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Document made available under the Patent Cooperation Treaty (PCT)

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International filing date: 02 May 2006 (02.05.2006)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/748,919
Filing date: 08 December 2005 (08.12.2005)

Date of receipt at the International Bureau: 28 September 2006 (28.09.2006)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/JP 2006/309606

07.09.2006

PA 1486198

THE UNITED STATES OF AMERICA

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UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

July 05, 2006

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APPLICATION NUMBER: ***60/748,919***
FILING DATE: ***December 08, 2005***

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS ***US60/748,919***

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

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60/748919
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV592416544US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Mark J.	Selby	San Francisco, California
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Bing	Chen	Alameda, California
Tseng-Hui Timothy	Chen	Burlingame, California
Changyu	Wang	Fremont, California
Haichun	Huang	Fremont, California

Additional inventors are being named on the _____ separately numbered sheets attached hereto

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**

[X] The address corresponding to Customer Number: 07278

OR

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Country	US	Telephone	(206) 262-8900	Email	

ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Application Data Sheet. See 37 CFR 1.76
[X] Specification *Number of Pages* 59
[X] Drawing(s) *Number of Sheets* 54
[ ] CD(s), Number of CDs _____
[X] Other (specify) Certificate of Express Mailing (1 page) 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).

METHOD OF PAYMENT OF FILING FEES AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.
[X] A check or money order is enclosed to cover the filing fee and application size fee (if applicable). TOTAL FEE AMOUNT ($) 450.00
[ ] Payment by credit card. Form PTO-2038 is attached.
[X] The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any overpayment to Deposit Account Number: 04-0100 A duplicative copy of this form is enclosed for fee processing.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No. [ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

SIGNATURE Jeffrey C. Pepe Date December 8, 2005

TYPED or PRINTED NAME Jeffrey C. Pepe REGISTRATION NO. *(if appropriate)* 46,985

TELEPHONE (206) 262-8911 Docket Number: 04280/1203401-US1

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

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 adverse events related to treatment with such antibodies individually.

BACKGROUND

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, 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. 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).

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

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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 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, 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 (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 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 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.

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

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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 mm^3. 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, X_{20}) (B) anti-PD-1 antibody (10 mg/kg) and mouse IgG (10 mg/kg) ($P_{10}X_{10}$), (C) anti-CTLA-4 antibody (10 mg/kg) and mouse IgG (10 mg/kg) ($C_{10}X_{10}$), (D) anti-CTLA-4 antibody and anti-PD-1 antibody (10 mg/kg each) ($C_{10}P_{10}$), (E) anti-CTLA-4 antibody and anti-PD-1 antibody (3 mg/kg each) (C_3P_3), and (F) anti-CTLA-4 antibody and anti-PD-1 antibody (1 mg/kg each) (C_1P_1). 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) ($C_{10}C_{10}P_{10}P_{10}$); 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) (10 mg/kg, day 10) ($P_{10}P_{10}C_{10}C_{10}$).

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.

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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 (F) 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 mm^3.

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 mm^3.

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 mm^3.

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

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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.

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. 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.

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 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 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. 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.

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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.

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.

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.

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.

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.

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.

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.

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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. 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).

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).

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).

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 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 reducing the incidence and/or severity of side effects of monotherapy by administering a

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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 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 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 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, 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 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.

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

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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 inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is

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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 (Clq) 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.

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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 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 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 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.

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 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 biosensor system such as a Biacore® system.

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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

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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

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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 IgGκ monoclonal (Lonberg *et al.*. (1994), *supra*; reviewed in Lonberg (1994) H*andbook 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,

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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 transchomosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. Such mice, referred to herein as "KM mice™", 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 discloure. 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 dislcosure. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome, 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

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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

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1×10^{-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 1×10^{-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 5×10^{-8} M or less, binds to human PD-1 with a K_D of 1×10^{-8} M or less, binds to human PD-1 with a K_D of 5×10^{-9} M or less, or binds to human PD-1 with a K_D of between 1×10^{-8} M and 1×10^{-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), cyclothosphamide, 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).

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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).

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 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* 3:207-212; 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 therapeutically include, but are not limited to, Iodine^{131}, Indium^{111}, Yttrium^{90} and Lutetium^{177}. Methods for preparing radioimmunconjugates 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.

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 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"),

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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.

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

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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.

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 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 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 alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts 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, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the invention also may include a pharmaceutically 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, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

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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.

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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 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.

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, 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 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 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.

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,

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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

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

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 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 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 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 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 concentration of about 1-1000 μg /ml and in some methods about 25-300 μg /ml.

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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

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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.

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 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

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 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

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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

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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

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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

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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 Fcα or Fcγ 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 by 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® (eprtuzumab), 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

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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.

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 another exmaple, 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 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: 2160-2169), 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 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: 546-51). These methods may also be used to activate T cell responses to infectious agents such

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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

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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 includes 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.

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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.

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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 EC_{50} (50% effective concentration) of about 0.38 μg (Figure 23) and a K_D of 4.7×10^{-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 EC_{50} 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

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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 10^6 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)

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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 mm^3 (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 mm^3 for the IgG control group; about 655 mm^3 for the CTLA-4 antibody alone group, about 510 mm^3 for the PD-1 antibody alone group, and about 280 mm^3 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 mm^3 for the IgG group; about 625 mm^3 for the CTLA-4 antibody alone group; about 525 mm^3 for the PD-1 antibody alone group; and about 10 mm^3 for the CTLA-4 antibody and PD-1 antibody combination group (and down to 0 mm^3 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 10^6 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 mm^3) into 11 groups for subsequent antibody therapy. At day

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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 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 mm^3), and on days 3, 6, 10, 13, 17 and 20 post-antibody injection. Mice were euthanized when the tumors reached a designated tumor end-point (a particular tumor volume such as 1500 mm^3 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 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 mm^3 for the IgG control group; about 1010 mm^3 for the CTLA-4 antibody alone group; about 695 mm^3 for the PD-1 antibody alone group; and about 80 mm^3 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 mm^3 for the IgG group; about 1060 mm^3 for the anti–CTLA-4 antibody alone group; about 480 mm^3 for the anti–PD-1 antibody alone group; and about 15 mm^3 for the anti–CTLA-4 antibody and anti–PD-1 antibody combination group (which was down to 0 mm^3 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.

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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 10^6 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 mm^3), 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 mm^3 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 mm^3 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 mm^3 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 mm^3 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 mm^3 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,

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5 mice with tumors having a volume of about 1100 mm^3 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 mm^3 for the IgG control group; about 470 mm^3 for the PD-1 antibody with the IgG control; about 290 mm^3 for the CTLA-4 antibody with the IgG control (measured at day 6); about 40 mm^3 for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 165 mm^3 for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 400 mm^3 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 mm^3 for the IgG control group; about 400 mm^3 for the PD-1 antibody with the IgG control; about 660 mm^3 for the CTLA-4 antibody with the IgG control; 0 mm^3 for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 90 mm^3 for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 650 mm^3 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.

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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 x 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 mm^3 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 mm^3 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 mm^3 and 3 with an ulcerated tumor), one mouse with a small tumor (volume of about 70 mm^3) 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 mm^3 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 mm^3 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.

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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.

† 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. 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 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 (2×10^6 cells/mouse) on day 0 for a time sufficient (about 7 days) to permit the establishment of a tumor. On days 7, 10, 13 and 16 post-implantation, ten groups of 8 mice having an average tumor volume of 110 mm^3 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

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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 mm^3 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 mm^3 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 mm^3 (*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 mm^3 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 mm^3, 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,

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2 mice with a tumor volume less than 500 mm^3, 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).

Figure 16 shows the response to immunostimulatory antibody treatment in mice with larger tumors, those having an initial volume of about 250 mm^3 (*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 mm^3 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 mg/kg anti-CTLA-4 antibody (Group L) had one mouse with an ulcerated tumor, 4 mice with a tumor volume greater than 1500 mm^3, 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.

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.

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† 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

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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 (2×10^6 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 2×10^7 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 2×10^7 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.

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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 antigens were generated by recombinant transfection methods in a CHO cell line.

Transgenic HuMab and KM mice™

Fully human monoclonal antibodies to PD-1 were prepared using the HCo7 strain of HuMab transgenic mice and the KM strain of transgenic transchromosomic mice, each of which express human antibody genes. In each of these mouse strains, the endogenous mouse kappa 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 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 mice™ were immunized with purified recombinant PD-1 fusion protein and PD-1-transfected CHO cells 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 - $10x10^6$ cells were used to immunize the HuMab mice and KM mice™ intraperitonealy, 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 of anti-PD-1 human immunoglobulin were used for fusions. Mice were boosted intravenously

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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 Mice™ Producing Anti-PD-1 Antibodies:

To select HuMab or KM mice™ producing antibodies that bound PD-1, sera from 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. 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 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 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 1x10 ^{5}/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-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 hybridomas were replated, screened again and, if still positive for human IgG, anti-PD-1

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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.

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

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.

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 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 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.

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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.

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 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 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.

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 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 CD3 regions as shown in Figures 32A and 35, and in SEQ ID NOs: 13, 18 and 23, respectively.

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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. 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.

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 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 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 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.

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

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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

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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 x 10^{-9} (M)	On rate k_{on} x 10^5 (1/Ms)	Off rate k_{off} x 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 μg/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 bindig 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 μg/ml. Goat-anti-human IgG (kappa

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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).

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 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.

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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.

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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.

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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.

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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).

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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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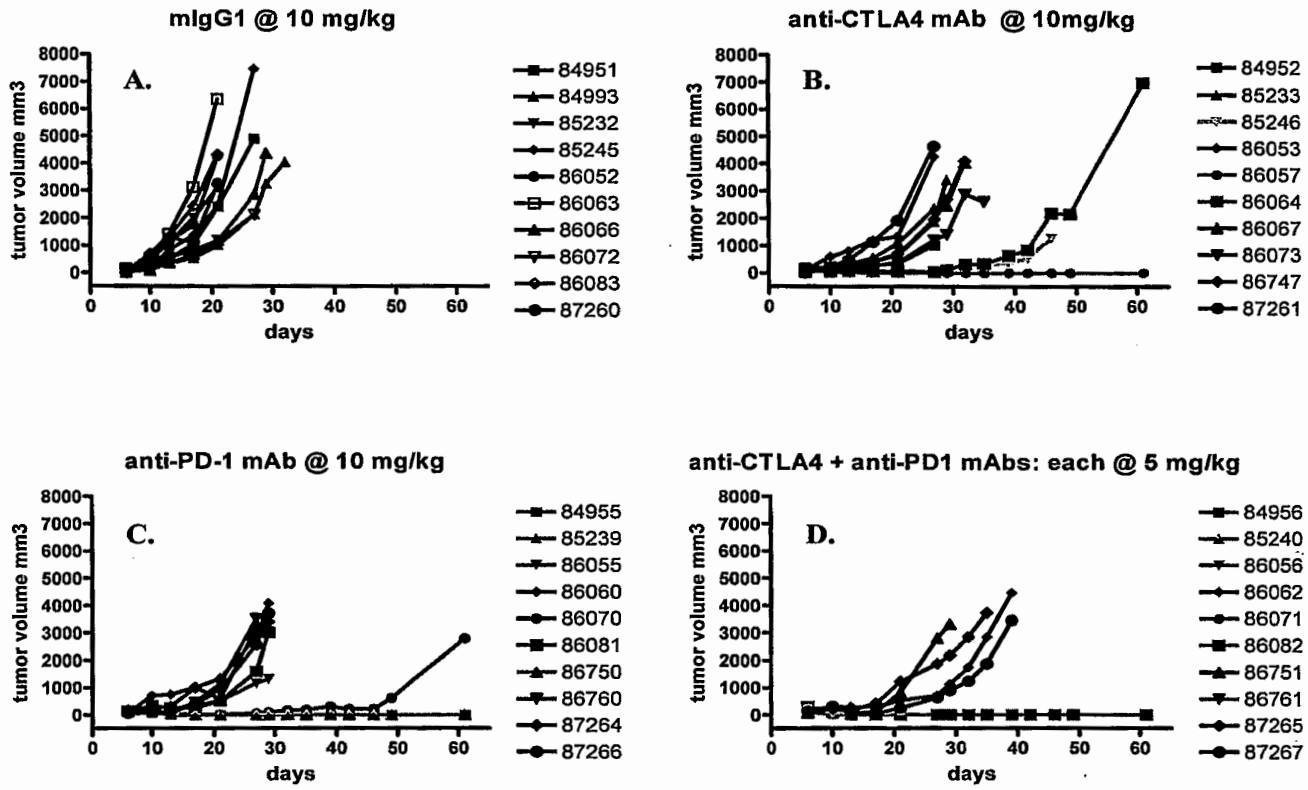


Figure 1

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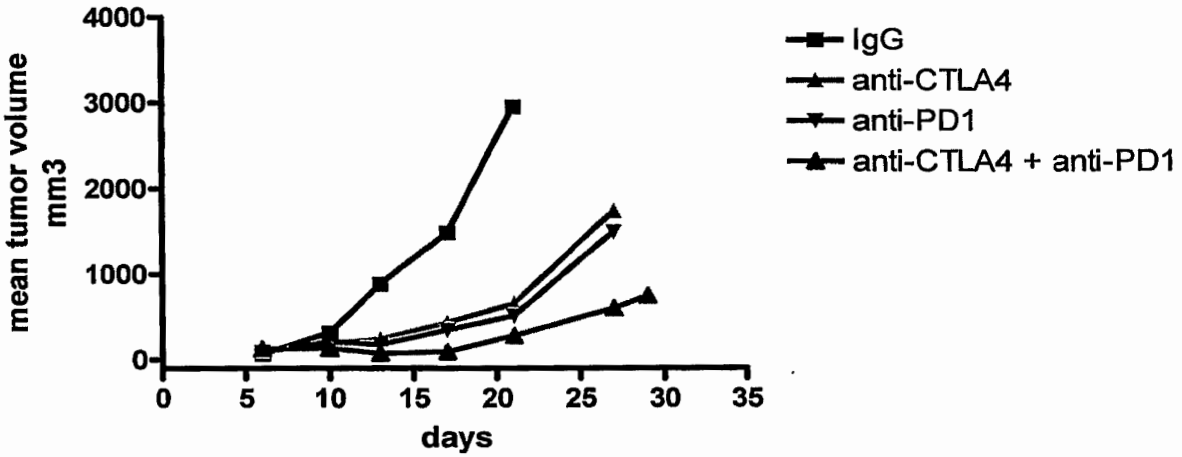


Figure 2

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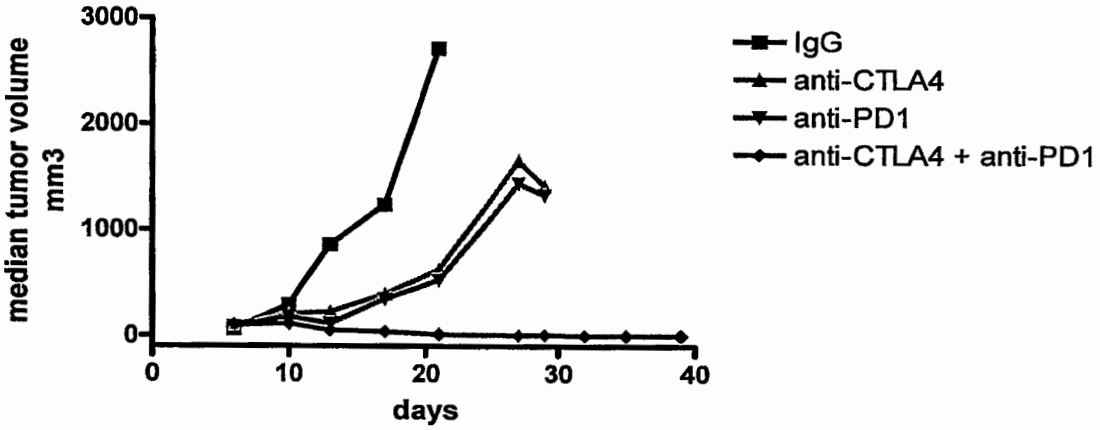


Figure 3

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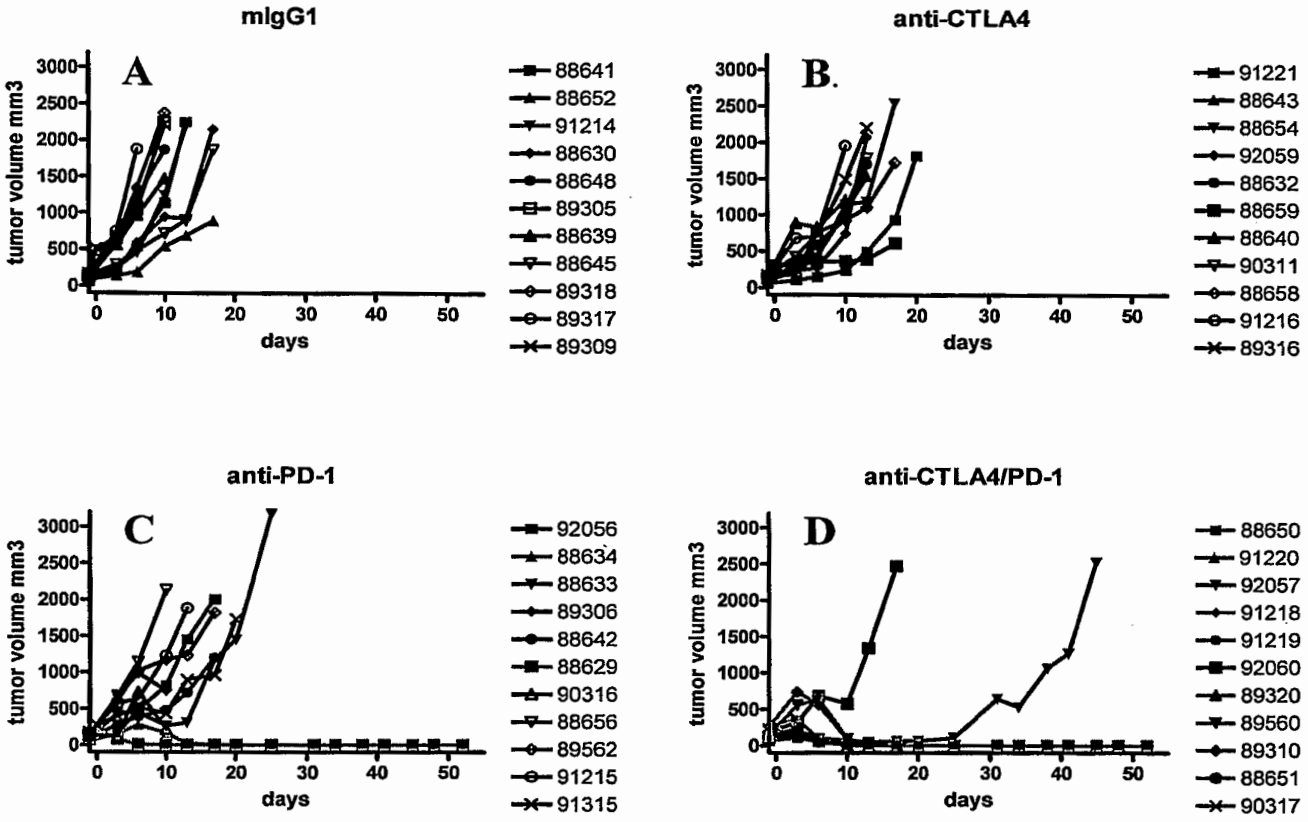


Figure 4

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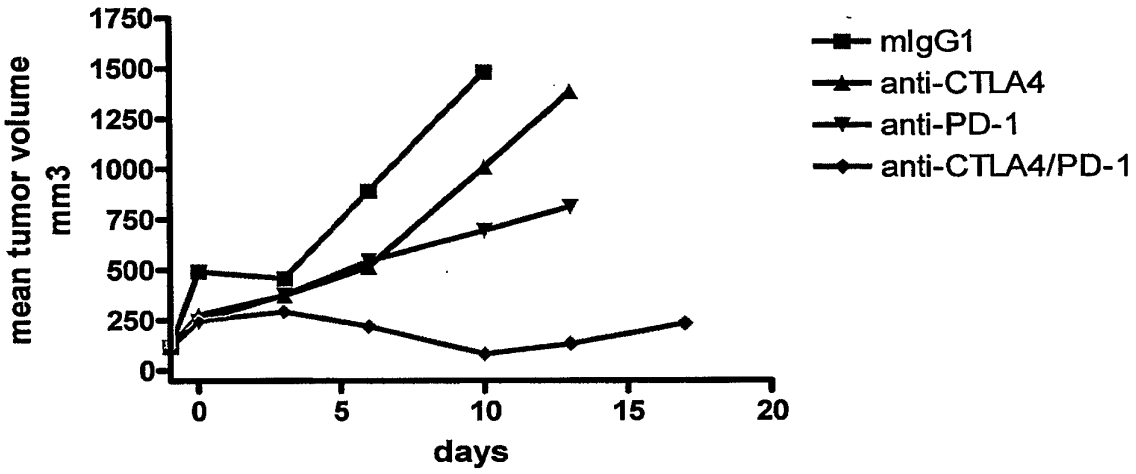


Figure 5

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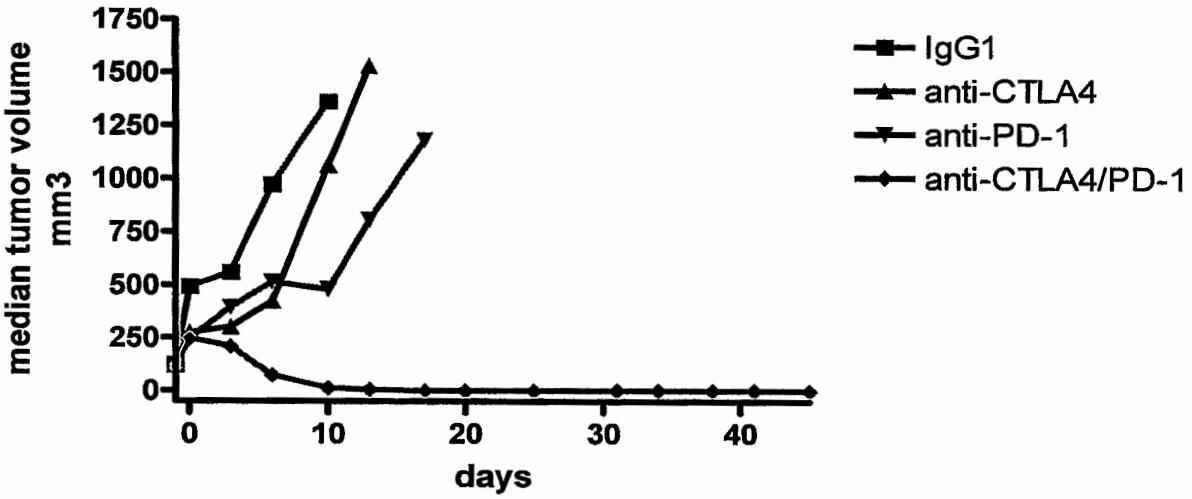


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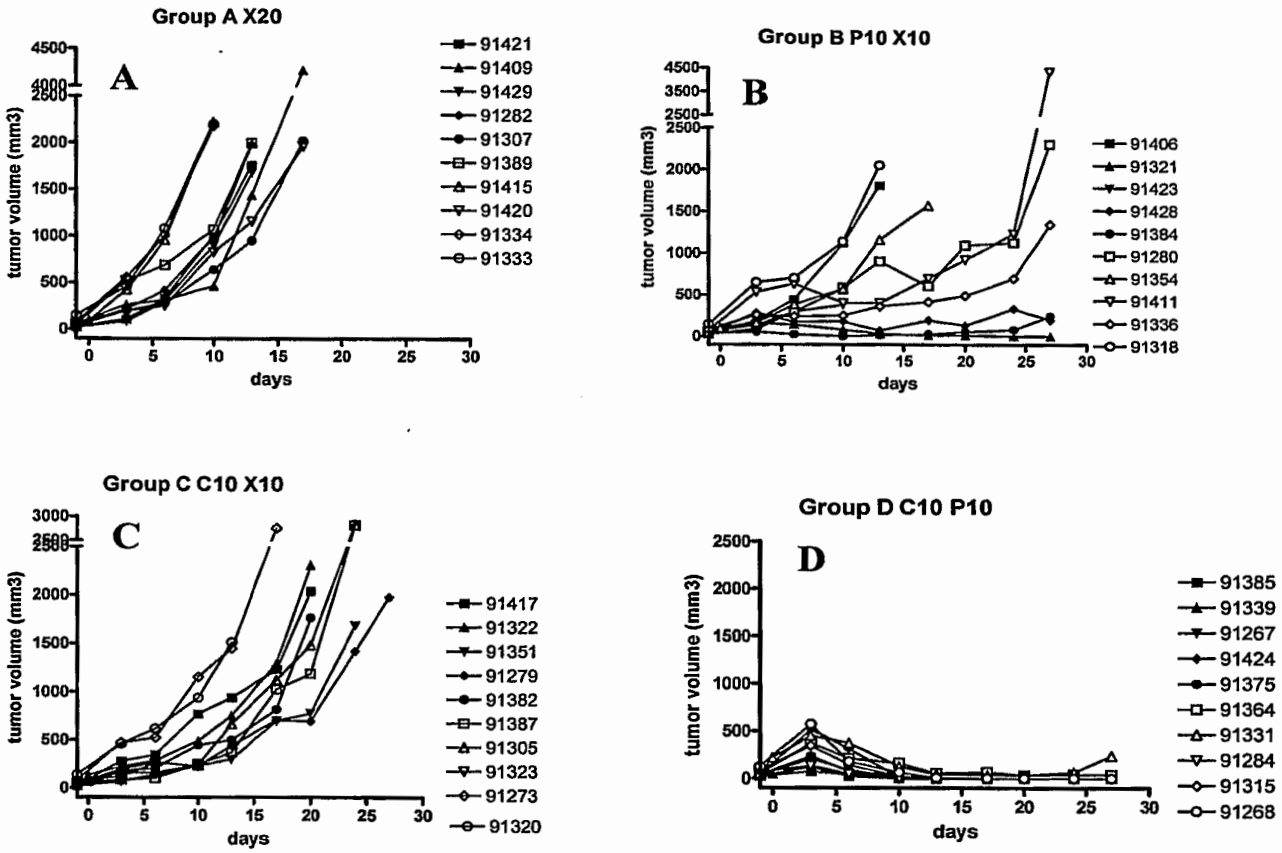


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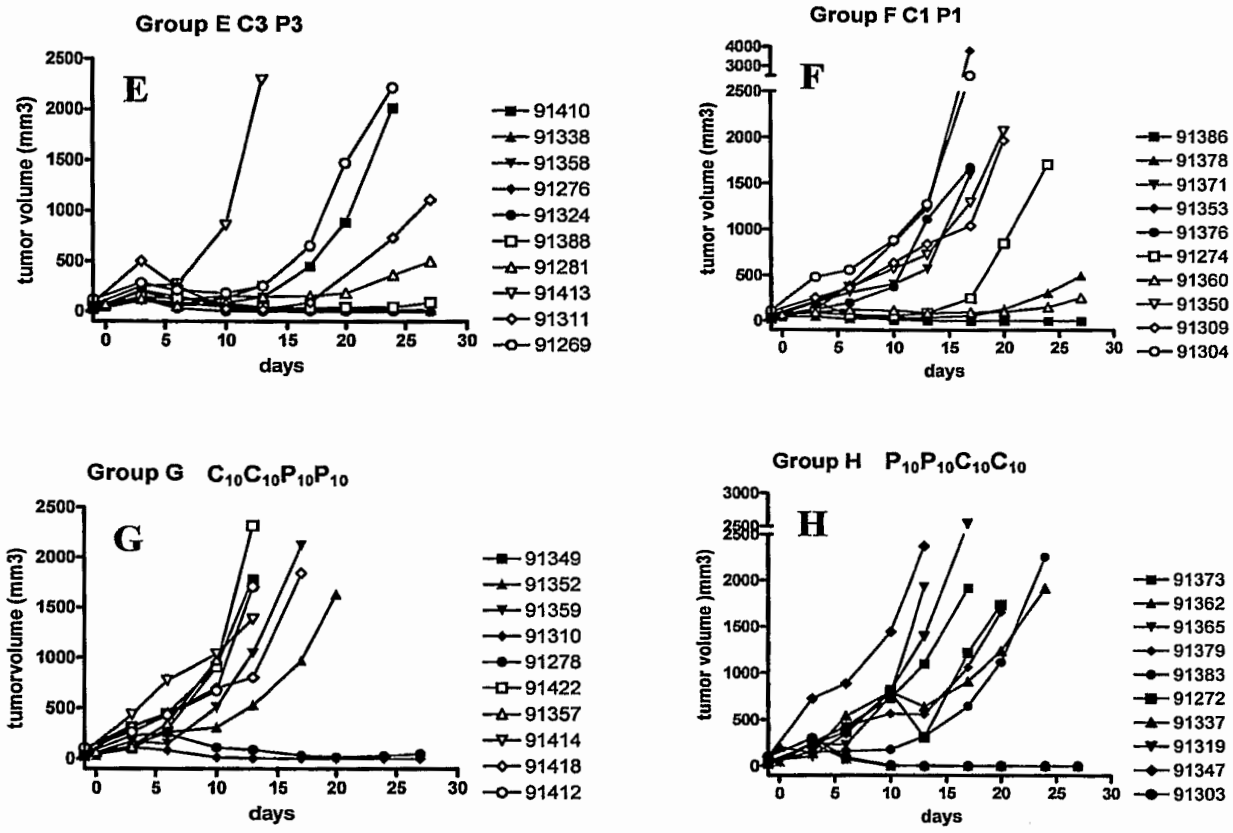


Figure 7 (cont.)

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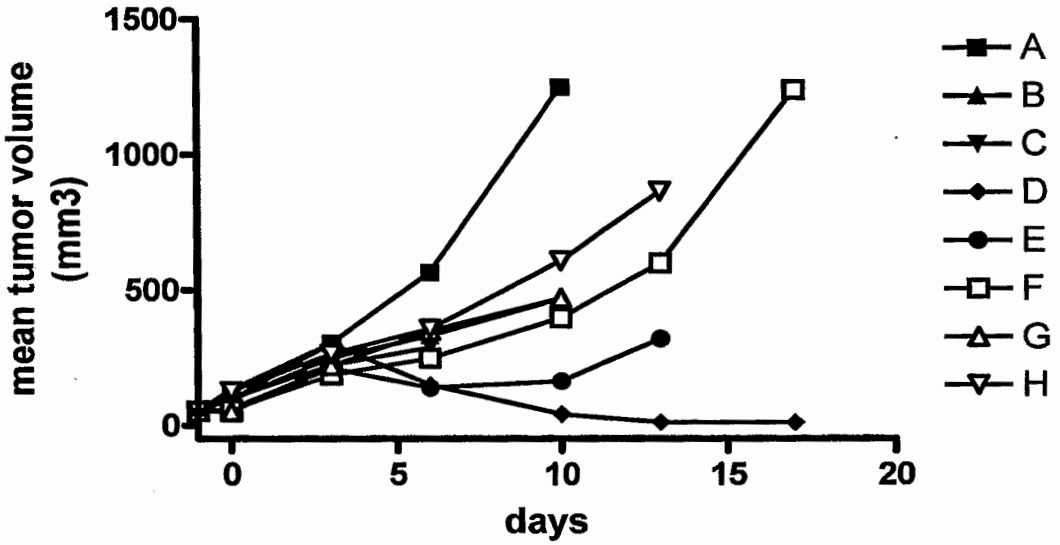


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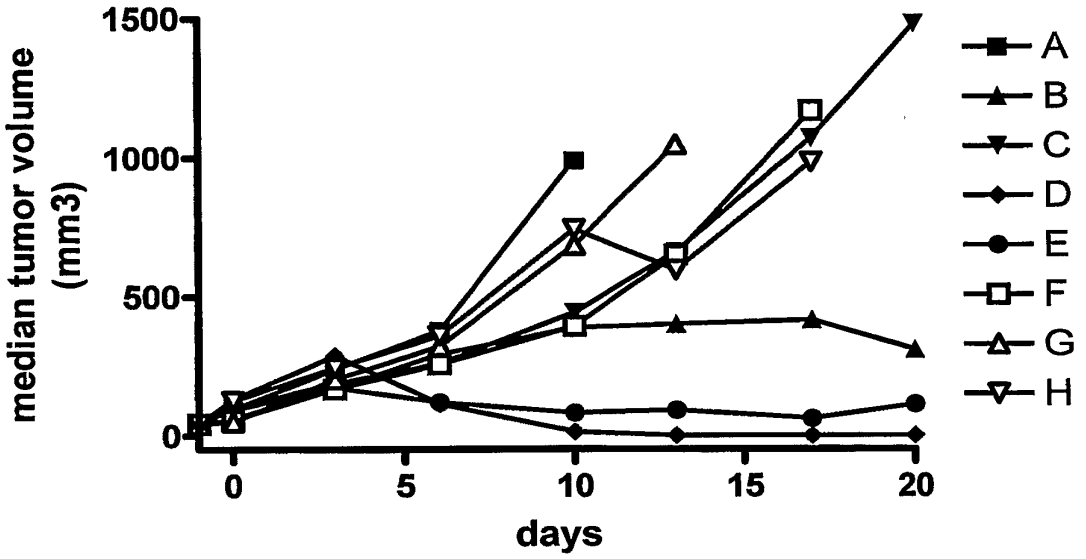


Figure 9

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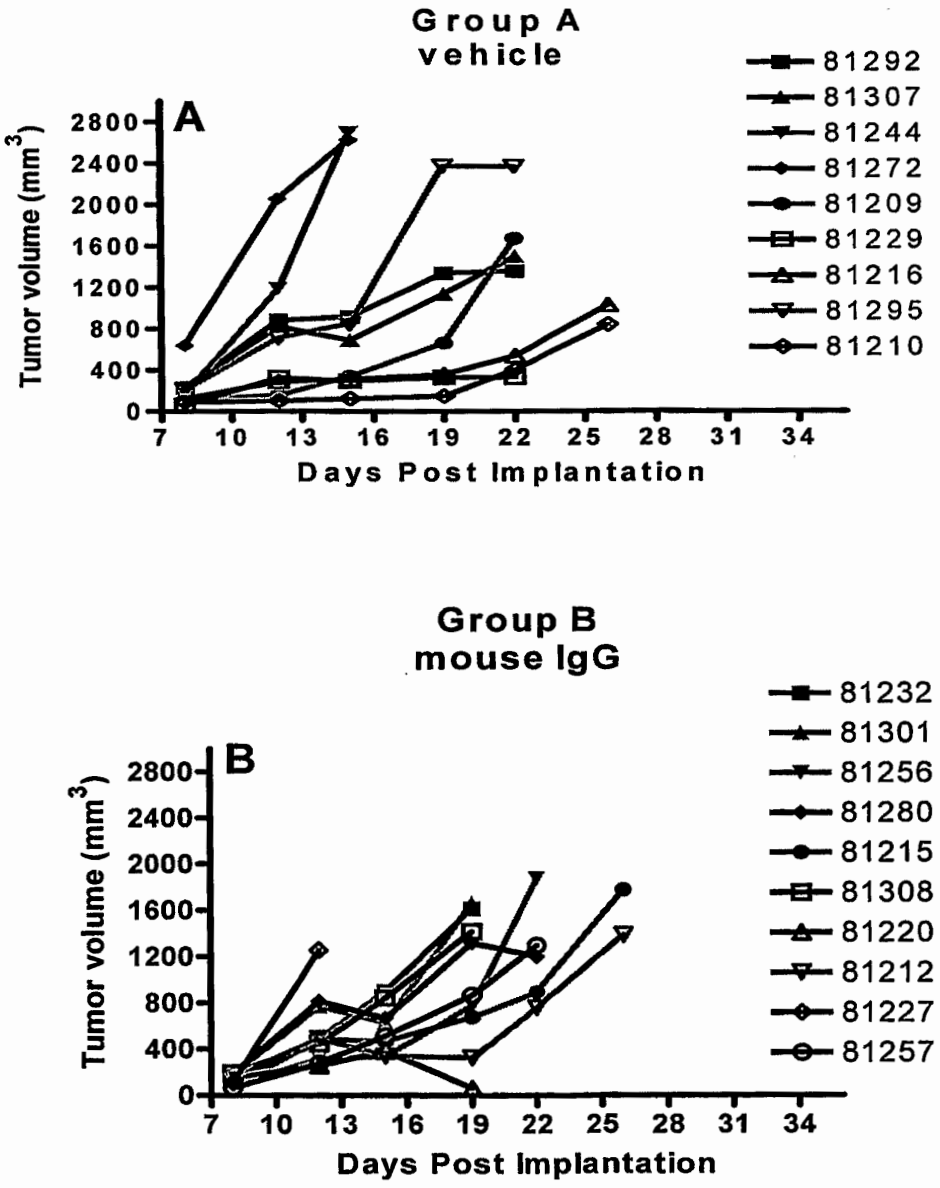
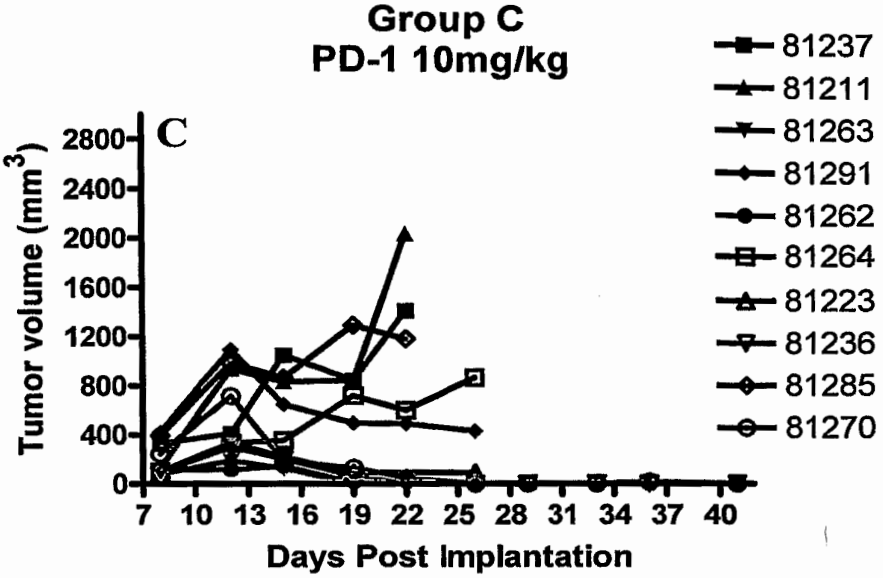


Figure 10

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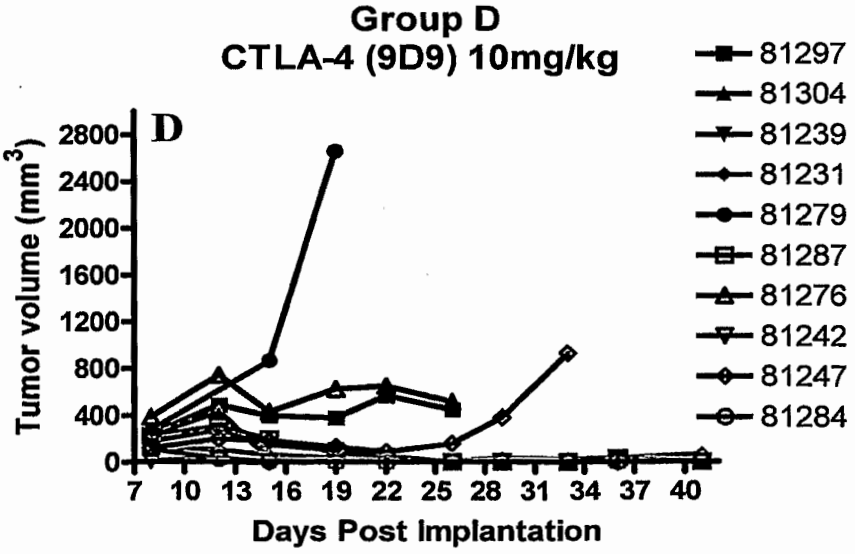
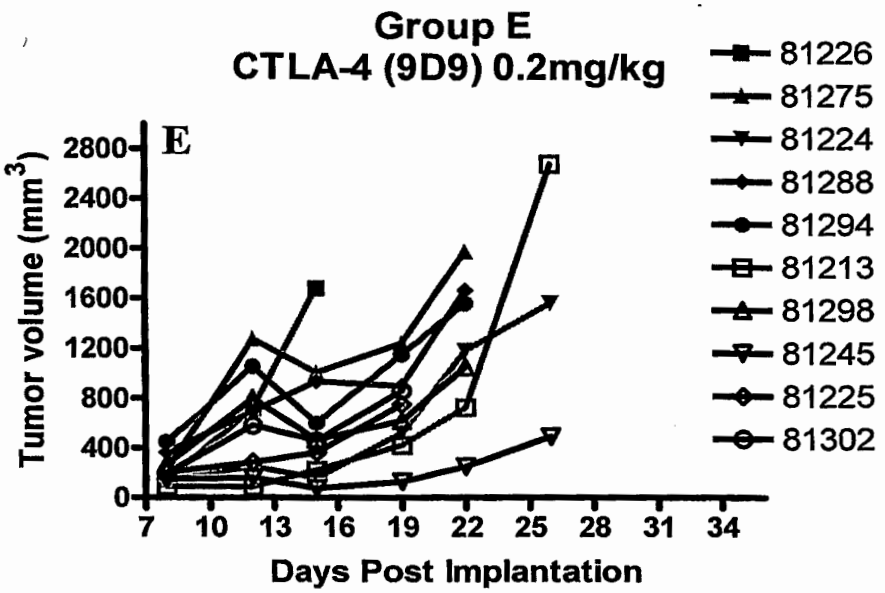


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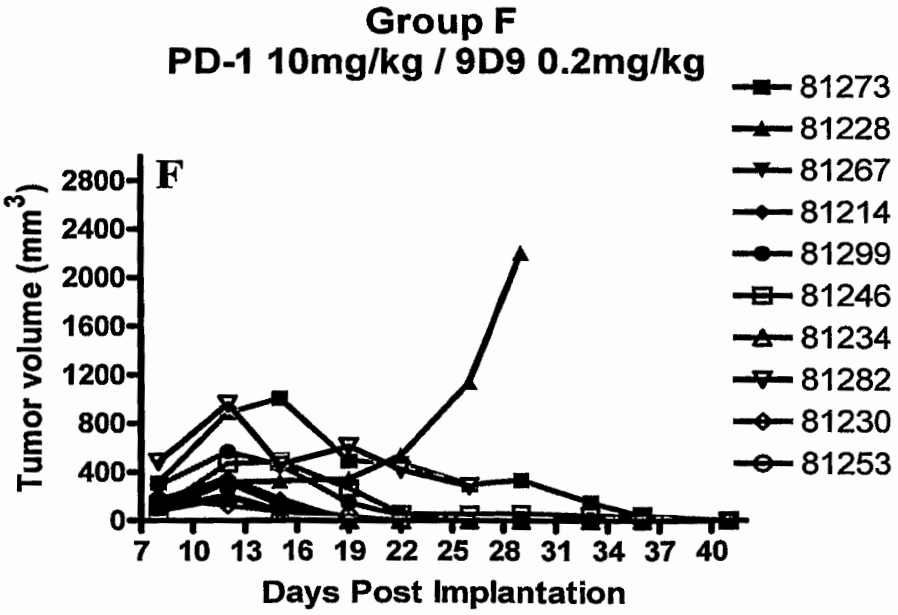


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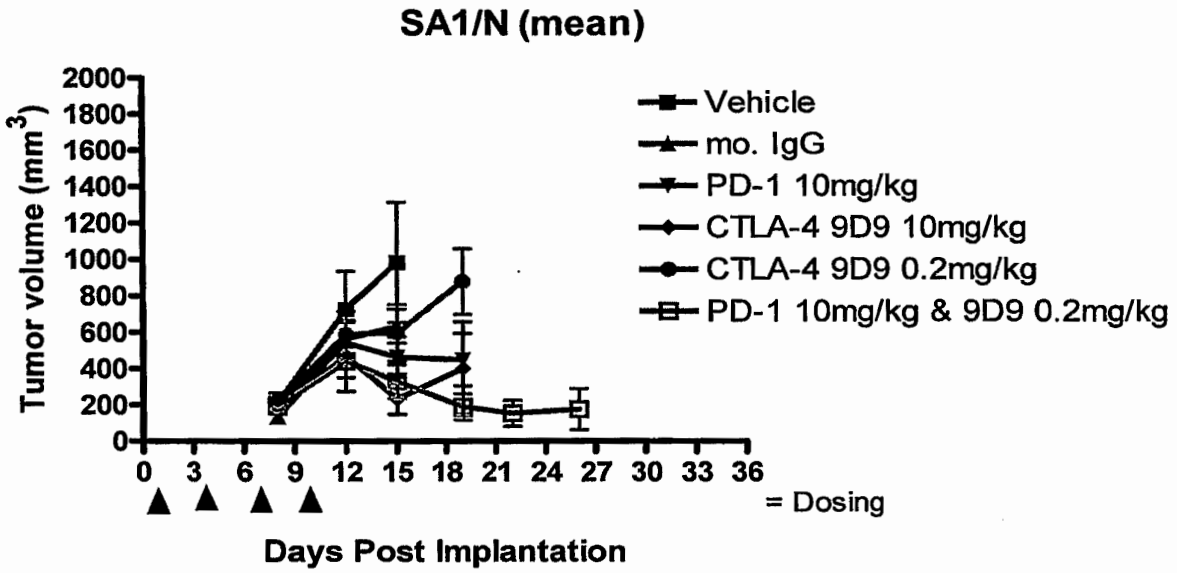


Figure 11

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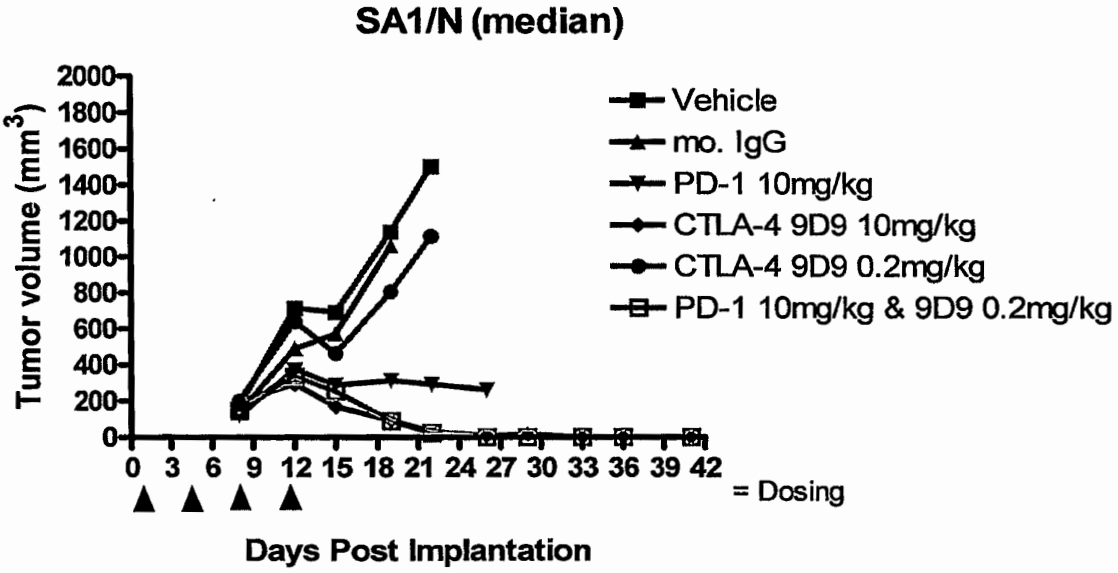


