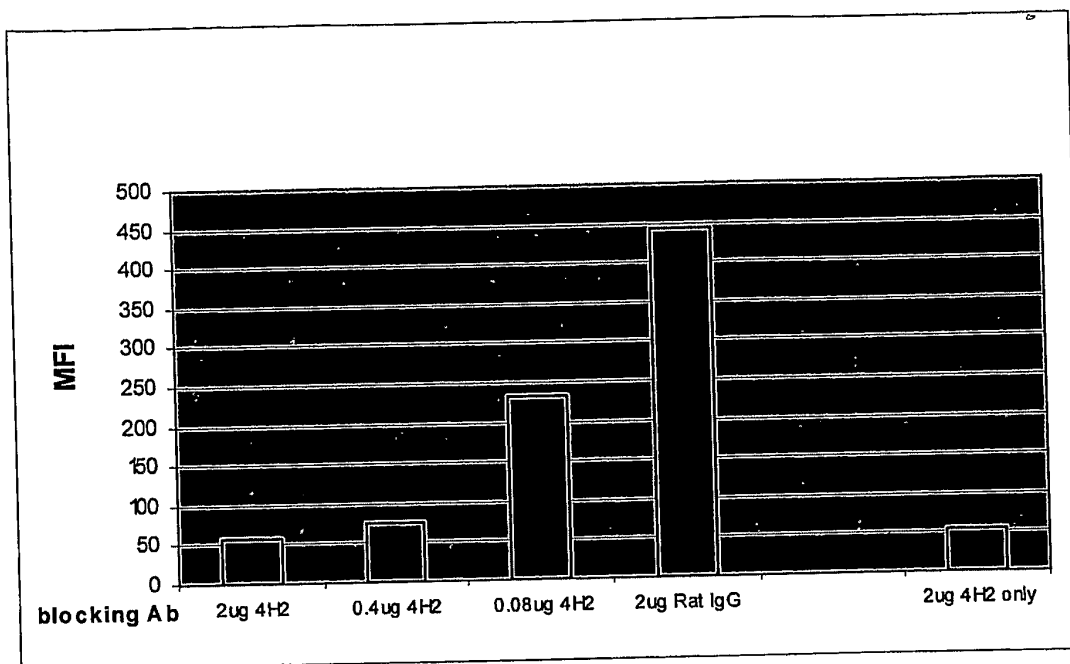


Figure 21

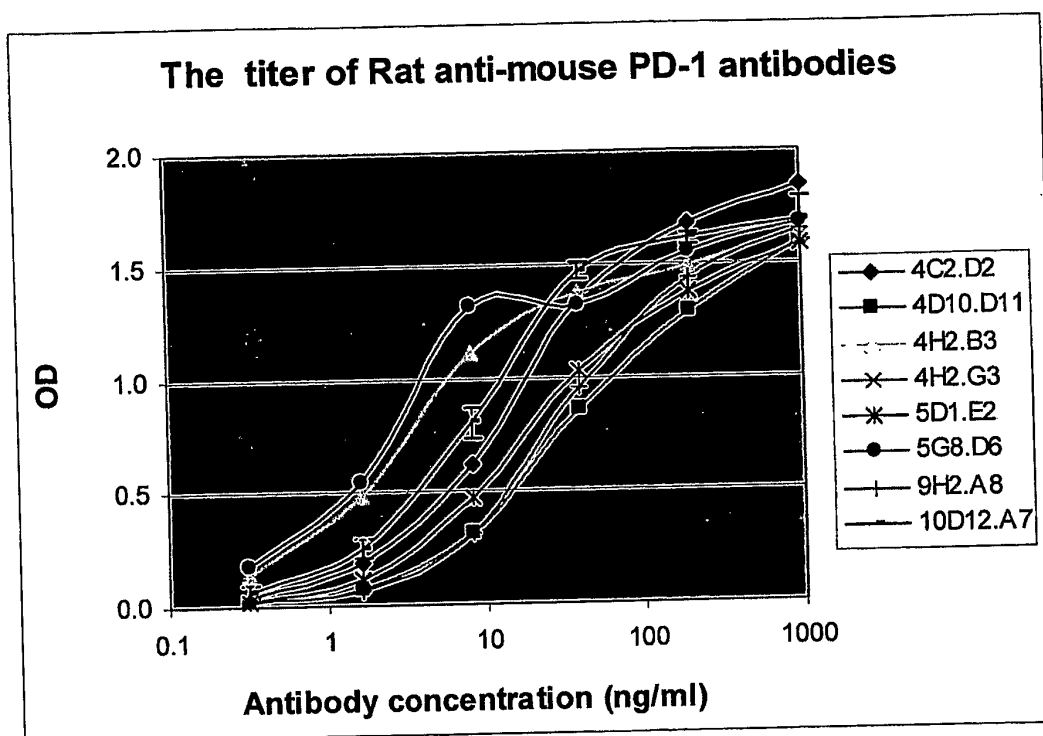


Figure 22

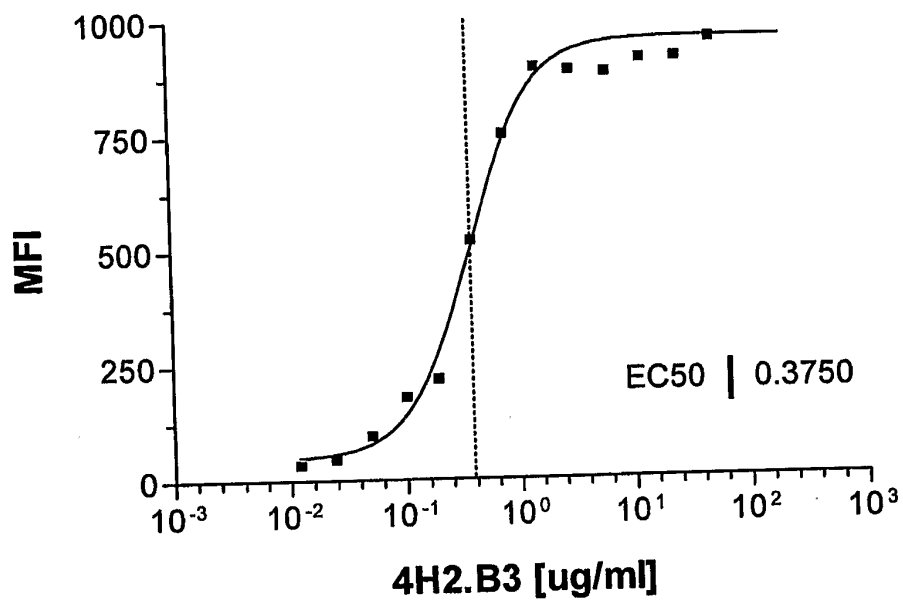


Figure 23

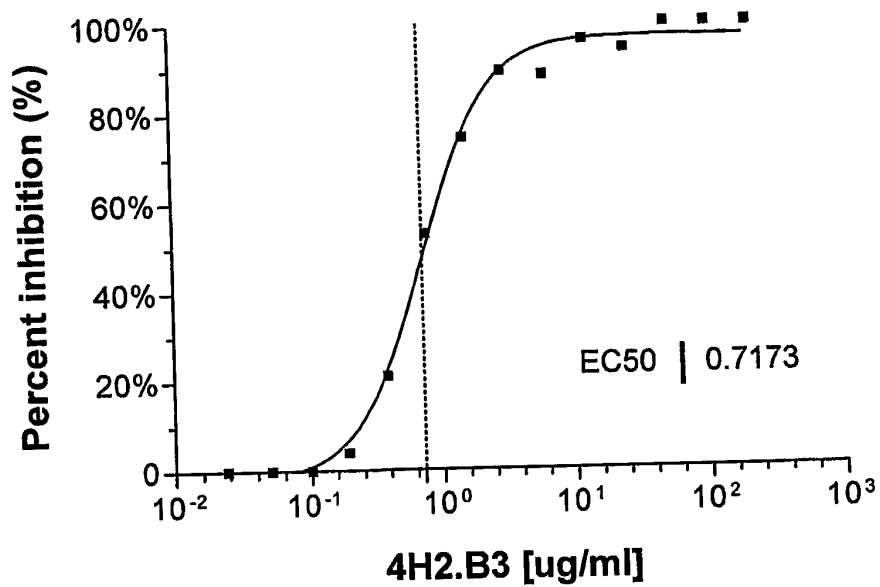


Figure 24

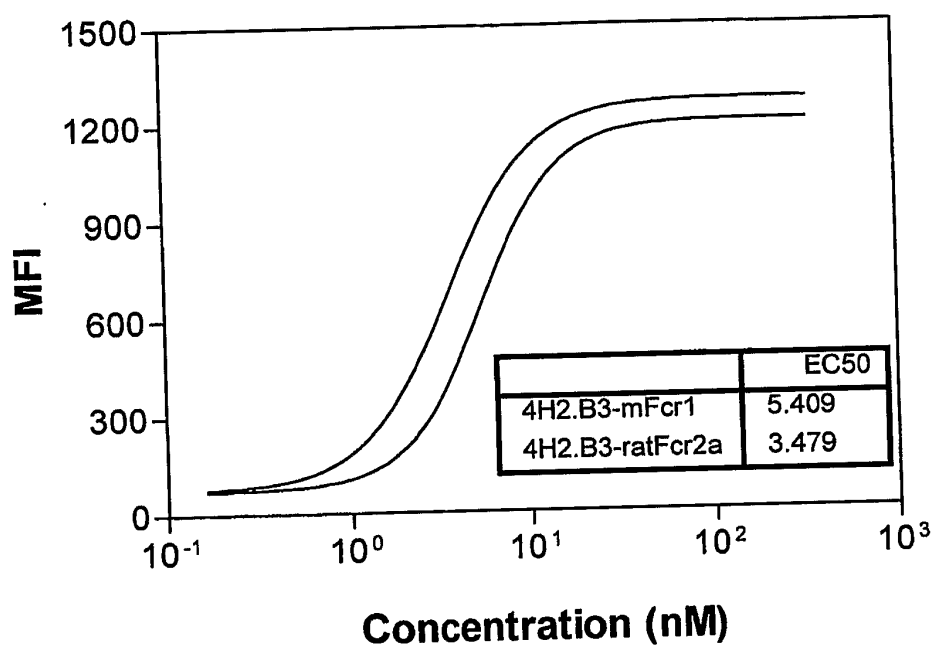


Figure 25

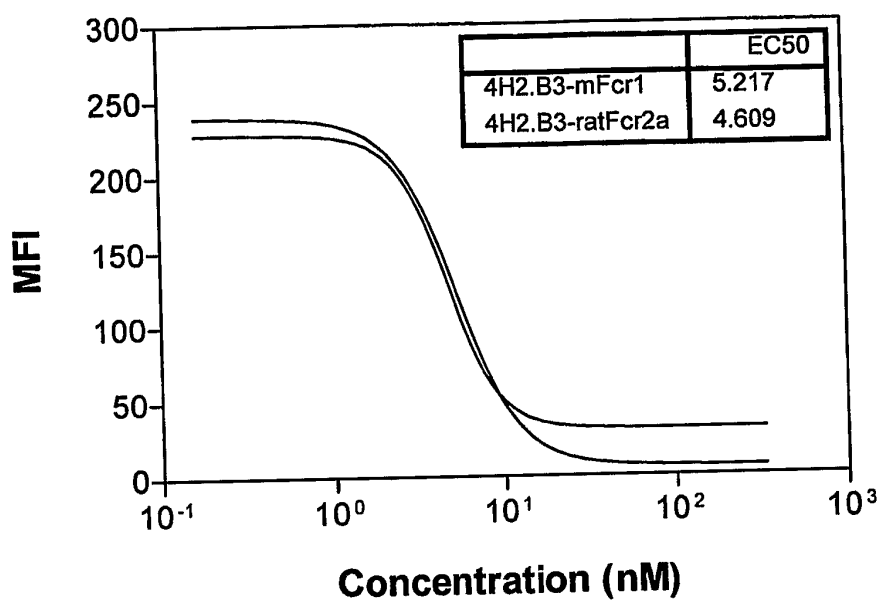


Figure 26

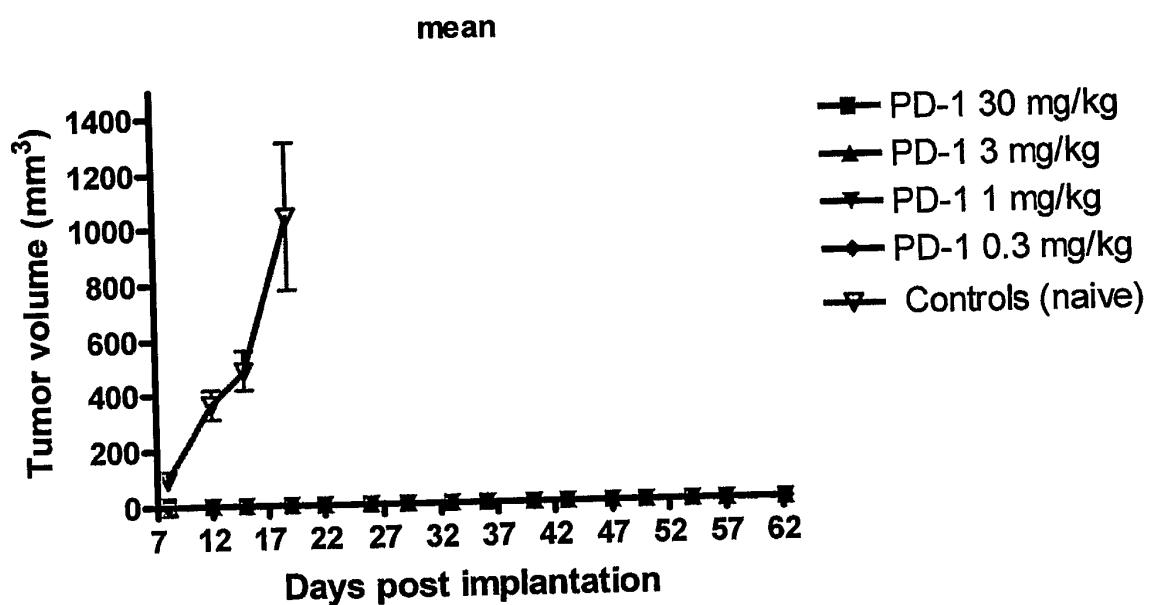


Figure 27

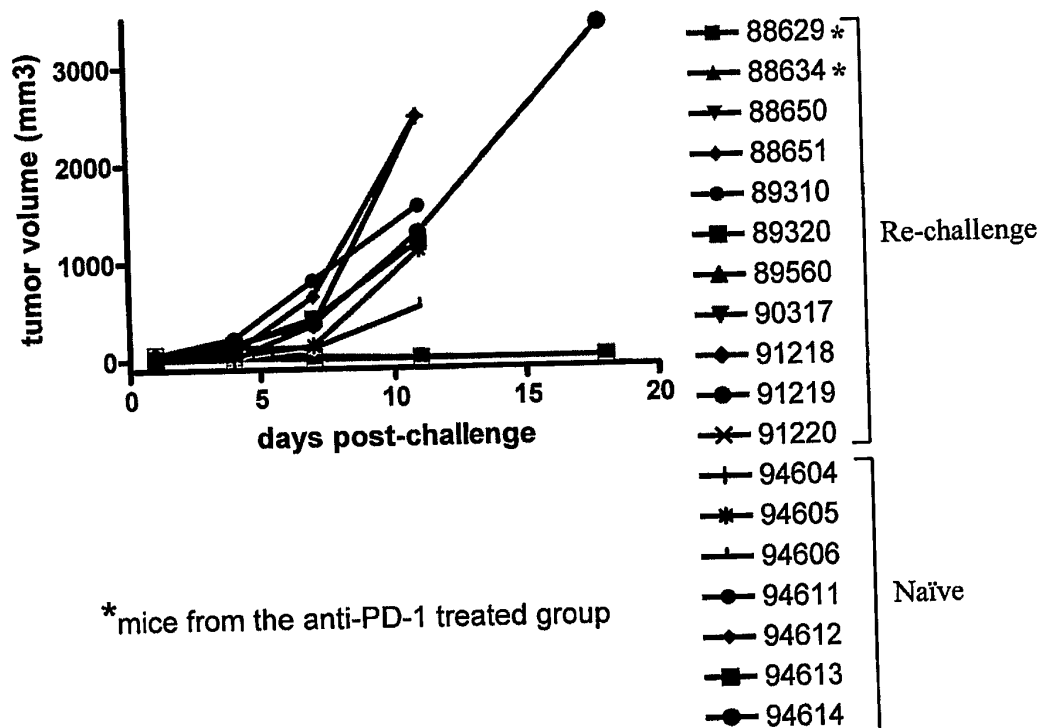


Figure 28

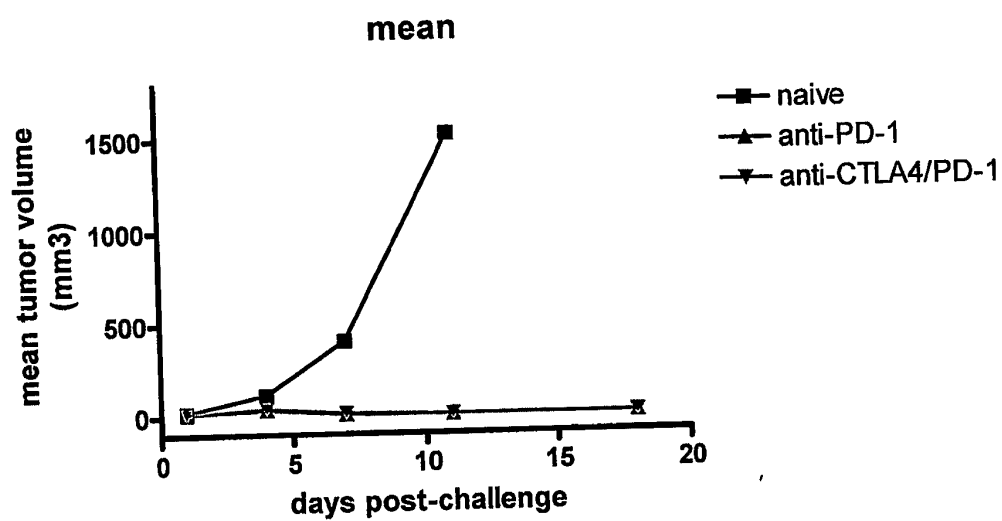


Figure 29

Anti-PD1 17D8 VH

V segment: 3-33
D segment: unknown
J segment: JH4b

```
1      Q  V  Q  L  V  E  S  G  G  D  V  V  Q  P  G  G  S  L
      CAG GTG CAG CTG GTG GAG TCT GGG GGA GAC GTG GTC CAG CCT GGG GGG TCC CTG

                                         CDR1
                                         ~~~~~
55      R  L  S  C  A  A  S  G  V  A  F  S  N  Y  G  M  H  W
      AGA CTC TCC TGT GCA GCG TCT GGA GTC GCC TTC AGT AAC TAT GGC ATG CAC TGG

                                         CDR2
                                         ~~~~~
109     V  R  Q  A  P  G  K  G  L  E  W  V  A  V  I  W  Y  D
      GTC CGC CAG GCT CCC GGC AAG GGG CTG GAG TGG GTG GCA GTT ATC TGG TAT GAT

                                         CDR2
                                         ~~~~~