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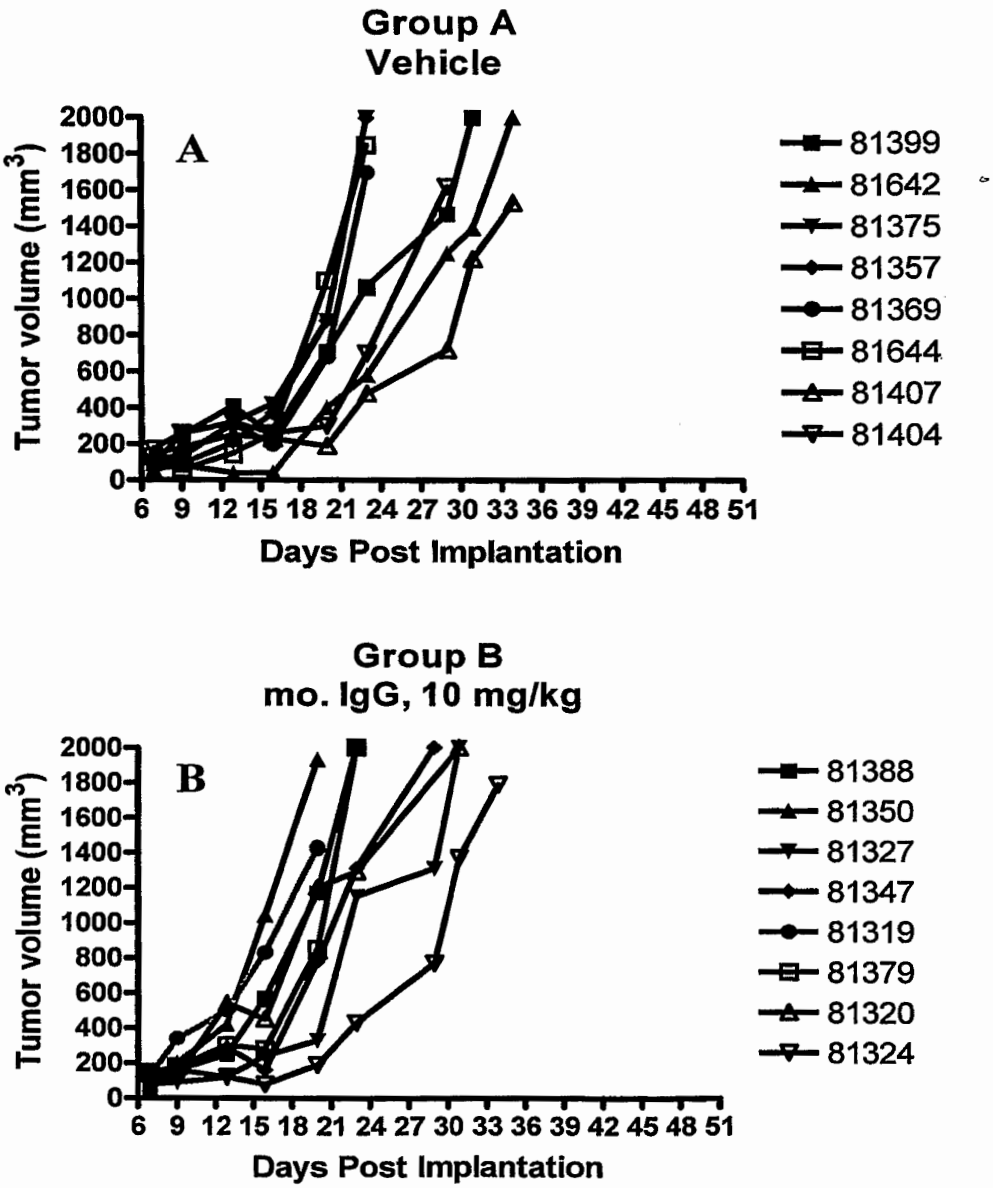


Figure 13

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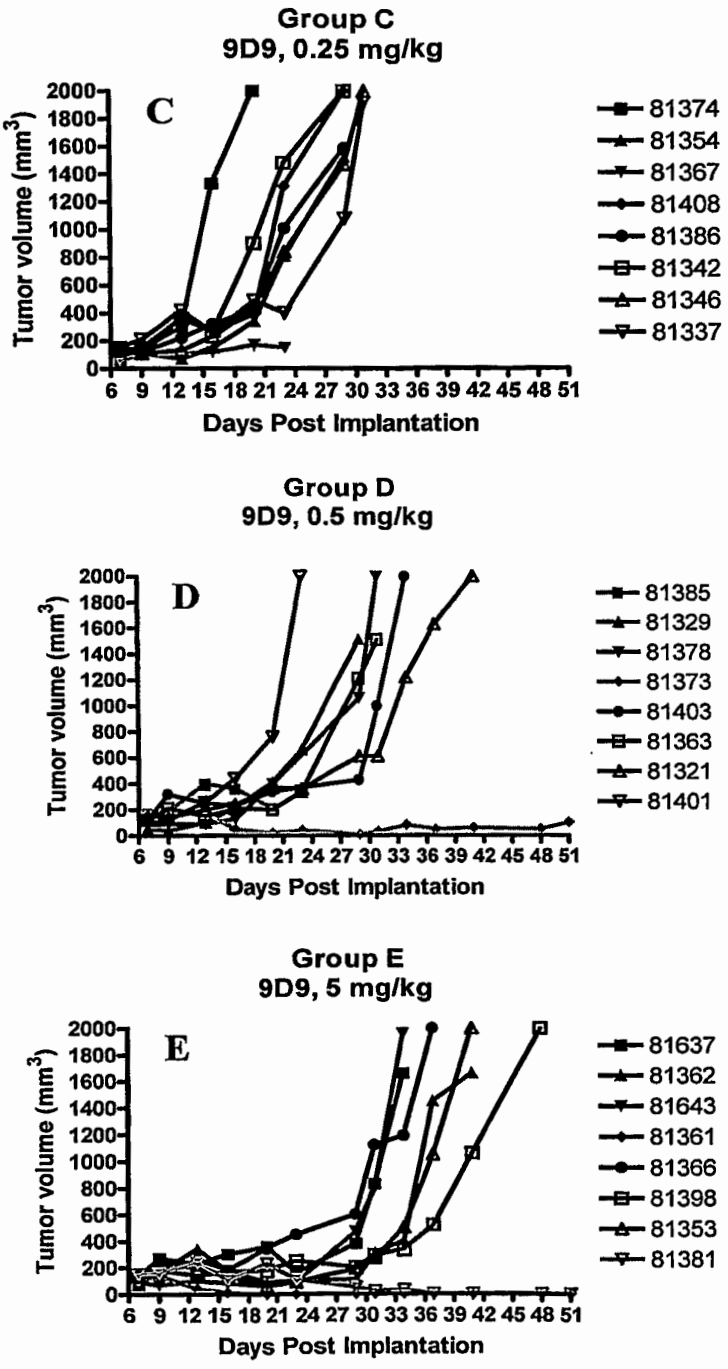
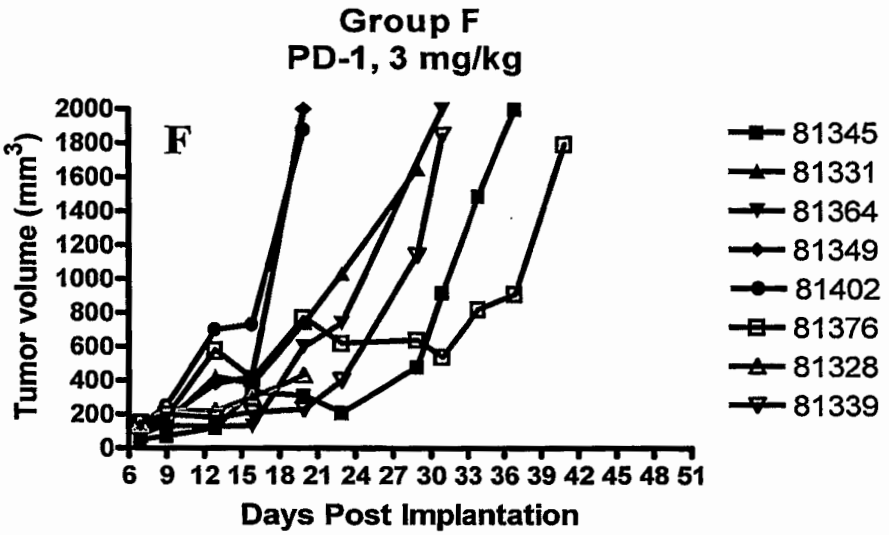


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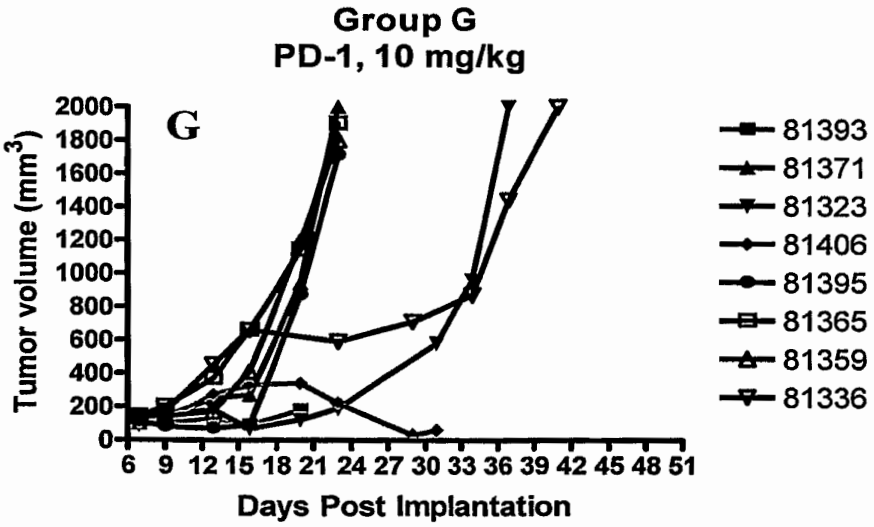


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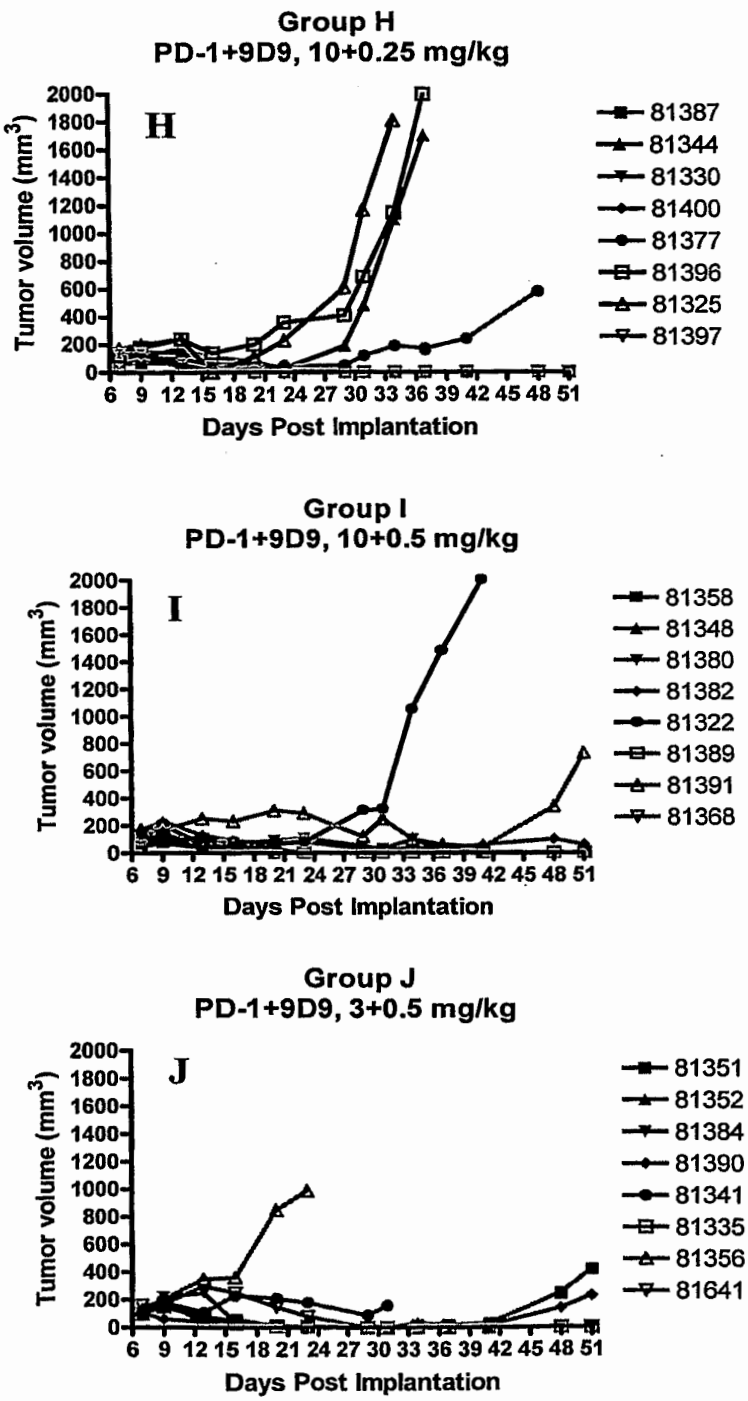


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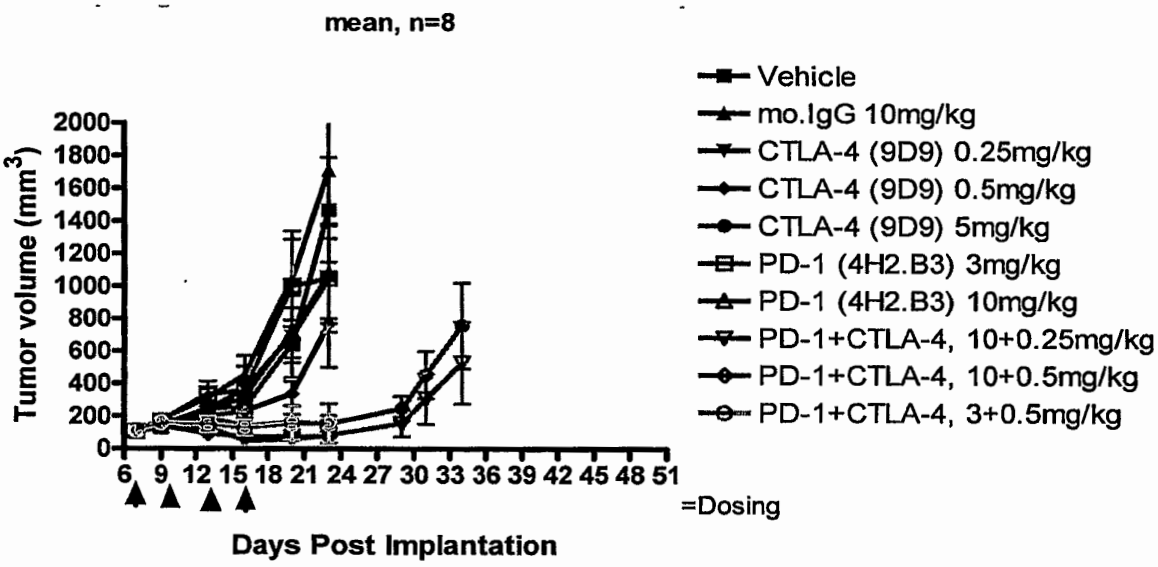


Figure 14

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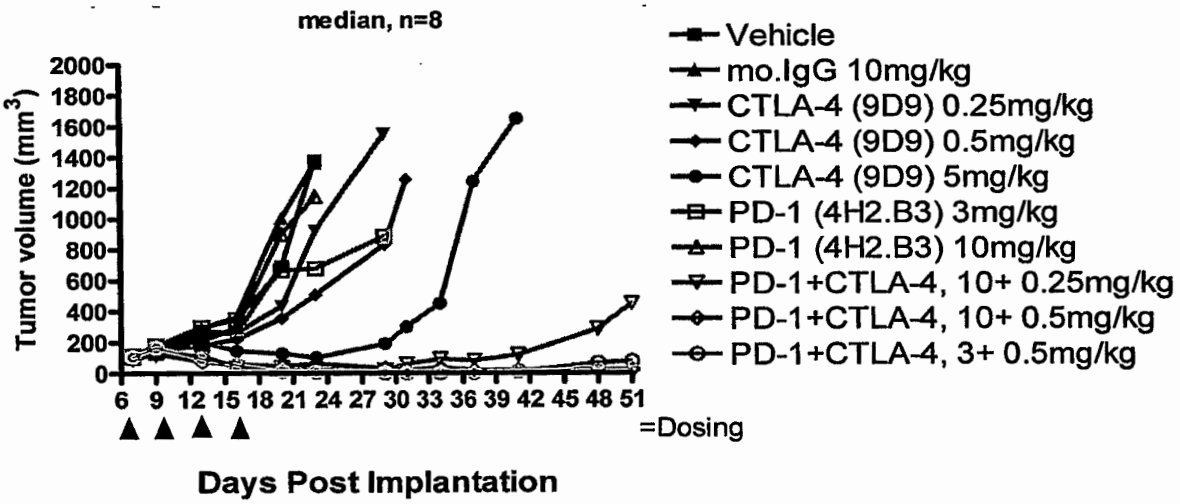
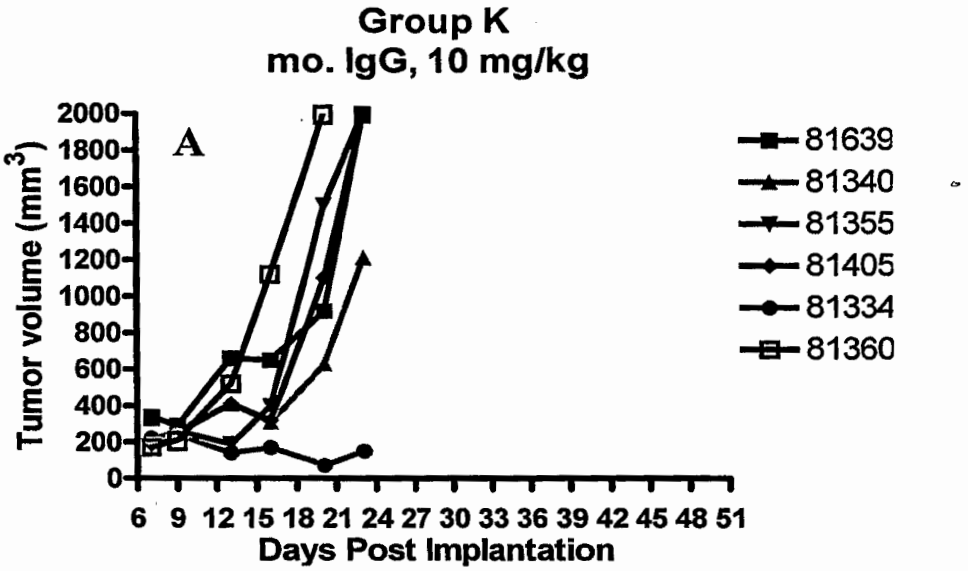


Figure 15

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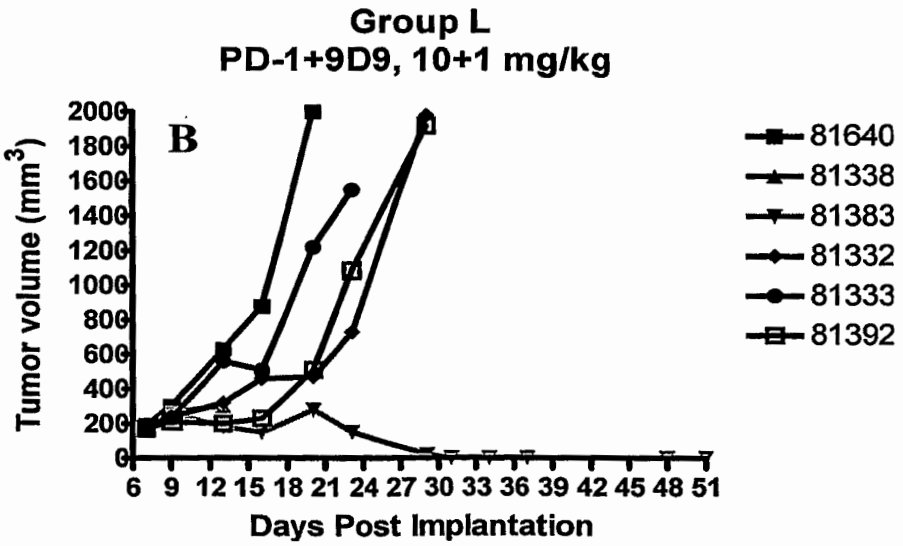


Figure 16

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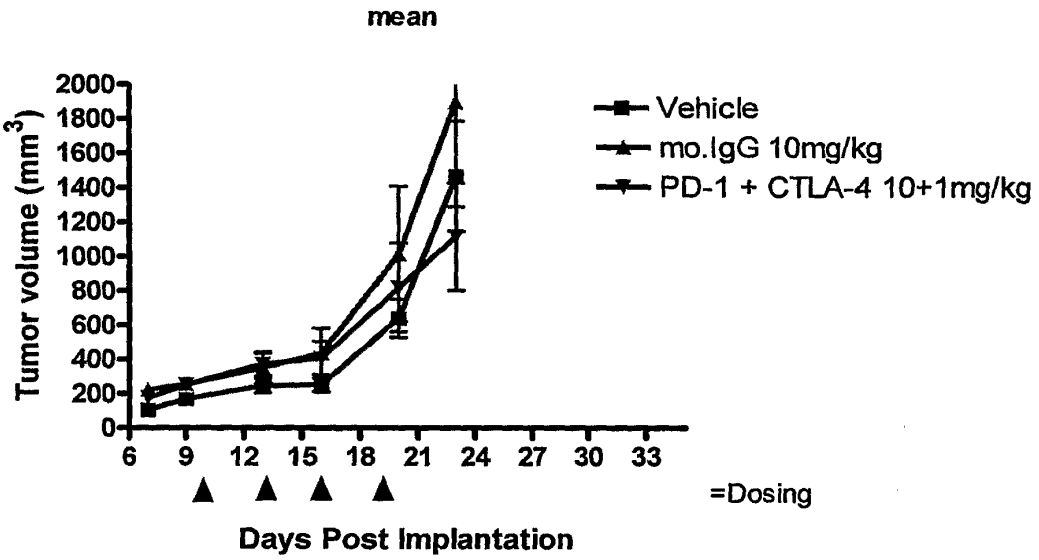


Figure 17

...USPTO from the IFW Image Database on 06/29/2006

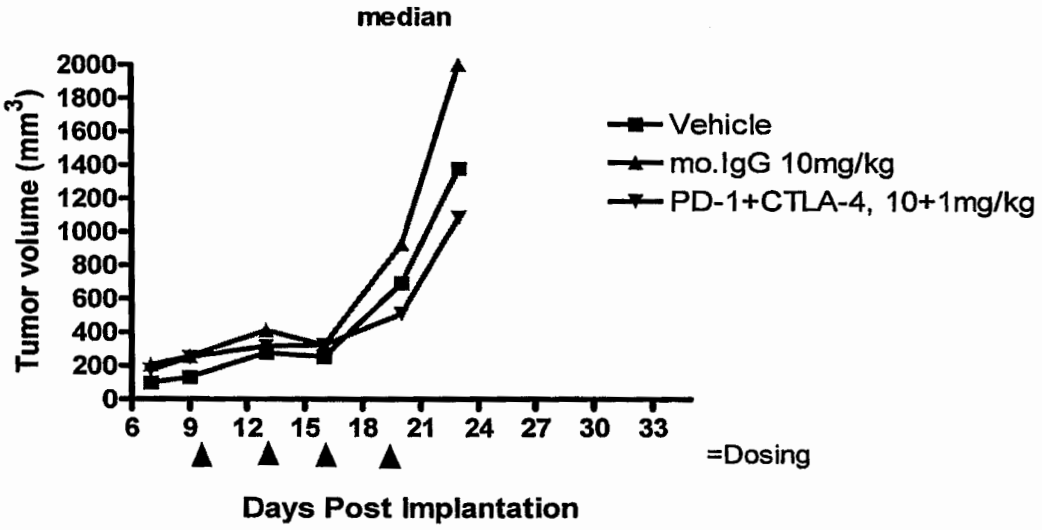


Figure 18

Copy provided by USPTO from the IFW Image Database on 06/29/2006

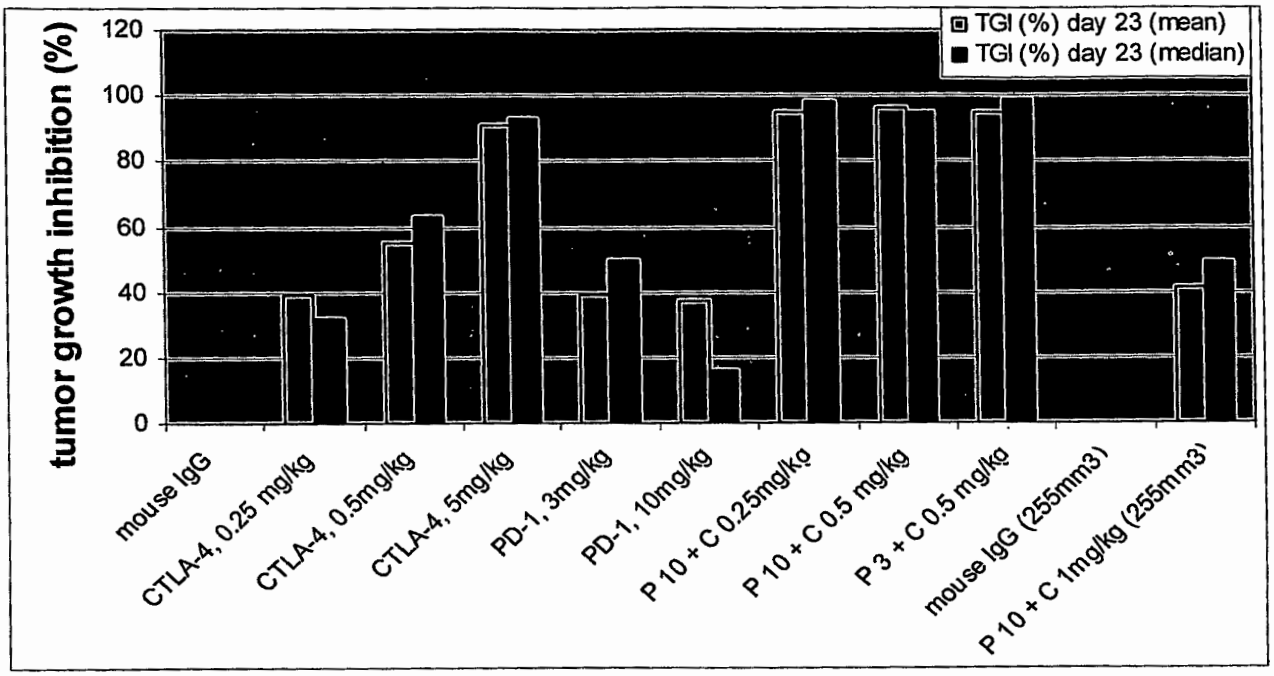
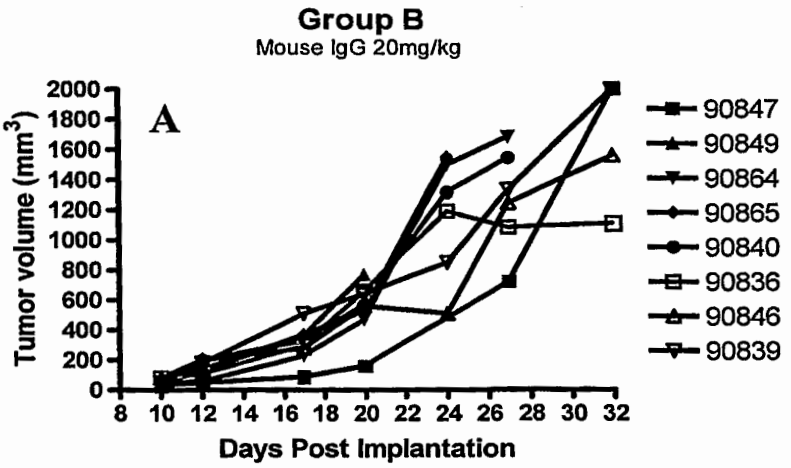


Figure 19

Copy provided by USPTO from the IFW Image Database on 06/29/2006



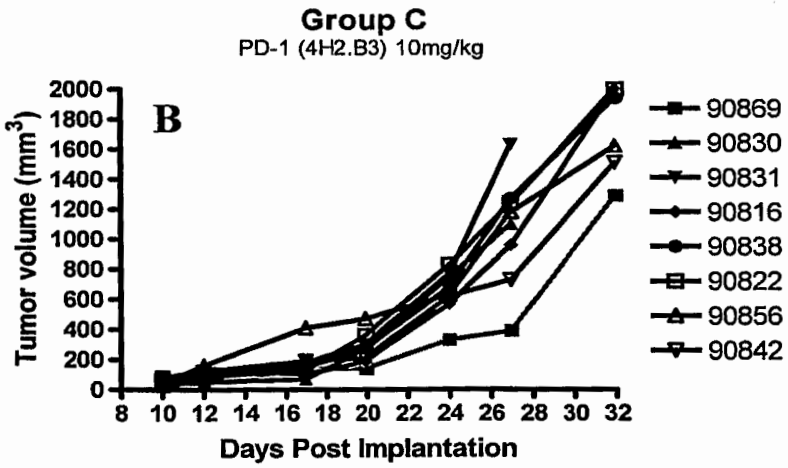
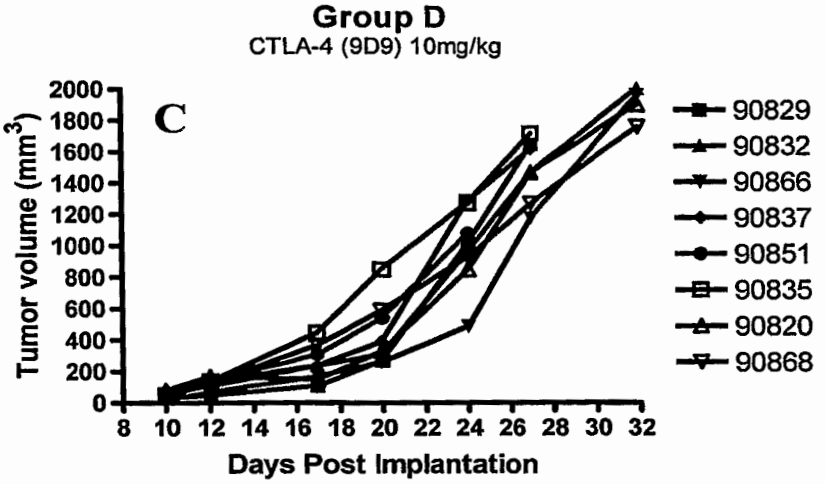


Figure 20

ravided by USPTO from the IFW Image Database on 06/29/2006



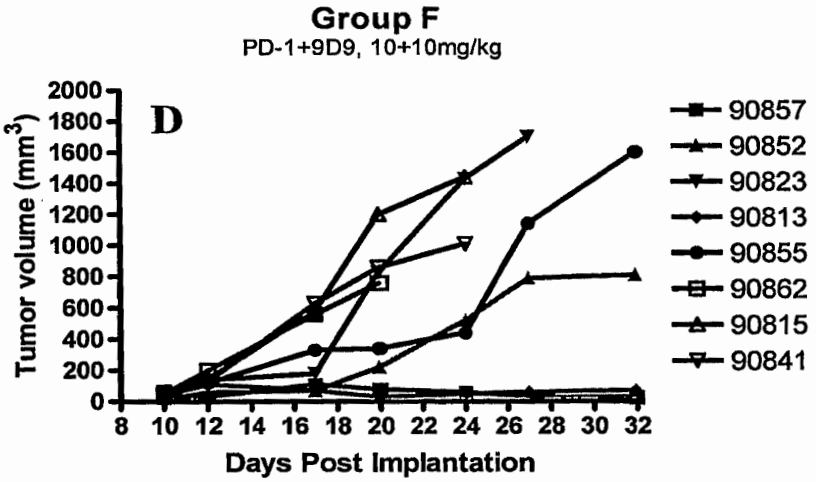


Figure 20 (cont.)

Copy provided by USPTO from the IFW Image Database on 06/29/2006

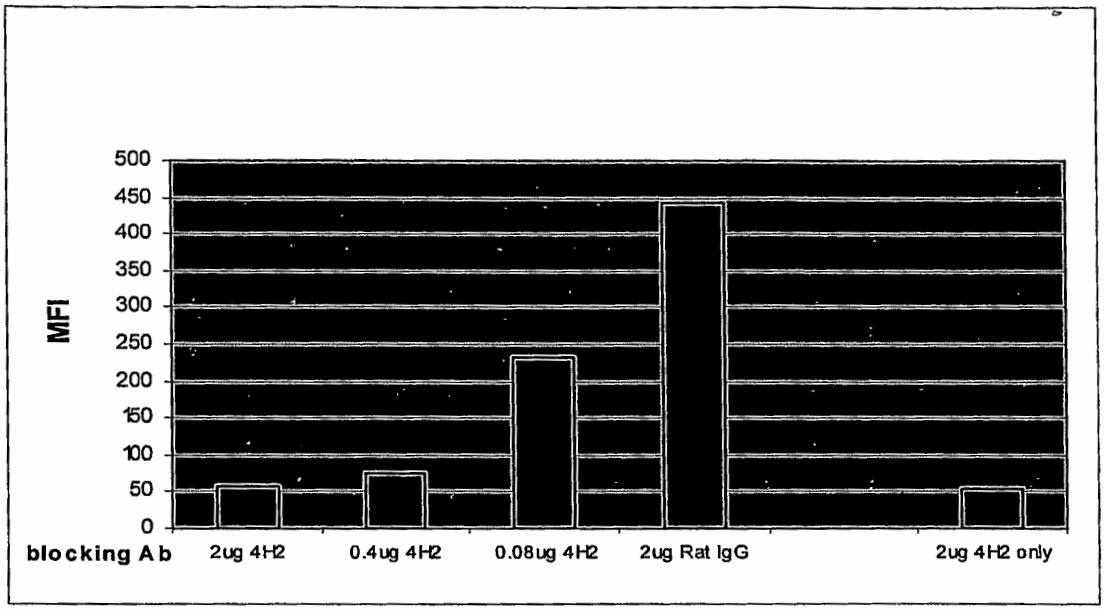


Figure 21

Copy provided by USPTO from the IFW Image Database on 06/29/2006

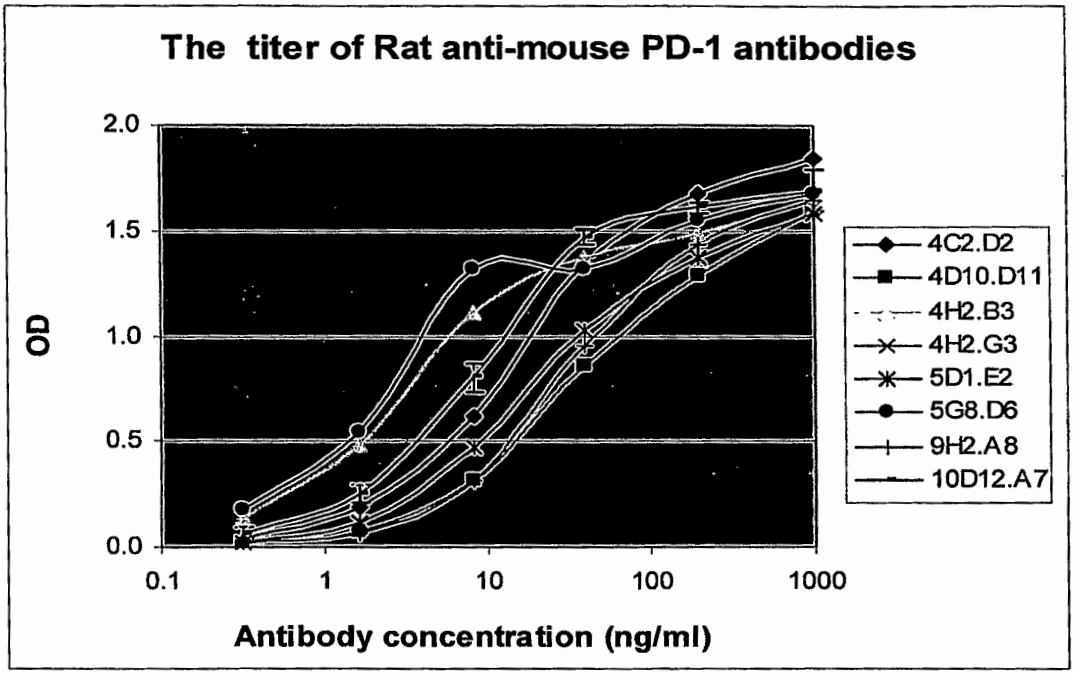


Figure 22

Copy provided by USPTO from the IFW Image Database on 06/29/2006

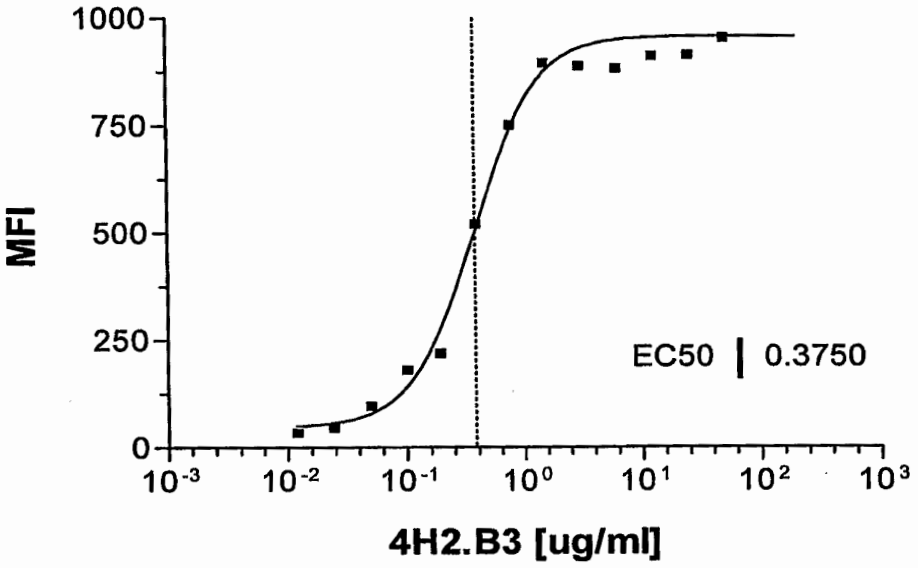


Figure 23

Copy provided by USPTO from the IFW Image Database on 06/29/2006

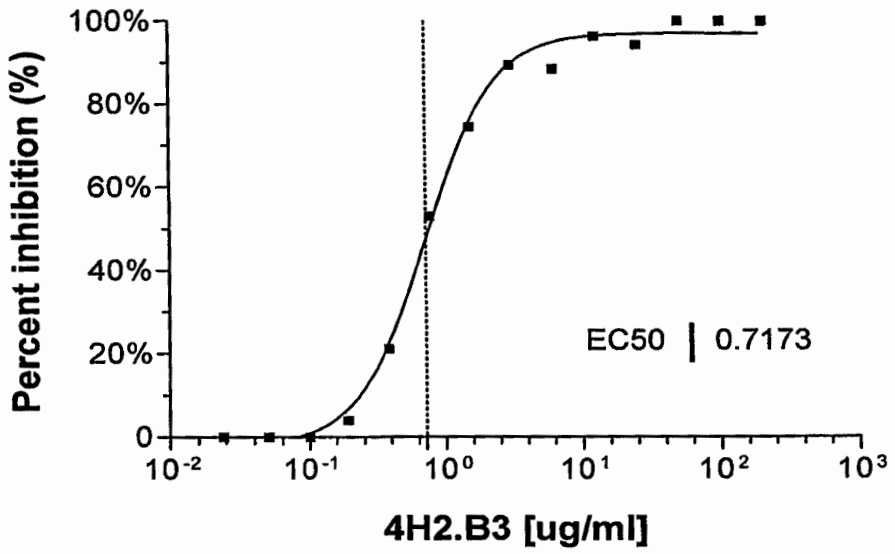


Figure 24

Copy provided by USPTO from the IFW Image Database on 06/29/2006

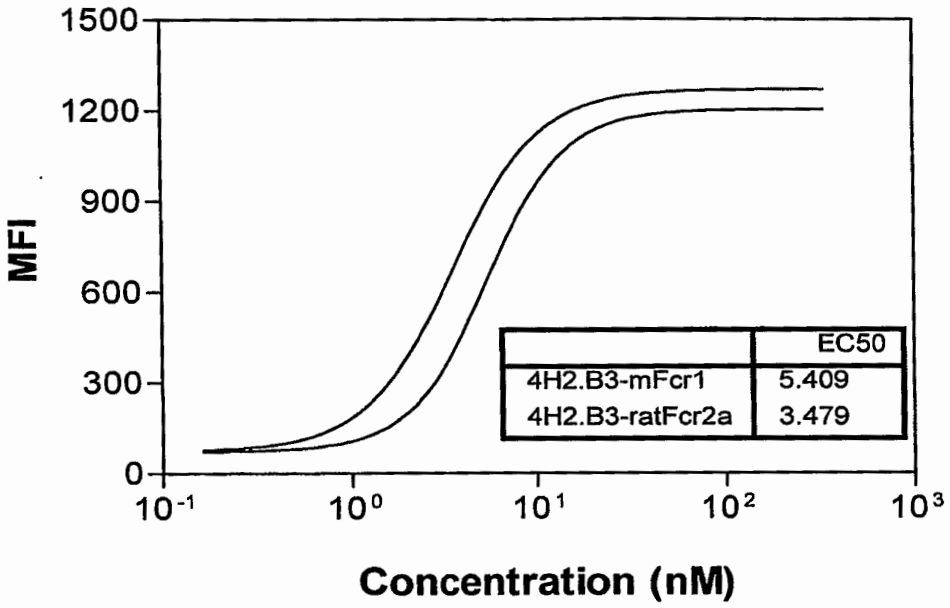


Figure 25

Copy provided by USPTO from the IFW Image Database on 06/29/2006

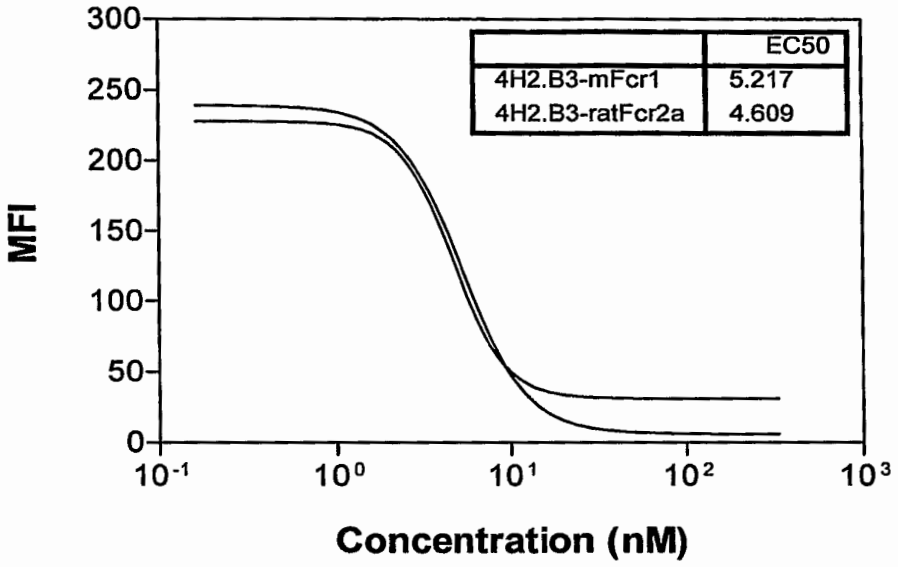


Figure 26

Copy provided by USPTO from the IFW Image Database on 06/29/2006

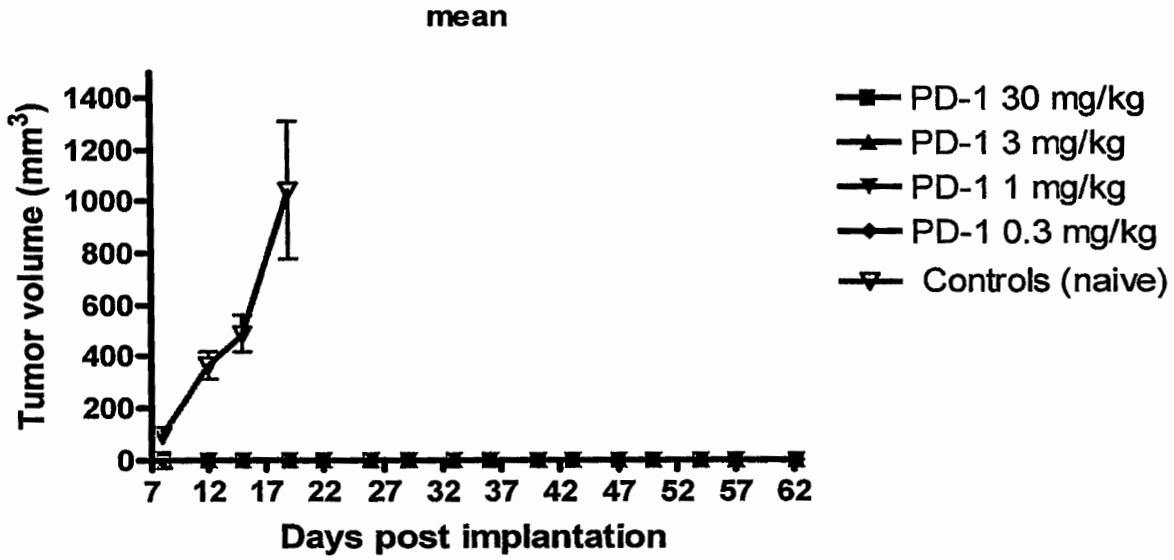


Figure 27

Copy provided by USPTO from the IFW Image Database on 06/29/2006

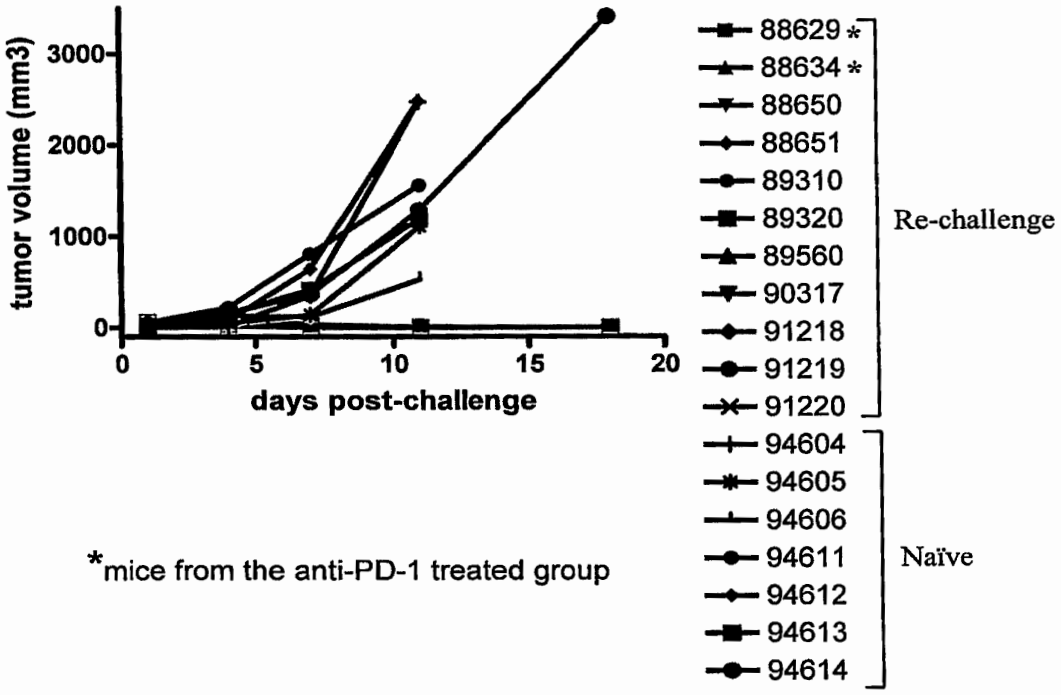


Figure 28

Copy provided by USPTO from the IFW Image Database on 06/29/2006

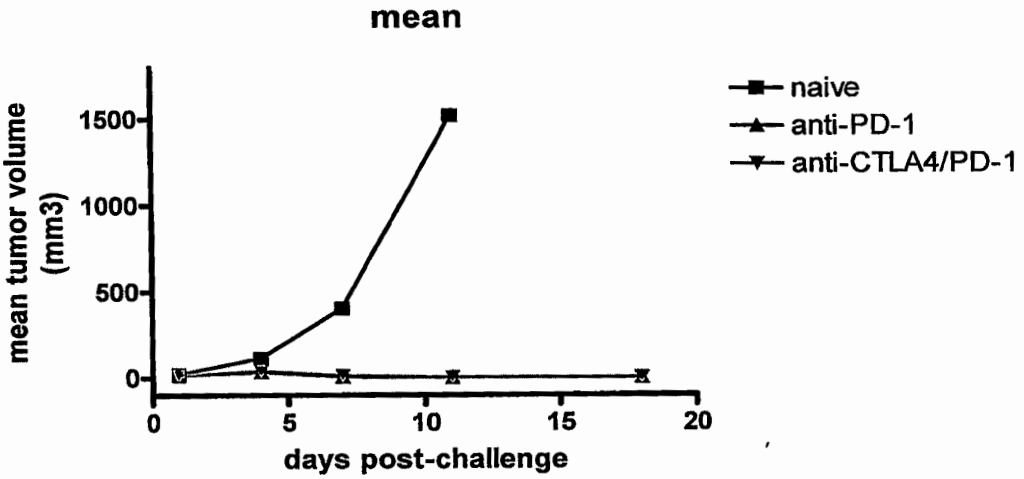


Figure 29

...ovided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD1 17D8 VH

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

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

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

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

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

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

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

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

Figure 30A

C... provided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD1 17D8 VK

V segment: L6
J segment: JK4

```
      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   I   S   S   Y   L   A   W   Y
55    GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT ATT AGC AGC TAC 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

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

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

Figure 30B

...ided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD-1 2D3 VH

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

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

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

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

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

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

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

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

Figure 31A

Document provided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD-1 2D3 VK

V segment: L6
J segment: JK4

```
      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   S   S   Y   L   A   W   Y
55    GCC ACC CTG TCC TGC AGG GCC AGT CAG AGT GTT AGC AGC TAC TTA GCC TGG TAC

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

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

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

Figure 31B

...ided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD-1 4H1 VH

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

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

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

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

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

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

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

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

Figure 32A

...vided by USPTO from the IFW Image Database on 06/29/2006

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

...vided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD1 5C4 VH

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

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

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

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

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

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

                                        CDR3
                                    ~~~~~~~~~~~~~~~~
      T   A   V   Y   Y   C   A   T . N   D   D   Y   W   G   Q   G   T   L
271   ACG GCT GTG TAT TAC TGT GCG ACA AAC GAC GAC TAC TGG GGC CAG GGA ACC CTG
                                      ↳
                                                    JH4b
      V   T   V   S   S
325   GTC ACC GTC TCC TCA
```

Figure 33A

...vided by USPTO from the IFW Image Database on 06/29/2006

Anti-PD1 5C4 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   S   S   Y   L   A   W   Y
55    GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AGT AGT TAC 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

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

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

Figure 33B

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Anti-PD-1 4A11 VH

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

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

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

          CDR1                                                            CDR2
          ~~~~                                                            ~~~~~~~~~~~~
          G   W   I   R   Q   P   P   G   K   G   L   E   W   I   G   S   I   Y
109       GGC TGG ATC CGT CAG CCC CCA GGG AAG GGA CTG GAG TGG ATT GGG AGT ATC TAT

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

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

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

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

Figure 34A

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Anti-PD-1 4A11 VK

V segment: L15
J segment: JK1

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

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

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

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

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

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

Figure 34B

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Anti-PD1 VH region alignment to VH 3-33

```
                ____CDR1__
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 - - - - - - -

                _____CDR2_____
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
2D3 VH:         - - - - - - - - - - - - - - - - - - K - - - - - - -
- - - - - - - - - - - - - -
4H1 VH:         - - - - - - - - - - L - - - - - - - - - - - - - - - -
- - - - - - - - - - - - - -
5C4 VH:         - - - - - - - - - - - - - - - - - - K R - - - - - -
- - - - - - - - - - - - - -
                                                    _CDR3__
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

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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

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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 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

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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 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

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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

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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 -


          CDR2
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          - - - - - - - - - - - - - - - - - - - - - - - -
- - - - - - - - - - - -


      CDR3
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

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

```
     CDR1
L15 germline    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
4A11 VK         - - - - - - - - - - - - - - - - - - - - S - - - -
- - - - - - -

     CDR2
L15 germline    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
4A11 VK         - - - - - - - - - - - - - - - - - - - - N - R
- - - - - - -

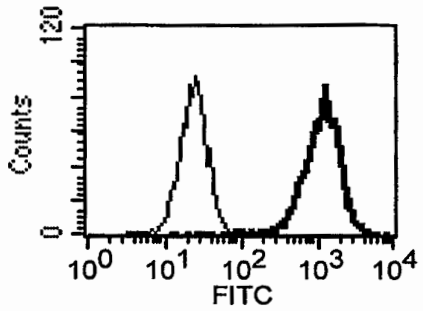
   CDR3
L15 germline    S G S G S G T D F T L T I S S L Q P E D F A T Y
Y C Q Q Y N S
4A11 VK         - - - - - - - - - - - - - - - - - - - - - - - -
- - - - - Y -

L15 germline    Y P
JK1 germline          T F G Q G T K V E I K
4A11 VK         - - R - - - - - - - - - - -     (JK1)
```

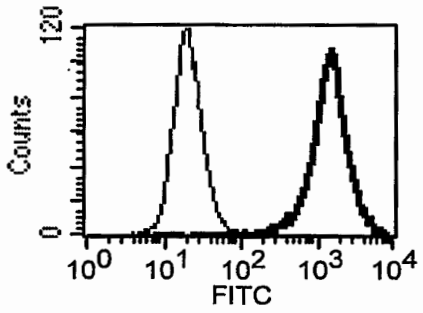
Figure 41

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(A)



(B)



---- Binding to CHO cells

--- Binding to CHO hPD-1 cells

Figure 42

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FILING DATE: ***May 09, 2005***

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. _____

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)

Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

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ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Application Data Sheet. See 37 CFR 1.76
[X] Specification *Number of Pages* 107
[X] Drawing(s) *Number of Sheets* 30
[ ] CD(s), Number of CDs _____
[ ] Other (specify)

Application Size Fee: If the specification and drawings exceed 100 sheets of paper, the application size fee due 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).

METHOD OF PAYMENT OF FILING FEES AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.
[X] A check or money order is enclosed to cover the filing fee and application size fee (if applicable). TOTAL FEE AMOUNT ($): 450.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
[X] No. [ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

SIGNATURE _____ Date May 9, 2005

TYPED or PRINTED NAME Paul M. Zagar, M.D. REGISTRATION NO. *(if appropriate)* 52,392

TELEPHONE (212) 527-7700 Docket Number: 04280/0202908-US0

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[Number 1 of 1]

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HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

Background of the Invention

The protein Programmed Death 1 (PD-1) is a member of the CD28 family of receptors, that also includes CD28, CTLA-4, ICOS and BTLA. The initial members of the family, CD28 and ICOS, were discovered by functional effects on augmenting T cell proliferation following the addition of monoclonal antibodies (Hutloff *et al.* (1999) *Nature* 397:263-266; Hansen *et al.* (1980) *Immunogenics* 10:247-260). PD-1 was discovered through screening for differential expression in apototic cells (Ishida *et al.* (1992) *EMBO J* 11:3887-95). The other members of the family, CTLA-4, and BTLA were discovered through screening for differential expression in cytotoxic T lymphocytes and TH1 cells, respectively. CD28, ICOS and CTLA-4 all have an unpaired cysteine residue allowing for homodimerization. In contrast, PD-1 is suggested to exist as a monomer, lacking the unpaired cysteine residue characteristic in other CD28 family members.

The PD-1 gene is a 55 kDa type I transmembrane protein that is part of the Ig gene superfamily (Agata *et al.* (1996) *Int Immunol* 8:765-72). PD-1 contains a membrane proximal immunoreceptor tyrosine inhibitory motif (ITIM) and a membrane distal tyrosine-based switch motif (ITSM) (Thomas, M.L. (1995) *J Exp Med* 181:1953-6; Vivier, E and Daeron, M (1997) *Immunol Today* 18:286-91). Although structurally similar to CTLA-4, PD-1 lacks the MYPPPY motif that is critical for B7-1 and B7-2 binding. Two ligands for PD-1 have been identified, PD-L1 and PD-L2, that have been shown to downregulate T cell activation upon binding to PD-1 (Freeman *et al.* (2000) *J Exp Med* 192:1027-34; Latchman *et al.* (2001) *Nat Immunol* 2:261-8; Carter *et al.* (2002) *Eur J Immunol* 32:634-43). Both PD-L1 and PD-L2 are B7 homologs that bind to PD-1, but do not bind to other CD28 family members.

PD-1 is an inhibitory member of the CD28 family 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). PD-1 deficient animals

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develop various autoimmune phenotypes, including autoimmune cardiomyopathy and a lupus-like syndrome with arthritis and nephritis (Nishimura *et al.* (1999) *Immunity* 11:141-51; Nishimura *et al.* (2001) *Science* 291:319-22). Additionally, PD-1 has been found to play a role in autoimmune encephalomyelitis, systemic lupus erythematosus, graft-versus-host disease (GVHD), type I diabetes, and rheumatoid arthritis (Salama *et al.* (2003) *J Exp Med* 198:71-78; Prokunina and Alarcon-Riquelme (2004) *Hum Mol Genet* 13:R143; Nielsen *et al.* (2004) *Lupus* 13:510). In a murine B cell tumor line, the ITSM of PD-1 was shown to be essential to block BCR-mediated Ca^{2+}-flux and tyrosine phosphorylation of downstream effector molecules (Okazaki *et al.* (2001) *PNAS* 98:13866-71).

Accordingly, agents that recognize PD-1, and methods of using such agents, are desired.

Summary of the Invention

The present invention provides isolated monoclonal antibodies, in particular human monoclonal antibodies, that bind to PD-1 and that exhibit numerous desirable properties. These properties include, for example, high affinity binding to human PD-1, but lacking substantial cross-reactivity with either human CD28, CTLA-4 or ICOS. Still further, antibodies of the invention have been shown to modulate immune responses. Accordingly, another aspect of the invention pertains to methods of modulating immune responses using anti-PD-1 antibodies. In particular, the invention provides a method of inhibiting growth of tumor cells *in vivo* using anti-PD-1 antibodies.

In one aspect, the invention pertains to an isolated monoclonal antibody, or an antigen-binding portion thereof, wherein the antibody exhibits at least one of the following properties:

(a) binds to human PD-1 with a K_D of 1×10^{-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;

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(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;

(j) inhibits tumor cell growth *in vivo*.

Preferably the antibody is a human antibody, although in alternative embodiments the antibody can be, for example, a murine antibody, a chimeric antibody or humanized antibody.

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

In another embodiment, the invention provides an isolated monoclonal antibody, or antigen binding protion thereof, wherein the antibody cross-competes for binding to PD-1 with a reference antibody comprising:

(a) a human heavy chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5; and

(b) a human light chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10.

In various embodiments, the reference antibody comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 6;

or the reference antibody comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 7;

or the reference antibody comprises:

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(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8;

or the reference antibody comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 4; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 9;

or the reference antibody comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 5; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10.

In another aspect, the invention pertains to an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human V_H 3-33 gene, wherein the antibody specifically binds PD-1. The invention further provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human V_H 4-39 gene, wherein the antibody specifically binds PD-1. The invention further provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human V_K L6 gene, wherein the antibody specifically binds PD-1. The invention further provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human V_K L15 gene, wherein the antibody specifically binds PD-1.

In a preferred embodiment, the invention provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising:

(a) a heavy chain variable region of a human V_H 3-33 gene; and

(b) a light chain variable region of a human V_K L6 gene;

wherein the antibody specifically binds to PD-1.

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In another preferred embodiment, the invention provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising:

(a) a heavy chain variable region of a human V_H 4-39 gene; and

(b) a light chain variable region of a human V_K L15 gene;

wherein the antibody specifically binds to PD-1.

In another aspect, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof, comprising:

a heavy chain variable region that comprises CDR1, CDR2, and CDR3 sequences; and a light chain variable region that comprises CDR1, CDR2, and CDR3 sequences, wherein:

(a) the heavy chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25, and conservative modifications thereof;

(b) the light chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40, and conservative modifications thereof; and

(c) the antibody specifically binds to human PD-1.

Preferably, the heavy chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 16, 17, 18, 19, and 20, and conservative modifications thereof; and the light chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 31, 32, 33, 34, and 35, and conservative modifications thereof. Preferably, the heavy chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 11, 12, 13, 14, and 15, and conservative modifications thereof; and the light chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 26, 27, 28, 29, and 30, and conservative modifications thereof.

In yet another aspect, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region and a light chain variable region, wherein:

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(a) the heavy chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5;

(b) the light chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10;

(c) the antibody binds to human PD-1 with a K_D of 1×10^{-7} M or less; and

(d) the antibody does not substantially bind to human CD28, CTLA-4 or ICOS.

In a preferred embodiment, the antibodies additionally comprise at least one of the following properties:

(a) the antibody increases T-cell proliferation in an MLR assay;

(b) the antibody increases interferon-gamma production in an MLR assay; or

(c) the antibody increases IL-2 secretion in an MLR assay.

Additionally or alternatively, the antibody may comprise one or more of the other features listed above.

In preferred embodiments, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof, comprising:

(a) a heavy chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15;

(b) a heavy chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20;

(c) a heavy chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25;

(d) a light chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30;

(e) a light chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35; and

(f) a light chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40;

wherein the antibody specifically binds PD-1.

A preferred combination comprises:

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(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 11;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 16;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 21;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 26;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 31; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 36.

Another preferred combination comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 12;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 17;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 22;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 27;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 32; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 37.

Another preferred combination comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 13;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 18;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 23;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 28;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 33; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 38.

Another preferred combination comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 14;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 19;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 24;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 29;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 34; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 39.

Another preferred combination comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 15;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 20;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 25;

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(d) a light chain variable region CDR1 comprising SEQ ID NO: 30;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 35; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 40.

Other preferred antibodies of the invention, or antigen binding portions thereof, comprise:

(a) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5; and

(b) a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10;

wherein the antibody specifically binds PD-1.

A preferred combination comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 6.

Another preferred combination comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 7.

Another preferred combination comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8.

Another preferred combination comprises:

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 4; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 9.

Another preferred combination comprises:

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(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 5; and

(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10.

The antibodies of the invention can be, for example, full-length antibodies, for example of an IgG1 or IgG4 isotype. Alternatively, the antibodies can be antibody fragments, such as Fab or Fab'2 fragments, or single chain antibodies.

The invention also provides an immunoconjugate comprising an antibody of the invention, or antigen-binding portion thereof, linked to a therapeutic agent, such as a cytotoxin or a radioactive isotope. The invention also provides a bispecific molecule comprising an antibody, or antigen-binding portion thereof, of the invention, linked to a second functional moiety having a different binding specificity than said antibody, or antigen binding portion thereof.

Compositions comprising an antibody, or antigen-binding portion thereof, or immunoconjugate or bispecific molecule of the invention, and a pharmaceutically acceptable carrier, are also provided.

Nucleic acid molecules encoding the antibodies, or antigen-binding portions thereof, of the invention are also encompassed by the invention, as well as expression vectors comprising such nucleic acids and host cells comprising such expression vectors. Moreover, the invention provides a transgenic mouse comprising human immunoglobulin heavy and light chain transgenes, wherein the mouse expresses an antibody of the invention, as well as hybridomas prepared from such a mouse, wherein the hybridoma produces the antibody of the invention.

In yet another aspect, the invention provides a method of modulating an immune response in a subject comprising administering to the subject the antibody, or antigen-binding portion thereof, of the invention such that the immune response in the subject is modulated. Preferably, the antibody of the invention enhances, stimulates or increases the immune response in the subject.

In a further aspect, the invention provides a method of inhibiting growth of tumor cells in a subject, comprising administering to a subject a therapeutically effective amount of an anti-PD-1 antibody, or antigen-binding portion thereof. The antibodies of the

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invention are preferred for use in the method although other anti-PD-1 antibodies can be used instead (or in combination with an anti-PD-1 antibody of the invention). For example, a chimeric, humanized or fully human anti-PD-1 antibody can be used in the method of inhibiting tumor growth.

In a further aspect, the invention provides a method of treating an infectious disease in a subject, comprising administering to a subject a therapeutically effective amount of an anti-PD-1 antibody, or antigen-binding portion thereof. The antibodies of the invention are preferred for use in the method although other anti-PD-1 antibodies can be used instead (or in combination with an anti-PD-1 antibody of the invention). For example, a chimeric, humanized or fully human anti-PD-1 antibody can be used in the method of treating an infectious disease.

Still further, the invention provides a method of enhancing an immune response to an antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) an anti-PD-1 antibody, or antigen-binding portion thereof, such that an immune response to the antigen in the subject is enhanced. The antigen can be, for example, a tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen. The antibodies of the invention are preferred for use in the method although other anti-PD-1 antibodies can be used instead (or in combination with an anti-PD-1 antibody of the invention). For example, a chimeric, humanized or fully human anti-PD-1 antibody can be used in the method of enhancing an immune response to an antigen in a subject.

The invention also provides methods for making "second generation" anti-PD-1 antibodies based on the sequences of the anti-PD-1 antibodies provided herein. For example, the invention provides a method for preparing an anti-PD-1 antibody comprising:

(a) providing: (i) a heavy chain variable region antibody sequence comprising a CDR1 sequence that is selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15, and/or a CDR2 sequence that is selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20; and/or a CDR3 sequence that is selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25; or (ii) a light chain variable region antibody sequence comprising a CDR1 sequence that is selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30, and/or a CDR2 sequence that is

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selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35, and/or a CDR3 sequence that is selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40;

(b) altering at least one amino acid residue within at least one variable region antibody sequence, said sequence being selected from the heavy chain variable region antibody sequence and the light chain variable region antibody sequence, to create at least one altered antibody sequence; and

(c) expressing the altered antibody sequence as a protein.

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

Brief Description of the Drawings

Figure 1A 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. 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 1B 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.

Figure 2A 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

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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.

Figure 2B 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.

Figure 3A 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.

Figure 3B 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 4A 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.

Figure 4B 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

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CDR3 (SEQ ID NO: 39) regions are delineated and the V and J germline derivations are indicated.

Figure 5A 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 5B 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. 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 6 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).

Figure 7 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 8 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).

Figure 9 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).

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Figure 10 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 11 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).

Figure 12 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 13A-13B 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 13A shows to the flow cytometry plot for 5C4. Figure 13B shows the flow cytometry plot for 4H1.

Figure 14 shows a graph demonstrating that the human monoclonal antibodies 17D8, 2D3, 4H1, 5C4, and 4A11, directed against human PD-1, bind specifically to PD-1, and not to other members of the CD28 family.

Figures 15A-15C show the results of flow cytometry experiments demonstrating that the human monoclonal antibodies 4H1 and 5C4, directed against human PD-1, binds to PD-1 on the cell surface. Figure 15A shows binding to activated human T-cells. Figure 15B shows the binding to cynomolgous monkey T-cells. Figure 15C shows the binding to CHO transfected cells expressing PD-1.

Figures 16A-16C show the results of experiments demonstrating that human monoclonal antibodies against human PD-1 promote T-cell proliferation, IFN-gamma secretion and IL-2 secretion in a mixed lymphocyte reaction assay. Figure 16A is a bar

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graph showing concentration dependent T-cell proliferation; Figure 16B is a bar graph showing concentration dependent IFN-gamma secretion; Figure 16C is a bar graph showing concentration dependent IL-2 secretion.

Figures 17A-17B show the results of flow cytometry experiments demonstrating that human monoclonal antibodies against human PD-1 block the binding of PD-L1 and PD-L2 to CHO transfected cells expressing PD-1. Figure 17A is a graph showing inhibition of binding of PD-L1; Figure 17B is a graph showing inhibition of binding of PD-L2.

Figure 18 shows the results of flow cytometry experiments demonstrating that human monoclonal antibodies against human PD-1 do not promote T-cell apoptosis.

Figure 19 shows the results of experiments demonstrating that anti-PD-1 HuMabs have a concentration dependent effect on IFN gamma secretion by PBMCs from CMV-positive donors when PBMCs were stimulated with a CMV lysate and anti-PD-1.

Figure 20 shows the results of tumor growth experiments in a mouse model system demonstrating that treatment *in vivo* of mouse tumors with anti-PD-1 antibodies inhibits the growth of tumors.

Detailed Description of the Invention

In one aspect, the present invention relates to isolated monoclonal antibodies, particularly human monoclonal antibodies, that bind specifically to PD-1. In certain embodiments, the antibodies of the invention exhibit one or more desireable functional properties, such as high affinity binding to PD-1, lack of cross-reactivity to other CD28 family members, the ability to stimulate T cell proliferation, IFN-γ and/or IL-2 secretion in mixed lymphocyte reactions, the abilty to inhibit binding of one or more PD-1 ligands (*e.g.*, PD-L1 and/or PD-L2), the ability to cross-react with cynomolgus monkey PD-1, the

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ability to stimulate antigen-specific memory responses, the ability to stimulate antibody responses and/or the ability to inhibit growth of tumor cells *in vivo*. Additionally or alternatively, the antibodies of the invention are derived from particular heavy and light chain germline sequences and/or comprise particular structural features such as CDR regions comprising particular amino acid sequences.

The invention provides, for example, isolated antibodies, methods of making such antibodies, immunoconjugates and bispecific molecules comprising such antibodies and pharmaceutical compositions containing the antibodies, immunconjugates or bispecific molecules of the invention.

In another aspect, the invention pertains to methods of inhibiting growth of tumor cells in a subject using anti-PD-1 antibodies. As demonstrated herein, anti-PD-1 antibodies are capable of inhibiting tumor cell growth *in vivo*. The invention also relates to methods of using the antibodies to modify an immune response, as well as to treat diseases such as cancer or infectious disease, or to stimulate a protective autoimmune response or to stimulate antigen-specific immune reponses (e.g., by coadministration of anti-PD-1 with an antigen of interest).

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

The term "immune response" refers to the action of, for example, 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.

A "signal transduction pathway" refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. As used herein, the phrase "cell surface receptor" includes, for example, molecules and complexes of molecules capable

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of receiving a signal and the transmission of such a signal across the plasma membrane of a cell. An example of a "cell surface receptor" of the present invention is the PD-1 receptor.

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 inter-connected 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 (Clq) 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),

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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.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds PD-1 is substantially free of antibodies that specifically bind antigens other than PD-1). An isolated antibody that specifically binds PD-1 may, however, have cross-reactivity to other antigens, such as PD-1 molecules from other species. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

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 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 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

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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 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.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by the heavy chain constant region genes.

The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

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The term "human antibody derivatives" refers to any modified form of the human antibody, *e.g.*, a conjugate of the antibody and another agent or antibody.

The term "humanized antibody" is intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences.

The term "chimeric antibody" is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

As used herein, an antibody that "specifically binds to human PD-1" is intended to refer to an antibody that binds to human PD-1 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, 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 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 by using surface plasmon resonance, preferably using a 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.

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As used herein, the term "subject" includes any human or nonhuman animal. The term "nonhuman animal" includes all vertebrates, *e.g.*, mammals and non-mammals, such as nonhuman primates, sheep, dogs, cats, horses, cows chickens, amphibians, reptiles, *etc.*

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

Anti-PD-1 Antibodies

The antibodies of the invention are characterized by particular functional features or properties of the antibodies. For example, the antibodies bind specifically to PD-1 (e.g., bind to human PD-1 and may cross-react with PD-1 from other species, such as cynomolgus monkey). Preferably, an antibody of the invention binds to PD-1 with high affinity, for example with a K_D of 1×10^{-7} M or less. The anti-PD-1 antibodies of the invention preferably exhibit one or more of the following characteristics:

(a) binds to human PD-1 with a K_D of 1×10^{-7} M or less;

(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;

(j) inhibits tumor cell growth *in vivo*.

Preferrably, the antibody binds to human PD-1 with a K_D of 5×10^{-8} M or less, binds to human PD-1 with a K_D of 1×10^{-8} M or less, binds to human PD-1 with a K_D of 5×10^{-9} M or less, or binds to human PD-1 with a K_D of between 1×10^{-8} M and 1×10^{-10} M or less.

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An antibody of the invention may exhibit any combination of the above-listed features, such as two, three, four, five or more of the above-listed features.

Standard assays to evaluate the binding ability of the antibodies toward PD-1 are known in the art, including for example, ELISAs, Western blots and RIAs. The binding kinetics (*e.g.*, binding affinity) of the antibodies also can be assessed by standard assays known in the art, such as by Biacore analysis. Suitable assays for evaluating any of the above-described characteristics are described in detail in the Examples.

Monoclonal Antibodies 17D8, 2D3, 4H1, 5C4 and 4A11

Preferred antibodies of the invention are the human monoclonal antibodies 17D8, 2D3, 4H1, 5C4, and 4A11, isolated and structurally characterized as described in Examples 1 and 2. The V_H amino acid sequences of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 1, 2, 3, 4, and 5, respectively. The V_L amino acid sequences of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 6, 7, 8, 9, and 10, respectively.

Given that each of these antibodies can bind to PD-1, the V_H and V_L sequences can be "mixed and matched" to create other anti-PD-1 binding molecules of the invention. PD-1 binding of such "mixed and matched" antibodies can be tested using the binding assays described above and in the Examples (*e.g.*, ELISAs). Preferably, when V_H and V_L chains are mixed and matched, a V_H sequence from a particular V_H/V_L pairing is replaced with a structurally similar V_H sequence. Likewise, preferably a V_L sequence from a particular V_H/V_L pairing is replaced with a structurally similar V_L sequence.

Accordingly, in one aspect, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof comprising:

(a) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5; and

(b) a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10;

wherein the antibody specifically binds PD-1, preferably human PD-1.

Preferred heavy and light chain combinations include:

(a) a heavy chain variable region comprising the amino acid sequence of

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SEQ ID NO: 1; and (b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 6; or

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2; and (b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 7; or

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and (b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8; or

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 4; and (b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 9; or

(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 5; and (b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10.

In another aspect, the invention provides antibodies that comprise the heavy chain and light chain CDR1s, CDR2s and CDR3s of 17D8, 2D3, 4H1, 5C4, and 4A11, or combinations thereof. The amino acid sequences of the V_H CDR1s of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 11, 12, 13, 14, and 15, respectively. The amino acid sequences of the V_H CDR2s of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 16, 17, 18, 19, and 20, respectively. The amino acid sequences of the V_H CDR3s of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 21, 22, 23, 24, and 25, respectively. The amino acid sequences of the V_k CDR1s of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 26, 27, 28, 29, and 30, respectively. The amino acid sequences of the V_k CDR2s of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 31, 32, 33, 34, and 35, respectively. The amino acid sequences of the V_k CDR3s of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 36, 37, 38, 39, and 40, respectively. The CDR regions are delineated using the Kabat system (Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242).

Given that each of these antibodies can bind to PD-1 and that antigen-binding specificity is provided primarily by the CDR1, CDR2, and CDR3 regions, the V_H CDR1,

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CDR2, and CDR3 sequences and V_k CDR1, CDR2, and CDR3 sequences can be "mixed and matched" (*i.e.*, CDRs from different antibodies can be mixed and match, although each antibody must contain a V_H CDR1, CDR2, and CDR3 and a V_k CDR1, CDR2, and CDR3) to create other anti-PD-1 binding molecules of the invention. PD-1 binding of such "mixed and matched" antibodies can be tested using the binding assays described above and in the Examples (*e.g.*, ELISAs, Biacore analysis). Preferably, when V_H CDR sequences are mixed and matched, the CDR1, CDR2 and/or CDR3 sequence from a particular V_H sequence is replaced with a structurally similar CDR sequence(s). Likewise, when V_k CDR sequences are mixed and matched, the CDR1, CDR2 and/or CDR3 sequence from a particular V_k sequence preferably is replaced with a structurally similar CDR sequence(s). It will be readily apparent to the ordinarily skilled artisan that novel V_H and V_L sequences can be created by substituting one or more V_H and/or V_L CDR region sequences with structurally similar sequences from the CDR sequences disclosed herein for monoclonal antibodies antibodies 17D8, 2D3, 4H1, 5C4, and 4A11.

Accordingly, in another aspect, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof comprising:

(a) a heavy chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15;

(b) a heavy chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20;

(c) a heavy chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25;

(d) a light chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30;

(e) a light chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35; and

(f) a light chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40;

wherein the antibody specifically binds PD-1, preferably human PD-1.

In a preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 11;

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(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 16;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 21;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 26;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 31; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 36.

In another preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 12;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 17;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 22;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 27;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 32; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 37.

In another preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 13;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 18;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 23;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 28;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 33; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 38.

In another preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 14;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 19;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 24;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 29;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 34; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 39.

In another preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 15;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 20;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 25;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 30;

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(e) a light chain variable region CDR2 comprising SEQ ID NO: 35; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 40.

Antibodies Having Particular Germline Sequences

In certain embodiments, an antibody of the invention comprises a heavy chain variable region from a particular germline heavy chain immunoglobulin gene and/or a light chain variable region from a particular germline light chain immunoglobulin gene.

For example, in a preferred embodiment, the invention provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human V_H 3-33 gene, wherein the antibody specifically binds PD-1, preferably human PD-1. In another preferred embodiment, the invention provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human V_H 4-39 gene, wherein the antibody specifically binds PD-1, preferably human PD-1. In yet another preferred embodiment, the invention provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human V_K L6 gene, wherein the antibody specifically binds PD-1, preferably human PD-1. In yet another preferred embodiment, the invention provides an isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human V_K L15 gene, wherein the antibody specifically binds PD-1, preferably human PD-1. In yet another preferred embodiment, the invention provides an isolated monoclonal antibody, or antigen-binding portion thereof, wherein the antibody:

(a) comprises a heavy chain variable region that is the product of or derived from a human V_H 3-33 or 4-39 gene (which gene encodes the amino acid sequence set forth in SEQ ID NO: 51 or 53, respectively);

(b) comprises a light chain variable region that is the product of or derived from a human V_K L6 or L15 gene (which gene encodes the amino acid sequence set forth in SEQ ID NO: 52 or 54, respectively); and

(c) specifically binds to PD-1.

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Examples of antibodies having V_H and V_K of V_H 3-33 and V_K L6, respectively, are 17D8, 2D3, 4H1, and 5C4. An example of an antibody having V_H and V_K of V_H 4-39 and V_K L15, respectively is 4A11.

As used herein, a human antibody comprises heavy or light chain variable regions that is "the product of" or "derived from" a particular germline sequence if the variable regions of the antibody are obtained from a system that uses human germline immunoglobulin genes. Such systems include immunizing a transgenic mouse carrying human immunoglobulin genes with the antigen of interest or screening a human immunoglobulin gene library displayed on phage with the antigen of interest. A human antibody that is "the product of" or "derived from" a human germline immunoglobulin sequence can be identified as such by comparing the amino acid sequence of the human antibody to the amino acid sequences of human germline immunoglobulins and selecting the human germline immunoglobulin sequence that is closest in sequence (*i.e.*, greatest % identity) to the sequence of the human antibody. A human antibody that is "the product of" or "derived from" a particular human germline immunoglobulin sequence may contain amino acid differences as compared to the germline sequence, due to, for example, naturally-occurring somatic mutations or intentional introduction of site-directed mutation. However, a selected human antibody typically is at least 90% identical in amino acids sequence to an amino acid sequence encoded by a human germline immunoglobulin gene and contains amino acid residues that identify the human antibody as being human when compared to the germline immunoglobulin amino acid sequences of other species (*e.g.*, murine germline sequences). In certain cases, a human antibody may be at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a human antibody derived from a particular human germline sequence will display no more than 10 amino acid differences from the amino acid sequence encoded by the human germline immunoglobulin gene. In certain cases, the human antibody may display no more than 5, or even no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

Homologous Antibodies

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In yet another embodiment, an antibody of the invention comprises heavy and light chain variable regions comprising amino acid sequences that are homologous to the amino acid sequences of the preferred antibodies described herein, and wherein the antibodies retain the desired functional properties of the anti-PD-1 antibodies of the invention.

For example, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region and a light chain variable region, wherein:

(a) the heavy chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5;

(b) the light chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10; and

the antibody exhibits one or more of the following properties:

(c) the antibody binds to human PD-1 with a K_D of 1 x 10^{-7} M or less;

(d) the antibody does not substantially bind to human CD28, CTLA-4 or ICOS;

(e) the antibody increases T-cell proliferation in an MLR assay;

(f) the antibody increases interferon-gamma production in an MLR assay;

(g) the antibody increases Il-2 secretion in an MLR assay;

(h) the antibody binds to human PD-1 and cynomolgus monkey PD-1;

(i) the antibody inhibits the binding of PD-L1 and/or PD-L2 to PD-1;

(j) the antibody stimulates antigen-specific memory responses;

(k) the antibody stimulates antibody responses;

(l) the antibody inhibits tumor cell growth *in vivo*.

In other embodiments, the V_H and/or V_L amino acid sequences may be 85%, 90%, 95%, 96%, 97%, 98% or 99% homologous to the sequences set forth above. An antibody having V_H and V_L regions having high (*i.e.*, 80% or greater) homology to the V_H and V_L regions of the sequences set forth above, can be obtained by mutagenesis (*e.g.*, site-

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directed or PCR-mediated mutagenesis) of nucleic acid molecules encoding SEQ ID NOs: 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50, followed by testing of the encoded altered antibody for retained function (*i.e.*, the functions set forth in (c) through (g) above) using the functional assays described herein.

As used herein, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

Additionally or alternatively, the protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the XBLAST program (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the antibody molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. (See www.ncbi.nlm.nih.gov).

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Antibodies with Conservative Modifications

In certain embodiments, an antibody of the invention comprises a heavy chain variable region comprising CDR1, CDR2 and CDR3 sequences and a light chain variable region comprising CDR1, CDR2 and CDR3 sequences, wherein one or more of these CDR sequences comprise specified amino acid sequences based on the preferred antibodies described herein (*e.g.*, 17D8, 2D3, 4H1, 5C4, or 4A11), or conservative modifications thereof, and wherein the antibodies retain the desired functional properties of the anti-PD-1 antibodies of the invention. Accordingly, the invention provides an isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences and a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein:

(a) the heavy chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 21, 22, 23, 24, and 25, and conservative modifications thereof;

(b) the light chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequence of SEQ ID NOs: 36, 37, 38, 39, and 40, and conservative modifications thereof; and

the antibody exhibits one or more of the following properties:

(c) the antibody binds to human PD-1 with a K_D of 1×10^{-7} M or less;

(d) the antibody does not substantially bind to human CD28, CTLA-4 or ICOS;

(e) the antibody increases T-cell proliferation in an MLR assay;

(f) the antibody increases interferon-gamma production in an MLR assay;

(g) the antibody increases Il-2 secretion in an MLR assay;

(h) the antibody binds to human PD-1 and cynomolgus monkey PD-1;

(i) the antibody inhibits the binding of PD-L1 and/or PD-L2 to PD-1;

(j) the antibody stimulates antigen-specific memory responses;

(k) the antibody stimulates antibody responses;

(l) the antibody inhibits tumor cell growth *in vivo*.

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In a preferred embodiment, the heavy chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 16, 17, 18, 19, and 20, and conservative modifications thereof; and the light chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 31, 32, 33, 34, and 35, and conservative modifications thereof. In another preferred embodiment, the heavy chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 11, 12, 13, 14, and 15, and conservative modifications thereof; and the light chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 26, 27, 28, 29, and 30, and conservative modifications thereof.

As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of an antibody of the invention can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for retained function (*i.e.*, the functions set forth in (c) through (g) above) using the functional assays described herein.

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Antibodies that Bind to the Same Epitope as Anti-PD-1 Antibodies of the Invention

In another embodiment, the invention provides antibodies that bind to the same epitope on human PD-1 as any of the PD-1 monoclonal antibodies of the invention (*i.e.*, antibodies that have the ability to cross-compete for binding to PD-1 with any of the monoclonal antibodies of the invention). In preferred embodiments, the reference antibody for cross-competition studies can be the monoclonal antibody 17D8 (having V_H and V_L sequences as shown in SEQ ID NOs: 1 and 6, respectively), or the monoclonal antibody 2D3 (having V_H and V_L sequences as shown in SEQ ID NOs: 2 and 7, respectively), or the monoclonal antibody 4H1 (having V_H and V_L sequences as shown in SEQ ID NOs: 3 and 8, respectively), or the monoclonal antibody 5C4 (having V_H and V_L sequences as shown in SEQ ID NOs: 4 and 9, respectively), or the monoclonal antibody 4A11 (having V_H and V_L sequences as shown in SEQ ID NOs: 5 and 10, respectively). Such cross-competing antibodies can be identified based on their ability to cross-compete with 17D8, 2D3, 4H1, 5C4, or 4A11 in standard PD-1 binding assays. For example, BIAcore analysis, ELISA assays or flow cytometry may be used to demonstrate cross-competition with the antibodies of the current invention. The ability of a test antibody to inhibit the binding of, for example, 17D8, 2D3, 4H1, 5C4, or 4A11, to human PD-1 demonstrates that the test antibody can compete with 17D8, 2D3, 4H1, 5C4, or 4A11 for binding to human PD-1 and thus binds to the same epitope on human PD-1 as 17D8, 2D3, 4H1, 5C4, or 4A11. In a preferred embodiment, the antibody that binds to the same epitope on human PD-1 as 17D8, 2D3, 4H1, 5C4, or 4A11 is a human monoclonal antibody. Such human monoclonal antibodies can be prepared and isolated as described in the Examples.

Engineered and Modified Antibodies

An antibody of the invention further can be prepared using an antibody having one or more of the V_H and/or V_L sequences disclosed herein as starting material to engineer a modified antibody, which modified antibody may have altered properties from

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the starting antibody. An antibody can be engineered by modifying one or more residues within one or both variable regions (*i.e.*, V_H and/or V_L), for example within one or more CDR regions and/or within one or more framework regions. Additionally or alternatively, an antibody can be engineered by modifying residues within the constant region(s), for example to alter the effector function(s) of the antibody.

One type of variable region engineering that can be performed is CDR grafting. Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.* (1998) *Nature* 332:323-327; Jones, P. *et al.* (1986) *Nature* 321:522-525; Queen, C. *et al.* (1989) *Proc. Natl. Acad. See. U.S.A.* 86:10029-10033; U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*)

Accordingly, another embodiment of the invention pertains to an isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15, SEQ ID NOs: 16, 17, 18, 19, and 20, and SEQ ID NOs: 21, 22, 23, 24, and 25, respectively, and a light chain variable region comprising CDR1, CDR2, and CDR3 sequences comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30, SEQ ID NOs: 31, 32, 33, 34, and 35, and SEQ ID NOs: 36, 37, 38, 39, and 40, respectively. Thus, such antibodies contain the V_H and V_L CDR sequences of monoclonal antibodies 17D8, 2D3, 4H1, 5C4, or 4A11 yet may contain different framework sequences from these antibodies.

Such framework sequences can be obtained from public DNA databases or published references that include germline antibody gene sequences. For example,

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germline DNA sequences for human heavy and light chain variable region genes can be found in the "VBase" human germline sequence database (available on the Internet at www.mrc-cpe.cam.ac.uk/vbase), as well as in Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I. M., *et al.* (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J. P. L. *et al.* (1994) "A Directory of Human Germ-line V_H Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference.

Preferred framework sequences for use in the antibodies of the invention are those that are structurally similar to the framework sequences used by selected antibodies of the invention, *e.g.*, similar to the V_H 3-33 framework sequences (SEQ ID NO: 51) and/or the V_H 4-39 framework sequences (SEQ ID NO: 53) and/or the V_K L6 framework sequences (SEQ ID NO: 52) and/or the V_K L15 framework sequences (SEQ ID NO: 54) used by preferred monoclonal antibodies of the invention. The V_H CDR1, CDR2, and CDR3 sequences, and the V_K CDR1, CDR2, and CDR3 sequences, can be grafted onto framework regions that have the identical sequence as that found in the germline immunoglobulin gene from which the framework sequence derive, or the CDR sequences can be grafted onto framework regions that contain one or more mutations as compared to the germline sequences. For example, it has been found that in certain instances it is beneficial to mutate residues within the framework regions to maintain or enhance the antigen binding ability of the antibody (see *e.g.*, U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al*).

Another type of variable region modification is to mutate amino acid residues within the V_H and/or V_K CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties (*e.g.*, affinity) of the antibody of interest. Site-directed mutagenesis or PCR-mediated mutagenesis can be performed to introduce the mutation(s) and the effect on antibody binding, or other functional property of interest, can be evaluated in *in vitro* or *in vivo* assays as described herein and provided in the Examples. Preferably conservative modifications (as discussed above) are introduced. The

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mutations may be amino acid substitutions, additions or deletions, but are preferably substitutions. Moreover, typically no more than one, two, three, four or five residues within a CDR region are altered.

Accordingly, in another embodiment, the invention provides isolated anti-PD-1 monoclonal antibodies, or antigen binding portions thereof, comprising a heavy chain variable region comprising: (a) a V_H CDR1 region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 11, 12, 13, 14, and 15; (b) a V_H CDR2 region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 16, 17, 18, 19, and 20; (c) a V_H CDR3 region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 21, 22, 23, 24, and 25; (d) a V_K CDR1 region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 26, 27, 28, 29, and 30; (e) a V_K CDR2 region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 31, 32, 33, 34, and 35; and (f) a V_K CDR3 region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 36, 37, 38, 39, and 40.

Engineered antibodies of the invention include those in which modifications have been made to framework residues within V_H and/or V_K, *e.g.* to improve the properties of the antibody. Typically such framework modifications are made to decrease the immunogenicity of the antibody. For example, one approach is to "backmutate" one or more framework residues to the corresponding germline sequence. More specifically, an

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antibody that has undergone somatic mutation may contain framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody framework sequences to the germline sequences from which the antibody is derived.

For example, Table 1 below shows a number of amino acid changes in the framework regions of the anti-PD-1 antibodies 17D8, 2D3, 4H1, 5C4, and 4A11 that differ from the parent germline sequence. To return one or more of the amino acid residues in the framework region sequences to their germline configuration, the somatic mutations can be "backmutated" to the germline sesquence by, for example, site-directed mutagenesis or PCR-mediated mutagenesis. The alignment of V_H regions for 17D8, 2D3, 4H1, and 5C4, against the parent germline V_H 3-33 sequence is shown in Figure 6. The alignment of V_H regions for 4A11 against the parent germline V_H 4-39 sequence is shown in Figure 11.

Table 1. Modifications to antibodies 17D8, 2D3, 4H1, 5C4, and 4A11 from the germline configuration.

Anti-PD-1 Ab	Amino acid position	Amino acid of antibody	Original amino acid of germline configuration
17D8	10	D	G
	16	G	R
	27	V	F
	28	A	T
	78	M	T
	93	M	V
2D3	10	D	G
	27	L	F
	30	T	S
	85	N	S
	98	T	R
4H1	3	Y	Q
	84	T	N
	88	V	A
	98	S	R
5C4	21	D	S
	23	K	A
	27	I	F
	80	F	Y
	98	T	R

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4A11	29	L	I
	79	Q	H
	98	V	A

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell epitopes to thereby reduce the potential immunogenicity of the antibody. This approach is also referred to as "deimmunization" and is described in futher detail in U.S. Patent Publication No. 20030153043 by Carr *et al.*

In addition or alternative to modifications made within the framework or CDR regions, antibodies of the invention may be engineered to include modifications within the Fc region, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody of the invention may be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody. Each of these embodiments is described in further detail below. The numbering of residues in the Fc region is that of the EU index of Kabat.

In one embodiment, the hinge region of CH1 is modified such that the number of cysteine residues in the hinge region is altered, *e.g.*, increased or decreased. This approach is described further in U.S. Patent No. 5,677,425 by Bodmer *et al.* The number of cysteine residues in the hinge region of CH1 is altered to, for example, facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.

In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half life of the antibody. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcyl protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent No. 6,165,745 by Ward *et al.*

In another embodiment, the antibody is modified to increase its biological half life. Various approaches are possible. For example, one or more of the following

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mutations can be introduced: T252L, T254S, T256F, as described in U.S. Patent No. 6,277,375 to Ward. Alternatively, to increase the biological half life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022 by Presta *et al.*

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector function(s) of the antibody. For example, one or more amino acids selected from amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322 can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Patent Nos. 5,624,821 and 5,648,260, both by Winter *et al.*

In another example, one or more amino acids selected from amino acid residues 329, 331 and 322 can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent Nos. 6,194,551 by Idusogie *et al.*

In another example, one or more amino acid residues within amino acid positions 231 and 239 are altered to thereby alter the ability of the antibody to fix complement. This approach is described further in PCT Publication WO 94/29351 by Bodmer *et al.*

In yet another example, the Fc region is modified to increase the ability of the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fcγ receptor by modifying one or more amino acids at the following positions: 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439. This approach is described further in PCT Publication WO 00/42072 by Presta. Moreover, the binding sites on human IgG1 for FcγR1, FcγRII, FcγRIII and FcRn have

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been mapped and variants with improved binding have been described (see Shields, R.L. *et al.* (2001) *J. Biol. Chem.* 276:6591-6604). Specific mutations at positions 256, 290, 298, 333, 334 and 339 were shown to improve binding to FcγRIII. Additionally, the following combination mutants were shown to improve FcγRIII binding: T256A/S298A, S298A/E333A, S298A/K224A and S298A/E333A/K334A.

In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycoslated antibody can be made (*i.e.*, the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Patent Nos. 5,714,350 and 6,350,861 by Co *et al.*

Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (alpha (1,6) fucosyltransferase), such that antibodies expressed in the Ms704, Ms705, and Ms709 cell lines lack fucose on their carbohydrates. The Ms704, Ms705, and Ms709 $FUT8^{-/-}$ cell lines were created by the targeted disruption of the FUT8 gene in CHO/DG44 cells using two replacement vectors (see U.S. Patent Publication No. 20040110704 by Yamane *et al.* and Yamane-Ohnuki *et al.* (2004) *Biotechnol Bioeng* 87:614-22). As another example, EP 1,176,195 by Hanai *et al.* describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl

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transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation by reducing or eliminating the alpha 1,6 bond-related enzyme. Hanai *et al.* also describe cell lines which have a low enzyme activity for adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity, for example the rat myeloma cell line YB2/0 (ATCC CRL 1662). PCT Publication WO 03/035835 by Presta describes a variant CHO cell line, Lec13 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, R.L. *et al.* (2002) *J. Biol. Chem.* 277:26733-26740). PCT Publication WO 99/54342 by Umana *et al.* describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.*, beta(1,4)-N-acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana *et al.* (1999) *Nat. Biotech.* 17:176-180). Alternatively, the fucose residues of the antibody may be cleaved off using a fucosidase enzyme. For example, the fucosidase alpha-L-fucosidase removes fucosyl residues from antibodies (Tarentino, A.L. *et al.* (1975) *Biochem.* 14:5516-23).

Another modification of the antibodies herein that is contemplated by the invention is pegylation. An antibody can be pegylated to, for example, increase the biological (*e.g.*, serum) half life of the antibody. To pegylate an antibody, the antibody, or fragment thereof, typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. In certain embodiments, the antibody to be pegylated is an aglycosylated antibody. Methods for pegylating proteins are known in the art and can be applied to the antibodies of the invention. See for example, EP 0 154 316 by Nishimura *et al.* and EP 0 401 384 by Ishikawa *et al.*

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Methods of Engineering Antibodies

As discussed above, the anti-PD-1 antibodies having V_H and V_K sequences disclosed herein can be used to create new anti-PD-1 antibodies by modifying the VH and/or V_K sequences, or the constant region(s) attached thereto. Thus, in another aspect of the invention, the structural features of an anti-PD-1 antibody of the invention, *e.g.* 17D8, 2D3, 4H1, 5C4, or 4A11, are used to create structurally related anti-PD-1 antibodies that retain at least one functional property of the antibodies of the invention, such as binding to human PD-1. For example, one or more CDR regions of 17D8, 2D3, 4H1, 5C4, or 4A11, or mutations thereof, can be combined recombinantly with known framework regions and/or other CDRs to create additional, recombinantly-engineered, anti-PD-1 antibodies of the invention, as discussed above. Other types of modifications include those described in the previous section. The starting material for the engineering method is one or more of the V_H and/or V_K sequences provided herein, or one or more CDR regions thereof. To create the engineered antibody, it is not necessary to actually prepare (*i.e.*, express as a protein) an antibody having one or more of the V_H and/or V_K sequences provided herein, or one or more CDR regions thereof. Rather, the information contained in the sequence(s) is used as the starting material to create a "second generation" sequence(s) derived from the original sequence(s) and then the "second generation" sequence(s) is prepared and expressed as a protein.