163     G  S  N  K  Y  Y  A  D  S  V  K  G  R  F  T  I  S  R
      GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG AAG GGC CGG TTC ACC ATC TCC AGA

217     D  N  S  K  N  M  L  Y  L  Q  M  N  S  L  R  A  E  D
      GAC AAT TCC AAG AAC ATG CTC TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC

                                         CDR3
                                         ~~~~~
271     T  A  M  Y  Y  C  A  R  N  D  D  Y  W  G  Q  G  T  L
      ACG GCT ATG TAT TAC TGT GCG AGG AAC GAT GAC TAC TGG GGC CAG GGA ACC CTG

                                         L → JH4b

325     V  T  V  S  S
      GTC ACC GTC TCC TCA
```

Figure 30A

Anti-PD1 17D8 VK

V segment: L6
J segment: JK4

```
1      E I V L T Q S P A T L S L S P G E R
      GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

                                CDR1
                                ~~~~~
55      A T L S C R A S Q S I S S Y L A W Y
      GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT ATT AGC AGC TAC TTA GCC TGG TAC

                                CDR2
                                ~~~~~
109     Q Q K P G Q A P R L L I Y D A S N R
      CAA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG

      CDR2
      ~~~~~
163     A T G I P A R F S G S G S G T D F T
      GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

                                CDR3
                                ~~~~~
217     L T I S S L E P E D F A V Y Y C Q Q
      CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG

      CDR3
      ~~~~~
271     R S N W P L G G G T K V E I K
      CGT AGC AAC TGG CCT CTC GGC GGA GGG ACC AAG GTG GAG ATC AAA
```



Figure 30B

Anti-PD-1 2D3 VH

V segment: 3-33
D segment: 7-27
J segment: JH4b

```
1      Q V Q L V E S G G D V V Q P G R S L
      CAG GTG CAG CTG GTG GAA TCT GGG GGA GAC GTG GTC CAG CCT GGG AGG TCC CTG

                                CDR1
                                ~~~~~
55     R L S C A A S G L T F T N Y G F H W
      AGA CTC TCC TGT GCA GCG TCT GGA TTA ACC TTC ACT AAC TAT GGC TTC CAC TGG

                                CDR2
                                ~~~~~
109    V R Q A P G K G L E W V A V I W Y D
      GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCT GTT ATA TGG TAT GAT

                                CDR2
                                ~~~~~
163    G S K K Y Y A D S V K G R F T I S R
      GGA AGT AAG AAA TAT TAT GCA GAC TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA

217    D N S K N T L Y L Q M N N L R A E D
      GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AAC CTG AGA GCC GAG GAC

                                CDR3
                                ~~~~~
271    T A V Y Y C A T G D D Y W G Q G T L
      ACG GCT GTG TAT TAC TGT GCG ACT GGG GAT GAC TAC TGG GGC CAG GGA ACC CTG

325    V T V S S
      GTC ACC GTC TCC TCA
```

Figure 31A

Anti-PD-1 2D3 VK

V segment: L6
J segment: JK4

```

1      E I V L T Q S P A T L S L S P G E R
      GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

                                CDR1
                                ~~~~~
55      A T L S C R A S Q S V S S Y L A W Y
      GCC ACC CTG TCC TGC AGG GCC AGT CAG AGT GTT AGC AGC TAC TTA GCC TGG TAC

                                CDR2
                                ~~~~~
109     Q Q K P G Q A P R L L I Y D T S N R
      CAA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT ACA TCC AAC AGG

      CDR2
      ~~~~~
163     A T G I P A R F S G S G S G T D F T
      GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

                                CDR3
                                ~~~~~
217     L T I S S L E P E D F A V Y Y C Q Q
      CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG

      CDR3
      ~~~~~
271     R S N W P L T F G G G T K V E I K
      CGT AGC AAC TGG CCG CTC ACT TTC GGC GGA GGG ACC AAG GTG GAG ATC AAA
```

Figure 31B

Anti-PD-1 4H1 VH

V segment: 3-33
D segment: undetermined
J segment: JH4b

```

1   Q V Y L V E S G G G V V Q P G R S L
   CAG GTG TAC TTG GTA GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG TCC CTG

                                CDR1
                                ~~~~~
55  R L S C A A S G F T F S N Y G M H W
   AGA CTC TCC TGT GCA GCG TCT GGA TTC ACC TTC AGT AAC TAT GGC ATG CAC TGG

                                CDR2
                                ~~~~~
109 V R Q A P G K G L E W V A L I W Y D
   GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA CTT ATA TGG TAT GAT

                                CDR2
                                ~~~~~
163 G S N K Y Y A D S V K G R F T I S R
   GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA

217 D N S K N T L Y L Q M T S L R V E D
   GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG ACC AGT CTG AGA GTC GAG GAC

                                CDR3
                                ~~~~~
271 T A V Y Y C A S N V D H W G Q G T L
   ACG GCT GTG TAT TAT TGT GCG AGC AAC GTT GAC CAT TGG GGC CAG GGA ACC CTG

325 V T V S S
   GTC ACC GTC TCC TCA
```