Accordingly, in another embodiment, the invention provides a method for preparing an anti-PD-1 antibody comprising:

(a) providing: (i) a heavy chain variable region antibody sequence comprising a CDR1 sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20, and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25; and/or (ii) a light chain variable region antibody sequence comprising a CDR1 sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35, and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40;

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(b) altering at least one amino acid residue within the heavy chain variable region antibody sequence and/or the light chain variable region antibody sequence to create at least one altered antibody sequence; and

(c) expressing the altered antibody sequence as a protein.

Standard molecular biology techniques can be used to prepare and express the altered antibody sequence.

Preferably, the antibody encoded by the altered antibody sequence(s) is one that retains one, some or all of the functional properties of the anti-PD-1 antibodies described herein, which functional properties include, but are not limited to:

(a) the antibody binds to human PD-1 with a K_D of 1×10^{-7} M or less;

(b) the antibody does not substantially bind to human CD28, CTLA-4 or ICOS;

(c) the antibody increases T-cell proliferation in an MLR assay;

(d) the antibody increases interferon-gamma production in an MLR assay;

(e) the antibody increases Il-2 secretion in an MLR assay;

(f) the antibody binds to human PD-1 and cynomolgus monkey PD-1;

(g) the antibody inhibits the binding of PD-L1 and/or PD-L2 to PD-1;

(h) the antibody stimulates antigen-specific memory responses;

(i) the antibody stimulates antibody responses;

(j) the antibody inhibits tumor cell growth *in vivo*.

The functional properties of the altered antibodies can be assessed using standard assays available in the art and/or described herein, such as those set forth in the Examples (*e.g.*, flow cytometry, binding assays).

In certain embodiments of the methods of engineering antibodies of the invention, mutations can be introduced randomly or selectively along all or part of an anti-PD-1 antibody coding sequence and the resulting modified anti-PD-1 antibodies can be screened for binding activity and/or other functional properties as described herein. Mutational methods have been described in the art. For example, PCT Publication WO 02/092780 by Short describes methods for creating and screening antibody mutations using saturation mutagenesis, synthetic ligation assembly, or a combination thereof.

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Alternatively, PCT Publication WO 03/074679 by Lazar *et al.* describes methods of using computational screening methods to optimize physiochemical properties of antibodies.

Nucleic Acid Molecules Encoding Antibodies of the Invention

Another aspect of the invention pertains to nucleic acid molecules that encode the antibodies of the invention. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. *See*, F. Ausubel, *et al.*, ed. (1987) Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York. A nucleic acid of the invention can be, for example, DNA or RNA and may or may not contain intronic sequences. In a preferred embodiment, the nucleic acid is a cDNA molecule.

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

Preferred nucleic acids molecules of the invention are those encoding the VH and VL sequences of the 17D8, 2D3, 4H1, 5C4, or 4A11 monoclonal antibodies. DNA sequences encoding the VH sequences of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 41, 42, 43, 44, and 45, respectively. DNA sequences encoding the VL sequences of 17D8, 2D3, 4H1, 5C4, and 4A11 are shown in SEQ ID NOs: 46, 47, 48, 49, and 50, respectively.

Once DNA fragments encoding VH and VL segments are obtained, these DNA fragments can be further manipulated by standard recombinant DNA techniques, for

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example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E. A., el al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see *e.g.*, Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl. Acad.*

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Sci. USA 85:5879-5883; McCafferty *et al.*, (1990) *Nature* 348:552-554).

Production of Monoclonal Antibodies of the Invention

Monoclonal antibodies (mAbs) of the present 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. Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed *e.g.*, viral or oncogenic transformation of B lymphocytes.

The preferred 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.

Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody prepared as described above. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (*e.g.*, human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art (see *e.g.*, U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*).

In a preferred embodiment, the antibodies of the invention are human monoclonal antibodies. Such human monoclonal antibodies directed against PD-1 can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include

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mice referred to herein as HuMAb mice and KM mice™, respectively, and are collectively referred to herein as "human Ig mice."

The HuMAb mouse® (Medarex, Inc.) contains human immunoglobulin gene miniloci that encode 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 (see *e.g.*, Lonberg, *et al.* (1994) Nature 368(6474): 856-859). 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 IgGκ monoclonal (Lonberg, N. *et al.* (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci.* 764:536-546). The preparation and use of HuMab mice, and the genomic modifications carried by such mice, is further described in Taylor, L. *et al.* (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. *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; Taylor, L. *et al.* (1994) *International Immunology* 6: 579-591; and Fishwild, D. *et al.* (1996) *Nature Biotechnology* 14: 845-851, the contents of all of which are hereby specifically incorporated by reference in their entirety. See further, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent No. 5,545,807 to Surani *et al.*; PCT Publication Nos. WO 92/03918, WO 93/12227, WO 94/25585, WO 97/13852, WO 98/24884 and WO 99/45962, all to Lonberg and Kay; and PCT Publication No. WO 01/14424 to Korman *et al.*

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

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Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-PD-1 antibodies of the invention. 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-PD-1 antibodies of the invention. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome, 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-PD-1 antibodies of the invention.

Human monoclonal antibodies of the invention 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.*

Immunization of Human Ig Mice

When human Ig mice are used to raise human antibodies of the invention, such mice can be immunized with a purified or enriched preparation of PD-1 antigen and/or

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recombinant PD-1, or an PD-1 fusion protein, as described by 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 and WO 01/14424. Preferably, the mice will be 6-16 weeks of age upon the first infusion. For example, a purified or recombinant preparation (5-50 μg) of PD-1 antigen can be used to immunize the human Ig mice intraperitoneally.

Detailed procedures to generate fully human monoclonal antibodies to PD-1 are described in Example 1 below. Cumulative experience with various antigens has shown that the transgenic mice respond when initially immunized intraperitoneally (IP) with antigen in complete Freund's adjuvant, followed by every other week IP immunizations (up to a total of 6) with antigen in incomplete Freund's adjuvant. However, adjuvants other than Freund's are also found to be effective. In addition, whole cells in the absence of adjuvant are found to be highly immunogenic. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by ELISA (as described below), and mice with sufficient titers of anti-PD-1 human immunoglobulin can be used for fusions. Mice can be boosted intravenously with antigen 3 days before sacrifice and removal of the spleen. It is expected that 2-3 fusions for each immunization may need to be performed. Between 6 and 24 mice are typically immunized for each antigen. Usually both HCo7 and HCo12 strains are used. In addition, both HCo7 and HCo12 transgene can be bred together into a single mouse having two different human heavy chain transgenes (HCo7/HCo12). Alternatively or additionally, the KM mouse™ strain can be used, as described in Example 1.

Generation of Hybridomas Producing Human Monoclonal Antibodies of the Invention

To generate hybridomas producing human monoclonal antibodies of the invention, splenocytes and/or lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can be screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from immunized mice can

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be fused to one-sixth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (ATCC, CRL 1580) with 50% PEG. Cells are plated at approximately 2 x 10^5 in flat bottom microtiter plate, followed by a two week incubation in selective medium containing 20% fetal Clone Serum, 18% "653" conditioned media, 5% origen (IGEN), 4 mM L-glutamine, 1 mM sodium pyruvate, 5mM HEPES, 0.055 mM 2-mercaptoethanol, 50 units/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml gentamycin and 1X HAT (Sigma; the HAT is added 24 hours after the fusion). After approximately two weeks, cells can be cultured in medium in which the HAT is replaced with HT. Individual wells can then be screened by ELISA for human monoclonal IgM and IgG antibodies. Once extensive hybridoma growth occurs, medium can be observed usually after 10-14 days. The antibody secreting hybridomas can be replated, screened again, and if still positive for human IgG, the monoclonal antibodies can be subcloned at least twice by limiting dilution. The stable subclones can then be cultured *in vitro* to generate small amounts of antibody in tissue culture medium for characterization.

To purify human monoclonal antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (Pharmacia, Piscataway, N.J.). Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by OD_{280} using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80° C.

Generation of Transfectomas Producing Monoclonal Antibodies of the Invention

Antibodies of the invention also can be produced in a host cell transfectoma using, for example, a combination of recombinant DNA techniques and gene transfection methods as is well known in the art (*e.g.*, Morrison, S. (1985) Science 229:1202).

For example, to express the antibodies, or antibody fragments thereof, DNAs encoding partial or full-length light and heavy chains, can be obtained by standard molecular biology techniques (*e.g.*, PCR amplification or cDNA cloning using a hybridoma that expresses the antibody of interest) and the DNAs can be inserted into

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expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). The light and heavy chain variable regions of the antibodies described herein can be used to create full-length antibody genes of any antibody isotype by inserting them into expression vectors already encoding heavy chain constant and light chain constant regions of the desired isotype such that the V_H segment is operatively linked to the C_H segment(s) within the vector and the V_K segment is operatively linked to the C_L segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel (Gene Expression Technology. Methods in Enzymology 185, Academic Press, San Diego, CA (1990)). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences, may depend on such factors as the choice of the

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host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV), Simian Virus 40 (SV40), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP) and polyoma. Alternatively, nonviral regulatory sequences may be used, such as the ubiquitin promoter or β-globin promoter. Still further, regulatory elements composed of sequences from different sources, such as the SRα promoter system, which contains sequences from the SV40 early promoter and the long terminal repeat of human T cell leukemia virus type 1 (Takebe, Y. *et al.* (1988) *Mol. Cell. Biol.* 8:466-472).

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see, *e.g.*, U.S. Pat. Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr- host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been

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reported to be ineffective for production of high yields of active antibody (Boss, M. A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R. J. Kaufman and P. A. Sharp (1982) *Mol. Biol. 159*:601-621), NSO myeloma cells, COS cells and SP2 cells. In particular, for use with NSO myeloma cells, another preferred expression system is the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338,841. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Characterization of Antibody Binding to Antigen

Antibodies of the invention can be tested for binding to PD-1 by, for example, standard ELISA. Briefly, microtiter plates are coated with purified PD-1 at 0.25 μg/ml in PBS, and then blocked with 5% bovine serum albumin in PBS. Dilutions of antibody (*e.g.*, dilutions of plasma from PD-1-immunized mice) are added to each well and incubated for 1-2 hours at 37°C. The plates are washed with PBS/Tween and then incubated with secondary reagent (*e.g.*, for human antibodies, a goat-anti-human IgG Fc-specific polyclonal reagent) conjugated to alkaline phosphatase for 1 hour at 37°C. After washing, the plates are developed with pNPP substrate (1 mg/ml), and analyzed at OD of 405-650. Preferably, mice which develop the highest titers will be used for fusions.

An ELISA assay as described above can also be used to screen for hybridomas that show positive reactivity with PD-1 immunogen. Hybridomas that bind with high avidity to PD-1 are subcloned and further characterized. One clone from each

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hybridoma, which retains the reactivity of the parent cells (by ELISA), can be chosen for making a 5-10 vial cell bank stored at -140 °C, and for antibody purification..

To purify anti-PD-1 antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (Pharmacia, Piscataway, NJ). Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by OD_{280} using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80 °C.

To determine if the selected anti-PD-1 monoclonal antibodies bind to unique epitopes, each antibody can be biotinylated using commercially available reagents (Pierce, Rockford, IL). Competition studies using unlabeled monoclonal antibodies and biotinylated monoclonal antibodies can be performed using PD-1 coated-ELISA plates as described above. Biotinylated mAb binding can be detected with a strep-avidin-alkaline phosphatase probe.

To determine the isotype of purified antibodies, isotype ELISAs can be performed using reagents specific for antibodies of a particular isotype. For example, to determine the isotype of a human monoclonal antibody, wells of microtiter plates can be coated with 1 μg/ml of anti-human immunoglobulin overnight at 4° C. After blocking with 1% BSA, the plates are reacted with 1 μg /ml or less of test monoclonal antibodies or purified isotype controls, at ambient temperature for one to two hours. The wells can then be reacted with either human IgG1 or human IgM-specific alkaline phosphatase-conjugated probes. Plates are developed and analyzed as described above.

Anti-PD-1 human IgGs can be further tested for reactivity with PD-1 antigen by Western blotting. Briefly, PD-1 can be prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the separated antigens are transferred to nitrocellulose membranes, blocked with 10% fetal calf serum, and probed with the monoclonal antibodies to be tested. Human IgG binding can be detected using anti-human IgG alkaline phosphatase and developed with BCIP/NBT substrate tablets (Sigma Chem. Co., St. Louis, Mo.).

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Immunoconjugates

In another aspect, the present invention features an anti-PD-1 antibody, or a 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) 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), cyclothosphamide, 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).

Cytoxins 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, such as proteases preferentially expressed in tumor tissue such as cathepsins (*e.g.*, cathepsins B, C, D).

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For further discussion of types of cytotoxins, linkers and methods for conjugating therapeutic agents to antibodies, see also Saito, G. *et al.* (2003) *Adv. Drug Deliv. Rev.* 55:199-215; Trail, P.A. *et al.* (2003) *Cancer Immunol. Immunother.* 52:328-337; Payne, G. (2003) *Cancer Cell* 3:207-212; Allen, T.M. (2002) *Nat. Rev. Cancer* 2:750-763; Pastan, I. and Kreitman, R. J. (2002) *Curr. Opin. Investig. Drugs* 3:1089-1091; Senter, P.D. and Springer, C.J. (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 therapeutically include, but are not limited to, iodine^{131}, indium^{111}, yttrium^{90} and lutetium^{177}. Method for preparing radioimmunconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including Zevalin™ (IDEC Pharmaceuticals) and Bexxar™ (Corixa Pharmaceuticals), and similar methods can be used to prepare radioimmunoconjugates using the antibodies of the invention.

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 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

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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.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

Bispecific Molecules

In another aspect, the present invention features bispecific molecules comprising an anti-PD-1 antibody, or a fragment thereof, of the invention. An antibody of the invention, or antigen-binding portions thereof, can be derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, another antibody or ligand for a receptor) to generate a bispecific molecule that binds to at least two different binding sites or target molecules. The antibody of the invention may in fact be derivatized or linkd to more than one other functional molecule to generate multispecific molecules that bind to more than two different binding sites and/or target molecules; such multispecific molecules are also intended to be encompassed by the term "bispecific molecule" as used herein. To create a bispecific molecule of the invention, an antibody of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, antibody fragment, peptide or binding mimetic, such that a bispecific molecule results.

Accordingly, the present invention includes bispecific molecules comprising at least one first binding specificity for PD-1 and a second binding specificity for a second target epitope. In a particular embodiment of the invention, the second target epitope is an Fc receptor, *e.g.*, human FcγRI (CD64) or a human Fcα receptor (CD89). Therefore, the invention includes bispecific molecules capable of binding both to FcγR or FcαR expressing effector cells (*e.g.*, monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing PD-1. These bispecific molecules target PD-1 expressing cells to effector cell and trigger Fc receptor-mediated effector cell activities,

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such as phagocytosis of an PD-1 expressing cells, antibody dependent cell-mediated cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

In an embodiment of the invention in which the bispecific molecule is multispecific, the molecule can further include a third binding specificity, in addition to an anti-Fc binding specificity and an anti-PD-1 binding specificity. In one embodiment, the third binding specificity is an anti-enhancement factor (EF) portion, *e.g.*, a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. The "anti-enhancement factor portion" can be an antibody, functional antibody fragment or a ligand that binds to a given molecule, *e.g.*, an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the F_c receptor or target cell antigen. The "anti-enhancement factor portion" can bind an F_c receptor or a target cell antigen. Alternatively, the anti-enhancement factor portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T-cell (*e.g.* via CD2, CD3, CD8, CD28, CD4, CD40, ICAM-1 or other immune cell that results in an increased immune response against the target cell).

In one embodiment, the bispecific molecules of the invention comprise as a binding specificity at least one antibody, or an antibody fragment thereof, including, *e.g.*, an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner *et al.* U.S. Patent No. 4,946,778, the contents of which is expressly incorporated by reference.

In one embodiment, the binding specificity for an Fcγ receptor is provided by a monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ-chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fcγ receptor classes: FcγRI (CD64), FcγRII(CD32), and FcγRIII (CD16). In one preferred embodiment, the Fcγ

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receptor a human high affinity FcγRI. The human FcγRI is a 72 kDa molecule, which shows high affinity for monomeric IgG (10^8 - 10^9 M^{-1}).

The production and characterization of certain preferred anti-Fcγ monoclonal antibodies are described by Fanger *et al.* in PCT Publication WO 88/00052 and in U.S. Patent No. 4,954,617, the teachings of which are fully incorporated by reference herein. These antibodies bind to an epitope of FcγRI, FcγRII or FcγRIII at a site which is distinct from the Fcγ binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-FcγRI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. The hybridoma producing mAb 32 is available from the American Type Culture Collection, ATCC Accession No. HB9469. In other embodiments, the anti-Fcγ receptor antibody is a humanized form of monoclonal antibody 22 (H22). The production and characterization of the H22 antibody is described in Graziano, R.F. *et al.* (1995) *J. Immunol* 155 (10): 4996-5002 and PCT Publication WO 94/10332. The H22 antibody producing cell line was deposited at the American Type Culture Collection under the designation HA022CL1 and has the accession no. CRL 11177.

In still other preferred embodiments, the binding specificity for an Fc receptor is provided by an antibody that binds to a human IgA receptor, *e.g.*, an Fc-alpha receptor (FcαRI (CD89)), the binding of which is preferably not blocked by human immunoglobulin A (IgA). The term "IgA receptor" is intended to include the gene product of one α-gene (FcαRI) located on chromosome 19. This gene is known to encode several alternatively spliced transmembrane isoforms of 55 to 110 kDa. FcαRI (CD89) is constitutively expressed on monocytes/macrophages, eosinophilic and neutrophilic granulocytes, but not on non-effector cell populations. FcαRI has medium affinity ($\approx 5 \times 10^7$ M^{-1}) for both IgA1 and IgA2, which is increased upon exposure to cytokines such as G-CSF or GM-CSF (Morton, H.C. *et al.* (1996) *Critical Reviews in Immunology* 16:423-440). Four FcαRI-specific monoclonal antibodies, identified as A3, A59, A62 and A77, which bind FcαRI outside the IgA ligand binding domain, have been described (Monteiro, R.C. *et al.* (1992) *J. Immunol.* 148:1764).

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FcαRI and FcγRI are preferred trigger receptors for use in the bispecific molecules of the invention because they are (1) expressed primarily on immune effector cells, *e.g.*, monocytes, PMNs, macrophages and dendritic cells; (2) expressed at high levels (*e.g.*, 5,000-100,000 per cell); (3) mediators of cytotoxic activities (*e.g.*, ADCC, phagocytosis); (4) mediate enhanced antigen presentation of antigens, including self-antigens, targeted to them.

While human monoclonal antibodies are preferred, other antibodies which can be employed in the bispecific molecules of the invention are murine, chimeric and humanized monoclonal antibodies.

The bispecific molecules of the present invention can be prepared by conjugating the constituent binding specificities, *e.g.*, the anti-FcR and anti-PD-1 binding specificities, using methods known in the art. For example, each binding specificity of the bispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohaxane-1-carboxylate (sulfo-SMCC) (see *e.g.*, Karpovsky *et al.* (1984) *J. Exp. Med.* 160:1686; Liu, MA *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:8648). Other methods include those described in Paulus (1985) Behring Ins. Mitt. No. 78, 118-132; Brennan *et al.* (1985) *Science* 229:81-83), and Glennie *et al.* (1987) *J. Immunol.* 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies, they can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific molecule is a mAb x mAb, mAb x Fab, Fab x $F(ab')_2$ or ligand x Fab

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fusion protein. A bispecific molecule of the invention can be a single chain molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific molecules may comprise at least two single chain molecules. Methods for preparing bispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Binding of the bispecific molecules to their specific targets can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (*e.g.*, growth inhibition), or Western Blot assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using *e.g.*, an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

Pharmaceutical Compositions

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of monoclonal antibodies, or antigen-binding portion(s) thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of (*e.g.*, two or more different) antibodies, or immunoconjugates or bispecific molecules of the invention. For example, a pharmaceutical composition of the

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invention can comprise a combination of antibodies (or immunoconjugates or bispecifics) 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 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 uses of the 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 compound, *i.e.*, antibody, immunoconjuage, or bispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

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 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 alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts 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, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the invention also may include a pharmaceutically acceptable anti-oxidant. Examples of pharmaceutically acceptable

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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, 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,

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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 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.

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, 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

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increased as 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 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.

For administration of the antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages 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 within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months. Preferred dosage regimens for an anti-PD-1 antibody of the invention include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the antibody being 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 some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be, for example, weekly, monthly, every three monthgs 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 concentration of about 1-1000 μg /ml and in some methods about 25-300 μg /ml.

Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary

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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.

A "therapeutically effective dosage" of an anti-PD-1 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 compound to inhibit tumor growth can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to

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inhibit, such inhibition *in vitro* by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms 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.

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 of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

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

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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 medicants 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.

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. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (*see, e.g.*, V.V. 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 K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273.

Uses and Methods of the Invention

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The antibodies, antibody compositions and methods of the present invention have numerous *in vitro* and *in vivo* utilities involving, for example, detection of PD-1 or enhancement of immune response by blockade of PD-1. 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 of modifying an immune response in a subject comprising administering to the subject the antibody, or antigen-binding portion thereof, of the invention such that the immune response in the subject is modified. Preferebly, the response is enhanced, stimulated or up-regulated.

As used herein, the term "subject" is intended to include human and non-human animals. Non-human animals includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, amphibians, and reptiles, although mammals are preferred, such as non-human primates, sheep, dogs, cats, cows and horses. Preferred subjects include human patients in need of enhancement of an immune response. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting the T-cell mediated immune response. In a particular embodiment, the methods are particularly suitable for treatment of cancer cells *in vivo*. To achieve antigen-specific enhancement of immunity, the anti-PD-1 antibodies can be administered together with an antigen of interest. When antibodies to PD-1 are administered together with another agent, the two can be administered in either order or simultaneously.

The invention further provides methods for detecting the presence of human PD-1 antigen in a sample, or measuring the amount of human PD-1 antigen, comprising contacting the sample, and a control sample, with a human monoclonal antibody, or an antigen binding portion thereof, which specifically binds to human PD-1, under conditions that allow for formation of a complex between the antibody or portion thereof and human PD-1. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of human PD-1 antigen in the sample.

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Given the specific binding of the antibodies of the invention for PD-1, compared to CD28, ICOS and CTLA-4, the antibodies of the invention can be used to specifically detect PD-1 expression on the surface of cells and, moreover, can be used to purify PD-1 via immunoaffinity purification.

Cancer

Blockade of PD-1 by antibodies can enhance the immune response to cancerous cells in the patient. The ligand for PD-1, PD-L1, is not expressed in normal human cells, but 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, 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 to PD-L1 and the effect is additive when the interaction of PD-1 to PD-L2 is blocked as well (Iwai *et al.* (2002) *PNAS* 99:12293-7; Brown *et al.* (2003) *J. Immunol.* 170:1257-66). While previous studies have shown that T-cell proliferation can be restored by inhibiting the interaction of PD-1 to PD-L1, there have been no reports of a direct effect on cancer tumor growth *in vivo* by blocking the PD-1/PD-L1 interaction. In one aspect, the present invention relates to treatment of a subject *in vivo* using an anti-PD-1 antibody such that growth of cancerous tumors is inhibited. An anti-PD-1 antibody may be used alone to inhibit the growth of cancerous tumors. Alternatively, an anti-PD-1 antibody may be used in conjunction with other immunogenic agents, standard cancer treatments, or other antibodies, as described below.

Accordingly, in one embodiment, the invention provides a method of inhibiting growth of tumor cells in a subject, comprising administering to the subject a therapeutically effective amount of an anti-PD-1 antibody, or antigen-binding portion thereof. Preferably, the antibody is a human anti-PD-1 antibody (such as any of the human anti-human PD-1 antibodies described herein). Additionally or alternatively, the antibody may be a chimeric or humanized anti-PD-1 antibody.

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Preferred cancers whose growth may be inhibited using the antibodies of the invention include cancers typically responsive to immunotherapy. Non-limiting examples of preferred cancers for treatment 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 invention 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, especially metastatic cancers that express PD-L1 (Iwai *et al.* (2005) *Int. Immunol.* 17:133-144).

Optionally, antibodies to PD-1 can be 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).

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In humans, some tumors have been shown to be immunogenic such as melanomas. It is anticipated that by raising the threshold of T cell activation by PD-1 blockade, we may expect to activate tumor responses in the host.

PD-1 blockade is likely to be most effective when 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, N. and Sznol, M., Cancer Vaccines, Ch. 61, pp. 3023-3043 in DeVita, V. *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, SA (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. PD-1 blockade 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, N *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

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alter protein sequence or create fusion proteins between two unrelated sequences (ie. 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, R & Srivastava, P (1995) *Science* 269:1585-1588; Tamura, Y. *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, F. *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, A. *et al.* (2000) *Nature Medicine* 6:332-336). As a method of vaccination, DC immunization may be effectively combined with PD-1 blockade to activate more potent anti-tumor responses.

PD-1 blockade may also be combined with standard cancer treatments. PD-1 blockade may be effectively combined with chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered (Mokyr, M. *et al.* (1998) *Cancer Research* 58: 5301-5304). An example of such a combination is an anti-PD-1 antibody in combination with decarbazine for the treatment of melanoma. Another example of such a combination is an anti-PD-1 antibody in combination with interleukin-2 (IL-2) for the treatment of melanoma. The scientific rationale behind the combined use of PD-1 blockade and chemotherapy is that cell death, that 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 PD-1 blockade through cell death are radiation, surgery, and hormone deprivation. Each of these protocols creates a source of tumor antigen in the host. Angiogenesis inhibitors may also be combined with

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PD-1 blockade. Inhibition of angiogenesis leads to tumor cell death which may feed tumor antigen into host antigen presentation pathways.

PD-1 blocking antibodies can also be used in combination with bispecific antibodies that target Fc alpha or Fc gamma receptor-expressing effectors 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 by augmented by the use of PD-1 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.

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-beta (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). Antibodies to each of these entities may be used in combination with anti-PD-1 to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

Other antibodies which may be used to activate host immune responsiveness can be used in combination with anti-PD-1. These include molecules on the surface of dendritic cells which 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 conjuction with PD-1 antibodies (Ito, N. *et al.* (2000) *Immunobiology* 201 (5) 527-40). Activating antibodies to T cell costimulatory molecules such as CTLA-4 (e.g., US Patent No. 5,811,097), OX-40 (Weinberg, A. *et al.* (2000) *Immunol* 164: 2160-2169), 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,

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therapeutic benefit may be obtained from graft vs. tumor responses. PD-1 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: 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 antibodies may be expected to increase the frequency and activity of the adoptively transferred T cells.

Infectious Diseases

Other methods of the invention are used to treat patients that have been exposed to particular toxins or pathogens. Accordingly, another aspect of the invention provides a method of treating an infectious disease in a subject comprising administering to the subject an anti-PD-1 antibody, or antigen-binding portion thereof, such that the subject is treated for the infectious disease. Preferably, the antibody is a human anti-human PD-1 antibody (such as any of the human anti-PD-1 antibodies described herein). Additionally or alternatively, the antibody can be a chimeric or humanized antibody.

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

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Some examples of pathogenic viruses causing infections treatable by methods of the invention include HIV, hepatitis (A, B, or C), herpes virus (*e.g.*, VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus.

Some examples of pathogenic bacteria causing infections treatable by methods of the invention include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lymes disease bacteria.

Some examples of pathogenic fungi causing infections treatable by methods of the invention include Candida (albicans, krusei, glabrata, tropicalis, etc.), Cryptococcus neoformans, Aspergillus (fumigatus, niger, etc.), Genus Mucorales (mucor, absidia, rhizophus), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis and Histoplasma capsulatum.

Some examples of pathogenic parasites causing infections treatable by methods of the invention include Entamoeba histolytica, Balantidium coli, Naegleriafowleri, Acanthamoeba sp., Giardia lambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondi, Nippostrongylus brasiliensis.

In all of the above methods, PD-1 blockade can be combined with other forms of immunotherapy such as cytokine treatment (*e.g.*, interferons, GM-CSF, G-CSF, IL-2), or bispecific antibody therapy, which provides for enhanced presentation of tumor antigens (see, *e.g.*, Holliger (1993) *Proc. Natl. Acad. Sci.* USA 90:6444-6448; Poljak (1994) *Structure* 2:1121-1123).

Autoimmune reactions

Anti-PD-1 antibodies may provoke and amplify autoimmune responses. Indeed, induction of anti-tumor responses using tumor cell and peptide vaccines reveals that

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many anti-tumor responses involve anti-self reactivities (depigmentation observed in anti-CTLA-4 + GM-CSF-modified B16 melanoma in van Elsas *et al.* supra; depigmentation in Trp-2 vaccinated mice (Overwijk, W. *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96: 2982-2987); autoimmune prostatitis evoked by TRAMP tumor cell vaccines (Hurwitz, A. (2000) *supra*), melanoma peptide antigen vaccination and vitilago observed in human clinical trials (Rosenberg, SA and White, DE (1996) *J. Immunother Emphasis Tumor Immunol* 19 (1): 81-4).

Therefore, it is possible to consider using anti-PD-1 blockade in conjunction with various self proteins in order to devise vaccination protocols to efficiently generate immune responses against these self proteins for disease treatment. For example, Alzheimers disease involves inappropriate accumulation of Aβ peptide in amyloid deposits in the brain; antibody responses against amyloid are able to clear these amyloid deposits (Schenk *et al.*, (1999) *Nature* 400: 173-177).

Other self proteins may also be used as targets such as IgE for the treatment of allergy and asthma, and TNFα for rhematoid arthritis. Finally, antibody responses to various hormones may be induced by the use of anti-PD-1 antibody. Neutralizing antibody responses to reproductive hormones may be used for contraception. Neutralizing antibody response to hormones and other soluble factors that are required for the growth of particular tumors may also be considered as possible vaccination targets.

Analogous methods as described above for the use of anti-PD-1 antibody can be used for induction of therapeutic autoimmune responses to treat patients having an inappropriate accumulation of other self-antigens, such as amyloid deposits, including Aβ in Alzheimer's disease, cytokines such as TNFα, and IgE.

Vaccines

Anti-PD-1 antibodies may be used to stimulate antigen-specific immune responses by coadministration of an anti-PD-1 antibody with an antigen of interest (*e.g.*, a vaccine). Accordingly, in another aspect the invention provides a method of enhancing an immune response to an antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) an anti-PD-1 antibody, or antigen-binding portion thereof, such

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that an immune response to the antigen in the subject is enhanced. Preferably, the antibody is a human anti-human PD-1 antibody (such as any of the human anti-PD-1 antibodies described herein). Additionally or alternatively, the antibody can be a chimeric or humanized antibody. The antigen can be, for example, a tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen. Non-limiting examples of such antigens include those discussed in the sections above, such as the tumor antigens (or tumor vaccines) discussed above, or antigens from the viruses, bacteria or other pathogens described above.

Suitable routes of administering the antibody compositions (*e.g.*, human monoclonal antibodies, multispecific and bispecific molecules and immunoconjugates) of the invention *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (*e.g.*, intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the antibody composition.

As previously described, human anti-PD-1 antibodies of the invention can be co-administered with one or other more therapeutic agents, *e.g.*, a cytotoxic agent, a radiotoxic agent or an immunosuppressive agent. The antibody can be linked to the agent (as an immunocomplex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.*, an anti-cancer therapy, *e.g.*, radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin (adriamycin), cisplatin bleomycin sulfate, carmustine, chlorambucil, decarbazine and cyclophosphamide hydroxyurea which, by themselves, are only effective at levels which are toxic or subtoxic to a patient. Cisplatin is intravenously administered as a 100 mg/ dose once every four weeks and adriamycin is intravenously administered as a 60-75 mg/ml dose once every 21 days. Co-administration of the human anti-PD-1 antibodies, or antigen binding fragments thereof, of the present invention with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such co-administration

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can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the antibody.

Also within the scope of the present invention are kits comprising the antibody compositions of the invention (*e.g.*, human antibodies, bispecific or multispecific molecules, or immunoconjugates) and instructions for use. The kit can further contain a least one additional reagent, or one or more additional human antibodies of the invention (e.g., a human antibody having a complementary activity which binds to an epitope in PD-1 antigen distinct from the first human antibody). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

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 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 antigens were generated by recombinant transfection methods in a CHO cell line.

Transgenic HuMab and KM mice™

Fully human monoclonal antibodies to PD-1 were prepared using the HCo7 strain of HuMab transgenic mice and the KM strain of transgenic transchromosomic mice, each of which express human antibody genes. In each of these mouse strains, the endogenous

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mouse kappa 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 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 mice™ were immunized with purified recombinant PD-1 fusion protein and PD-1-transfected CHO cells 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 -10x10^6 cells were used to immunize the HuMab mice and KM mice™ intraperitonealy, 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 of anti-PD-1 human immunogolobulin 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.

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

To select HuMab or KM mice™ producing antibodies that bound PD-1, sera from 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. 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 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 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 $1x10^5$/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-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

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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 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.

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

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 1A 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 1B and in SEQ ID NO: 46 and 6, respectively.

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 6. Further analysis of the 17D8 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 1A and 6, and in SEQ ID NOs: 11, 16 and 21, respectively.

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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 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 1B and 7, 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 2A 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 2B and in SEQ ID NO: 47 and 7, respectively.

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 6. Further analysis of the 2D3 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 2A and 6, 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 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 2B and 8, and in SEQ ID NOs: 27, 32 and 37, respectively.

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

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The nucleotide and amino acid sequences of the light chain variable region of 4H1 are shown in Figure 3B and in SEQ ID NO: 48 and 8, respectively.

Comparison of the 4H1 heavy chain immunoglobulin sequence to the known human 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 6. 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 CD3 regions as shown in Figures 3A and 6, 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 9. 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 3B and 9, 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 4A and in SEQ ID NO: 44 and 4, respectively.

The nucleotide and amino acid sequences of the light chain variable region of 5C4 are shown in Figure 4B 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 from human germline JH 4b. The alignment of the 5C4 VH sequence to the germline VH 3-33 sequence is shown in Figure 6. 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 4A and 6, and in SEQ ID NOs: 14, 19 and 24, respectively.

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Comparison of the 5C4 light chain immunoglobulin sequence to the known human 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 10. 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 4B and 10, 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 5A 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 5B and in SEQ ID NO: 50 and 10, respectively.

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 11. 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 11, 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 12. 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 5B and 12, and in SEQ ID NOs: 30, 35 and 40, respectively.

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Example 3: **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 2.

Table 2. Biacore binding data for PD-1 human monoclonal antibodies.

Sample #	Sample ID	Affinity K_D x 10^{-9} (M)	On rate k_{on} x 10^5 (1/Ms)	Off rate k_{off} x 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

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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 μg/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 13A (5C4) and 13B (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 bindig 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 μg/ml. Goat-anti-human IgG (kappa chain-specific) polyclonal antibody conjugated with horseradish peroxidase (HRP) was used as secondary antibody. The results are shown in Figure 14. 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.

Example 4: Characterization of anti-PD-1 antibody binding to PD-1 expressed on the surface of human and monkey cells

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Anti-PD-1 antibodies were tested for binding to cells expressing PD-1 on their cell surface by flow cytometry.

Activated human T-cells, monkey peripheral blood mononuclear cells (PBMC), and CHO cells transfected with PD-1 were each tested for antibody binding. Human T cells and cynomolgous PBMC were activated by anti-CD3 antibody to induce PD-1 expression on T cells prior to binding with a human anti-PD-1 monoclonal antibody. Binding of the 5C4 and 4H1 anti-PD-1 human monoclonal antibodies was assessed by incubating the transfected cells with either IgG1 or IgG4 forms of the anti-PD-1 human monoclonal antibodies at different concentrations. 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 shown in Figures 15A (activated human T cells), 15B (cynomolgous monkey PBMC) and 15C (PD-1-transfected CHO cells). The anti-PD-1 monoclonal antibodies 5C4 and 4H1 bound to activated human T cells, activated monkey PBMCs, and CHO cells transfected with human PD-1, as measured by the mean fluorescent intensity (MFI) of staining. These data demonstrate that the anti-PD-1 HuMAbs bind to both human and cynomolgous monkey cell surface PD-1.

Example 5: Effect of human anti-PD-1 antibodies on cell proliferation and cytokine production in a Mixed Lymphocyte Reaction

A mixed lymphocyte reaction was employed to demonstrate the effect of blocking the PD-1 pathway to lymphocyte effector cells. T cells in the assay were tested for proliferation, IFN-gamma secretion and IL-2 secretion in the presence or absence of an anti-PD-1 HuMAb antibody.

Human T-cells were purified from PBMC using a human CD4+ T cell enrichment column (R&D systems). Each culture contained 10^5 purified T-cells and 10^4 allogeneic dendritic cells in a total volume of 200 µl. Anti-PD-1 monoclonal antibody 5C4, 4H1, 17D8, 2D3 or a Fab fragment portion of 5C4 was added to each culture at different antibody concentrations. Either no antibody or an isotype control antibody was used as a

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negative control. The cells were cultured for 5 days at 37°C. After day 5, 100 µl of medium was taken from each culture for cytokine measurement. The levels of IFN-gamma and other cytokines were measured using OptEIA ELISA kits (BD Biosciences). The cells were labeled with ^{3}H-thymidine, cultured for another 18 hours, and analyzed for cell proliferation. The results are shown in Figures 16A (T cell proliferation), 16B (IFN-γ secretion) and 16C (IL-2 secretion). The anti-PD-1 human monoclonal antibodies promoted T-cell proliferation, IFN-gamma secretion and IL-2 secretion in a concentration dependent manner. The 5C4-Fab fragment also promoted T-cell proliferation, IFN-gamma secretion and IL-2 secretion in a concentration dependent manner. In contrast, cultures containing the isotype control antibody did not show an increase in T cell proliferation, IFN-gamma or IL-2 secretion.

Example 6: Blocking of ligand binding to PD-1 by human anti-PD-1 antibodies

Anti-PD-1 HuMAbs were tested for the ability to block binding of the ligands PD-L1 and PD-L2 to PD-1 expressed on transfected CHO cells by using a flow cytometry assay.

PD-1 expressing CHO cells were suspended in FACS buffer (PBS with 4% fetal calf serum). Various concentrations of the anti-PD-1 HuMAbs 5C4 and 4H1 were added to the cell suspension and incubated at 4°C for 30 minutes. Unbound antibody was washed off and either FITC-labeled PD-L1 fusion protein or FITC-labeled PD-L2 fusion protein was added into the tubes and incubated at 4°C for 30 minutes. Flow cytometric analyses were performed using a FACScan flowcytometer (Becton Dickinson, San Jose, CA). The results are depicted in Figures 17A (blocking of PD-L1) and 17B (blocking of PD-L2). The anti-PD-1 monoclonal antibodies 5C4 and 4H1 blocked binding of PD-L1 and PD-L2 to CHO cells transfected with human PD-1, as measured by the mean fluorescent intensity (MFI) of staining. These data demonstrate that the anti-PD-1 HuMAbs block binding of ligand (both PD-L1 and PD-L2) to cell surface PD-1.

Example 7: Effect of human anti-PD-1 antibodies on the release of

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cytokines in human blood

The anti-PD-1 HuMAbs were mixed with fresh human whole blood in order to determine whether the anti-PD-1 HuMAbs alone stimulated the release of certain cytokines from human blood cells.

500 μl of heparinized-fresh human whole blood, was added into each well. Either 10 μg or 100 μg of an anti-PD-1 HuMAb (4H1 or 5C4,the latter either as an IgG1 or IgG4 isotype) was added to each well. Some wells were incubated with anti-CD3 antibody as a positive control, or a human IgG1 or human IgG4 antibody as isotype-matched negative controls. The cells were incubated at 37° C for either 6 or 24 hours. The cells were spun down and the plasma was collected for measurement of the cytokines IFN-gamma, TNF-alpha, IL-2, IL-4, IL-6, IL-10 and IL-12 using a cytokine cytometric bead array assay (BD Biosciences). The concentration of each cytokine (pg/ml) is shown in Tables 3a, with a 6 hour incubation, and 3b, with a 24 hour incubation, below. The results show that treatment with the human anti-PD-1 antibodies 5C4 and 4H1 alone did not stimulate human blood cells to release any of the cytokines IFN-gamma, TNF-alpha, IL-2, IL-4, IL-6, IL-10 and IL-12.

Table 3a. Cytokine production following 6 hour incubation

Ab	IFN-gamma (pg/ml)	TNF-alpha (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	IL-4 (pg/ml)	IL-2 (pg/ml)
No Ab	12.3	2	3	5	3.6	1.9
10 mg/ml anti-CD3	5000	530	82.6	510.4	37.2	467.9
100 mg/ml anti-CD3	5000	571	91.3	530	43.9	551.5
10 mg/ml hIgG1	7	1.8	2.8	4.4	2.6	1.5
100 mg/ml hIgG1	0	2.2	2.7	6	2.6	1.4
10 mg/ml hIgG4	5.4	1.4	2.5	4.5	2.1	1.3
100 mg/ml hIgG4	6.4	2.3	3	32.6	2.9	1.4
10 mg/ml	6.2	1.8	2.4	4.1	2.8	1.6

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4H1						
100 mg/ml 4H1	11.8	2	2.6	3.5	2.6	1.7
10 mg/ml 5C4 IgG1	4.2	1.6	2.3	3.9	2.5	1.3
100 mg/ml 5C4 IgG1	0	1.4	2.2	3.6	2.1	1.2
10 mg/ml 5C4 IgG4	8.3	2.5	1.9	4.8	1.6	1.5
100 mg/ml 5C4 IgG4	3.6	1.7	2.4	3.9	2.3	1.5

Table 3b. Cytokine production following 24 hour incubation

Ab	IFN-gamma (pg/ml)	TNF-alpha (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	IL-4 (pg/ml)	IL-2 (pg/ml)
No Ab	11.2	2	6.1	5.9	2.6	1.7
10 mg/ml anti-CD3	5000	565.9	432	5000	64.5	1265.3
100 mg/ml anti-CD3	5000	535	461	5000	73.8	1334.9
10 mg/ml hIgG1	0	0	0	0	0	0
100 mg/ml hIgG1	11.5	1.7	7.9	60.8	2.9	1.5
10 mg/ml hIgG4	24.6	3.1	8.3	63.4	3.1	2.3
100 mg/ml hIgG4	11.2	1.8	8	27.7	3.1	2.4
10 mg/ml 4H1	27.3	2.9	8	13.9	5.3	2.6
100 mg/ml 4H1	17.5	2.5	4.4	7	4	2.1
10 mg/ml 5C4 IgG1	9.1	2	7.6	68.5	3.5	1.8
100 mg/ml 5C4 IgG1	12.9	1.9	6.1	25.3	2.9	1.7
10 mg/ml 5C4 IgG4	14	1.9	4.4	3.3	2.6	1.9
100 mg/ml 5C4 IgG4	0	0	0	0	0	0

Example 8: Effect of anti-PD-1 antibodies on the apoptosis of T-cells

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The effect of anti-PD-1 antibodies on the induction of apoptosis of T-cells was measured using an annexin V staining test.

T cells were cultured in a mixed lymphocyte reaction, as described above in Example 5. The anti-PD-1 antibody 5C4 was added to the tube at a concentration of 25 μg/ml. A non-specific antibody was used as a control. Annexin V and propidium iodide were added according to standard protocol (BD Biosciences). The mixture was incubated for 15 minutes in the dark at room temperature and then analyzed using a FACScan flowcytometer (Becton Dickinson, San Jose, CA). The results are shown in Figures 18. The anti-PD-1 antibody 5C4 did not have an effect on T-cell apoptosis.

Example 9: Effect of anti-PD-1 antibodies on cytokine secretion by viral-stimulated PBMC cells from a virus positive donor

In this example, peripheral blood mononuclear cells (PBMC) from a donor positive for CMV were isolated and exposed to a CMV lysate in the presence or absence of anti-PD-1 antibodies to examine the effect of the antibodies on cytokine secretion simulated by antigen.

$2x10^5$ human PMBCs from a CMV positive donor were cultured in a total volume of 200 μl and added into each well along with a lysate of CMV-infected cells. The anti-PD-1 HuMAb 5C4 was added to each well in various concentrations for 4 days. After day 4, 100 μl of medium was taken from each culture for cytokine measurement. The level of IFN-gamma was measured using OptEIA ELISA kits (BD Biosciences). The cells were labeled with ^{3}H-thymidine, cultured for another 18 hours, and analyzed for cell proliferation. The cell proliferation was analyzed using the Cell Titer-Glo reagent (Promega). The results are shown in Figure 19. The anti-PD-1 HuMab 5C4 increased IFN gamma secretion in a concentration dependent manner. These results shows that anti-PD-1 HuMAbs can stimulate IFN-gamma release in a memory T cell response from PBMC cells previously stimulated against an antigen.

Example 10: Effect of anti-PD-1 antibody on secondary antibody response to antigen

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Mice were immunized and rechallenged with a TI -antigen (DNP-Ficoll) and also treated with a rat anti-mouse-PD-1 antibody, or a control antibody to examine the effect of the anti-PD-1 antibody on antibody titers.

Female C57BL6 mice were divided into two groups, with 6 mice/group. One group was treated with a control rat IgG and the other with a rat anti-mouse PD-1 antibody. The mice were immunized with 5μg of DNP-Ficoll (a T1-antigen) in 50μl CFA by i.p. at day 0. Either the control rat IgG antibody or the rat-mPD-1 antibody (200μg/mouse) was given by i.p. at days -1, 0 and 2. Four weeks later, mice were rechallenged with 5μg of DNP-Ficoll in 50μl IFA by i.p. at day 0. Rat anti-mPD-1 antibody or control antibody (200ug/mouse) was given by i.p. at days 0 and 1. Antibody titers were measured by standard ELISA assay at day 7 following the boost. The results are shown in Table 4 below. In the mice treated with the anti-mPD-1 antibody, both IgM and IgG3 isotypes showed the greatest increase in titer following challenge with the T1-antigen, as compared to mice treated with a control antibody. These results demonstrate that anti-PD-1 treatment can increase antibody titers in response to T1-antigen.

Table 4. Murine secondary response following treatment with anti-PD-1 antibody

Antibody Isotype	Control group	Rat anti-mouse PD-1 antibody	P value
IgM	606	1200	0.026
IgG	9	15.55	0.18
IgG1	1.2	1.1	0.83
IgG2b	5.05	9.26	0.18
IgG3	21.9	81.2	0.03

* Results shown are average concentration of antibody isotype (μg/ml)

Example 11: Treatment of *in vivo* tumor model using anti-PD-1 antibodies

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Mice implanted with a cancerous tumor were treated *in vivo* with anti-PD-1 antibodies to examine the *in vivo* effect of the antibodies on tumor growth. As a positive control, an anti-CTLA-4 antibody was used, since such antibodies have been shown to inhibit tumor growth *in vivo*.

In this experiment, the anti-PD-1 antibody used was a chimeric rat anti-mouse-PD-1 antibody generated using well known laboratory techniques. To generate the rat anti-mouse PD-1 antibody, rats were immunized with mouse cells transfected to express a recombinant mouse PD-1 fusion protein (R&D Systems Catalog No. 1021-PD) and monoclonal antibodies were screened for binding to mouse PD-1 antigen by ELISA assay. The rat anti-PD-1 antibody V regions were then recombinantly linked to a murine IgG1 constant region using standard molecular biology techniques and rescreened for binding to mouse PD-1 by ELISA and FACS. The chimeric rat anti-mouse-PD-1 antibody used herein is referred to as 4H2.

For the tumor studies, female AJ mice between 6-8 weeks of age (Harlan Laboratories) were were randomized by weight into 6 groups. The mice were implanted subcutaneously in the right flank with 2 x 10^6 SA1/N fibrosarcoma cells dissolved in 200 µl of DMEM media on day 0. The mice were treated with PBS vehicle, or antibodies at 10 mg/kg. The animals were dosed by intraperitoneal injection with approximately 200 µl of PBS containing antibody or vehicle on days 1, 4, 8 and 11. Each group contained 10 animals and the groups consisted of: (i) a vehicle group, (ii) control mouse IgG, (iii) control hamster IgG, (iv) hamster anti-mouse CTLA4 antibody and (v) the chimeric anti-PD-1 antibody 4H2 . The mice were monitored twice weekly for tumor growth for approximately 6 weeks. 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 tumor end point (1500 mm^3) or show greater than 15% weight loss. The results are shown in Figure 20. The anti-PD-1 antibody extended the mean time to reaching the tumor end point volume (1500 mm^3) from ~25 days in the control groups to ~40 days. Thus, treatment with an anti-PD-1 antibody has a direct *in vivo* inhibitory effect on tumor growth.