Figure 32A

Anti-PD-1 4H1 VK

V segment: L6
J segment: JK1

```

      E I V L T Q S P A T L S L S P G E R
1  GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

                                CDR1
                                ~~~~~
      A T L S C R A S Q S V N S N L A W Y
55  GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AAC AGT AAC TTA GCC TGG TAC

                                CDR2
                                ~~~~~
      Q Q K P G Q A P R L L I Y D A S N R
109 CAA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG

      CDR2
      ~~~~~
      A T G I P A R F S G S G S G T D F T
163  GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

      L T I S S L E P E D F A V Y Y C H C
217  CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAC TGT

      CDR3
      ~~~~~
      Q Q R S N W W T F G Q G T K V E I K
271  CAG CAG CGT AGC AAC TGG TGG ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA
```

Figure 32B

Anti-PD1 5C4 VH

V segment: 3-33
D segment: unknown
J segment: JH4b

```
1      Q  V  Q  L  V  E  S  G  G  G  V  V  Q  P  G  R  S  L
      CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG TCC CTG

                                         CDR1
                                         ~~~~~
55      R  L  D  C  K  A  S  G  I  T  F  S  N  S  G  M  H  W
      AGA CTC GAC TGT AAA GCG TCT GGA ATC ACC TTC AGT AAC TCT GGC ATG CAC TGG

                                         CDR2
                                         ~~~~~
109     V  R  Q  A  P  G  K  G  L  E  W  V  A  V  I  W  Y  D
      GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG GCA GTT ATT TGG TAT GAT

                                         CDR2
                                         ~~~~~
163     G  S  K  R  Y  Y  A  D  S  V  K  G  R  F  T  I  S  R
      GGA AGT AAA AGA TAC TAT GCA GAC TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA

217     D  N  S  K  N  T  L  F  L  Q  M  N  S  L  R  A  E  D
      GAC AAT TCC AAG AAC ACG CTG TTT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC

                                         CDR3
                                         ~~~~~
271     T  A  V  Y  Y  C  A  T  N  D  D  Y  W  G  Q  G  T  L
      ACG GCT GTG TAT TAC TGT GCG ACA AAC GAC GAC TAC TGG GGC CAG GGA ACC CTG

                                         JH4b
                                         ~~~~~
325     V  T  V  S  S
      GTC ACC GTC TCC TCA
```

Figure 33A

Anti-PD1 5C4 VK

V segment: L6
J segment: JK1

```

1      E I V L T Q S P A T L S L S P G E R
    GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

                                CDR1
                                ~~~~~
55      A T L S C R A S Q S V S S Y L A W Y
    GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AGT AGT TAC TTA GCC TGG TAC

                                CDR2
                                ~~~~~
109     Q Q K P G Q A P R L L I Y D A S N R
    CAA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG

    CDR2
    ~~~~~
163     A T G I P A R F S G S G S G T D F T
    GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

                                CDR3
                                ~~~~~
217     L T I S S L E P E D F A V Y Y C Q Q
    CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG

    CDR3
    ~~~~~
271     S S N W P R T F G Q G T K V E I K
    AGT AGC AAC TGG CCT CGG ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA
        ↓

```

Figure 33B

Anti-PD-1 4A11 VH

V segment: 4-39
D segment: 3-9
J segment: JH4b

```
1      Q L Q L Q E S G P G L V K P S E T L
      CAG CTG CAG CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCG GAG ACC CTG

                                         CDR1
                                         ~~~~~
55     S L T C T V S G G S L S R S S F F W
      TCC CTC ACC TGC ACT GTC TCT GGT GGC TCC CTC AGC AGG AGT AGT TTC TTC TGG

                                         CDR2
                                         ~~~~~
109    G W I R Q P P G K G L E W I G S I Y
      GGC TGG ATC CGT CAG CCC CCA GGG AAG GGA CTG GAG TGG ATT GGG AGT ATC TAT

                                         CDR2
                                         ~~~~~
163    Y S G S T Y Y N P S L K S R V T I S
      TAT AGT GGG AGC ACC TAC TAC AAC CCG TCC CTC AAG AGT CGA GTC ACC ATA TCC

217    V D T S K N Q F S L K L S S V T A A
      GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCA

                                         CDR3
                                         ~~~~~
271    D T A V Y Y C V R D Y D I L T G D E
      GAC ACG GCT GTG TAT TAC TGT GTG AGA GAT TAC GAT ATT TTG ACT GGC GAC GAG

                                         CDR3
                                         ~~~~~
325    D Y W G Q G T L V T V S S
      GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTG TCC TCA
```

Figure 34A

Anti-PD-1 4A11 VK

V segment: L15
J segment: JK1

```

1      D I Q M T Q S P S S L S A S V G D R
      GAC ATC CAG ATG ACC CAG TCT CCA TCC TCA CTG TCT GCA TCT GTG GGA GAC AGA

                                CDR1
                                ~~~~~
55     V S I T C R A S Q G I S S W L A W Y
      GTC TCC ATC ACT TGT CGG GCG AGT CAG GGT ATT AGC AGC TGG TTA GCC TGG TAT

                                CDR2
                                ~~~~~
109    Q Q K P E K A P K S L I Y A A S N L
      CAG CAG AAA CCA GAG AAA GCC CCT AAG TCC CTG ATC TAT GCT GCA TCC AAT TTA

      CDR2
      ~~~~~
163    R S G V P S R F S G S G S G T D F T
      CGA AGT GGG GTC CCA TCA AGG TTC AGC GGC AGT GGA TCT GGG ACA GAT TTC ACT

                                CDR3
                                ~~~~~
217    L T I S S L Q P E D F A T Y Y C Q Q
      CTC ACC ATC AGC AGC CTG CAG CCT GAA GAT TTT GCA ACT TAT TAC TGC CAA CAG

      CDR3
      ~~~~~
271    Y Y S Y P R T F G Q G T K V E I K
      TAT TAT AGT TAC CCT AGG ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA
```

Figure 34B

Anti-PD1 VH region alignment to VH 3-33

	<u>CDR1</u>
3-33 Germline:	Q V Q L V E S G G G V V Q P G R S L R L S C A A S
	G F T F S S Y G M H W V R Q
17D8 VH:	- - - - - D - - - - - G - - - - -
	- V A - - N - - - - -
2D3 VH:	- - - - - D - - - - -
	- L - - T N - - F - - - - -
4H1 VH:	- - Y - - - - -
	- - - - - N - - - - -
5C4 VH:	- - - - - D - K - - -
	- I - - - N S - - - - -

	<u>CDR2</u>
3-33 Germline:	A P G K G L E W V A V I W Y D G S N K Y Y A D S V
	K G R F T I S R D N S K N T
17D8 VH:	- - - - - M - - - - -
	- - - - - K - - - - -
2D3 VH:	- - - - -
	- - - - - L - - - - -
4H1 VH:	- - - - -
	- - - - - K R - - - - -
5C4 VH:	- - - - -

	<u>CDR3</u>
3-33 Germline:	L Y L Q M N S L R A E D T A V Y Y C A R
17D8 VH:	- - - - - M - - - - -
	- - - - - (JH4b)
2D3 VH:	- - - - - N - - - - - T G - - - - -
	- - - - - (JH4b)
4H1 VH:	- - - - - T - - - - - V - - - - - S - V - H -
	- - - - - (JH4b)
5C4 VH:	- F - - - - - T N D D Y W
	G Q G T L V T V S S (JH4b)

Figure 35

Anti-PD1 17D8 VK region alignment to Vk L6

CDR1
L6 germline: E I V L T Q S P A T L S L S P G E R A T L S
C R A S Q S V S S Y L A
17D8: - - - - -
- - - - - I - - - - -

CDR2
L6 germline: W Y Q Q K P G Q A P R L L I Y D A S N R A T
G I P A R F S G S G S
17D8: - - - - -
- - - - -

L6 germline: G T D F T L T I S S L E P E D F A V Y Y C
17D8: - - - - -

CDR3
L6 germline: Q Q R S N W
17D8: - - - - - P L G G G T K V E I K

Figure 36

Anti-PD1 2D3 VK region alignment to Vk L6

```

      CDR1
L6 germline:  E I V L T Q S P A T L S L S P G E R A T L S C R A
S Q S V S S Y L A
2D3 VK:      - - - - -
- - - - -

      CDR2
L6 germline:  W Y Q Q K P G Q A P R L L I Y D A S N R A T G I P
A R F S G S G S G
2D3 VK:      - - - - - T - - - - -
- - - - -

      CDR3
L6 germline:  T D F T L T I S S L E P E D F A V Y Y C Q Q R S N
W P
JK4 germline:
L T F G G G T
2D3 VK:      - - - - -
- - - - -

JK4 germline:  K V E I K
2C3 VK:      - - - - - (JK4)
```

Figure 37

Anti-PD1 4H1 VK region alignment to Vk L6

```

      CDR1
L6 germline:  E I V L T Q S P A T L S L S P G E R A T L S C R A
S Q S V S S Y L A
4H1 VK:      - - - - -
- - - - -

      CDR2
L6 germline:  W Y Q Q K P G Q A P R L L I Y D A S N R A T G I P
A R F S G S G S G
4H1 VK:      - - - - -
- - - - -

      CDR3
L6 germline:  T D F T L T I S S L E P E D F A V Y Y C Q Q R S N
W P
JK1 germline:
W T F G Q G T
4H1 VK:      - - - - - S - -
- - R - - - -

JK4 germline:  K V E I K
4H1 VK:      - - - - - (JK1)
```