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SEQ ID NO:	SEQUENCE	SEQ ID NO:	SEQUENCE
1	VH a.a. 17D8	26	VK CDR1 a.a. 17D8
2	VH a.a. 2D3	27	VK CDR1 a.a. 2D3
3	VH a.a. 4H1	28	VK CDR1 a.a. 4H1
4	VH a.a. 5C4	29	VK CDR1 a.a. 5C4
5	VH a.a. 4A11	30	VK CDR1 a.a. 4A11
6	VK a.a. 17D8	31	VK CDR2 a.a. 17D8
7	VK a.a. 2D3	32	VK CDR2 a.a. 2D3
8	VK a.a. 4H1	33	VK CDR2 a.a. 4H1
9	VK a.a. 5C4	34	VK CDR2 a.a. 5C4
10	VK a.a. 4A11	35	VK CDR2 a.a. 4A11
11	VH CDR1 a.a. 17D8	36	VK CDR3 a.a. 17D8
12	VH CDR1 a.a. 2D3	37	VK CDR3 a.a. 2D3
13	VH CDR1 a.a. 4H1	38	VK CDR3 a.a. 4H1
14	VH CDR1 a.a. 5C4	39	VK CDR3 a.a. 5C4
15	VH CDR1 a.a. 4A11	40	VK CDR3 a.a. 4A11
16	VH CDR2 a.a. 17D8	41	VH n.t. 17D8
17	VH CDR2 a.a. 2D3	42	VH n.t. 2D3
18	VH CDR2 a.a. 4H1	43	VH n.t. 4H1
19	VH CDR2 a.a. 5C4	44	VH n.t. 5C4
20	VH CDR2 a.a. 4A11	45	VH n.t. 4A11
21	VH CDR3 a.a. 17D8	46	VK n.t. 17D8
22	VH CDR3 a.a. 2D3	47	VK n.t. 2D3
23	VH CDR3 a.a. 4H1	48	VK n.t. 4H1
24	VH CDR3 a.a. 5C4	49	VK n.t. 5C4
25	VH CDR3 a.a. 4A11	50	VK n.t. 4A11
51	VH 3-33 germline a.a.	53	VH 4-39 germline a.a.
52	VK L6 germline a.a.	54	VK L15 germline a.a.

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We claim:

1. An isolated human monoclonal antibody, or an antigen-binding portion thereof, wherein the antibody binds to PD-1 and wherein the antibody exhibits at least one of the following properties:

a) binds to human PD-1 with a K_D of 1×10^{-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; or

e) increases interleukin-2 (IL-2) secretion in an MLR assay.

2. The antibody of claim 1 which is full-length antibody of an IgG1 or IgG4 isotype.

3. The antibody of claim 1 which is an antibody fragment or a single chain antibody.

4. The antibody of claim 1, wherein the antibody binds to human PD-1 with a K_D of 5×10^{-8} M or less.

5. The antibody of claim 1, wherein the antibody binds to human PD-1 with a K_D of 1×10^{-8} M or less.

6. The antibody of claim 1, wherein the antibody binds to human PD-1 with a K_D of 5×10^{-9} M or less.

7. The antibody of claim 1, wherein the antibody binds to human PD-1 with a K_D of between 1×10^{-8} M and 1×10^{-10} M.

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8. An isolated human monoclonal antibody, or an antigen-binding portion thereof, wherein the antibody cross-competes for binding to PD-1 with a reference antibody comprising:

a) a human heavy chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5; and

b) a human light chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10.

9. The antibody of claim 8, wherein the human heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 1 and the human light chain variable region comprises the amino acid sequence of SEQ ID NO: 6.

10. The antibody of claim 8, wherein the human heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 2 and the human light chain variable region comprises the amino acid sequence of SEQ ID NO: 7.

11. The antibody of claim 8, wherein the human heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 3 and the human light chain variable region comprises the amino acid sequence of SEQ ID NO: 8.

12. The antibody of claim 8, wherein the human heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 4 and the human light chain variable region comprises the amino acid sequence of SEQ ID NO: 9.

13. The antibody of claim 8, wherein the human heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 5 and the human light chain variable region comprises the amino acid sequence of SEQ ID NO: 10.

14. An isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human V_H 3-33 gene, wherein the antibody specifically binds to PD-1.

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15. An isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human V_H 4-39 gene, wherein the antibody specifically binds to PD-1.

16. An isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human V_K L6 gene, wherein the antibody specifically binds to PD-1.

17. An isolated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human V_K L15 gene, wherein the antibody specifically binds to PD-1.

18. An isolated monoclonal antibody, or an antigen-binding portion thereof, comprising:

a) a heavy chain variable region of a human V_H 3-33 gene; and

b) a light chain variable region of a human Vk L6 gene;

wherein the antibody specifically binds to PD-1.

19. An isolated monoclonal antibody, or an antigen-binding portion thereof, comprising:

a) a heavy chain variable region of a human V_H 4-39 gene; and

b) a light chain variable region of a human Vk L15 gene;

wherein the antibody specifically binds to PD-1.

20. An isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region that comprises CDR1, CDR2, and CDR3 sequences; and a light chain variable region that comprises CDR1, CDR2, and CDR3 sequences, wherein:

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a) the heavy chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25, and conservative modifications thereof;
b) the light chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40, and conservative modifications thereof; and
c) the antibody specifically binds to PD-1.

21. The antibody of claim 20, wherein the heavy chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 16, 17, 18, 19, and 20, and conservative modifications thereof; and the light chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 31, 32, 33, 34, and 35, and conservative modifications thereof.

22. The antibody of claim 21, wherein the heavy chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 11, 12, 13, 14, and 15, and conservative modifications thereof; and the light chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 26, 27, 28, 29, and 30, and conservative modifications thereof.

23. An isolated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region and a light chain variable region, wherein:
a) the heavy chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5;
b) the light chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10;
c) the antibody binds to human PD-1 with a K_D of 1×10^{-7} M or less; and

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d) the antibody does not substantially bind to human CD28, CTLA-4 or ICOS.

24. The antibody of claim 23, wherein the antibody further comprises at least one of the following properties:

a) the antibody increases T-cell proliferation in an Mixed Lymphocyte Reaction (MLR) assay;

b) the antibody increases interferon-gamma production in an MLR assay; or

c) the antibody increases interleukin-2 (IL-2) secretion in an MLR assay.

25. An isolated monoclonal antibody, or antigen binding portion thereof, comprising:

a) a heavy chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15;

b) a heavy chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20;

c) a heavy chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25;

d) a light chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30;

e) a light chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35; and

f) a light chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40;

wherein the antibody specifically binds PD-1.

26. The antibody of claim 25, which comprises:

a) a heavy chain variable region CDR1 comprising SEQ ID NO: 11;

b) a heavy chain variable region CDR2 comprising SEQ ID NO: 16;

c) a heavy chain variable region CDR3 comprising SEQ ID NO: 21;

d) a light chain variable region CDR1 comprising SEQ ID NO: 26;

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e) a light chain variable region CDR2 comprising SEQ ID NO: 31; and

f) a light chain variable region CDR3 comprising SEQ ID NO: 36.

27. The antibody of claim 25, which comprises:

a) a heavy chain variable region CDR1 comprising SEQ ID NO: 12;

b) a heavy chain variable region CDR2 comprising SEQ ID NO: 17;

c) a heavy chain variable region CDR3 comprising SEQ ID NO: 22;

d) a light chain variable region CDR1 comprising SEQ ID NO: 27;

e) a light chain variable region CDR2 comprising SEQ ID NO: 32; and

f) a light chain variable region CDR3 comprising SEQ ID NO: 37.

28. The antibody of claim 25, which comprises:

a) a heavy chain variable region CDR1 comprising SEQ ID NO: 13;

b) a heavy chain variable region CDR2 comprising SEQ ID NO: 18;

c) a heavy chain variable region CDR3 comprising SEQ ID NO: 23;

d) a light chain variable region CDR1 comprising SEQ ID NO: 28;

e) a light chain variable region CDR2 comprising SEQ ID NO: 33; and

f) a light chain variable region CDR3 comprising SEQ ID NO: 38.

29. The antibody of claim 25, which comprises:

a) a heavy chain variable region CDR1 comprising SEQ ID NO: 14;

b) a heavy chain variable region CDR2 comprising SEQ ID NO: 19;

c) a heavy chain variable region CDR3 comprising SEQ ID NO: 24;

d) a light chain variable region CDR1 comprising SEQ ID NO: 29;

e) a light chain variable region CDR2 comprising SEQ ID NO: 34; and

f) a light chain variable region CDR3 comprising SEQ ID NO: 39.

30. The antibody of claim 25, which comprises:

a) a heavy chain variable region CDR1 comprising SEQ ID NO: 15;

b) a heavy chain variable region CDR2 comprising SEQ ID NO: 20;

c) a heavy chain variable region CDR3 comprising SEQ ID NO: 25;

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d) a light chain variable region CDR1 comprising SEQ ID NO: 30;

e) a light chain variable region CDR2 comprising SEQ ID NO: 35; and

f) a light chain variable region CDR3 comprising SEQ ID NO: 40.

31. An isolated monoclonal antibody, or antigen binding portion thereof comprising:

a) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, and 5; and

b) a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 7, 8, 9, and 10;

wherein the antibody specifically binds PD-1.

32. The antibody of claim 31, which comprises:

a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1; and

b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 6.

33. The antibody of claim 31, which comprises:

a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2; and

b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 7.

34. The antibody of claim 31, which comprises:

a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and

b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8.

35. The antibody of claim 31, which comprises:

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a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 4; and

b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 9.

36. The antibody of claim 31, which comprises:

a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 5; and

b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 10.

37. A composition comprising the antibody, or antigen-binding portion thereof, of any one of claims 1-36, and a pharmaceutically acceptable carrier.

38. An immunoconjugate comprising the antibody, or antigen-binding portion thereof, of any one of claims 1-36, linked to a therapeutic agent.

39. A composition comprising the immunoconjugate of claim 38 and a pharmaceutically acceptable carrier.

40. The immunoconjugate of claim 38, wherein the therapeutic agent is a cytotoxin.

41. A composition comprising the immunoconjugate of claim 40 and a pharmaceutically acceptable carrier.

42. The immunoconjugate of claim 38, wherein the therapeutic agent is a radioactive isotope.

43. A composition comprising the immunoconjugate of claim 40 and a pharmaceutically acceptable carrier.

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44. A bispecific molecule comprising the antibody, or antigen-binding portion thereof, of any one of claims 1-36, linked to a second functional moiety having a different binding specificity than said antibody, or antigen binding portion thereof.

45. A composition comprising the bispecific molecule of claim 44, and a pharmaceutically acceptable carrier.

46. An isolated nucleic acid molecule encoding the antibody, or antigen-binding portion thereof, of any one of claims 1-36.

47. An expression vector comprising the nucleic acid molecule of claim 46.

48. A host cell comprising the expression vector of claim 47.

49. A transgenic mouse comprising human immunoglobulin heavy and light chain transgenes, wherein the mouse expresses the antibody of any one of claims 1-36.

50. A hybridoma prepared from the mouse of claim 49, wherein the hybridoma produces said antibody.

51. A method of modulating an immune response in a subject comprising administering to the subject the antibody, or antigen-binding portion thereof, of any one of claims 1-36 such that the immune response in the subject is modulated.

52. A method of inhibiting growth of tumor cells in a subject, comprising administering to the subject a therapeutically effective amount of an anti-PD-1 antibody, or antigen-binding portion thereof.

53. The method of claim 52, wherein the antibody is a chimeric antibody.

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54. The method of claim 52, wherein the antibody is a humanized antibody.

55. The method of claim 52, wherein the antibody is a fully human antibody.

56. The method of claim 52, wherein the tumor cells are of a cancer selected from the group consisting of melanoma, renal cancer, prostate cancer, breast cancer, colon cancer and lung cancer.

57. The method of claim 52, wherein the tumor cells are of a cancer selected from the list consisting of 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.

58. A method of inhibiting growth of tumor cells in a subject, comprising administering to a subject the antibody, or antigen-binding portion thereof, of any one of claims 1-36 in an amount effective to inhibit growth of the tumor cells.

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59. A method of treating an infectious disease in a subject comprising administering to the subject the antibody, or antigen-binding portion thereof, of any one of claims 1-36 such that the subject is treated for the infectious disease.

60. The method of claim 59, wherein the infectious disease is selected from the list consisting of: HIV, Influenza, Herpes, Giardia, Malaria, Leishmania, the pathogenic infection by the virus Hepatitis (A, B, & C), herpes virus (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus, pathogenic infection by the bacteria chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lymes disease bacteria, pathogenic infection by the fungi Candida (albicans, krusei, glabrata, tropicalis, etc.), Cryptococcus neoformans, Aspergillus (fumigatus, niger, etc.), Genus Mucorales (mucor, absidia, rhizophus), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis and Histoplasma capsulatum, and pathogenic infection by the parasites Entamoeba histolytica, Balantidium coli, Naegleriafowleri, Acanthamoeba sp., Giardia lambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondi, Nippostrongylus brasiliensis.

61. A method of enhancing an immune response to an antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) the antibody, or antigen-binding portion thereof, of any one of claims 1-36 such that an immune response to the antigen in the subject is enhanced.

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62. The method of claim 61, wherein the antigen is a tumor antigen, a viral antigen, a bacterial antigen or an antigen from a pathogen.

63. A method for preparing an anti-PD-1 antibody comprising:

providing: (i) a heavy chain variable region antibody sequence comprising a CDR1 sequence that is selected from the group consisting of SEQ ID NOs: 11, 12, 13, 14, and 15, a CDR2 sequence that is selected from the group consisting of SEQ ID NOs: 16, 17, 18, 19, and 20; and a CDR3 sequence that is selected from the group consisting of SEQ ID NOs: 21, 22, 23, 24, and 25; or (ii) a light chain variable region antibody sequence comprising a CDR1 sequence that is selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, and 30, a CDR2 sequence that is selected from the group consisting of SEQ ID NOs: 31, 32, 33, 34, and 35, and a CDR3 sequence that is selected from the group consisting of SEQ ID NOs: 36, 37, 38, 39, and 40;

altering at least one amino acid residue within at least one variable region antibody sequence, said sequence being selected from the heavy chain variable region antibody sequence and the light chain variable region antibody sequence, to create at least one altered antibody sequence; and

expressing the altered antibody sequence as a protein.

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HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

Abstract

The present invention provides isolated monoclonal antibodies, particularly human monoclonal antibodies, that specifically bind to PD-1 with high affinity. Nucleic acid molecules encoding the antibodies of the invention, expression vectors, host cells and methods for expressing the antibodies of the invention are also provided. Immunoconjugates, bispecific molecules and pharmaceutical compositions comprising the antibodies of the invention are also provided. The invention also provides methods for detecting PD-1, as well as methods for treating various diseases, including cancer and infectious diseases, using anti-PD-1 antibodies.

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Anti-PD1 17D8 VH

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

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

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

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

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

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

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

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

Figure 1A

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Anti-PD1 17D8 VK

V segment: L6
J segment: JK4

```
      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   I   S   S   Y   L   A   W   Y
55    GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT ATT AGC AGC TAC 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

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

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

Figure 1B

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Anti-PD-1 2D3 VH

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

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

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

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

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

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

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

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

Figure 2A

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Anti-PD-1 2D3 VK

V segment: L6
J segment: JK4

```
      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   S   S   Y   L   A   W   Y
55    GCC ACC CTG TCC TGC AGG GCC AGT CAG AGT GTT AGC AGC TAC TTA GCC TGG TAC

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

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

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

Figure 2B

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Anti-PD-1 4H1 VH

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

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

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

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

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

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

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

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

Figure 3A

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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 3B

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App No.: Not Yet Assigned Docket No.: 04280/0202908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

Anti-PD1 5C4 VH

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

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

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

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

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

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

                                      CDR3
                                      ~~~~~~~~~~~~~~~~
      T   A   V   Y   Y   C   A   T   N   D   D   Y   W   G   Q   G   T   L
271   ACG GCT GTG TAT TAC TGT GCG ACA AAC GAC GAC TAC TGG GGC CAG GGA ACC CTG
                                          ↳
                                                  JH4b
      V   T   V   S   S
325   GTC ACC GTC TCC TCA
```

Figure 4A

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App No.: Not Yet Assigned Docket No.: 04280/0202908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

Anti-PD1 5C4 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   S   S   Y   L   A   W   Y
55    GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AGT AGT TAC 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

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

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

Figure 4B

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Anti-PD-1 4A11 VH

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

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

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

          CDR1                                                          CDR2
          ~~~~                                                          ~~~~~~~~~~~
          G   W   I   R   Q   P   P   G   K   G   L   E   W   I   G   S   I   Y
109     GGC TGG ATC CGT CAG CCC CCA GGG AAG GGA CTG GAG TGG ATT GGG AGT ATC TAT

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


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

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

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

Figure 5A

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Anti-PD-1 4A11 VK

V segment: L15
J segment: JK1

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

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

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

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

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

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

Figure 5B

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Anti-PD1 VH region alignment to VH 3-33

```
                                                                  CDR1
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 - - - - - - -

                                                  CDR2
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
2D3 VH:         - - - - - - - - - - - - - - - - - K - - - - - - - - - - - - - - - - - - - - -
4H1 VH:         - - - - - - - - - - L - - - - - - - - - - - - - - - - - - - - - - - - - - - -
5C4 VH:         - - - - - - - - - - - - - - - - - K R - - - - - - - - - - - - - - - - - - - -

                                                    CDR3
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 6

App No.: Not Yet Assigned   Docket No.: 04280/020908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

App No.: Not Yet Assigned Docket No.: 04280/0202908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

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 7

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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 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 8

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App No.: Not Yet Assigned Docket No.: 04280/0202908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

Copy provided by USPTO from the IFW Image Database on 06/29/2006

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 9

App No.: Not Yet Assigned Docket No.: 04280/020908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO
PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR
TREATING CANCER USING ANTI-PD-1 ANTIBODIES

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Anti-PD1 5C4 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
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 10

App No.: Not Yet Assigned Docket No.: 04280/0202908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

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

```
                                                                                                  CDR1
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 -

                                                  CDR2
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         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

                                                                                  CDR3
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 11

App No.: Not Yet Assigned Docket No.: 04280/0202908-US0
Inventor:
Title: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES

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

```
                                                                    ____CDR1_____
L15 germline     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
4A11 VK          - - - - - - - - - - - - - - - - - - - S - - - - - - - - - - -

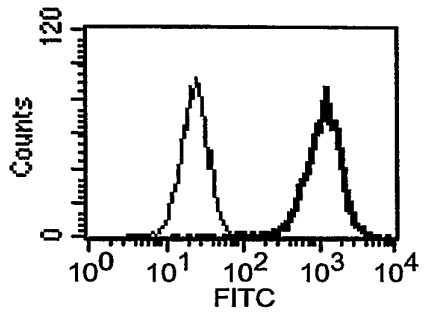
                 _____                                  ___CDR2_____
L15 germline     W L A W Y Q Q K P E K A P K S L I Y A A S S L Q S G V P S R F
4a11 VK          - - - - - - - - - - - - - - - - - - - N - R - - - - - - - -

                                                                  __CDR3___
L15 germline     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
4A11 VK          - - - - - - - - - - - - - - - - - - - - - - - - - - - - Y -

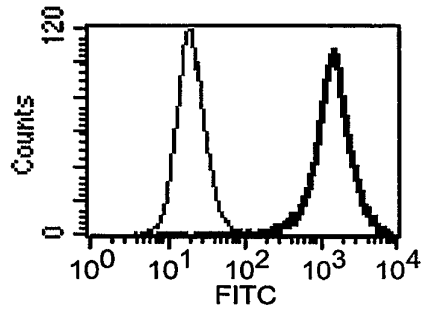
                 _______
L15 germline     Y P
JK1 germline           T F G Q G T K V E I K
4A11 VK          - - R - - - - - - - - - -    (JK1)
```

Figure 12

(A)



(B)



---- Binding to CHO cells

---- Binding to CHO hPD-1 cells

Figure 13

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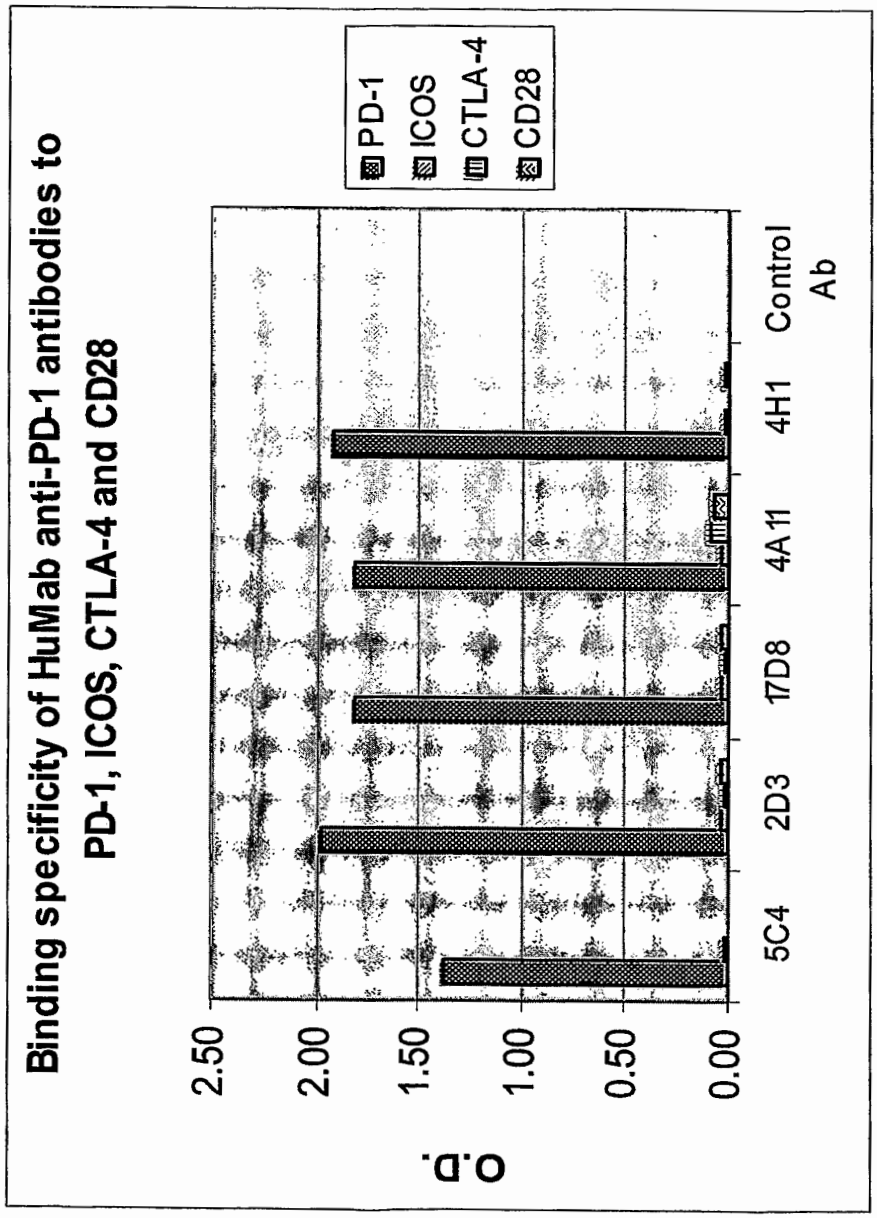


Figure 14

Copy provided by USPTO from the IFW Image Database on 06/29/2006

Binding of HuMab anti-hPD-1 Abs to activated human T cells

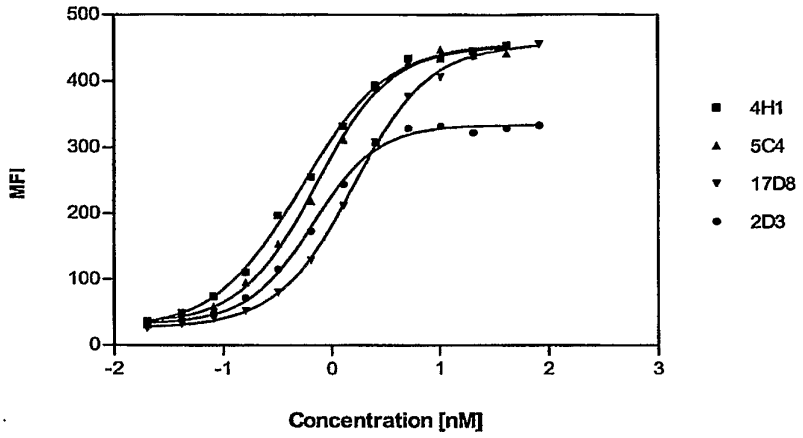


Figure 15A

Copy provided by USPTO from the IFW Image Database on 06/29/2006

Binding of HuMab anti-hPD-1 Abs to activated cynomolgous monkey PBMC

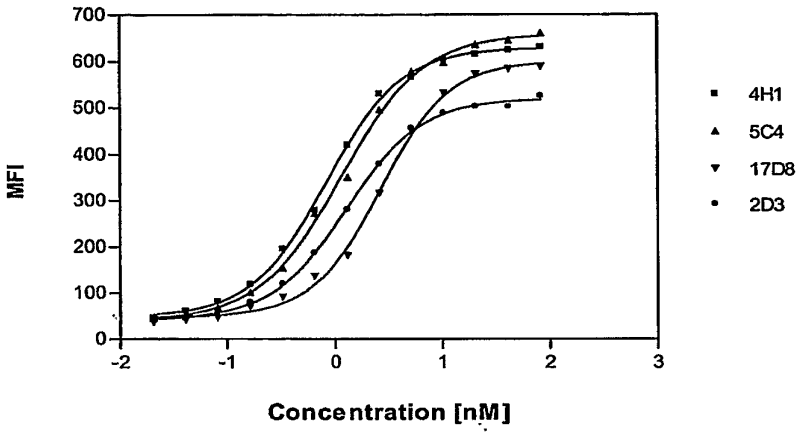


Figure 15B

Copy provided by USPTO from the IFW Image Database on 06/29/2006

Binding of HuMab anti-hPD-1 Abs to PD-1 expressing CHO cells

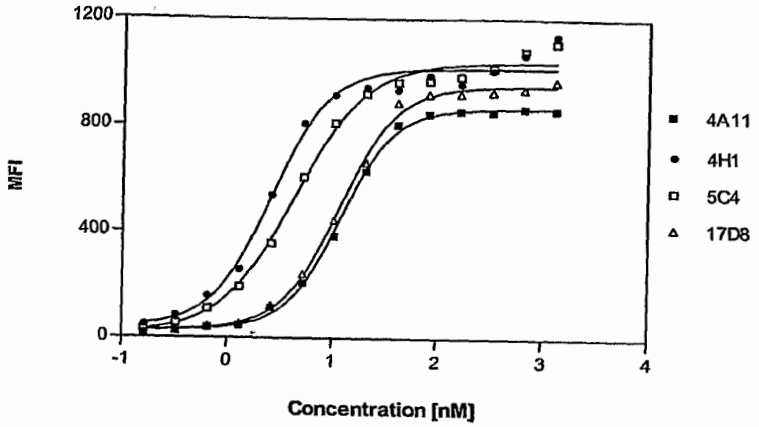


Figure 15C

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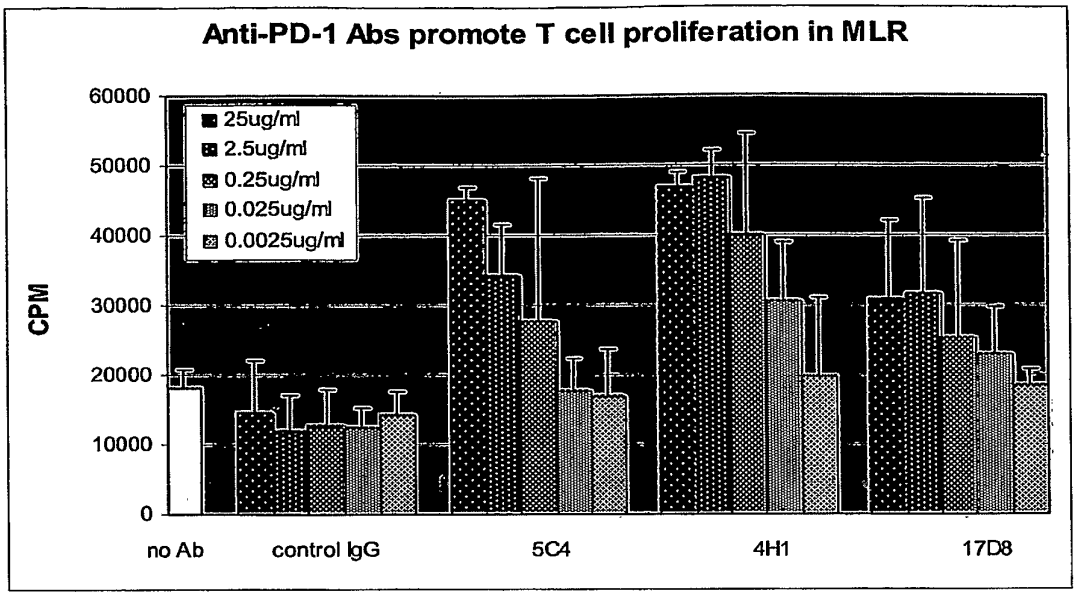


Figure 16A

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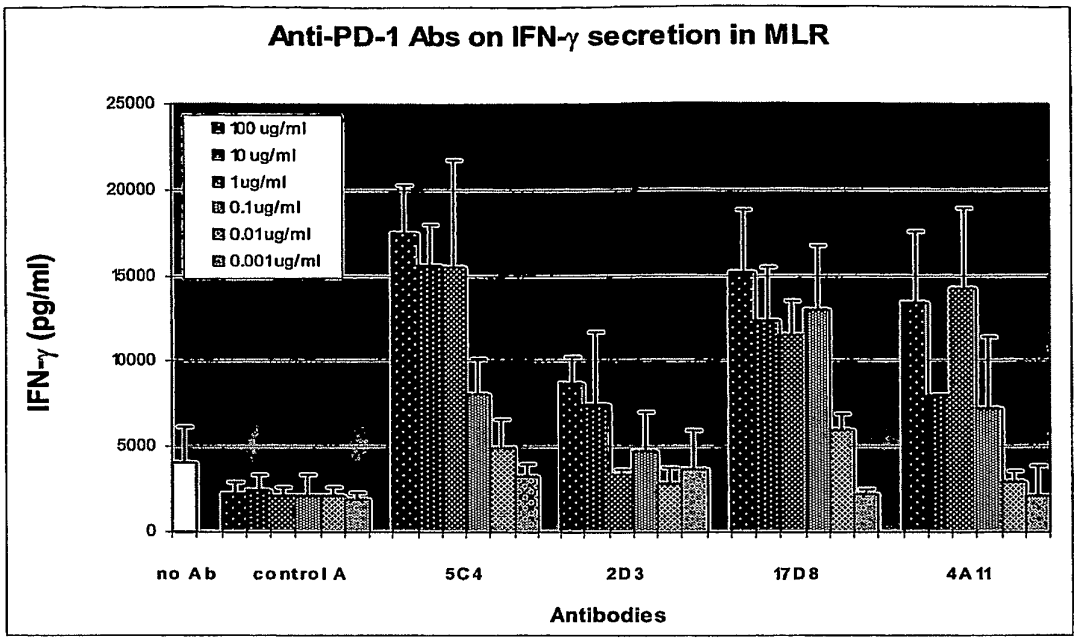


Figure 16B

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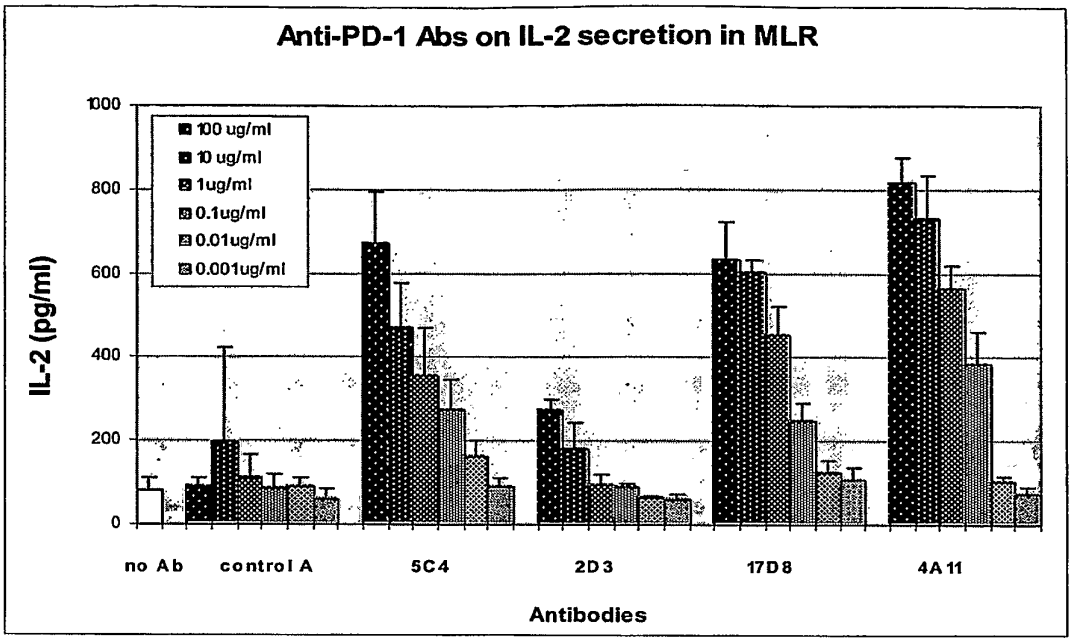


Figure 16C

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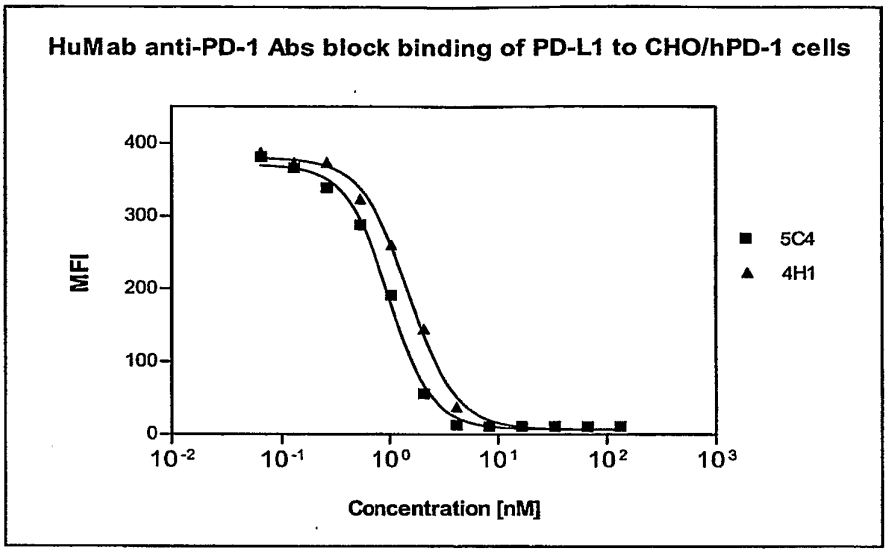


Figure 17A

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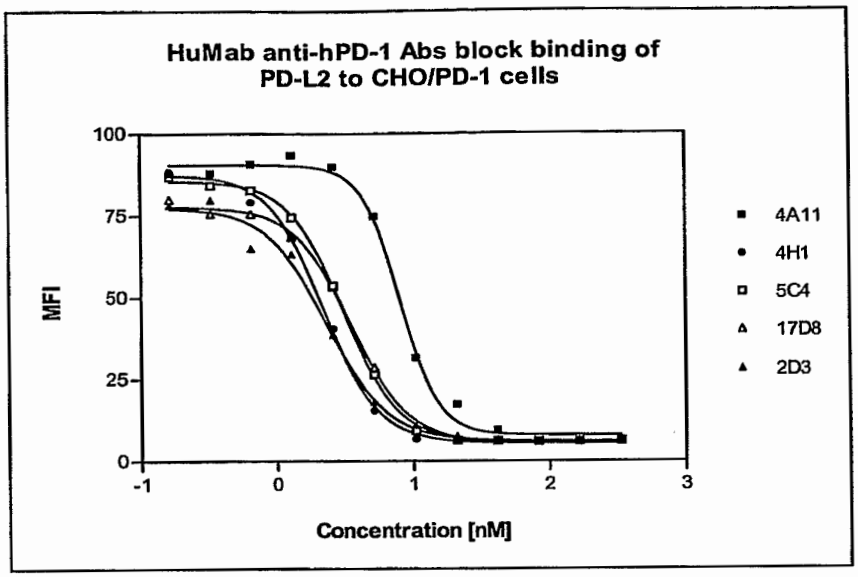


Figure 17B

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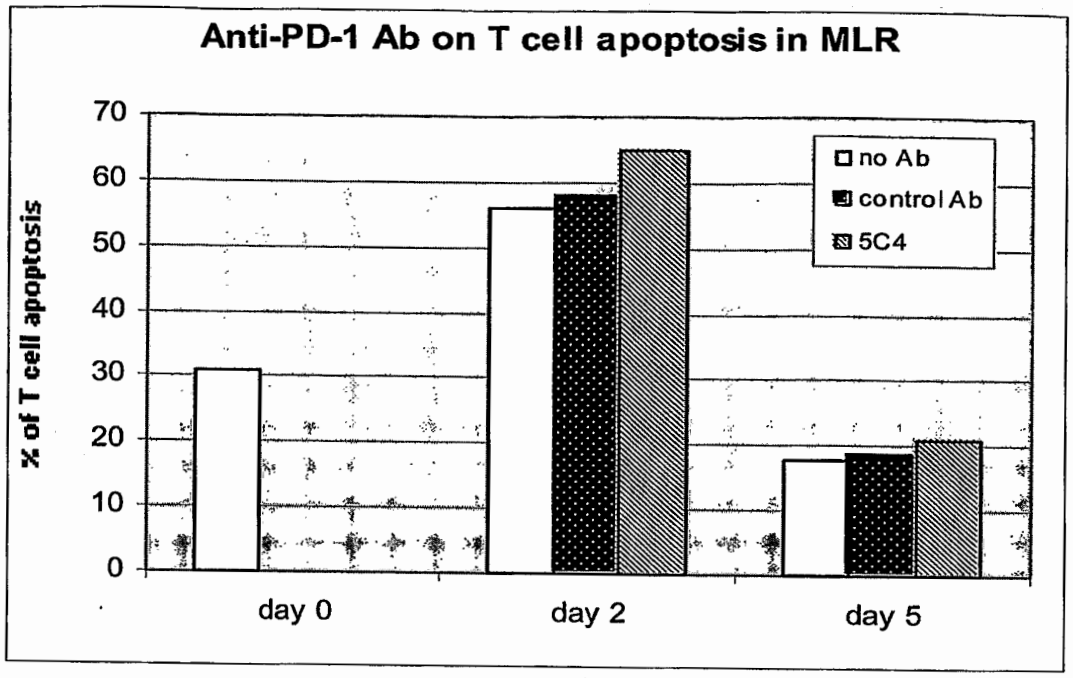


Figure 18

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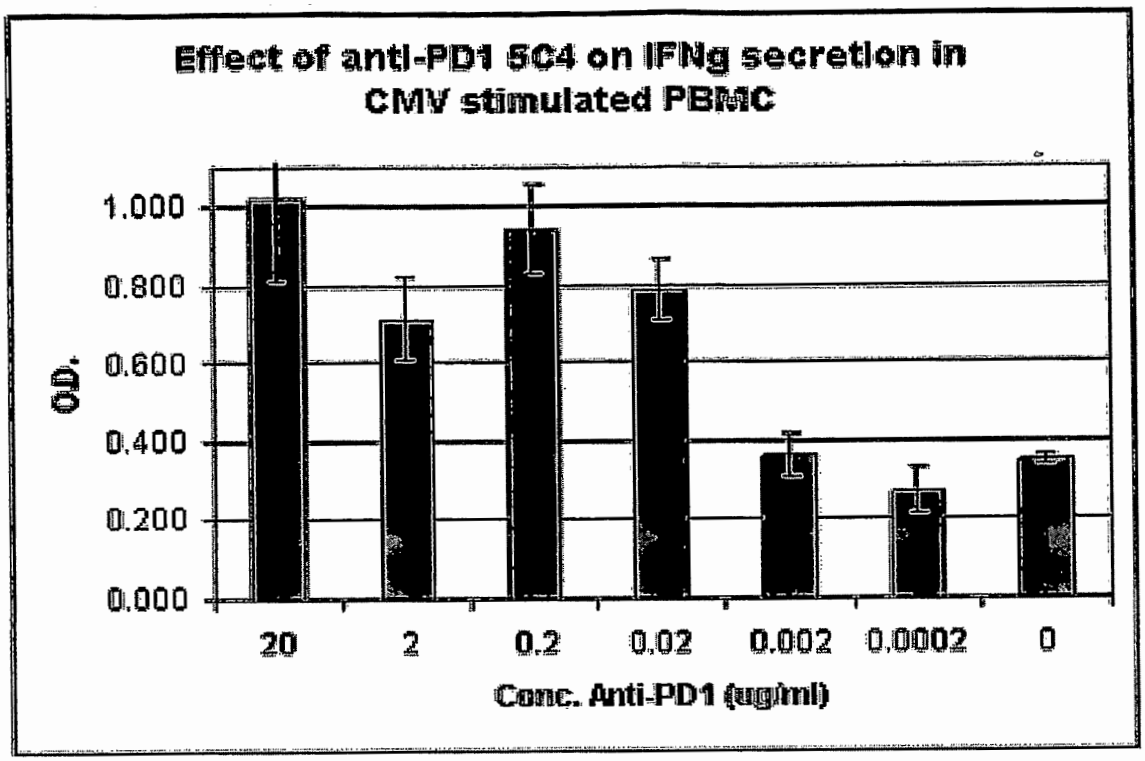


Figure 19

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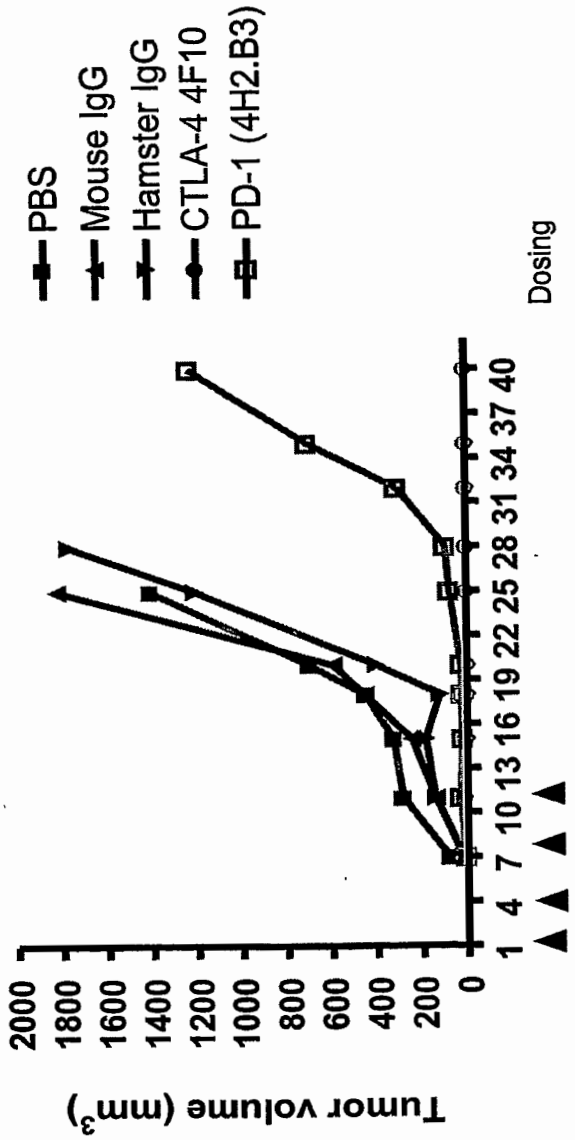


Figure 20

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Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/JP2006/309606

07.09.2006

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THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

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July 07, 2006

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APPLICATION NUMBER: ***60/738,434***
FILING DATE: ***November 21, 2005***

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS ***US60/738,434***

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV592417160US

INVENTOR(S)		
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Tseng-Hui Timothy	Chen	Burlingame, California
Changyu	Wang	Fremont, California
Haichun	Huang	Fremont, California

Additional inventors are being named on the _____ separately numbered sheets attached hereto

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**

[X] The address corresponding to Customer Number: 07278

OR

[ ] Firm or Individual Name DARBY & DARBY P.C.

Address P.O. Box 5257

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Country	US	Telephone	(206) 262-8900	Email	

ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Application Data Sheet. See 37 CFR 1.76
[X] Specification *Number of Pages* 47
[X] Drawing(s) *Number of Sheets* 33
[ ] CD(s), Number of CDs _____
[X] Other (specify) Fee Transmittal (1 page); Certificate of Express Mailing (1 page); 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).

METHOD OF PAYMENT OF FILING FEES AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.

TOTAL FEE AMOUNT ($)

[X] A check or money order is enclosed to cover the filing fee and application size fee (if applicable). 200.00

[ ] Payment by credit card. Form PTO-2038 is attached.

[X] The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any overpayment to Deposit Account Number: 04-0100 A duplicative copy of this form is enclosed for fee processing.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No. [ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

SIGNATURE _____ Date November 21, 2005

TYPED or PRINTED NAME Jeffrey C. Pepe REGISTRATION NO. *(if appropriate)* 46,985

TELEPHONE (206) 262-8911 Docket Number: 04280/0203401-US0

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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FEE TRANSMITTAL
For FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 200.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Alan J. Korman
Examiner Name	Not Yet Assigned
Art Unit	N/A
Attorney Docket No.	04280/0203401-US0

METHOD OF PAYMENT (check all that apply)

☒ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☐ Deposit Account Deposit Account Number: 04-0100 Deposit Account Name: Darby & Darby P.C.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	200.00

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____ - =	_____ x	_____ =	_____

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____ - =	_____ x	_____ =	_____

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due 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).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
80 - 100 =	_____	/50 _____ (round up to a whole number) x	_____	= _____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity	
Other (e.g., late filing surcharge): _____	_____

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Application No. (if known): Not Yet Assigned | Attorney Docket No.: 04280/0203401-US0

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Provisional Patent Application Transmittal (1 page)
Fee Transmittal (1 page)
Application Data Sheet (5 pages)
Specification (47 pages)
Drawings (33 sheets)
Return Receipt Postcard

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Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Group Art Unit::	N/A
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Title::	COMBINED USE OF CTLA-4 ANTIBODY AND PD-1 ANTIBODY FOR TREATMENT OF HYPERPROLIFERATIVE DISEASE
Attorney Docket Number::	04280/0203401-US0
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	33
Small Entity?::	No
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

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

TECHNICAL FIELD

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 adverse events related to treatment with such antibodies individually.

BACKGROUND

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, 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. 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).

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

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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 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, 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 (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 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 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 1 and tumor volume was monitored over 60 days.

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

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

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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 mm^3. The mice received subsequent antibody treatments on days 3, 6 and 10 as described in Example 2.

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 3) with one of the following therapies: (A) mouse IgG as a control (20 mg/kg, X_{20}) (B) anti-PD-1 antibody (10 mg/kg) and mouse IgG (10 mg/kg) ($P_{10}X_{10}$), (C) anti-CTLA-4 antibody (10 mg/kg) and mouse IgG (10 mg/kg) ($C_{10}X_{10}$), (D) anti-CTLA-4 antibody and anti-PD-1 antibody (10 mg/kg each) ($C_{10}P_{10}$), (E) anti-CTLA-4 antibody and anti-PD-1 antibody (3 mg/kg each) (C_3P_3), and (F) anti-CTLA-4 antibody and anti-PD-1 antibody (1 mg/kg each) (C_1P_1). 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) ($C_{10}C_{10}P_{10}P_{10}$); 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) (10 mg/kg, day 10) ($P_{10}P_{10}C_{10}C_{10}$).

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 4 and tumor volume was monitored over 41 days.

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

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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 5) 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 (F) 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 mm^3.

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 5) 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 mm^3.

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 mm^3.

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

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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.

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. 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.

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.

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 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 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

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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 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, 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 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.

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

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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 inter-connected 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

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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 (Clq) 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 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

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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 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.

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 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 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

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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 1 and 4) or after the tumor cells were implanted for a time sufficient to become an established tumor (Examples 2, 3 and 5). 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 6) 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 1, 3 and 5 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

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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

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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 IgGκ monoclonal (Lonberg *et al.*. (1994), *supra*; reviewed in Lonberg (1994) H*andbook 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.

In another embodiment, human antibodies of the instant disclosure can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchomosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. Such mice, referred to herein as "KM mice™", 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

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instant discloure. 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 dislcosure. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome, 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

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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 1×10^{-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 1×10^{-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.

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

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

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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* 3:207-212; 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 therapeutically include, but are not limited to, Iodine^{131}, Indium^{111}, Yttrium^{90} and Lutetium^{177}. Methods for preparing radioimmunconjugates 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.

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 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

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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.

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.

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

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formulated in separate pharmaceutical compositions or admixed as a single pharmaceutical 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 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 alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts 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, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the invention also may include a pharmaceutically 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, 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

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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 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.

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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, 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 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 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.

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.

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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.

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

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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 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 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 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 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,

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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.

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

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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

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 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

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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

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(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

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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

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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 Fcα or Fcγ 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 by 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® (eprtuzumab), 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.

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 another exmaple, antibodies to each of these entities may be further combined with an anti-PD-1

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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 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: 2160-2169), 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 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: 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

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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.

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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 includes 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.

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EXAMPLES

Example 1 *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 10^6 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 After 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 mm^3

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(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 mm^3 for the IgG control group; about 655 mm^3 for the CTLA-4 antibody alone group, about 510 mm^3 for the PD-1 antibody alone group, and about 280 mm^3 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 mm^3 for the IgG group; about 625 mm^3 for the CTLA-4 antibody alone group; about 525 mm^3 for the PD-1 antibody alone group; and about 10 mm^3 for the CTLA-4 antibody and PD-1 antibody combination group (and down to 0 mm^3 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 2 *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 (2×10^6 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 mm^3) 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

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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 mm^3), and on days 3, 6, 10, 13, 17 and 20 post-antibody injection. Mice were euthanized when the tumors reached a designated tumor end-point (a particular tumor volume such as 1500 mm^3 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 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 mm^3 for the IgG control group; about 1010 mm^3 for the CTLA-4 antibody alone group; about 695 mm^3 for the PD-1 antibody alone group; and about 80 mm^3 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 mm^3 for the IgG group; about 1060 mm^3 for the anti–CTLA-4 antibody alone group; about 480 mm^3 for the anti–PD-1 antibody alone group; and about 15 mm^3 for the anti–CTLA-4 antibody and anti–PD-1 antibody combination group (which was down to 0 mm^3 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 3 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 10^6 cells/mouse) for a time sufficient (about 6 to 7 days) to permit the formation of tumors as described in Example 2. Groups of 10 mice were injected IP at days 0, 3, 6 and 10 as follows:

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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 mm^3), 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 mm^3 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 4 mice that reached the tumor end–point and 6 mice with tumors having a volume of about 750 mm^3 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 mm^3 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 mm^3 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 mm^3 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 mm^3 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

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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. In each group, 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 mm^3 for the IgG control group; about 470 mm^3 for the PD-1 antibody with the IgG control; about 290 mm^3 for the CTLA-4 antibody with the IgG control (measured at day 6); about 40 mm^3 for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 165 mm^3 for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 400 mm^3 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 mm^3 for the IgG control group; about 400 mm^3 for the PD-1 antibody with the IgG control; about 660 mm^3 for the CTLA-4 antibody with the IgG control; 0 mm^3 for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 90 mm^3 for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 650 mm^3 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 the end 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 4 *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 x 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

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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 mm^3 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 mm^3 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 mm^3 and 3 with an ulcerated tumor), one mouse with a small tumor (volume of about 70 mm^3) 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 mm^3 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 mm^3 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 After 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

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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)
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.

† 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. 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 may also lead to inhibition of anti–tumor T cell responses (*see* Figure 20).

Example 5 *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 10^6 cells/mouse) on day 0 for a time sufficient (about 7 days) to permit the establishment of a tumor. On days 7, 10, 13 and 16 post-implantation, ten groups of 8 mice having an average tumor volume of 110 mm^3 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

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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 mm^3 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 mm^3 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 mm^3 (*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 mm^3 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 mm^3, 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 mm^3, and 4 mice that were tumor free. The unexpected synergistic effect of an anti–PD–1 antibody combined with an anti-CTLA-4 antibody,

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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).

Figure 16 shows the response to immunostimulatory antibody treatment in mice with larger tumors, those having an initial volume of about 250 mm^3 (*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 mm^3 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 mg/kg anti-CTLA-4 antibody (Group L) had one mouse with an ulcerated tumor, 4 mice with a tumor volume greater than 1500 mm^3, 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.

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.

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† 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 6 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 contacted 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 contacting with donkey–anti–rat IgG, FITC conjugated and measured by FACS. The anti-mPD-1 antibody had an EC_{50} (50% effective concentration) of about 0.38 μg (Figure 23) and a K_D of 4.7×10^{-9} M. To examine the inhibition of PD–L1 binding to PD–1, the same assay was performed except that the cells were also contacted with 0.16 μg mPD–L1-hFc fusion protein, then binding of PD–L1 to the PD–1 expressing CHO cells was detected by

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contacting 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 EC_{50} 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.

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 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.

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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

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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.

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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.

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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).

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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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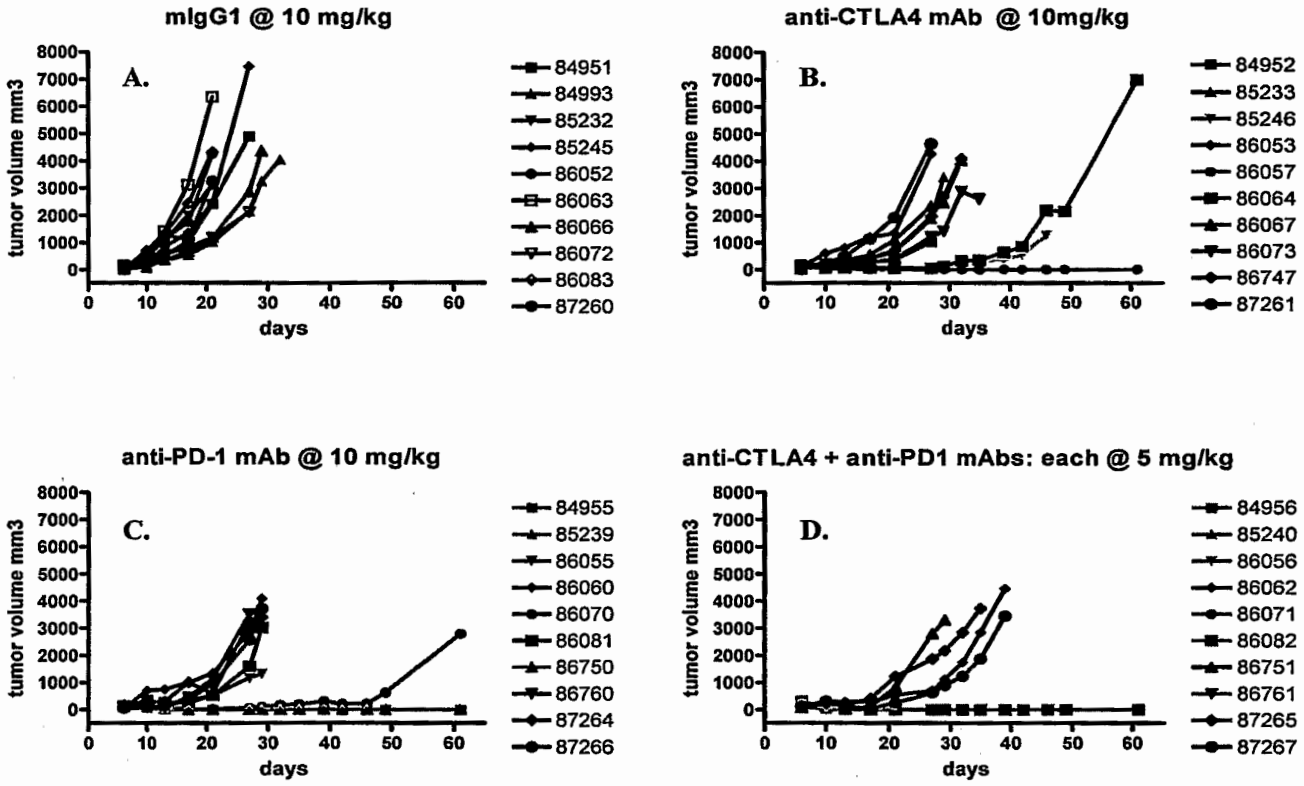


Figure 1.

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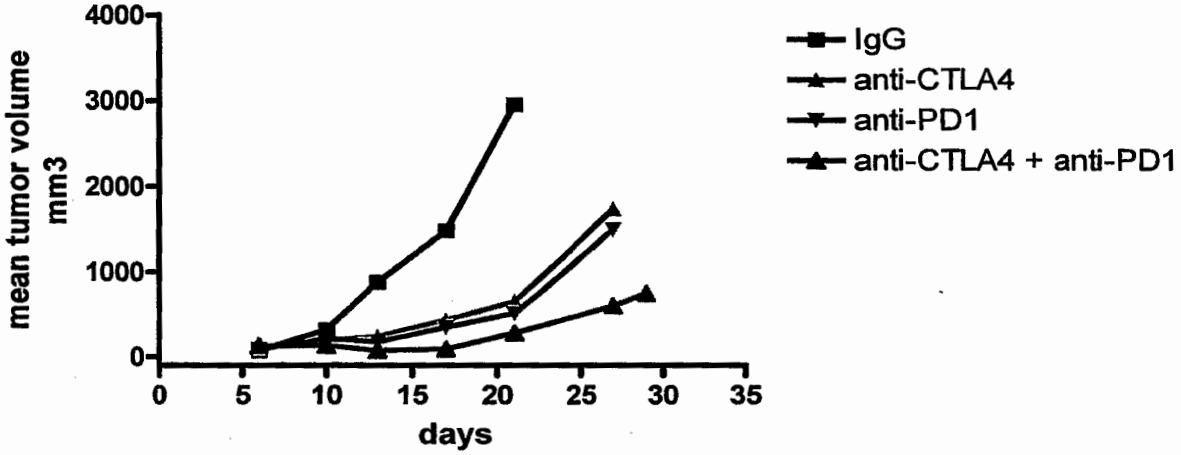


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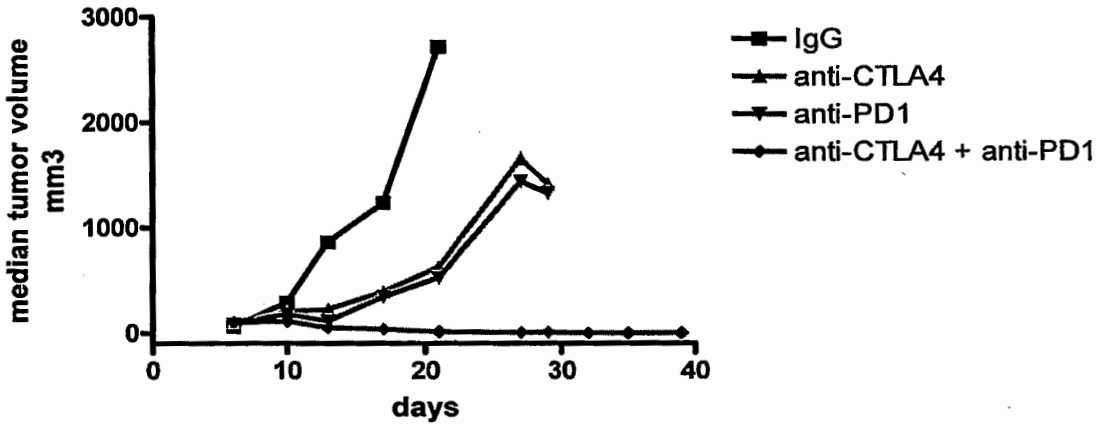


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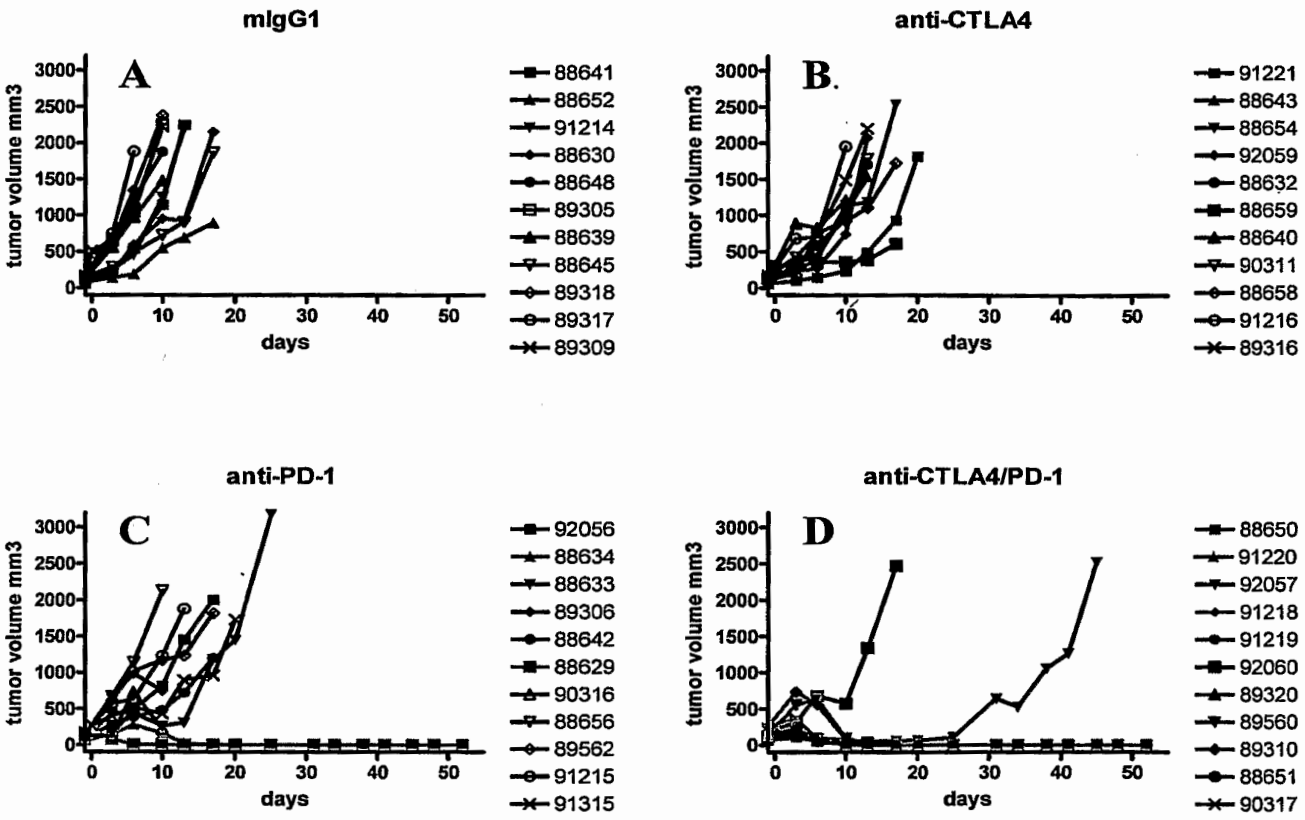


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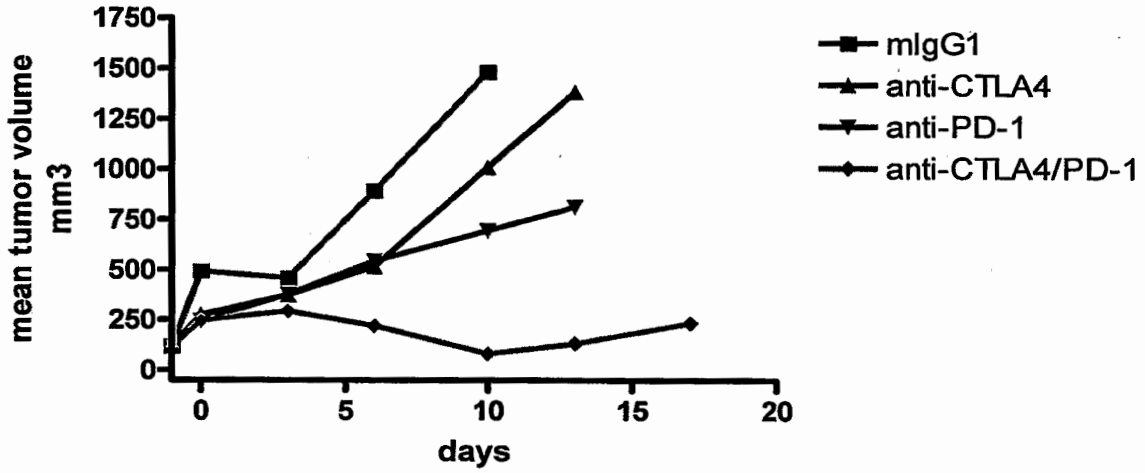


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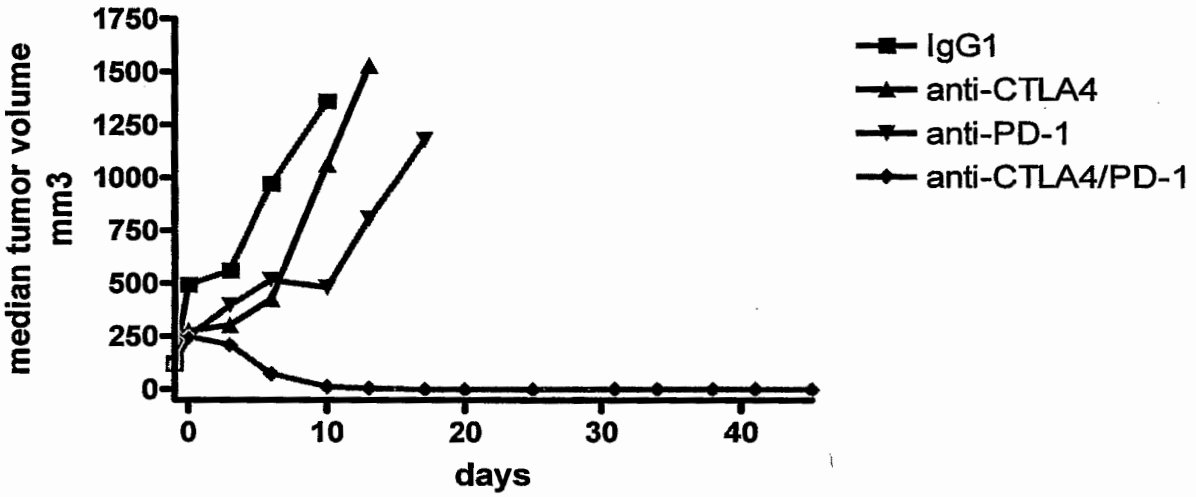


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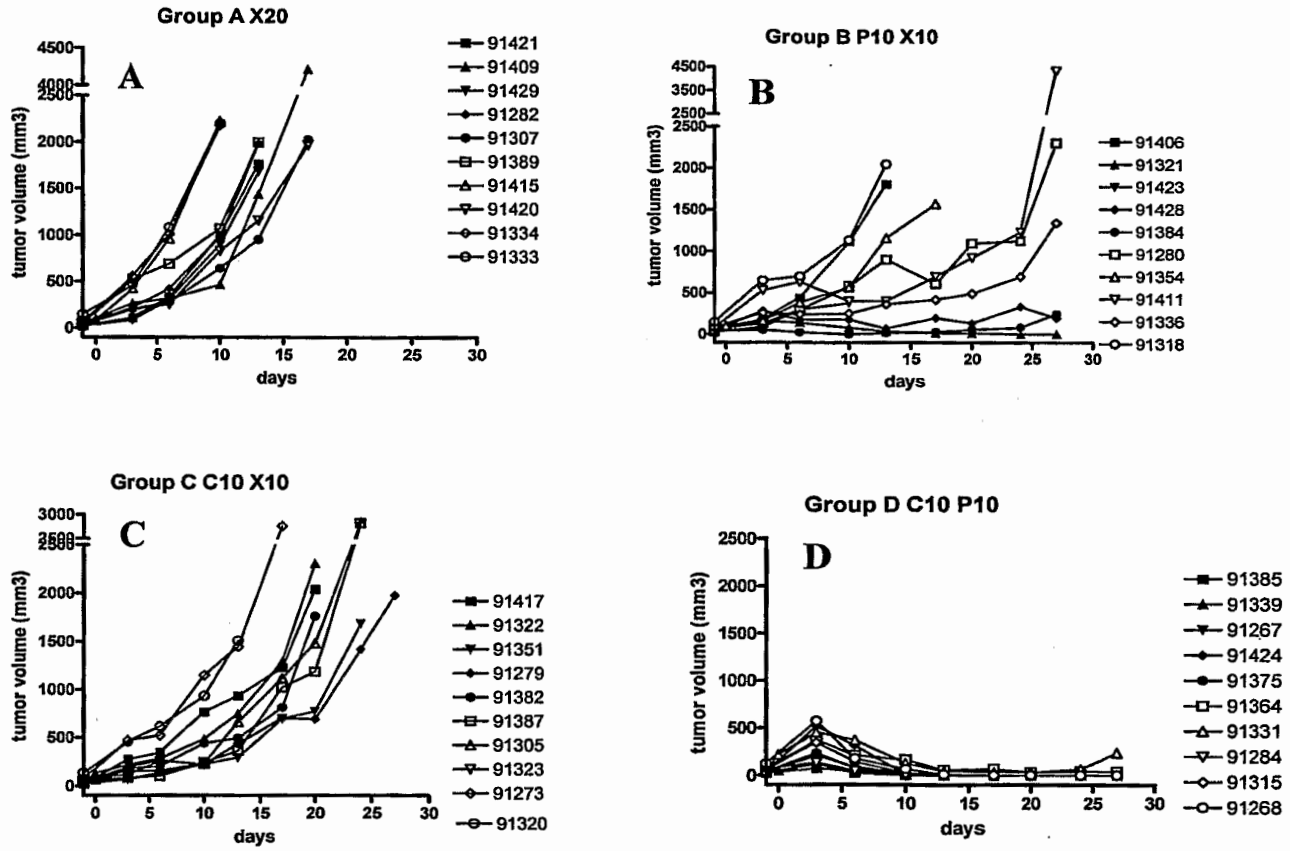


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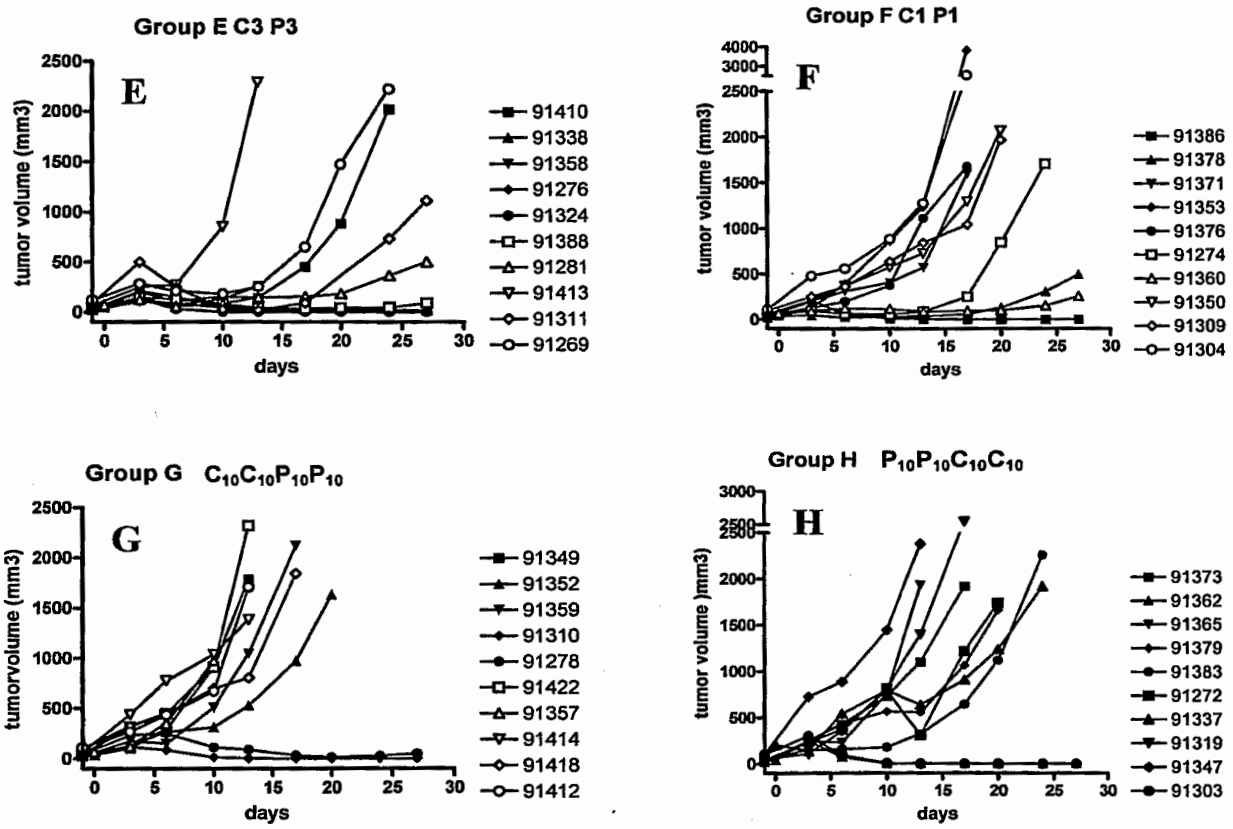


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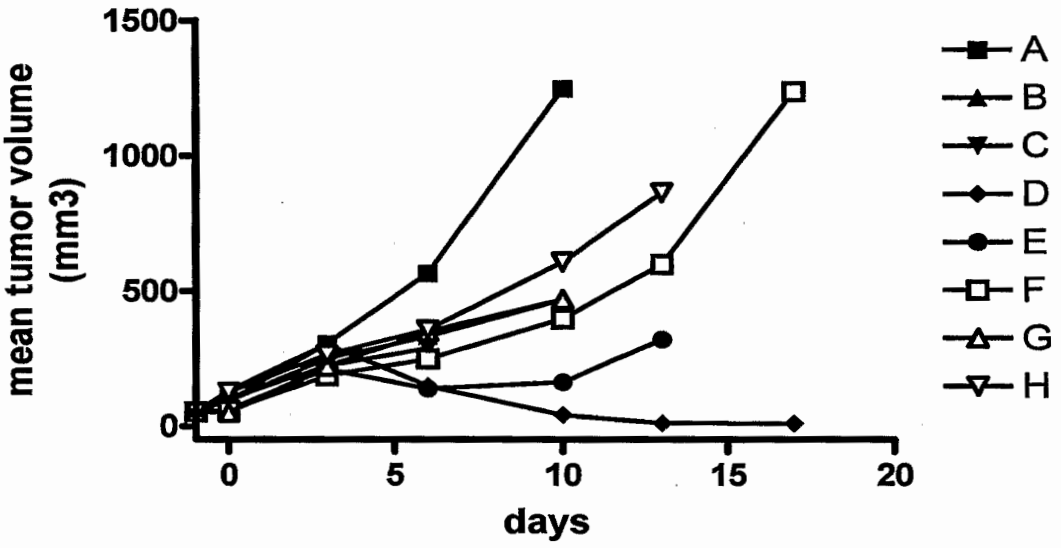


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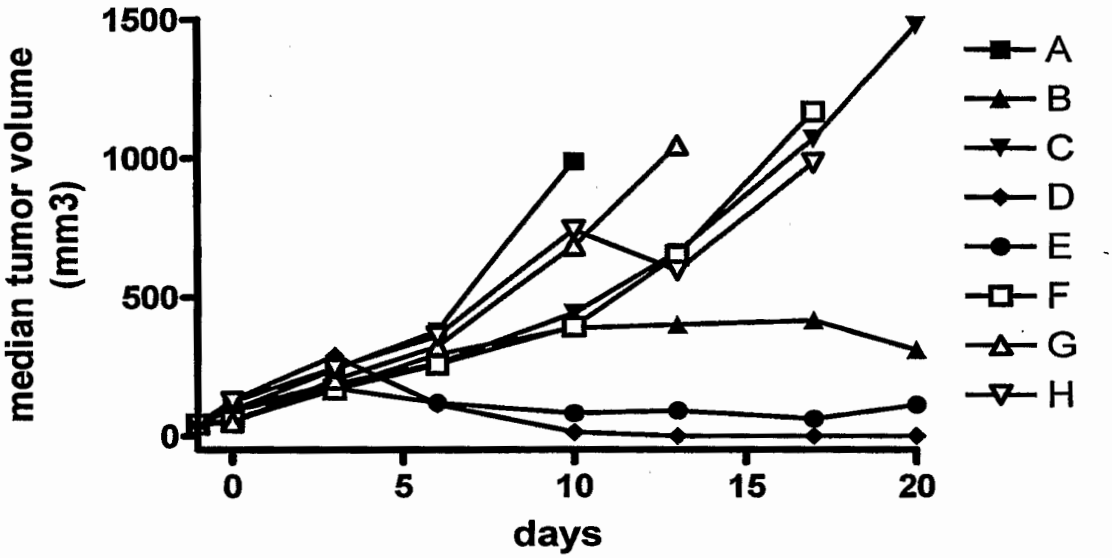
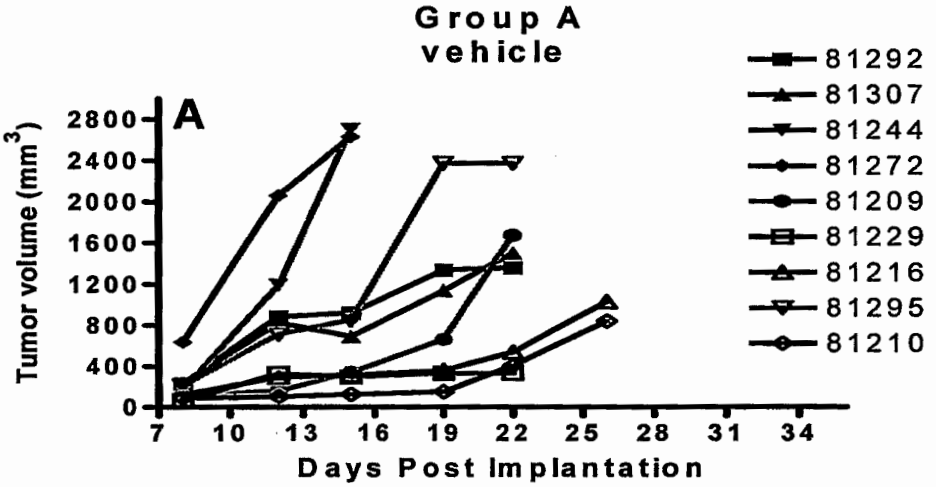


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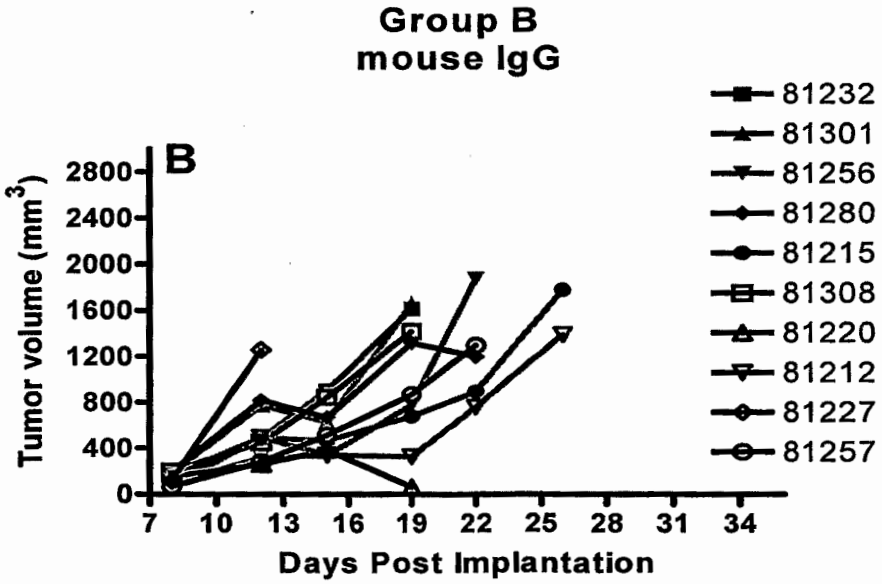
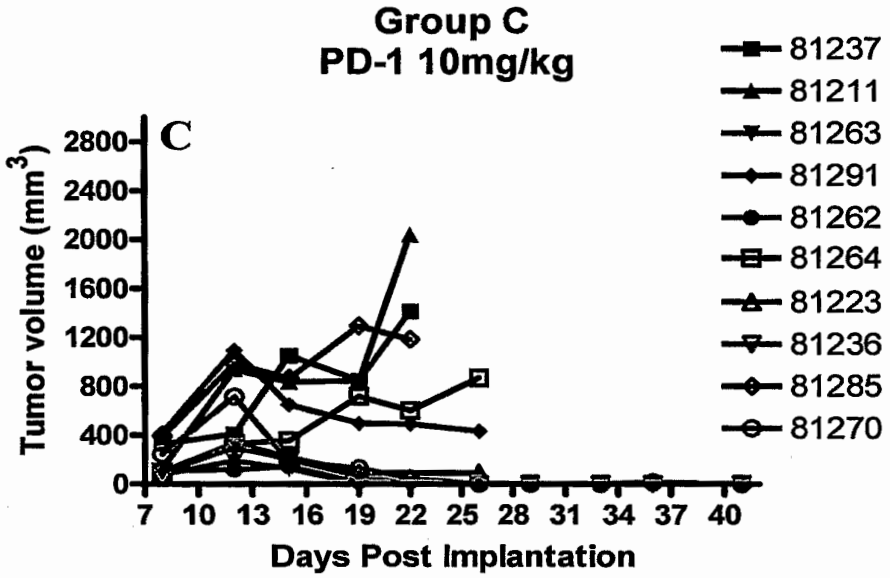


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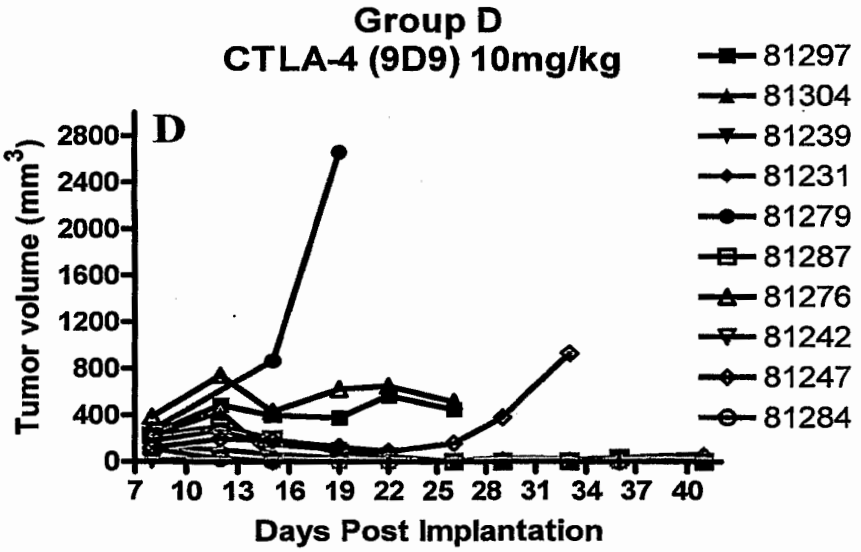
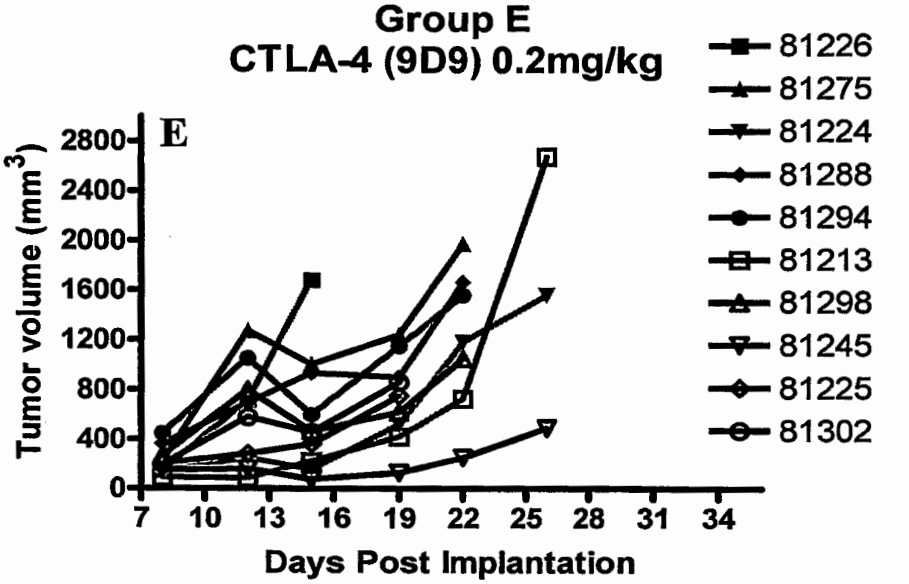


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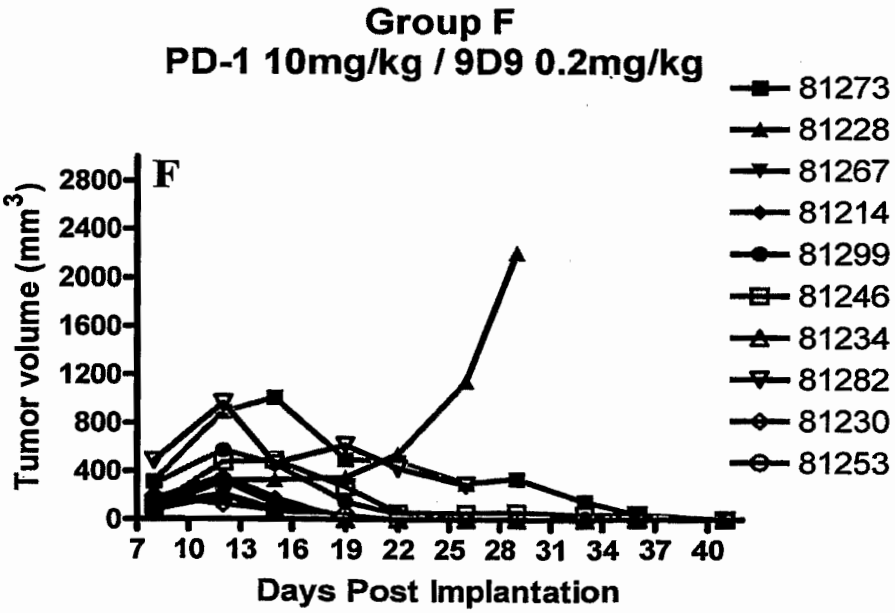


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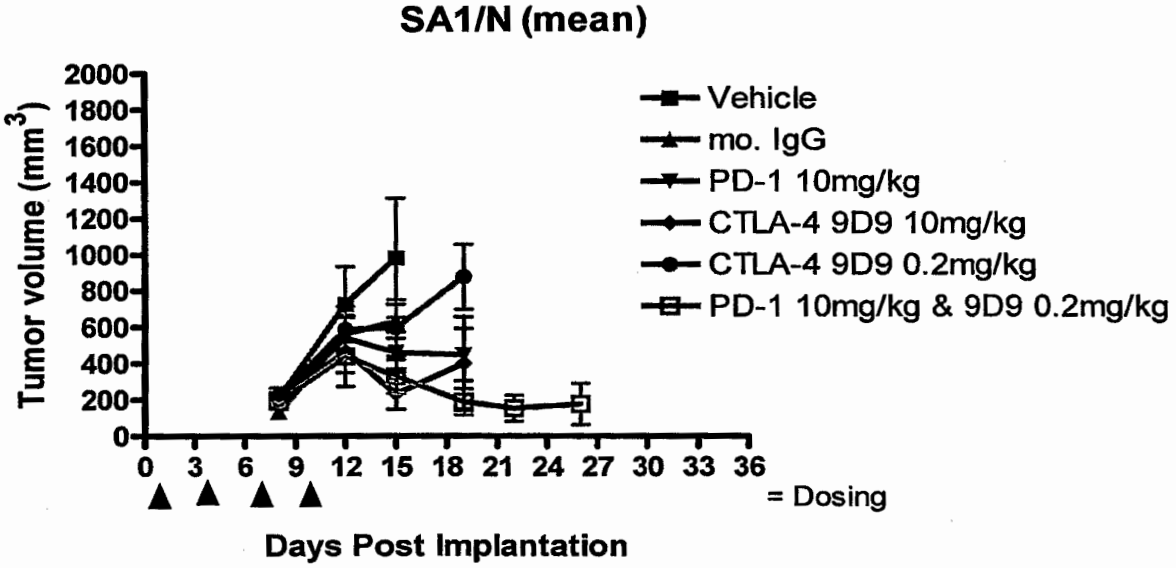


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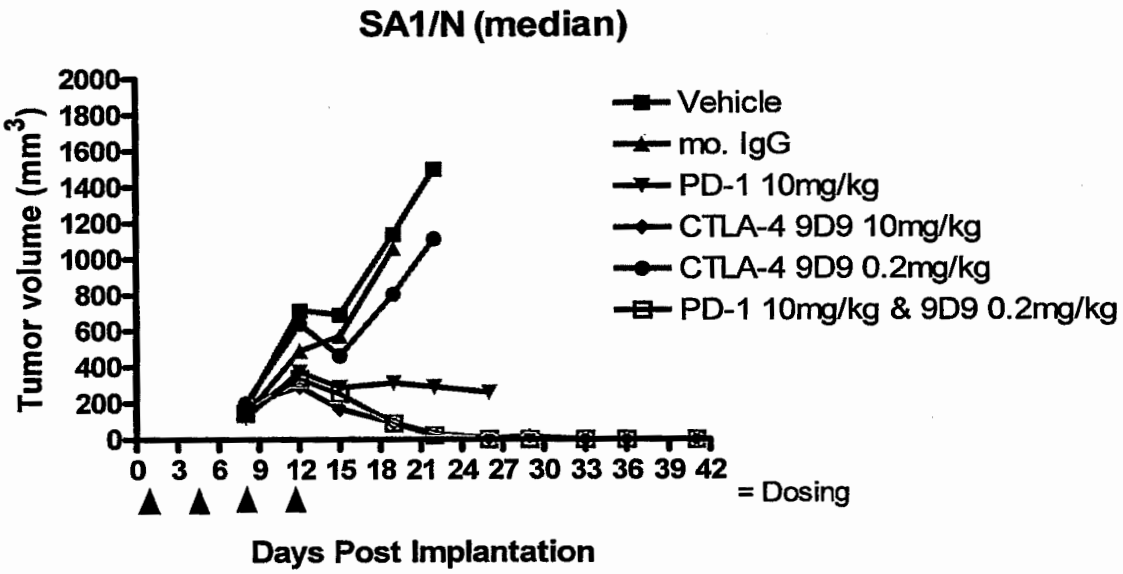


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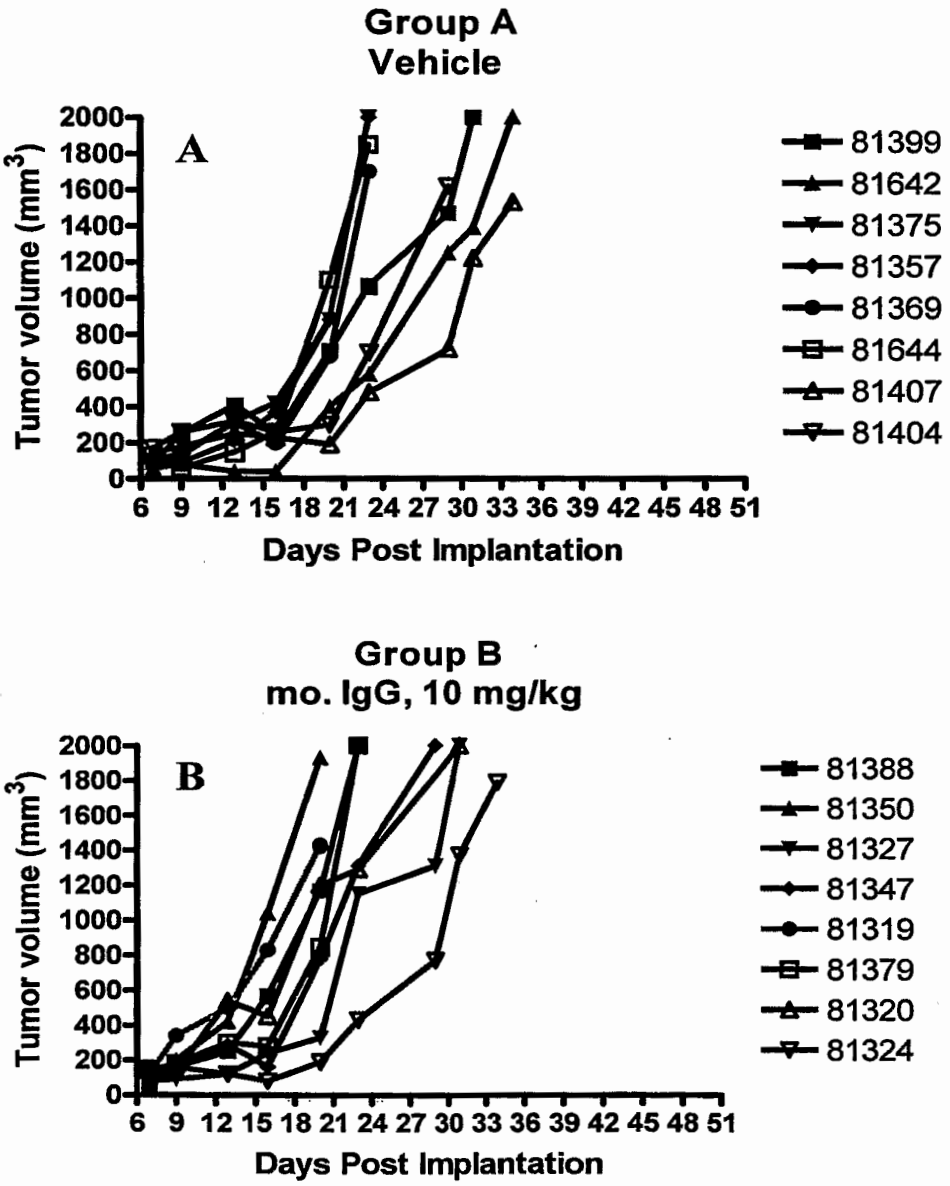
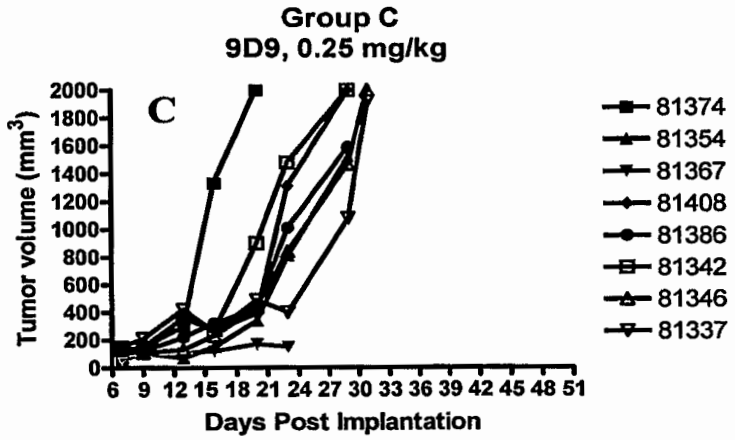
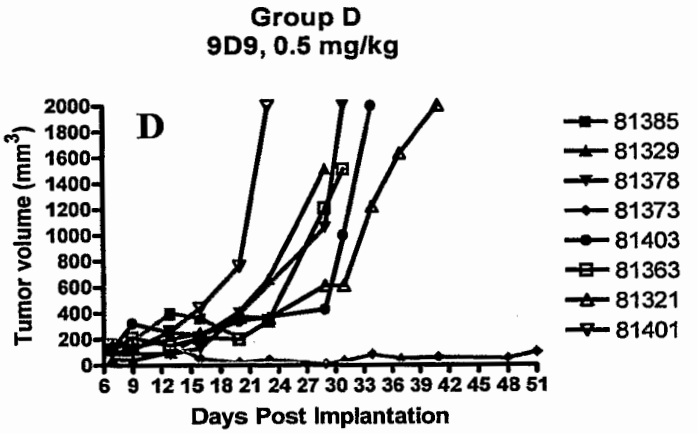


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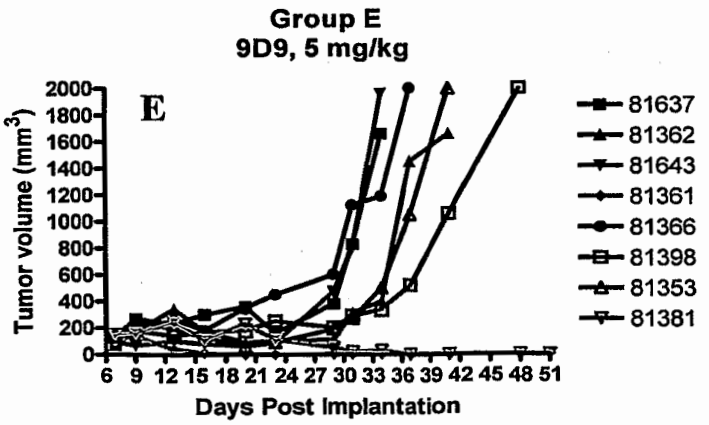
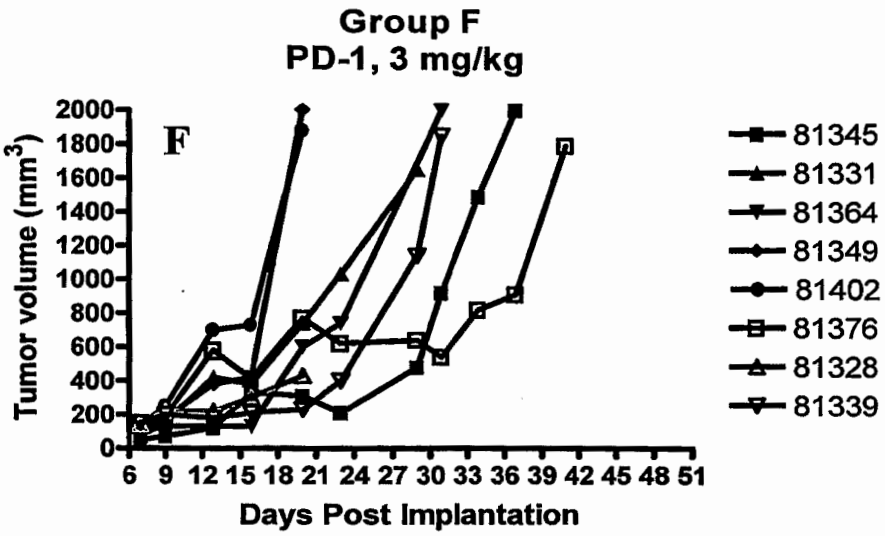


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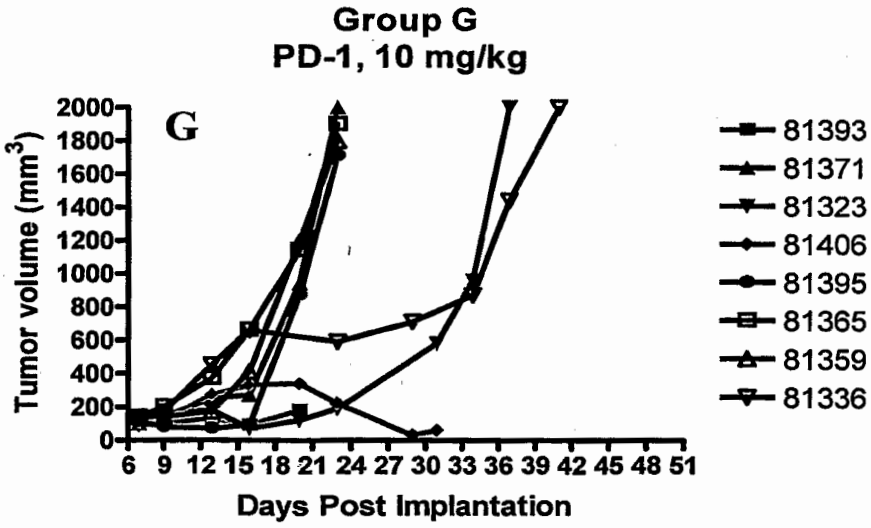


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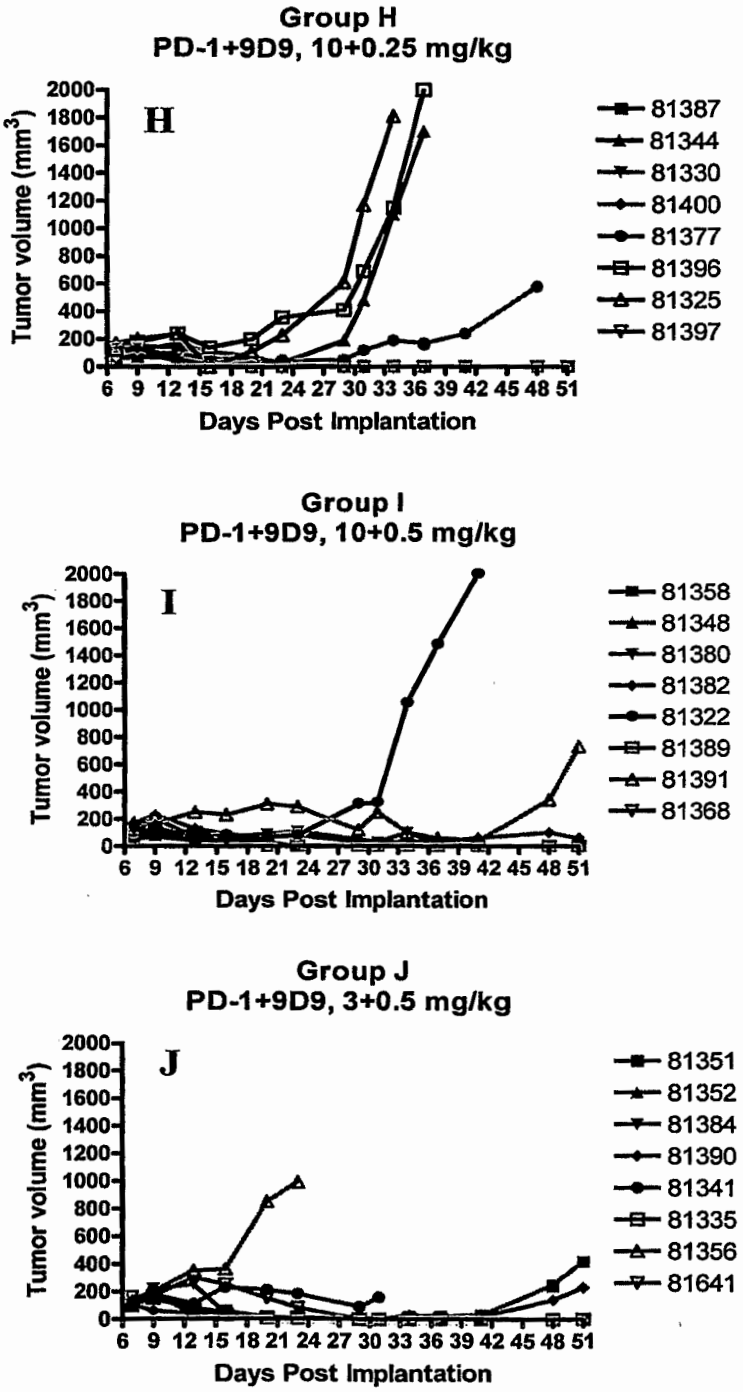


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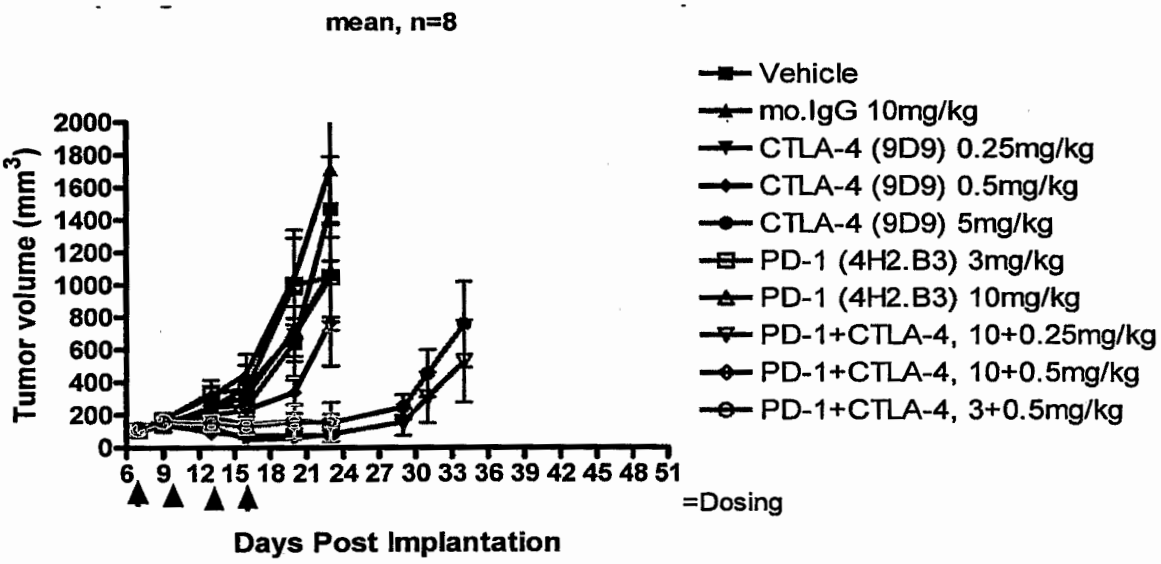


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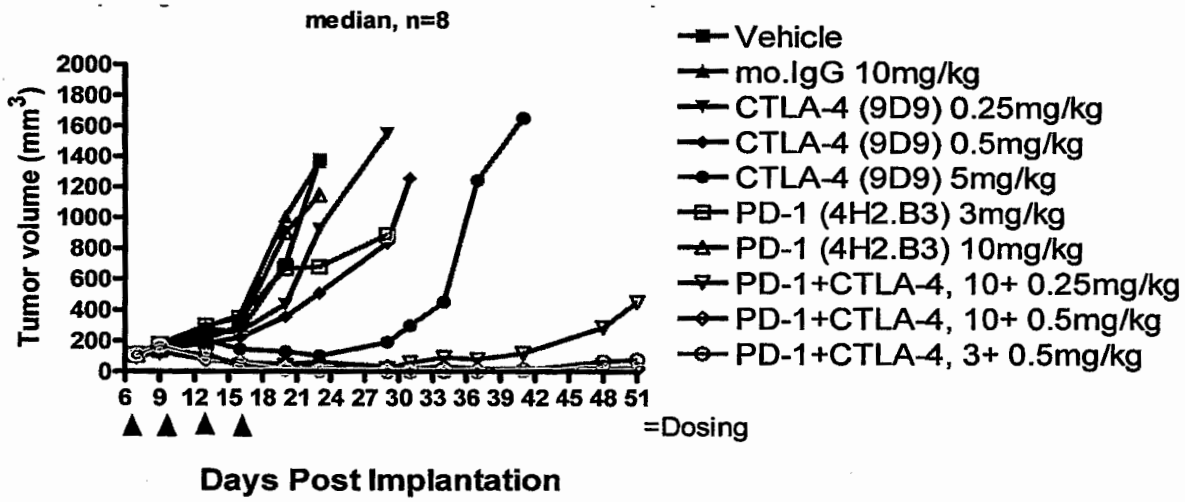
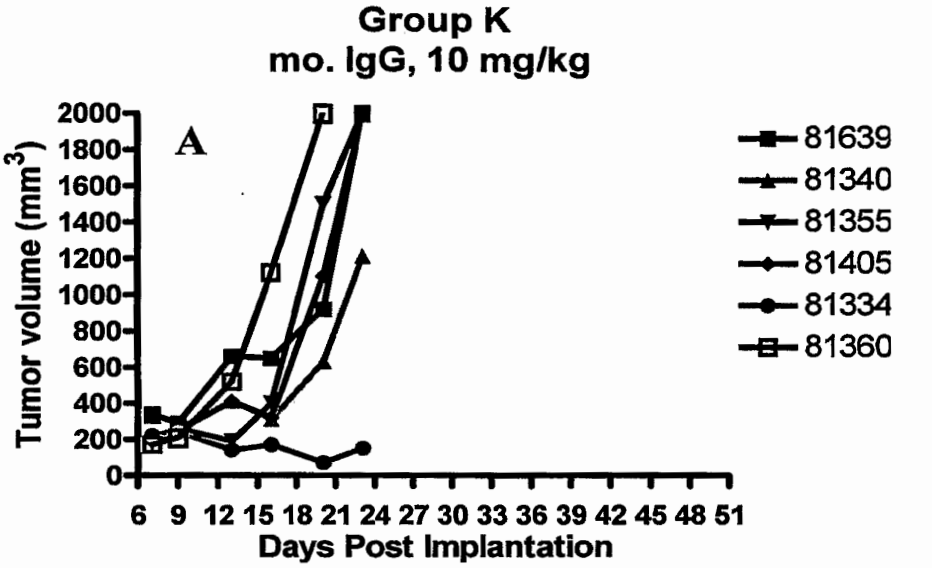


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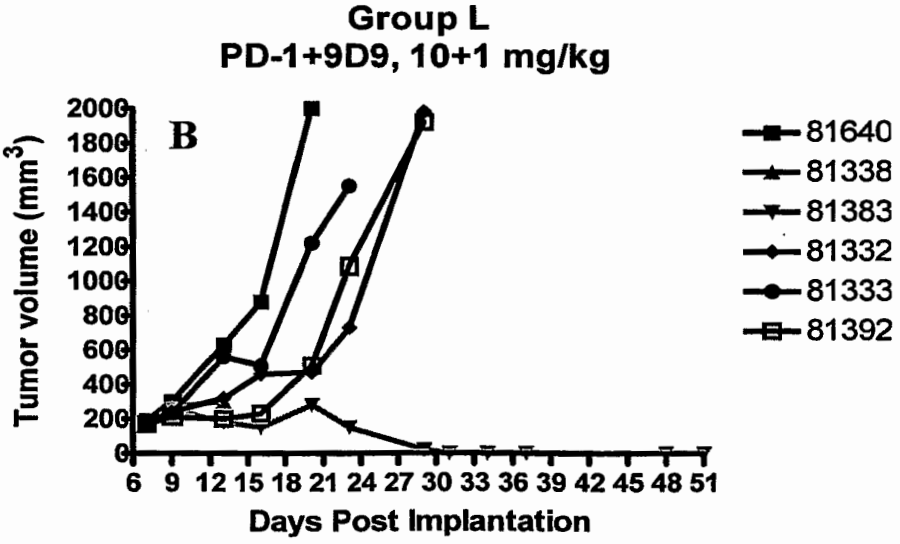


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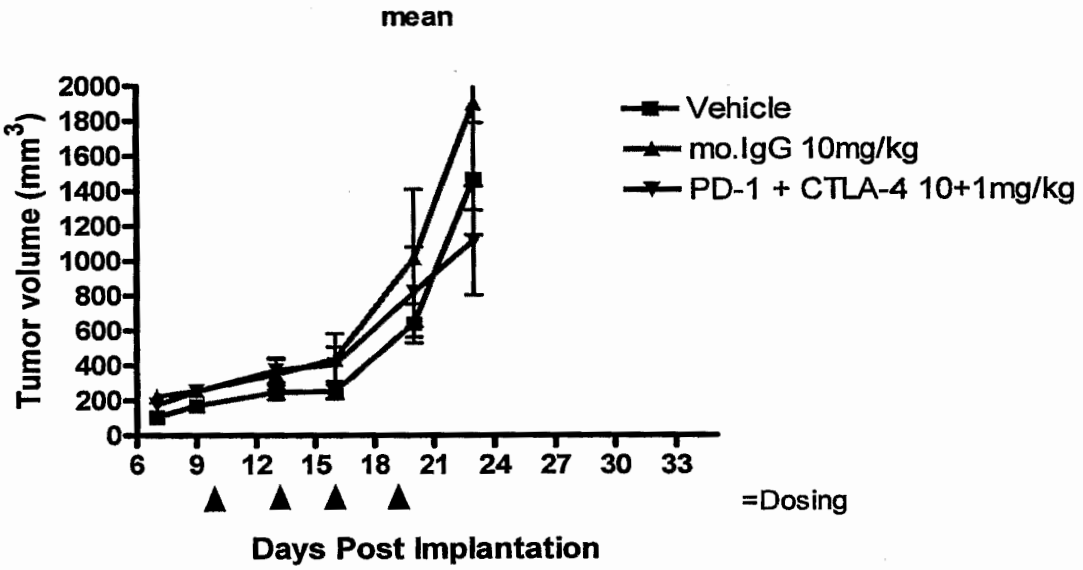


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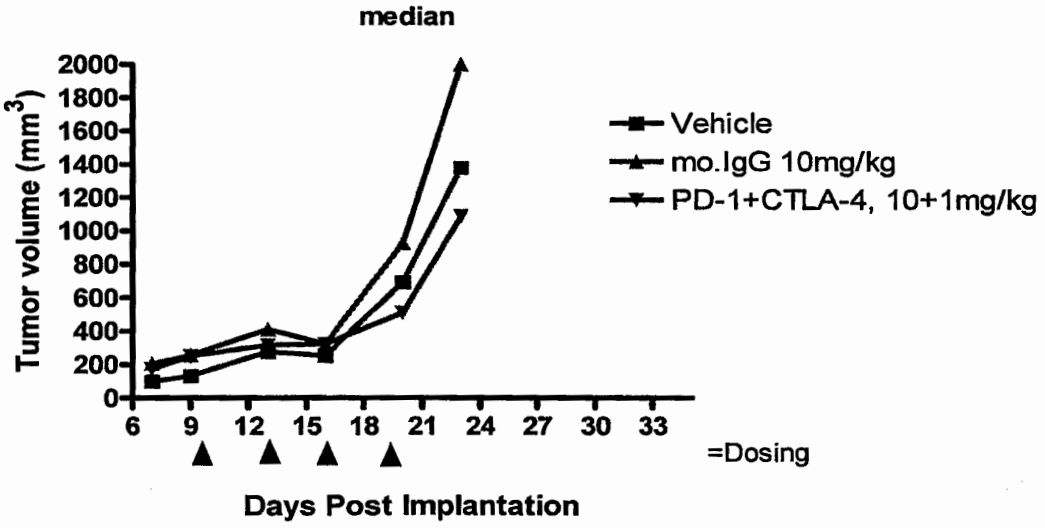


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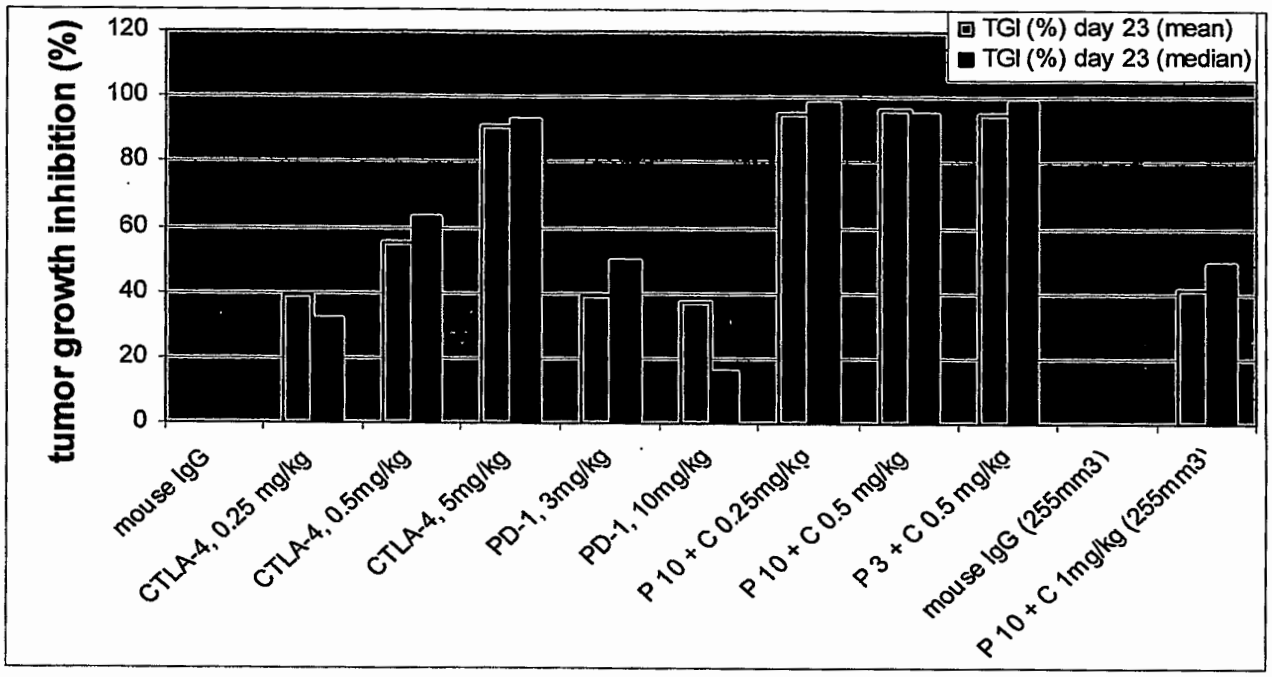
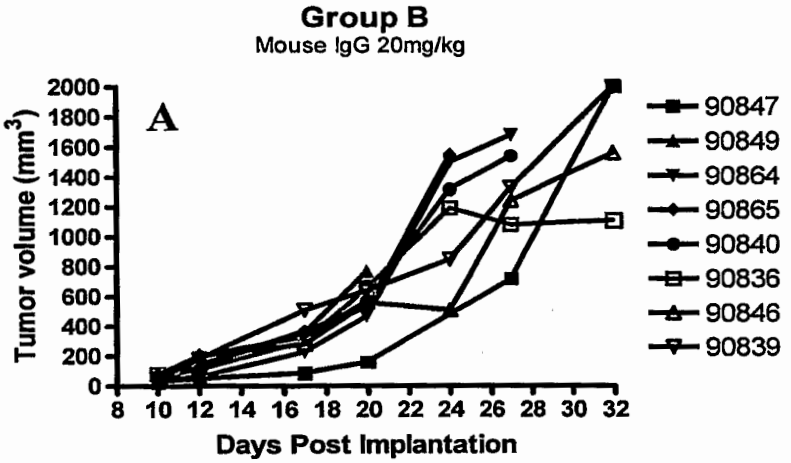


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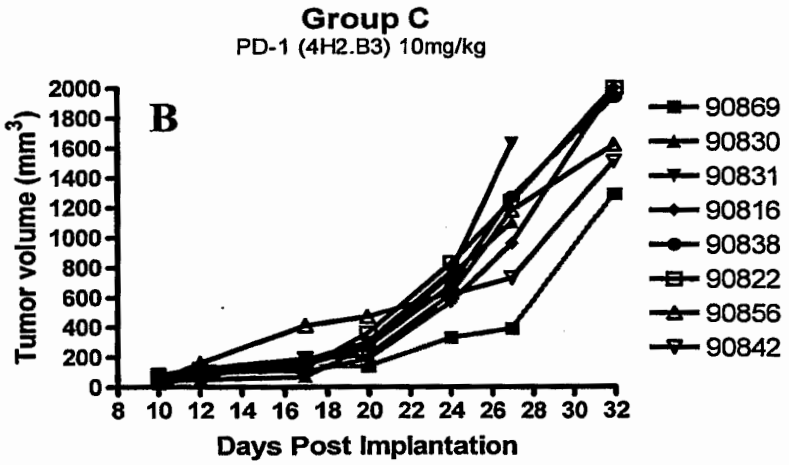
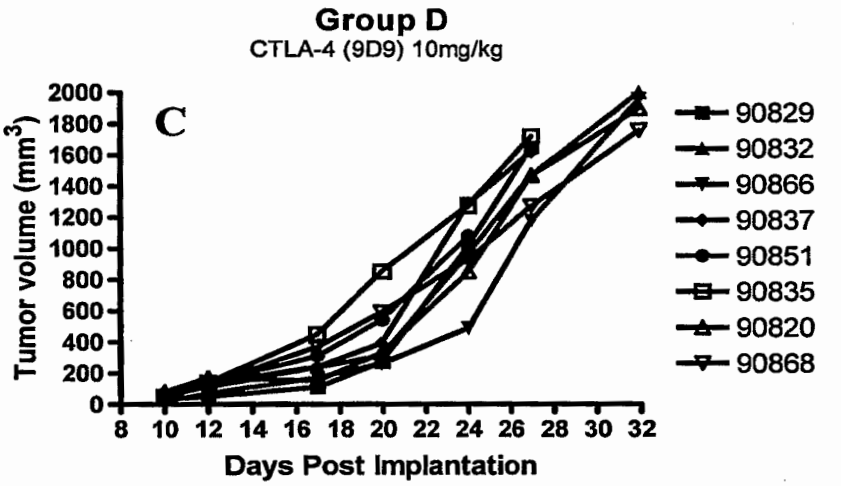


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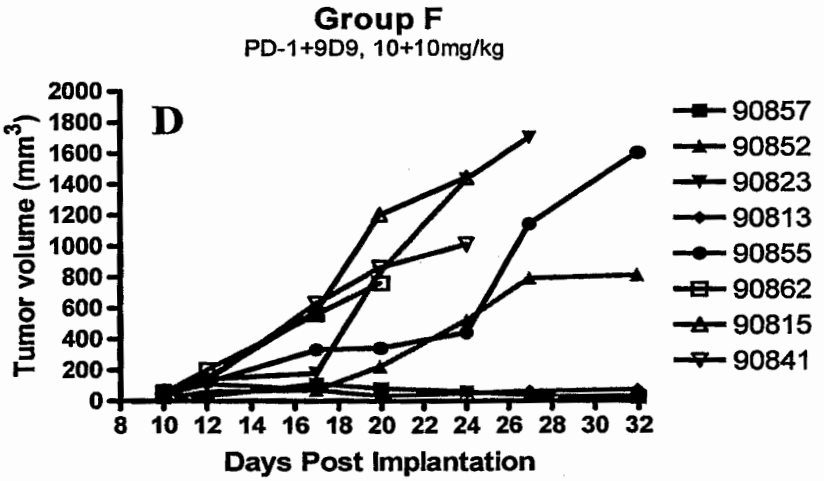


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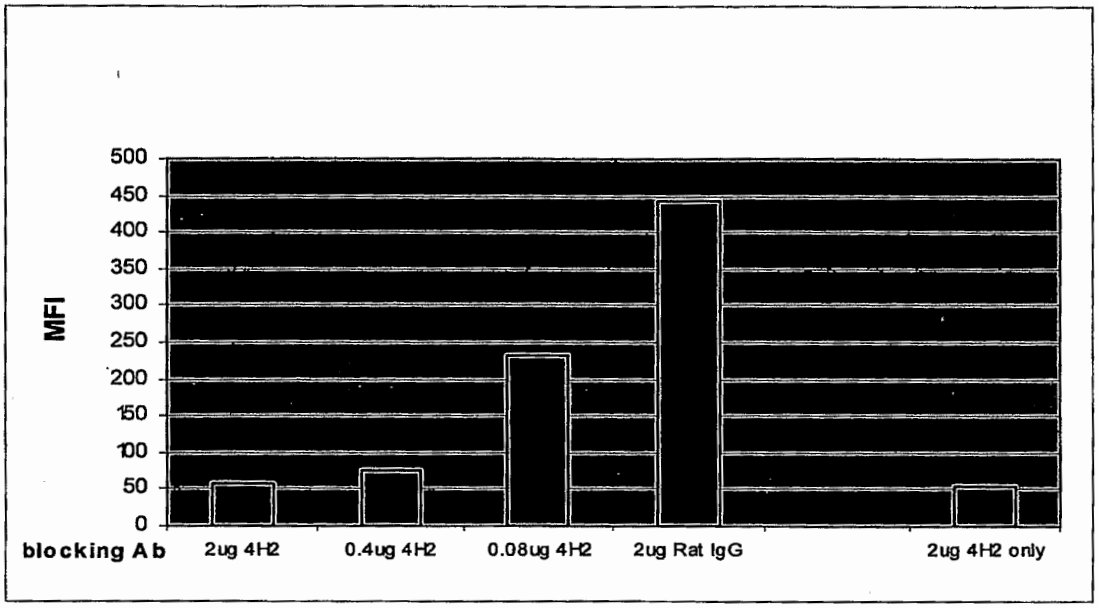


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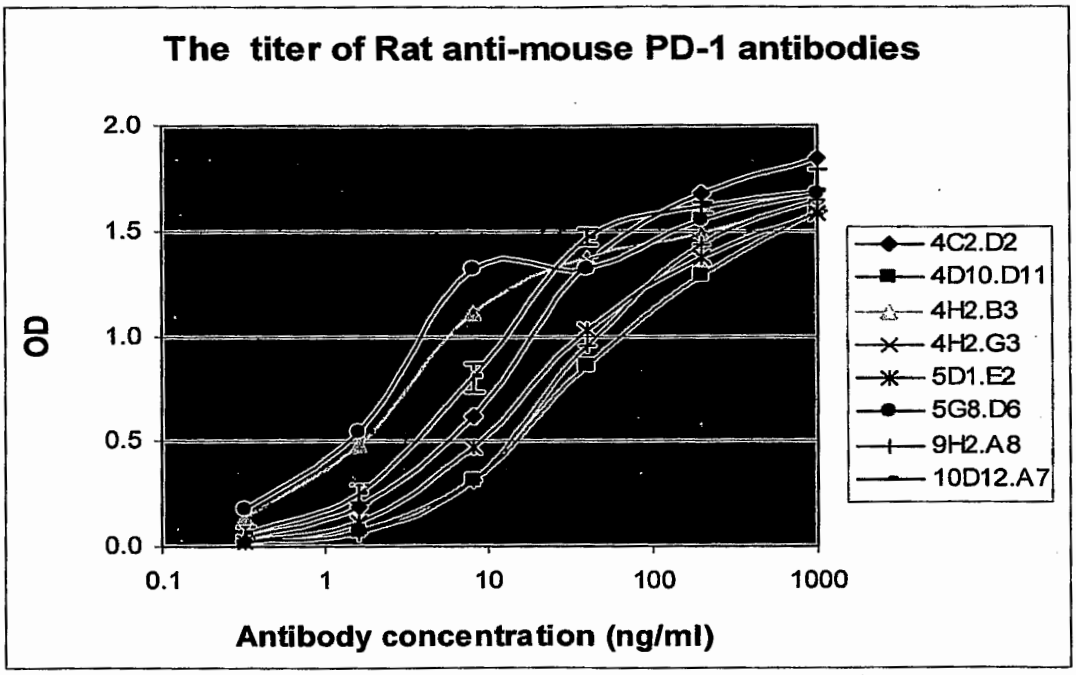


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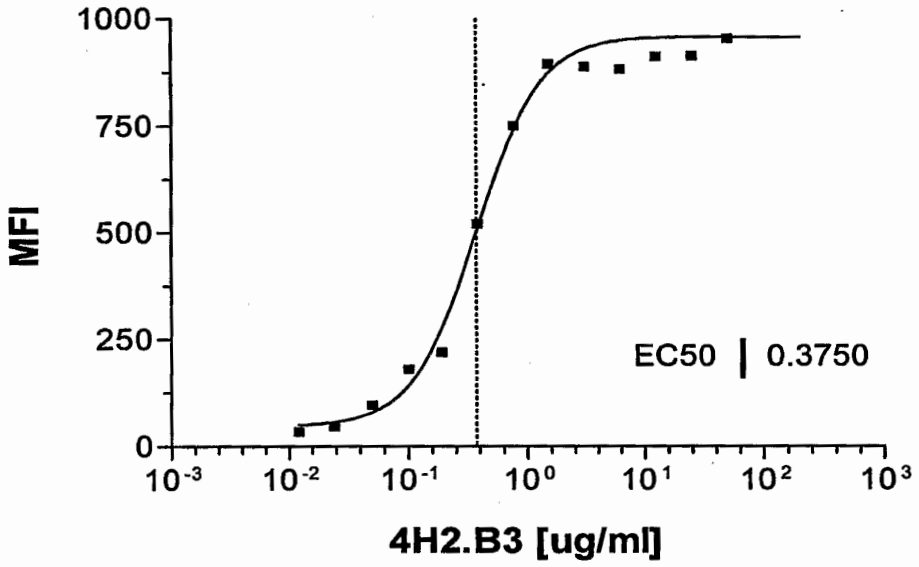


Figure 23.

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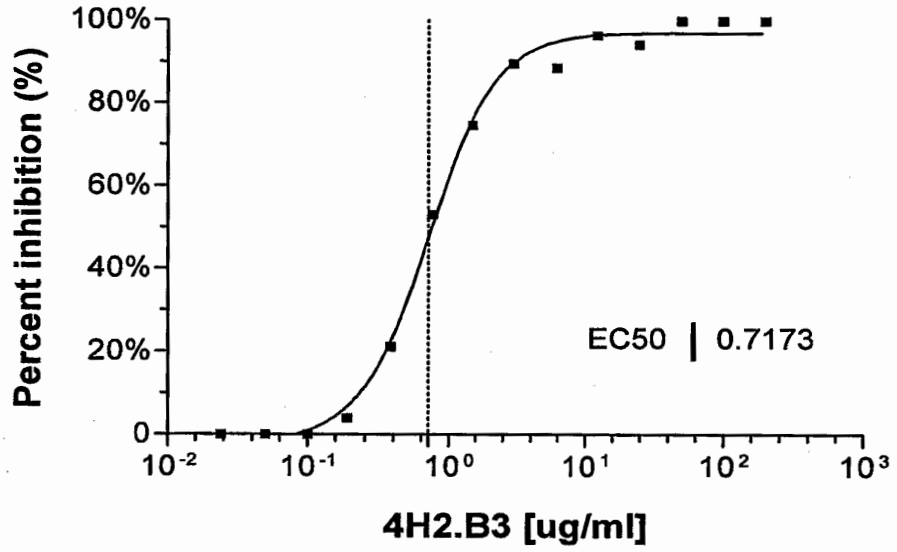


Figure 24.

Copy provided by USPTO from the IFW Image Database on 06/29/2006

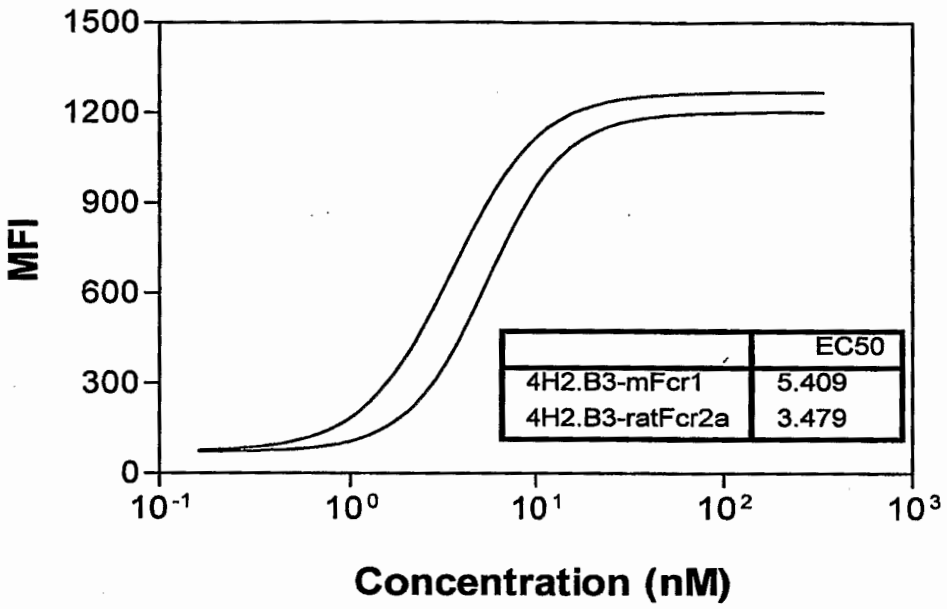


Figure 25.

Copy provided by USPTO from the IFW Image Database on 06/29/2006

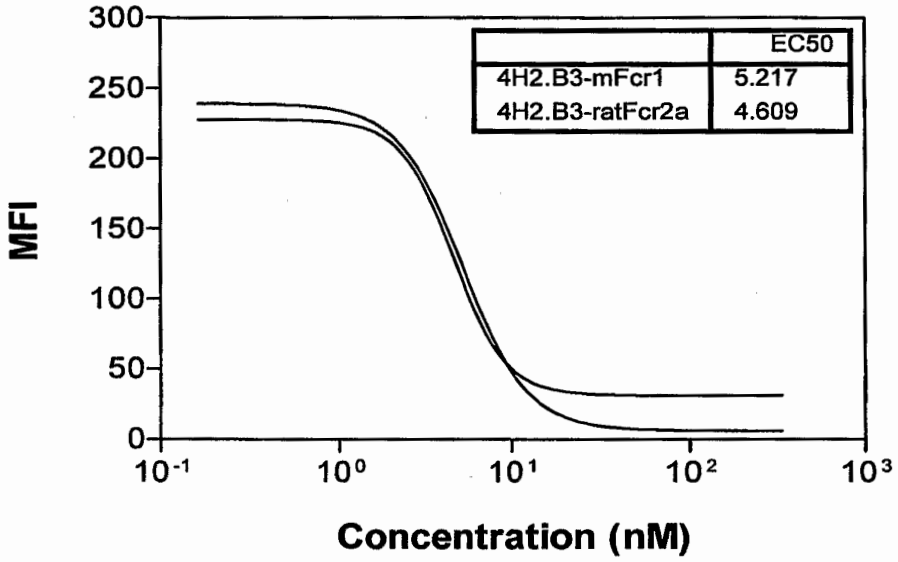


Figure 26.

Copy provided by USPTO from the IFW Image Database on 06/29/2006

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Sequence Listing could not be accepted due to errors.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866) 217-9197 (toll free).
Reviewer: Durreshwar Anjum
Timestamp: [year=2007; month=11; day=25; hr=7; min=38; sec=56; ms=489;]
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Reviewer Comments:
<110> Medarex, Inc.
<110> Ono Pharmaceutical Co., LTD.

please delete the extra <110> numeric identifier.

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<212> DNA
<213> Homo sapiens

Number of bases differ actually counted are 321 and input total above is 324.
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Validated By CRFValidator v 1.0.3

Application No: 11913217 Version No: 1.0

Input Set:

Output Set:

Started: 2007-10-31 12:51:15.862
Finished: 2007-10-31 12:51:17.104
Elapsed: 0 hr(s) 0 min(s) 1 sec(s) 242 ms
Total Warnings: 0
Total Errors: 2
No. of SeqIDs Defined: 74
Actual SeqID Count: 74

Error code	Error Description
E 248	Order Sequence Error <110> -> <110>; Expected Mandatory Tag: <120> in Header
E 253	The number of bases differs from <211> Input: 324 Calculated:321

SEQUENCE LISTING

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<110> Ono Pharmaceutical Co., LTD.

<120> HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1 (PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES ALONE OR IN COMBINATION WITH OTHER IMMUNOTHERAPEUTICS

<130> 077375.0499

<150> PCT/JP06/309606
<151> 2006-05-02

<150> US 60/679,466
<151> 2005-05-09

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35 40 45
Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
50 55 60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Met Leu Tyr
65 70 75 80
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        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Lys Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Asn Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
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Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Leu Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Thr Ser Leu Arg Val Glu Asp Thr Ala Val Tyr Tyr Cys
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Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Lys Arg Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65                  70                  75                  80
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Ser Phe Phe Trp Gly Trp Ile Arg Gln Pro Pro Gly Lys Gly Leu Glu
        35                  40                  45
Trp Ile Gly Ser Ile Tyr Tyr Ser Gly Ser Thr Tyr Tyr Asn Pro Ser
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Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe
65                  70                  75                  80
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Gly Phe His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
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Ala Val Ile Trp Tyr Asp Gly Ser Lys Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Leu Ser Arg Asp Asp Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
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        35                  40                  45
Trp Ile Ala Ser Ile Phe Tyr Ser Gly Glu Thr Tyr Phe Asn Pro Ser
    50                  55                  60
Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Arg Asn Gln Phe
65                  70                  75                  80
Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr
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        35                  40                  45
Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80
Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro Leu
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Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
            100                 105


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            20                  25                  30
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
        35                  40                  45
Tyr Asp Thr Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80
Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro Leu
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                85                  90                  95
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Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
        35                  40                  45
Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80
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Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
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Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