Figure 38

Anti-PD1 5C4 VK region alignment to VkL6

CDR1
L6 germline: E I V L T Q S P A T L S L S P G E R A T L S
C R A S Q S V S S Y L A
5C4: - - - - -
- - - - -

CDR2
L6 germline: W Y Q Q K P G Q A P R L L I Y D A S N R A T
G I P A R F S G S G S
5C4: - - - - -
- - - - -

L6 germline: G T D F T L T I S S L E P E D F A V Y Y C
5C4: - - - - -

CDR3
L6 germline: Q Q R S N W
5C4: - - S - - - P R T F G Q G T K V E I K

Figure 39

Anti-PD-1 4A11 VH region alignment to VH 4-39

4-39 germline	Q L Q L Q E S G P G L V K P S E T L S L T C T V
	S G G S I S S S S Y Y W
4A11 VH	- - - - -
	- - - L - R - - F F -

	<u>CDR2</u>	-
4-39 germline	G W I R Q P P G K G L E W I G S I Y Y S G S T Y	
	Y N P S L K S R V T I S	
4A11 VH	- - - - -	
	- - - - -	

	<u>CDR3</u>	
4-39 germline	V D T S K N H F S L K L S S V T A A D T A V Y Y	
	C A R	
4A11 VH	- - - - - Q - - - - -	
	- V - D Y D I L T G D E	

JH4b germline	D Y W G Q G T L V T V S S	
4A11 VH	- - - - -	(JH4b)

Figure 40

Anti-PD-1 4A11 VK region alignment to Vk L15

<u>CDR1</u>		
<u>L15 germline</u>		D I Q M T Q S P S S L S A S V G D R V T I T C R
A S Q G I S S		
<u>4A11 VK</u>		- - - - - S - - - -
- - - - -		
<u>CDR2</u>		
<u>L15 germline</u>		W L A W Y Q Q K P E K A P K S L I Y A A S S L Q
S G V P S R F		
<u>4A11 VK</u>		- - - - - N - R
- - - - -		
<u>CDR3</u>		
<u>L15 germline</u>		S G S G S G T D F T L T I S S L Q P E D F A T Y
Y C Q Q Y N S		
<u>4A11 VK</u>		- - - - -
- - - - - Y -		
<u>L15 germline</u>	<u>Y P</u>	
<u>JK1 germline</u>		T F G Q G T K V E I K
<u>4A11 VK</u>	- - R - - - - -	(JK1)

Figure 41

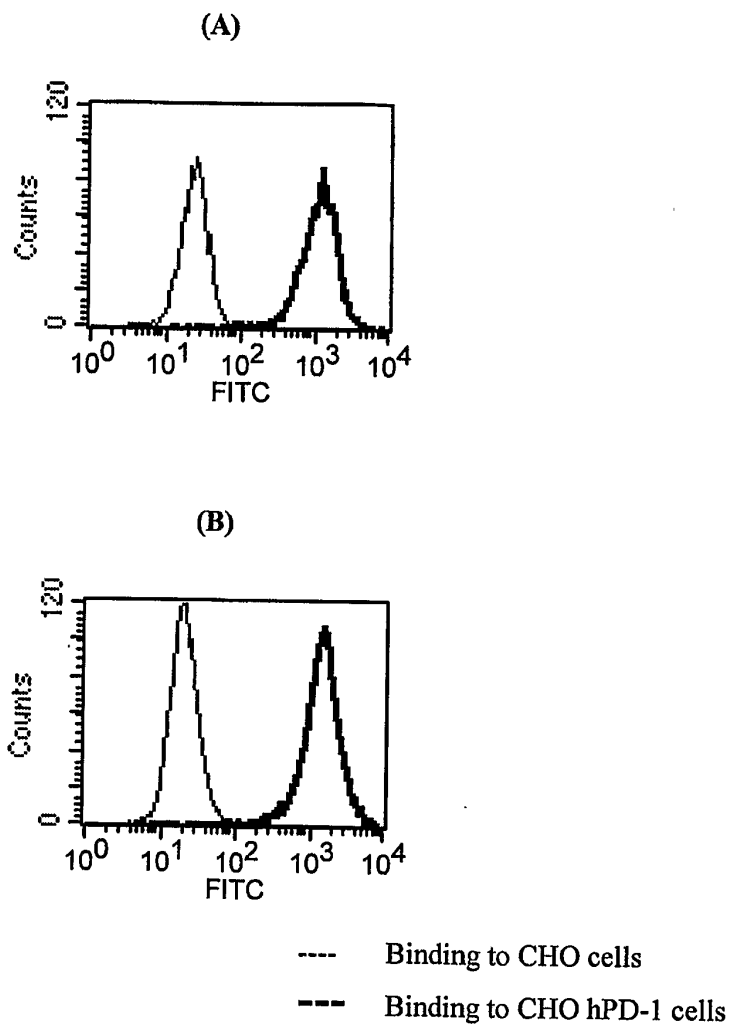


Figure 42