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Tyr Ala Ala Ser Asn Leu Arg Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
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Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
        35                  40                  45
Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80
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            100                 105


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Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
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Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Korman et al.	Conf. No.:	1207
Serial No.:	11/913,217	Examiner:	To Be Assigned
Filed:	October 31, 2007	Group Art Unit:	1656

For: Human Monoclonal Antibodies To Programmed Death 1(PD-1) And Methods For Treating Cancer Using Anti-PD-1 Antibodies Alone or in Combination with Other Immunotherapeutics

SUPPLEMENTAL PRELIMINARY AMENDMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Please replace the Sequence Listing filed on October 31, 2007, with the enclosed Substitute Sequence Listing in computer readable format submitted via EFS.

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

Dana-Farber EX1017
Dana-Farber v. BMS, IPR2023-00501
Page 787 of 2058

AMENDMENTS TO THE SPECIFICATION

Please cancel the previously filed Sequence Listing and replace it with the enclosed Substitute Sequence Listing filed via EFS.

Please replace the paragraph at page 1 before the section titled "Technical Field", with the following paragraph:

SEQUENCE LISTING

The specification further incorporates by reference the Substitute Sequence Listing submitted herewith via EFS on ~~October 31, 2007~~ February 28, 2008. Pursuant to 37 C.F.R. § 1.52(e)(5), the Sequence Listing text file, identified as ~~077375.0499SEQLISTING.txt~~ 0773750499.txt, is ~~48,642~~ 48,644 bytes and was created on ~~October 3, 2007~~ February 27, 2008. The Substitute Sequence Listing, electronically filed herewith, does not extend beyond the scope of the specification and thus does not contain new matter.

Dana-Farber EX1017
Dana-Farber v. BMS, IPR2023-00501
Page 788 of 2058

REMARKS

Applicants submit herewith a Substitute Sequence Listing in computer readable format in compliance with 37 C.F.R. §§ 1.821-1.825. By this amendment, the specification has been amended to incorporate by reference the Substitute Sequence listing submitted herewith. The Substitute Sequence Listing contains no new matter. Applicants believe no fee is due; however, if any fee is required in connection with this communication or any overpayment has been made, please charge any deficiency or credit any overpayment to Deposit Account No. 02-4377.

Respectfully submitted,

March 3, 2008
Date

Lisa D. Tyner
Patent Office Reg. No. 51,619

Attorney for Applicants
BAKER BOTTS L.L.P.
30 Rockefeller Plaza
New York NY 10112-4498
(212) 408-2500

Dana-Farber EX1017
Dana-Farber v. BMS, IPR2023-00501
Page 789 of 2058

Electronic Acknowledgement Receipt

EFS ID:	2940027
Application Number:	11913217
International Application Number:	
Confirmation Number:	1207
Title of Invention:	Human Monoclonal Antibodies To Programmed Death 1(PD-1) And Methods For Treating Cancer Using Anti-PD-1 Antibodies Alone or in Combination with Other Immunotherapeutics
First Named Inventor/Applicant Name:	Alan J Korman
Customer Number:	63367
Filer:	David W. Schalk
Filer Authorized By:	
Attorney Docket Number:	077375.0499
Receipt Date:	03-MAR-2008
Filing Date:	
Time Stamp:	14:33:46
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes) /Message Digest	Multi Part /.zip	Pages (if appl.)
1	Sequence Listing (Text File)	0773750499.txt	48644	no	0
Warnings:					
Information:					

2		0499.pdf	75915 037de05346e1f3c518763f33151c64aa2860cba8	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:	
Information:	
Total Files Size (in bytes):	124559

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 11913217 Document Date: 03/03/2008

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Sequence Listing

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions developed by SIRA.

At the time of document entry (noted above):

- Examiners may access SCORE content via the eDAN interface.
- Other USPTO employees can bookmark the current SCORE URL (http://es/ScoreAccessWeb/).
- External customers may access SCORE content via the Public and Private PAIR interfaces.

Form Revision Date: February 8, 2006

Dana-Farber EX1017
Dana-Farber v. BMS, IPR2023-00501
Page 792 of 2058

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/913,217	01/29/2009	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due 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).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	03/03/2008	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 30	Minus	** 30	= 0	X $ =		OR	X $50=	0
	Independent (37 CFR 1.16(h))	* 7	Minus	***7	= 0	X $ =		OR	X $210=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)					
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/ANDREW JAMES JR/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	: Korman *et al.*	Customer No.	: 63367
Serial No.	: 11/913,217	Confirmation No.	: 1207
Filing Date	: TBD	Group Art Unit	: 1656
Examiner	: TBD		
For	: HUMAN MONOCLONAL ANTIBODIES TO PROGRAMMED DEATH 1(PD-1) AND METHODS FOR TREATING CANCER USING ANTI-PD-1 ANTIBODIES ALONE OR IN COMBINATION WITH OTHER IMMUNOTHERAPEUTICS		

INFORMATION DISCLOSURE STATEMENT

FILED VIA EFS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to 37 C.F.R. §§ 1.56 and 1.97(b), applicants bring to the attention of the Examiner the documents listed on the attached Form PTO 1449 and respectfully request that the listed documents be considered by the Examiner and made of record in the above-captioned application.

This submission does not represent that a search has been made or that no better art exists and does not constitute an admission that the listed documents are material or constitute "prior art." If the Examiner applies the documents as prior art against any claim in the application and applicants determine that the cited documents do not constitute "prior art" under United States law, applicants reserve the right to present to the Office the relevant facts and law regarding the appropriate status of the documents.

Dana-Farber EX1017
Dana-Farber v. BMS, IPR2023-00501
Page 807 of 2058

Applicants further reserve the right to take appropriate action to establish the patentability of the disclosed invention over the listed documents, should the documents be applied against the claims of the present application.

Applicants believe no fee is due in connection with this submission. If any additional fee is due, or if any overpayment has been made, the Commissioner is authorized to charge any such fee or credit any overpayment, to our Deposit Account No. 02-4377.

Respectfully submitted,

BAKER BOTTS L.L.P.

October 7, 2008
Date

Steve Lendaris
Patent Office Reg. No. 53,202

30 Rockefeller Plaza
44th Floor
New York, NY 10012-4498
Attorney for Applicant(s)
212-408-2569

Dana-Farber EX1017
Dana-Farber v. BMS, IPR2023-00501
Page 808 of 2058

Substitute for form 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** **(Use as many sheets as necessary)**		
	Application Number	11/913,217
	Filing Date	TBD
	Inventor(s)	Korman, et al.
	Art Unit	1656
	Examiner Name	TBD
	Attorney Docket Number	077375.0499

U.S. PATENT DOCUMENTS

Exam Initial	No.	Document No.	Publication Date	Applicant(s)
	1.	2003/0232323	12/18/2003	FREEMAN, *et al.*
	2.	2004/0073957	04/15/2004	TOMIZUKA, *et al.*
	3.	2007/0065427	03/22/2007	FREEMAN, *et al.*
	4.	2007/0202100	08/30/2007	WOOD, *et al.*
	5.	2008/0025979	01/31/2008	HONJO, *et al.*
	6.	5,629,204	05/13/1997	HONJO, *et al.*
	7.	5,698,520	12/16/1997	HONJO, *et al.*
	8.	6,632,976	10/14/2003	TOMIZUKA, *et al.*
	9.	6,808,710	10/26/2004	WOOD, *et al.*
	10.	7,101,550	09/05/2006	WOOD, *et al.*
	11.	7,105,328	09/12/2006	WOOD, *et al.*

FOREIGN PATENT DOCUMENTS

Exam Initial	No.	Document No.	Publication Date	Applicant(s)
	12.	WO 01/14557	03/01/2001	WOOD, *et al.*
	13.	WO 02/079499	10/10/2002	WOOD, *et al.*
	14.	WO 03/042402	05/22/2003	FREEMAN, *et al.*

Examiner Signature		Date Considered	

NY02:637690.1

* Examiner: Initial citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute for form 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** **(Use as many sheets as necessary)**		
	Application Number	11/913,217
	Filing Date	TBD
	Inventor(s)	Korman, et al.
	Art Unit	1656
	Examiner Name	TBD
	Attorney Docket Number	077375.0499

Exam Initial	No.	Document No.	Publication Date	Applicant(s)
	15.	WO 2004/072286	08/26/2004	HONJO, *et al.*

Exam Initial	No.	**OTHER DOCUMENTS (including Author, Title, Date, Pertinent Pages, Etc.)**
	16	AGATA, *et al.*, 1996, "Expression of the PD-1 antigen on the surface of stimulation mouse T and B lymphocytes," *Int Immunol*, Vol. 8: p765-772
	17	BENNETT, *et al.*, 2003, "Program Death-1 Engagement Upon TCR Activation Has Distinct Effects on Costimulation and Cytokine-Driven Proliferation: Attenuation of ICOS, IL-4, and IL-21, But Not CD28, IL-7, and IL-15 Responses," *J Immunol*, Vol. 170: p711-718
	18	BLANK, *et al.*, 2005, "Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy," *Cancer Immunol. Immunother.*, Vol. 54: p307-314
	19	BROWN, *et al.*, 2003, "Blockade of Programmed Death-1 Ligands on Dendritic Cells Enhances T Cell Activation and Cytokine Production," *J. Immunol.*, Vol. 170: p1257-1266
	20	CARTER, *et al.*, 2002, "PD-1: PD-L inhibitory pathway affects both CD4+ and CD8+ T cells and is overcome by IL-2," *Eur J Immunol*, Vol. 32: p634-643
	21	DONG, *et al.*, 2002, "Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion," *Nat Med.*, Vol. 8: p783-800
	22	DONG, H. and CHEN, L., 2003, "B7-H1 pathway and its role in the evasion of tumor immunity," *J Mol Med*, Vol. 81: p281-287
	23	FINGER, et al., 1997, "The human PD-1 gene: complete cDNA, genomic organization, and developmentally regulated expression in B cell progenitors," Gene, Vol. 197: p177-187
	24	FREEMAN, *et al.*, 2000, "Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation," *J Exp Med*, Vol. 192: p1027-1034

Examiner Signature		Date Considered	

NY02:637690.1

* Examiner: Initial citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute for form 1449/PTO **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** **(Use as many sheets as necessary)**		
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(54) Title: PD-1, A RECEPTOR FOR B7-4, AND USES THEREFOR

(57) Abstract: The invention identifies PD-1 as a receptor for B7-4. B7-4 can inhibit immune cell activation upon binding to an inhibitory receptor on an immune cell. Accordingly, the invention provides agents for modulating PD-1, B7-4, and the interaction between B7-4 and PD-1 in order to modulate a costimulatory or an inhibitory signal in an immune cell resulting in modulation of the immune response.

PD-1, A RECEPTOR FOR B7-4, AND USES THEREFOR

Government Funding

Work described herein was supported under AI 39671, AI 44690, CA 84500 and AI 41584 awarded by the National Institutes of Health. The U.S. government, therefore, may have certain rights in this invention.

Related Applications

This claims priority to U.S.S.N. 60/150,390 filed on August 23, 1999. This application also claims priority to U.S.S.N. 60/164,897, filed on November 10, 1999. Both of these applications are incorporated herein in their entirety by this reference.

Background of the Invention

In order for T cells to respond to foreign proteins, two signals must be provided by antigen-presenting cells (APCs) to resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165:302-319; Mueller, D. L. *et al.* (1990) *J. Immunol.* 144:3701-3709). The first signal, which confers specificity to the immune response, is transduced via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Lenschow *et al.* (1996) *Annu. Rev. Immunol.* 14:233). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M. K. *et al.* (1988) *J. Immunol.* 140:3324-3330; Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D., *et al.* 1991 *Proc. Natl. Acad. Sci. USA* 88:6575-6579; Young, J. W. *et al.* (1992) *J. Clin. Invest.* 90:229-237; Koulova, L. *et al.* (1991) *J. Exp. Med.* 173:759-762; Reiser, H. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:271-275; van-Seventer, G. A. *et al.* (1990) *J. Immunol.* 144:4579-4586; LaSalle, J. M. *et al.* (1991) *J. Immunol.* 147:774-80; Dustin, M. I. *et al.* (1989) *J. Exp. Med.* 169:503; Armitage, R. J. *et al.* (1992) *Nature* 357:80-82; Liu, Y. *et al.* (1992) *J. Exp. Med.* 175:437-445).

The CD80 (B7-1) and CD86 (B7-2) proteins, expressed on APCs, are critical costimulatory molecules (Freeman *et al.* (1991) *J. Exp. Med.* 174:625; Freeman *et al.* (1989) *J. Immunol.* 143:2714; Azuma *et al.* (1993) *Nature* 366:76; Freeman *et al.* (1993) *Science* 262:909). B7-2 appears to play a predominant role during primary immune responses, while B7-1, which is upregulated later in the course of an immune response, may be important in prolonging primary T cell responses or costimulating secondary T cell responses (Bluestone (1995) *Immunity* 2:555).

One receptor to which B7-1 and B7-2 bind, CD28, is constitutively expressed on resting T cells and increases in expression after activation. After signaling through the T cell receptor, ligation of CD28 and transduction of a costimulatory signal induces T cells to proliferate and secrete IL-2 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 173:721-730; Gimmi, C. D. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:6575-6579; June, C. H. *et al.* (1990) *Immunol. Today.* 11:211-6; Harding, F. A. *et al.* (1992) *Nature* 356:607-609). A second receptor, termed CTLA4 (CD152) is homologous to CD28 but is not expressed on resting T cells and appears following T cell activation (Brunet, J. F. *et al.* (1987) *Nature* 328:267-270). CTLA4 appears to be critical in negative regulation of T cell responses (Waterhouse *et al.* (1995) *Science* 270:985). Blockade of CTLA4 has been found to remove inhibitory signals, while aggregation of CTLA4 has been found to provide inhibitory signals that downregulate T cell responses (Allison and Krummel (1995) *Science* 270:932). The B7 molecules have a higher affinity for CTLA4 than for CD28 (Linsley, P. S. *et al.* (1991) *J. Exp. Med.* 174:561-569) and B7-1 and B7-2 have been found to bind to distinct regions of the CTLA4 molecule and have different kinetics of binding to CTLA4 (Linsley *et al.* (1994) *Immunity* 1:793). A new molecule related to CD28 and CTLA4, ICOS, has been identified and seems to be important in IL-10 production (Hutloff *et al.* (1999) *Nature* 397:263; WO 98/38216), as has its ligand, which is a new B7 family member (Aicher A. *et al.* (2000) *J. Immunol.* 164:4689-96; Mages H.W. *et al.* (2000) *Eur. J. Immunol.* 30:1040-7; Brodie D. *et al.* (2000) *Curr. Biol.* 10:333-6; Ling V. *et al.* (2000) *J. Immunol.* 164:1653-7; Yoshinaga S.K. *et al.* (1999) *Nature* 402:827-32). If T cells are only stimulated through the T cell receptor, without receiving an additional costimulatory signal, they become nonresponsive, anergic, or die, resulting in downmodulation of the immune response.

The importance of the B7:CD28/CTLA4 costimulatory pathway has been demonstrated *in vitro* and in several *in vivo* model systems. Blockade of this costimulatory pathway results in the development of antigen specific tolerance in murine and human systems (Harding, F. A. *et al.* (1992) *Nature* 356:607-609; Lenschow, D. J. *et al.* (1992) *Science* 257:789-792; Turka, L. A. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:11102-11105; Gimmi, C. D. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6586-6590; Boussiotis, V. *et al.* (1993) *J. Exp. Med.* 178:1753-1763). Conversely, expression of B7 by B7 negative murine tumor cells induces T-cell mediated specific immunity accompanied by tumor rejection and long lasting protection to tumor challenge (Chen, L. *et al.* (1992) *Cell* 71:1093-1102; Townsend, S. E. and Allison, J. P. (1993) *Science* 259:368-370; Baskar, S. *et al.* (1993) *Proc. Natl. Acad. Sci.* 90:5687-5690.). Therefore, manipulation of the costimulatory pathways offers great potential to stimulate or suppress immune responses in humans.

Summary of the Invention

The present invention is based, at least in part, on the discovery that PD-1 is a receptor for B7-4 molecules expressed on antigen presenting cells. PD-1 transmits a negative signal to immune cells, similar to CTLA4. B7-4 molecules are expressed on the surface of antigen presenting cells and provide a costimulatory signal to immune cells and can transmit downmodulatory signals to immune cells, depending upon the molecule to which they bind. Thus, modulation of PD-1, B7-4, and/or the interaction between B7-4 and PD-1 results in modulation of the immune response.

Accordingly, in one aspect, the invention provides a method for modulating an immune response comprising contacting an immune cell with an agent that modulates signaling via PD-1 to thereby modulate the immune response.

In one embodiment, the immune response is downregulated.

In another embodiment, signaling via PD-1 is stimulated using an agent selected from the group consisting of: an activating antibody that recognizes PD-1, a form of B7-4 that binds to an inhibitory receptor, and a small molecule that binds to PD-1.

In one embodiment, the immune cell is selected from the group consisting of: a T cell, a B cell, and a myeloid cell.

In one embodiment, anergy is induced in the immune cell.

In one embodiment, the method further comprising contacting the immune cell with an additional agent that downregulates an immune response.

In one embodiment, the immune response is upregulated.

In one embodiment, the signaling via PD-1 is inhibited using an agent selected from the group consisting of: a blocking antibody that recognizes PD-1, a non-activating form of B7-4, an antibody that recognizes B7-4, and a soluble form of PD-1.

In one embodiment, the step of contacting occurs *in vivo*. In another embodiment, the step of contacting occurs *in vitro*.

In another aspect, the invention pertains to a method for modulating the interaction of B7-4 with an inhibitory receptor on an immune cell comprising contacting an antigen presenting cell which expresses B7-4 with an agent selected from the group consisting of: a form of B7-4, a form of PD-1, or an agent that modulates the interaction of B7-4 and PD-1 such that the interaction of B7-4 with an inhibitory receptor on an immune cell is modulated.

In one embodiment, the method further comprises contacting the immune cell or the antigen presenting cell with an additional agent that modulates an immune response.

In one embodiment, the step of contacting is performed *in vitro*. In another embodiment, the step of contacting is performed *in vivo*.

In one embodiment, the immune cell is selected from the group consisting of: a T cell, a B cell, and a myeloid cell.

In another aspect, the invention pertains to a method for inhibiting activation in an immune cell via a non-apoptotic mechanism comprising increasing the activity or expression of PD-1 in a immune cell such that immune cell activation is inhibited.

In another aspect, the invention pertains to vaccine comprising an antigen and an agent that inhibits signaling via PD-1 in an immune cell.

In another aspect, the invention pertains to a composition comprising an antigen and an agent that promotes signaling via PD-1 in an immune cell.

In another aspect, the invention pertains to a method for treating a subject having a condition that would benefit from upregulation of an immune response comprising administering an agent that inhibits signaling via PD-1 in a immune cell of the subject such that a condition that would benefit from upregulation of an immune response is treated.

In one embodiment, the agent comprises a soluble form of PD-1 or B7-4.

In one embodiment, the method further comprises administering a second agent that upregulates an immune response to the subject.

In one embodiment, the condition is selected from the group consisting of: a tumor, a neurological disease or an immunosuppressive disease.

In another aspect, the invention pertains to a method for treating a subject having a condition that would benefit from downregulation of an immune response comprising administering an agent that stimulates signaling via PD-1 in a immune cell of the subject such that a condition that would benefit from downregulation of an immune response is treated.

In one embodiment, said agent is selected from the group consisting of: an antibody that stimulates signaling via PD-1, a bispecific antibody, and soluble B7-4.

In one embodiment, the method further comprises administering a second agent that downregulates an immune response to the subject.

In one embodiment, the condition is selected from the group consisting of: a transplant, an allergy, and an autoimmune disorder.

In another aspect, the invention pertains to a cell-based assay for screening for compounds which modulate the activity of B7-4 or PD-1 comprising contacting a cell expressing a B7-4 target molecule or PD-1 target molecule with a test compound and determining the ability of the test compound to modulate the activity of the B7-4 or PD-1 target molecule.

In yet another aspect, the invention pertains to a cell-free assay for screening for compounds which modulate the binding of B7-4 or PD-1 to a target molecule comprising contacting a B7-4 or PD-1 protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to the B7-4 or PD-1 protein or biologically active portion thereof.

Brief Description of the Drawings

Figure 1 depicts the nucleotide sequence encoding a human secreted B7-4, B7-4S.

Figure 2 depicts the nucleotide sequence encoding a human B7-4, B7-4M.

Figure 3 depicts the amino acid sequence of human B7-4S and illustrates the signal, IgV, IgC, and hydrophilic tail domains.

Figure 4 depicts the amino acid sequence of human B7-4M and illustrates the signal, IgV, IgC, and transmembrane and cytoplasmic domains.

Figure 5 depicts the nucleotide sequence of murine B7-4.

Figure 6 depicts the amino acid sequence of murine B7-4.

Figure 7 depicts an alignment of the human and murine B7-4 amino acid sequences.

Figure 8 illustrates the results of FACS analysis of binding of CD28Ig, CTLA4-Ig, and control Ig by B7-4M-transfected COS cells.

Figure 9 illustrates the results FACS analysis of binding of IgG and murine ICOS-his fusion protein by B7-4M-transfected COS cells.

Figure 10 illustrates the results FACS analysis of binding of IgM, BB1 and 133 antibodies to B7-4M- transfected COS cells.

Figure 11 illustrates that COS cells transfected with B7-4M (292) can costimulate T cell proliferation.

Figure 12 illustrates that COS cells transfected with a B7-4M (292) can costimulate T cell proliferation.

Figure 13 illustrates the binding of PD-1 to B7-4M transfected COS cells.

Figure 14 illustrates the ability of added PD-1 and not Flt4 to compete for the binding of PD-1 to B7-4M transfected COS cells.

Figure 15 illustrates the ability of PD-1 to bind to B7-4 transfected CHO cells, as determined by flow cytometry.

Figure 16 illustrates the ability of PD-1 to bind to B7-4 transfected CHO cells, as determined by BIACORE analysis.

Figure 17 illustrates the ability of B7-4M to transmit a negative signal to T cells.

Figure 18 illustrates the inhibition of T cell proliferation and cytokine production in human T cell stimulated in the presence of B7-4.

Figure 19 illustrates that T cell receptor/B7-4 activation in the presence of CD28 costimulation results in inhibition of T cell proliferation.

Figure 20 illustrates the binding of PD-1 to CHO cells expressing B7-4.

Figure 21 illustrates the action of B7-4 in the inhibition of CD28 signals.

Figure 22 illustrates the inhibition of cytokine production by the PD-1:B7-4 pathway, as measured by cytokine ELISA.

Figure 23 illustrates the inhibition of cytokine production by the PD-1:B7-4 pathway, as measured by cytokine mRNA levels.

Figure 24 illustrates that the mechanism of action of the PD-1:B7-4 pathway is cell-cycle arrest.

Figure 25 illustrates the ability of antibodies to B7-4 to inhibit the interaction between B7-4 and PD-1.

Figure 26 illustrates the ability of antibodies to PD-1 to inhibit the interaction between B7-4 and PD-1.

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Figure 27 illustrates the ability of soluble B7-4Fc to exacerbate disease in a murine model of experimental autoimmune encephalomyelitis.

Detailed Description of the Invention

In addition to the previously characterized B lymphocyte activation antigens, *e.g.*, B7-1 and B7-2, there are other antigens on the surface of antigen presenting cells which modulate costimulation of immune cells. For example, B7-4 polypeptides have been isolated from keratinocyte and placental cDNA libraries. B7-4 has also been found to herein costimulate or inhibit T cells. The present invention identifies PD-1 as a receptor for B7-4.

Immune cells have receptors that transmit activating signals. For example, T cells have T cell receptors and the CD3 complex, B cells have B cell receptors, and myeloid cells have Fc receptors. In addition, immune cells bear receptors that transmit signals that provide costimulatory signals or receptors that transmit signals that inhibit receptor-mediated signaling. For example, CD28 transmits a costimulatory signal to T cells. After ligation of the T cell receptor, ligation of CD28 results in a costimulatory signal characterized by, *e.g.*, upregulation of IL-2rα, IL-2rβ, and IL-2rγ receptor, increased transcription of IL-2 messenger RNA, increased expression of cytokine genes (including IL-2, IFN-γ, GM-CSF, and TNF-a). Transmission of a costimulatory signal allows the cell to progress through the cell cycle and, thus, increases T cell proliferation (Greenfield *et al.* (1998) *Crit. Rev. Immunol.* 18:389). Binding of a receptor on a T cell which transmits a costimulatory signal to the cell (*e.g.*, ligation of a costimulatory receptor that leads to cytokine secretion and/or proliferation of the T cell) by a B7 family molecule, such as B7-4, results in costimulation. Thus, inhibition of an interaction between a B7 family molecule, such as B7-4, and a receptor that transmits a costimulatory signal on a immune cells results in a downmodulation of the immune response and/or specific unresponsiveness, termed immune cell anergy. Inhibition of this interaction can be accomplished using, *e.g.*, anti-CD28 Fab fragments, antibodies to B7-1, B7-2 and/or B7-4, or by using a soluble form of a receptor to which a B7 family member molecule can bind as a competitive inhibitor (*e.g.*, CTLA4Ig).

Inhibitory receptors that bind to costimulatory molecules have also been identified on immune cells. Activation of CTLA4, for example, transmits a negative signal to a T cell. Engagement of CTLA4 inhibits IL-2 production and can induce cell cycle arrest (Krummel and Allison (1996) *J. Exp. Med.* 183:2533). In addition, mice that lack CTLA4 develop lymphoproliferative disease (Tivol *et al.* (1995) *Immunity* 3:541; Waterhouse *et al.* (1995) *Science* 270:985). The blockade of CTLA4 with antibodies may remove an inhibitory signal, whereas aggregation of CTLA4 with antibody transmits an inhibitory signal. Therefore, depending upon the receptor to which a costimulatory molecule binds (*i.e.*, a costimulatory receptor such as CD28 or an inhibitory receptor such as CTLA4), certain B7 molecules including can promote T cell costimulation or inhibition.

PD-1 is a member of the immunoglobulin family of molecules (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704). PD-1 was previously identified using a subtraction cloning based approach designed to identify modulators of programmed cell death. (Ishida *et al.* (1992) *EMBO J.* 11:3887-95; Woronicz *et al.* (1995) *Curr. Top. Microbiol. Immunol.* 200:137). PD-1 is believed to play a role in lymphocyte survival, *e.g.*, during clonal selection (Honjo (1992) *Science* 258:591; Agata *et al.* (1996) *Int. Immunology.* 8:765; Nishimura *et al.* (1996) *Int. Immunology* 8:773). PD-1 was also implicated as a regulator of B cell responses (Nishimura (1998) *Int. Immunology* 10:1563). Unlike CTLA4, which is found only on T cells, PD-1 is also found on B cells and myeloid cells.

The instant discovery that PD-1 binds to B7-4 places PD-1 in a family of inhibitory receptors with CTLA4. While engagement of a costimulatory receptor results in a costimulatory signal in an immune cell, engagement of an inhibitory receptor, *e.g.*, CTLA4 or PD-1 (for example by crosslinking or by aggregation), leads to the transmission of an inhibitory signal in an immune cell, resulting in downmodulation of immune cell responses and/or in immune cell anergy. While transmission of an inhibitory signal leads to downmodulation in immune cell responses (and a resulting downmodulation in the overall immune response), the prevention of an inhibitory signal (*e.g.*, by using a non-activating antibody against PD-1) in immune cells leads to

upmodulation of immune cell responses (and a resulting upmodulation of an immune response).

The instant invention makes available agents useful for modulating the activity and/or expression of PD-1; the interaction between PD-1 and its natural ligand(s), (*e.g.*, B7-4); and agents for modulating the immune response via modulation of the interaction between PD-1 and its natural ligand, *e.g.*, B7-4. Exemplary modulatory agents for use in these methods are described further as follows.

B7-4 and PD-1 Nucleic Acid and Polypeptide Molecules

In one embodiment, a modulatory agent useful for modulating the activity and/or expression of PD-1 is a B7-4 and/or PD-1 nucleic acid molecule, preferably a human B7-4 and/or PD-1 nucleic acid molecule.

In one embodiment, the isolated nucleic acid molecules of the present invention encode eukaryotic B7-4 or PD-1 polypeptides. The B7-4 family of molecules share a number of conserved regions, including signal domains, IgV domains and the IgC domains. IgV domains and the IgC domains are art recognized Ig superfamily member domains. These domains correspond to structural units that have distinct folding patterns called Ig folds. Ig folds are comprised of a sandwich of two β sheets, each consisting of antiparallel β strands of 5-10 amino acids with a conserved disulfide bond between the two sheets in most, but not all, domains. IgC domains of Ig, TCR, and MHC molecules share the same types of sequence patterns and are called the C1-set within the Ig superfamily. Other IgC domains fall within other sets. IgV domains also share sequence patterns and are called V set domains. IgV domains are longer than C-domains and form an additional pair of β strands.

Two forms of humanB7-4 molecules have been identified. One form is a naturally occurring B7-4 soluble polypeptide, *i.e.*, having a short hydrophilic domain and no transmembrane domain, and is referred to herein as B7-4S (shown in SEQ ID NO:2). The second form is a cell-associated polypeptide, *i.e.*, having a transmembrane and cytoplasmic domain, referred to herein as B7-4M (shown in SEQ ID NO:4).

B7-4 proteins comprise a signal sequence, and an IgV domain and an IgC domain. The signal sequence of SEQ ID NO:2 is shown from about amino acid 1 to about amino acid 18. The signal sequence of SEQ ID NO:4 is shown from about amino acid 1 to about amino acid 18. The IgV domain of SEQ ID NO:2 is shown from about amino acid 19 to about amino acid 134 and the IgV domain of SEQ ID NO:4 is shown from about amino acid 19 to about amino acid 134. The IgC domain of SEQ ID NO:2 is shown from about amino acid 135 to about amino acid 227 and the IgC domain of SEQ ID NO:4 is shown from about amino acid 135 to about amino acid 227. The hydrophilic tail of the B7-4 exemplified in SEQ ID NO:2 comprises a hydrophilic tail shown from about amino acid 228 to about amino acid 245. The B7-4 polypeptide exemplified in SEQ ID NO:4 comprises a transmembrane domain shown from about amino acids 239 to about amino acid 259 of SEQ ID NO:4 and a cytoplasmic domain shown from about amino acid 260 to about amino acid 290 of SEQ ID NO:4.

Murine B7-4 molecules were also identified. The murine cDNA sequence is presented in Figure 5 and the murine B7-4 amino acid sequence is presented in Figure 6. The present invention also pertains to these murine B7-4 molecules.

PD-1 has been identified herein as a receptor which binds to B7-4. PD-1 molecules are members of the immunoglobulin gene superfamily. PD-1 (Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520) has an extracellular region containing immunoglobulin superfamily domain, a transmembrane domain, and an intracellular region including an immunoreceptor tyrosine-based inhibitory motif (ITIM). These features also define a larger family of molecules, called the immunoinhibitory receptors, which also includes gp49B, PIR-B, and the killer inhibitory receptors (KIRs) (Vivier and Daeron (1997) *Immunol. Today* 18:286). It is often assumed that the tyrosyl phosphorylated ITIM motif of these receptors interacts with SH2-domain containing phosphatases, which leads to inhibitory signals. A subset of these immunoinhibitory receptors bind to MHC molecules, for example the KIRs, and CTLA4 bind to B7-1 and B7-2. It has been proposed that there is a phylogenetic relationship between the MHC and B7 genes (Henry *et al.* (1999) *Immunol. Today* 20(6):285-8).

The nucleotide sequence of PD-1 is shown in SEQ ID NO:10 and 11 and the amino acid sequence of PD-1 is shown in SEQ ID NO:12 (see also Ishida *et al.* (1992) *EMBO J.* 11:3887; Shinohara *et al.* (1994) *Genomics* 23:704; U.S. Patent 5,698,520). PD-1 was previously identified using a subtraction cloning based approach to select for proteins involved in apoptotic cell death. PD-1 is identified herein as a member of the CD28/CTLA-4 family of molecules based on its ability to bind to B7-4. Like CTLA4, PD-1 is rapidly induced on the surface of T-cells in response to anti-CD3 (Agata *et al.* (1996) *Int. Immunol.* 8:765). In contrast to CTLA4, however, PD-1 is also induced on the surface of B-cells (in response to anti-IgM). PD-1 is also expressed on a subset of thymocytes and myeloid cells (Agata *et al.* (1996) *supra*; Nishimura *et al.* (1996) *Int. Immunol.* 8:773). The instant invention identifies B7-4 as a ligand of PD-1.

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

I. Definitions

As used herein, the term "immune cell" includes cells that are of hematopoietic origin and that play a role in the immune response. Immune cells include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

As used herein, the term "T cell" includes CD4+ T cells and CD8+ T cells. The term T cell also includes both T helper 1 type T cells and T helper 2 type T cells. The term "antigen presenting cell" includes professional antigen presenting cells (*e.g.*, B lymphocytes, monocytes, dendritic cells, Langerhans cells) as well as other antigen presenting cells (*e.g.*, keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes).

As used herein, the term "immune response" includes T cell mediated and/or B cell mediated immune responses that are influenced by modulation of T cell costimulation. Exemplary immune responses include T cell responses, *e.g.*, cytokine production, and cellular cytotoxicity. In addition, the term immune response includes immune responses that are indirectly effected by T cell activation, *e.g.*, antibody

production (humoral responses) and activation of cytokine responsive cells, *e.g.*, macrophages.

As used herein, the term "costimulatory receptor" includes receptors which transmit a costimulatory signal to a immune cell, *e.g.*, CD28. As used herein, the term "inhibitory receptors" includes receptors which transmit a negative signal to an immune cell (*e.g.*, CTLA4 or PD-1). An inhibitory signal as transduced by an inhibitory receptor can occur even if a costimulatory receptor (such as CD28) in not present on the immune cell and, thus, is not simply a function of competition between inhibitory receptors and costimulatory receptors for binding of costimulatory molecules (Fallarino *et al.* (1998) *J. Exp. Med.* 188:205). Transmission of an inhibitory signal to an immune cell can result in unresponsiveness or anergy or programmed cell death in the immune cell. Preferably transmission of an inhibitory signal operates through a mechanism that does not involve apoptosis. As used herein the term "apoptosis" includes programmed cell death which can be characterized using techniques which are known in the art. Apoptotic cell death can be characterized, *e.g.*, by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage.

Depending upon the form of the B7-4 molecule that binds to a receptor, either a signal can be transmitted (*e.g.*, by a multivalent form of a B7-4 molecule that results in crosslinking of receptor) or a signal can be inhibited (*e.g.*, by a soluble, monovalent form of a B7-4 molecule), *e.g.*, by competing with activating forms of B7-4 molecules for binding to the receptor. However, there are instances in which a soluble molecule can be stimulatory. The effects of the various modulatory agents can be easily demonstrated using routine screening assays as described herein.

As used herein, the term "costimulate" with reference to activated immune cells includes the ability of a costimulatory molecule to provide a second, non-activating receptor mediated signal (a "costimulatory signal") that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, *e.g.*, in a T cell that has received a T cell-receptor-mediated signal. Immune cells that have received a cell-receptor mediated signal, *e.g.*, via an activating receptor are referred to herein as "activated immune cells."

As used herein, the term "activating receptor" includes immune cell receptors that bind antigen, complexed antigen (*e.g.*, in the context of MHC molecules), or bind to antibodies. Such activating receptors include T cell receptors (TCR), B cell receptors (BCR), cytokine receptors, LPS receptors, complement receptors, and Fc receptors.

For example, T cell receptors are present on T cells and are associated with CD3 molecules. T cell receptors are stimulated by antigen in the context of MHC molecules (as well as by polyclonal T cell activating reagents). T cell activation via the TCR results in numerous changes, *e.g.*, protein phosphorylation, membrane lipid changes, ion fluxes, cyclic nucleotide alterations, RNA transcription changes, protein synthesis changes, and cell volume changes.

B cell receptors are present on B cells. B cell antigen receptors are a complex between membrane Ig (mIg) and other transmembrane polypeptides (*e.g.*, Igα and Igβ). The signal transduction function of mIg is triggered by crosslinking of receptor molecules by oligomeric or multimeric antigens. B cells can also be activated by anti-immunoglobulin antibodies. Upon BCR activation, numerous changes occur in B cells, including tyrosine phosphorylation.

Fc receptors are found on many cells which participate in immune responses. Fc receptors (FcRs) are cell surface receptors for the Fc portion of immunoglobulin molecules (Igs). Among the human FcRs that have been identified so far are those which recognize IgG (designated Fcγ R), IgE (Fcε R1), IgA (Fcα), and polymerized IgM/A (Fcμα R). FcRs are found in the following cell types: Fcε R I (mast cells), Fcε R.II (many leukocytes), Fcα R (neutrophils), and Fcμα R (glandular epithelium, hepatocytes) (Hogg, N. (1988) *Immunol. Today* 9:185-86). The widely studied FcγRs are central in

cellular immune defenses, and are responsible for stimulating the release of mediators of inflammation and hydrolytic enzymes involved in the pathogenesis of autoimmune disease (Unkeless, J. C. *et al.* (1988) *Annu. Rev. Immunol.* 6:251-81). The FcγRs provide a crucial link between effector cells and the lymphocytes that secrete Ig, since the macrophage/monocyte, polymorphonuclear leukocyte, and natural killer (NK) cell FcγRs confer an element of specific recognition mediated by IgG. Human leukocytes have at least three different receptors for IgG: h Fcγ RI (found on monocytes/macrophages), hFcγ RII (on monocytes, neutrophils, eosinophils, platelets, possibly B cells, and the K562 cell line), and Fcγ III (on NK cells, neutrophils, eosinophils, and macrophages).

With respect to T cells, transmission of a costimulatory signal to a T cell involves a signaling pathway that is not inhibited by cyclosporine A. In addition, a costimulatory signal can induce cytokine secretion (*e.g.*, IL-2 and/or IL-10) in a T cell and/or can prevent the induction of unresponsiveness to antigen, the induction of anergy, or the induction of cell death in the T cell.

As used herein, the term "inhibitory signal" refers to a signal transmitted via an inhibitory receptor (*e.g.*, CTLA4 or PD-1) for a molecule on a immune cell. Such a signal antagonizes a signal via an activating receptor (*e.g.*, via a TCR, CD3, BCR, or Fc molecule) and can result in, *e.g.*, inhibition of second messenger generation; an inhibition of proliferation; an inhibition of effector function in the immune cell, *e.g.*, reduced phagocytosis, reduced antibody production, reduced cellular cytotoxicity, the failure of the immune cell to produce mediators, (such as cytokines (*e.g.*, IL-2) and/or mediators of allergic responses); or the development of anergy.

As used herein, the term "unresponsiveness" includes refractivity of immune cells to stimulation, *e.g.*, stimulation via an activating receptor or a cytokine. Unresponsiveness can occur, *e.g.*, because of exposure to immunosuppressants or exposure to high doses of antigen. As used herein, the term "anergy" or "tolerance" includes refractivity to activating receptor-mediated stimulation. Such refractivity is generally antigen-specific and persists after exposure to the tolerizing antigen has ceased. For example, anergy in T cells (as opposed to unresponsiveness) is

characterized by lack of cytokine production, *e.g.*, IL-2. T cell anergy occurs when T cells are exposed to antigen and receive a first signal (a T cell receptor or CD-3 mediated signal) in the absence of a second signal (a costimulatory signal). Under these conditions, reexposure of the cells to the same antigen (even if reexposure occurs in the presence of a costimulatory molecule) results in failure to produce cytokines and, thus, failure to proliferate. Anergic T cells can, however, mount responses to unrelated antigens and can proliferate if cultured with cytokines (*e.g.*, IL-2). For example, T cell anergy can also be observed by the lack of IL-2 production by T lymphocytes as measured by ELISA or by a proliferation assay using an indicator cell line. Alternatively, a reporter gene construct can be used. For example, anergic T cells fail to initiate IL-2 gene transcription induced by a heterologous promoter under the control of the 5' IL-2 gene enhancer or by a multimer of the AP1 sequence that can be found within the enhancer (Kang *et al.* (1992) *Science* 257:1134).

The B7-4 protein and nucleic acid molecules comprise a family of molecules having certain conserved structural and functional features. Similarly, the PD-1 protein and nucleic acid molecules are members of a family of molecules having conserved structural and functional features. The term "family" when referring to the protein and nucleic acid molecules is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-human origin. Members of a family may also have common functional characteristics. The B7-4 molecules described herein are members of the B7 family of molecules. The term "B7 family" or "B7 molecules" as used herein includes costimulatory molecules that share sequence homology with B7 polypeptides, *e.g.*, with B7-1, B7-2, B7-3 (recognized by the antibody BB-1), B7h (Swallow *et al.* (1999) *Immunity* 11:423), and/or B7-4. For example, human B7-1 and B7-2 share approximately 26% amino acid sequence identity when compared using the BLAST program at NCBI with the default

parameters (Blosum62 matrix with gap penalties set at existence 11 and extension 1 (See http://www.ncbi.nlm.nih.gov)).

Preferred B7 polypeptides are capable of providing costimulatory or inhibitory signals to immune cells to thereby promote or inhibit immune cell responses. For example, when bound to a costimulatory receptor, B7-4 can induce costimulation of immune cells or can inhibit immune cell costimulation, e.g., when present in soluble form. When bound to an inhibitory receptor, B7-4 molecules can transmit an inhibitory signal to an immune cell. Preferred B7 family members include B7-1, B7-2 , B7-3 (recognized by the antibody BB-1), B7h, and B7-4 and soluble fragments or derivatives thereof. In one embodiment, B7 family members bind to one or more receptors on an immune cell, *e.g.*, CTLA4, CD28, ICOS, PD-1 and/or other receptors, and, depending on the receptor, have the ability to transmit an inhibitory signal or a costimulatory signal to an immune cell, preferably a T cell.

Preferred PD-1 molecules are capable of transmitting an inhibitory signal to an immune cell to thereby inhibit immune cell effector function or are capable of promoting costimulation (*e.g.*, by competitive inhibition) of immune cells, *e.g.*, when present in soluble, monomeric form. Preferred PD-1 family members bind to one or more receptors, *e.g.*, B7-1, B7-2, B7-4, and/or other molecules on antigen presenting cells, and share sequence identity with PD-1.

In addition, in one embodiment, proteins that are members of a protein family are bound by antibodies generated against one or more other family member proteins. For example, the anti-BB1 antibody recognizes B7-4 molecules.

As used herein, the term "activity" with respect to a B7-4 or PD-1 polypeptide includes activities which are inherent in the structure of a B7-4 or PD-1 protein. With regard to B7-4, the term "activity" includes the ability to modulate immune cell costimulation, *e.g.* by modulating a costimulatory signal in an activated immune cell, or to modulate inhibition by modulating an inhibitory signal in an immune cell, *e.g.*, by engaging a natural receptor on a immune cell. When an activating form of the B7-4 molecule binds to a costumulatory receptor, a costimulatory signal is generated in the immune cell. When an activating form of the B7-4 molecule binds to an inhibitory receptor, an inhibitory signal is generated in the immune cell.

Modulation of a costimulatory signal results in modulation of effector function of an immune cell. Thus, the term "B7-4 activity" includes the ability of a B7-4 polypeptide to bind its natural receptor(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

With respect to PD-1, the term "activity" includes the ability of a PD-1 polypeptide to modulate an inhibitory signal in an activated immune cell, *e.g.*, by engaging a natural ligand on an antigen presenting cell. PD-1 transmits an inhibitory signal to an immune cell in a manner similar to CTLA4. Modulation of an inhibitory signal in an immune cell results in modulation of proliferation of and/or cytokine secretion by an immune cell. PD-1 can also modulate a costimulatory signal by competing with a costimulatory receptor for binding of a B7 molecule. Thus, the term "PD-1 activity" includes the ability of a PD-1 polypeptide to bind its natural ligand(s), the ability to modulate immune cell costimulatory or inhibitory signals, and the ability to modulate the immune response.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

As used herein, an "antisense" nucleic acid molecule comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid molecule can hydrogen bond to a sense nucleic acid molecule.

As used herein, the term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (*e.g.*, 5' and 3' untranslated regions).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector,

wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid molecule of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, a "transgenic animal" refers to a non-human animal, preferably a mammal, more preferably a mouse, in which one or more of the cells of the animal includes a "transgene". The term "transgene" refers to exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, for example directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

As used herein, a "homologous recombinant animal" refers to a type of transgenic non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the

endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

As used herein, an "isolated protein" refers to a protein that is substantially free of other proteins, cellular material and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the B7-4 or PD-1 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of B7-4 or PD-1 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of B7-4 or PD-1 protein having less than about 30% (by dry weight) of non-B7-4 or PD-1 protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-B7-4 or PD-1 protein, still more preferably less than about 10% of non-B7-4 or PD-1 protein, and most preferably less than about 5% non-B7-4 or PD-1 protein. When the B7-4 or PD-1 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of B7-4 or PD-1 protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of B7-4 or PD-1 protein having less than about 30% (by dry weight) of chemical precursors or non-B7-4 or PD-1 chemicals, more preferably less than about 20% chemical precursors or non-B7-4 or PD-1 chemicals, still more preferably less than about 10% chemical precursors or non-B7-4 or PD-1 chemicals, and most preferably less than about 5% chemical precursors or non-B7-4 or PD-1 chemicals.

The term "antibody" as used herein also includes an "antigen-binding portion" of an antibody (or simply "antibody portion"). The term "antigen-binding 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.*, B7-4). 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 VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, 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 VL and VH 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; and Osbourn et al. 1998, Nature Biotechnology 16: 778). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Any VH and VL sequences of specific scFv can be linked to human immunoglobulin constant region cDNA or genomic sequences, in order to generate expression vectors encoding complete IgG molecules or other isotypes. VH and Vl can also be used in the generation of Fab , Fv or other fragments of immunoglobulins using either protein chemistry or recombinant DNA technology.Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R. J., *et al.* (1994) *Structure* 2:1121-1123).

Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecules, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S.M., *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S.M., *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein.

Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof, e.g. humanized, chimeric, etc. Preferably, antibodies of the invention bind specifically or substantially specifically to B7-4 molecules. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody composition, typically displays a single binding affinity for a particular antigen with which it immunoreacts.

The term "humanized antibody", as used herein, is intended to include antibodies made by a non-human cell having variable and constant regions which have been altered to more closely resemble antibodies that would be made by a human cell. For example, by altering the non-human antibody amino acid sequence to incorporate amino acids found in human germline immunoglobulin sequences. The humanized 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 mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs. The

term "humanized antibody", as used herein, also includes antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds B7-4 is substantially free of antibodies that specifically bind antigens other than B7-4). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

There is a known and definite correspondence between the amino acid sequence of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite correspondence between the nucleotide sequence of a particular nucleic acid molecule and the amino acid sequence encoded by that nucleic acid molecule, as defined by the genetic code.

GENETIC CODE	
Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
Cysteine (Cys, C)	TGC, TGT
Glutamic acid (Glu, E)	GAA, GAG
Glutamine (Gln, Q)	CAA, CAG
Glycine (Gly, G)	GGA, GGC, GGG, GGT
Histidine (His, H)	CAC, CAT
Isoleucine (Ile, I)	ATA, ATC, ATT
Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
Lysine (Lys, K)	AAA, AAG
Methionine (Met, M)	ATG
Phenylalanine (Phe, F)	TTC, TTT
Proline (Pro, P)	CCA, CCC, CCG, CCT
Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
Threonine (Thr, T)	ACA, ACC, ACG, ACT
Tryptophan (Trp, W)	TGG
Tyrosine (Tyr, Y)	TAC, TAT
Valine (Val, V)	GTA, GTC, GTG, GTT

Termination signal (end)	TAA, TAG, TGA

An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA molecule coding for a B7-4 or PD-1 polypeptide of the invention (or any portion thereof) can be used to derive the B7-4 or PD-1 amino acid sequence, using the genetic code to translate the DNA or RNA molecule into an amino acid sequence. Likewise, for any B7-4 or PD-1-amino acid sequence, corresponding nucleotide sequences that can encode B7-4 or PD-1 protein can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure herein of a B7-4 or PD-1 nucleotide sequence should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a B7-4 or PD-1 amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

II. Isolated Nucleic Acid Molecules

In one embodiment, modulating agents for use in the claimed methods comprise isolated nucleic acid molecules that encode B7-4 or PD-1 proteins or biologically active portions thereof. Nucleic acid fragments sufficient for use as hybridization probes to identify B7-4 or PD-1-encoding nucleic acids (*e.g.*, B7-4 or PD-1 mRNA) and fragments

for use as PCR primers for the amplification or mutation of B7-4 or PD-1 nucleic acid molecules are also provided. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid molecule (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid molecule is derived. For example, in various embodiments, the isolated B7-4 or PD-1 nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. An "isolated" B7-4 or PD-1 nucleic acid molecule may, however, be linked to other nucleotide sequences that do not normally flank the B7-4 or PD-1 sequences in genomic DNA (*e.g.*, the B7-4 or PD-1 nucleotide sequences may be linked to vector sequences). In certain preferred embodiments, an "isolated" nucleic acid molecule, such as a cDNA molecule, also may be free of other cellular material. However, it is not necessary for the B7-4 or PD-1 nucleic acid molecule to be free of other cellular material to be considered "isolated" (*e.g.*, a B7-4 or PD-1 DNA molecule separated from other mammalian DNA and inserted into a bacterial cell would still be considered to be "isolated").

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11 or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, using all or portion of the nucleic acid sequence of SEQ ID NO:1, 3, 10, or 11, as a hybridization probe, B7-4 or PD-1 nucleic acid molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, J. *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1, 3, 10, or 11 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence of SEQ ID NO:1, 3, 10, or 11, respectively.

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to B7-4 or PD-1 nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is complementary to the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, respectively, such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11, respectively, thereby forming a stable duplex.

In still another preferred embodiment, an isolated nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence (*e.g.*, to the entire length of the nucleotide sequence) shown in SEQ ID NO:1, 3, 10, or 11, or a portion of any of these nucleotide sequences.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the nucleic acid sequence of SEQ ID NO:1, 3, 10, or 11, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of a B7-4 or PD-1 protein. The nucleotide sequence determined from the cloning of the B7-4 or PD-1 genes allows for the generation of probes and primers designed for use in identifying and/or cloning other B7-4 or PD-1 family members, as well as B7-4 or PD-1 family homologues from other species. The probe/primer typically comprises a substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12 or 15, preferably about 20 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 consecutive nucleotides of a sense sequence of SEQ ID NO:1, 3, 10, or 11, or of a naturally occurring allelic variant or mutant of SEQ ID NO:1, 3, 10, or 11. In an exemplary embodiment, a nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least 350, 400, 450, 500, 550, 600, 650, 700, 750, or 800 nucleotides in length and hybridizes under stringent hybridization conditions to a nucleic acid molecule of SEQ ID NO:1, 3, 10, or 11.

In another embodiment, a second nucleic acid molecule comprises at least about 500, 600, 700, 800, 900, or 1000 contiguous nucleotides of SEQ ID NO:1, 3, 10, or 11.

In one embodiment, a nucleic acid molecule of the invention, *e.g.*, for use as a probe, does not include the portion of SEQ ID NO:1 from about nucleotides 815 to about 850 of SEQ ID NO:1 or about nucleotides 320 to 856 of SEQ ID NO:1. In another embodiment, a nucleic acid molecule of the invention does not include the portion of SEQ ID NO:3 from about nucleotides 314 to about 734, or from about nucleotides 835 to about 860, or from about nucleotides 1085 to about 1104 or from about nucleotides 1286 to about 1536 of SEQ ID NO:3.

In one embodiment, a nucleic acid molecule of the invention comprises at least about 500 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3. In a preferred embodiment, a nucleic acid molecule of the invention comprises at least about 600, at least about 700, at least about 800, at least about 900 or at least about 950 contiguous nucleotides of SEQ ID NO:1 or about 1000 contiguous nucleotides of SEQ ID NO:3. In another embodiment, a nucleic acid molecule of the invention comprises at least about 1500 or 1550 nucleotides of SEQ ID NO:3

Preferably, an isolated nucleic acid molecule of the invention comprises at least a portion of the coding region of SEQ ID NO:1 (shown in nucleotides 59-793) or SEQ ID NO:3 (shown in nucleotides 53-922). In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 1 to about nucleotide 319 of SEQ ID NO:1. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 855 to about nucleotide 968 of SEQ ID NO:1. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 1 to about nucleotide 314 of SEQ ID NO:3. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 955 to about nucleotide 1285 of SEQ ID NO:3. In another embodiment, a B7-4 nucleic acid molecule comprises from about nucleotide 1535 to about nucleotide 1552 of SEQ ID NO:3.

In other embodiments, a nucleic acid molecule of the invention has at least 70% identity, more preferably 80% identity, and even more preferably 90% identity with a nucleic acid molecule comprising: at least about 500, at least about 600, at least about 700, at least about 800, at least about 900 or at least about 1000 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3.

Probes based on the B7-4 or PD-1 nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissues which misexpress a B7-4 or PD-1 protein, such as by measuring a level of a B7-4 or PD-1-encoding nucleic acid in a sample of cells from a subject *e.g.*, detecting

B7-4 or PD-1 mRNA levels or determining whether a genomic B7-4 or PD-1 gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion of a B7-4 or PD-1 protein" can be prepared by isolating a portion of the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11 which encodes a polypeptide having a B7-4 or PD-1 biological activity (the biological activities of the B7-4 or PD-1 proteins are described herein), expressing the encoded portion of the B7-4 or PD-1 protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the B7-4 or PD-1 protein.

Nucleic acid molecules that differ from SEQ ID NO:1, 3, 10, or 11 due to degeneracy of the genetic code, and thus encode the same B7-4 or PD-1 protein as that encoded by SEQ ID NO:1, 3, 10, or 11, are encompassed by the invention. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO:2, 4, or 12. In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a B7-4 or PD-1 protein.

In addition to the B7-4 or PD-1 nucleotide sequences shown in SEQ ID NO:1, 3, 10, or 11 it should be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the B7-4or PD-1 proteins may exist within a population (*e.g.*, the human population). Such genetic polymorphism in the B7-4 or PD-1 genes may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding a B7-4 or PD-1 protein, preferably a mammalian B7-4 or PD-1 protein, and can further include non-coding regulatory sequences, and introns. Such natural allelic variations include both functional and non-functional B7-4 or PD-1 proteins and can typically result in 1-5% variance in the nucleotide sequence of a B7-4 or PD-1 gene. Such nucleotide variations and resulting amino acid polymorphisms in B7-4 or PD-1 genes that are the result of natural allelic variation and that do not alter the functional activity of a B7-4 or PD-1 protein are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding other B7-4 or PD-1 family members and, thus, which have a nucleotide sequence which differs from the B7-4 or PD-1 family sequences of SEQ ID NO:1, 3, 10, or 11 are intended to be within the scope of the invention. For example, another B7-4 or PD-1 cDNA can be identified based on the nucleotide sequence of human B7-4 or PD-1 . Moreover, nucleic acid molecules encoding B7-4 or PD-1 proteins from different species, and thus which have a nucleotide sequence which differs from the B7-4 or PD-1 sequences of SEQ ID NO:1, 3, 10, or 11 are intended to be within the scope of the invention. For example, a mouse B7-4 or PD-1 cDNA can be identified based on the nucleotide sequence of a human B7-4 or PD-1 molecule.

Nucleic acid molecules corresponding to natural allelic variants and homologues of the B7-4 or PD-1 cDNAs of the invention can be isolated based on their homology to the B7-4 or PD-1 nucleic acids disclosed herein using the cDNAs disclosed herein, or a portion thereof, as a hybridization probe according to standard hybridization techniques. For example, a B7-4 or PD-1 DNA can be isolated from a human genomic DNA library using all or portion of SEQ ID NO:1, 3, 10, or 11 as a hybridization probe and standard hybridization techniques (*e.g.*, as described in Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of a B7-4 or PD-1 gene can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO:1, 3, 10, or 11. For example, mRNA can be isolated from cells (*e.g.*, by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18:5294-5299) and cDNA can be prepared using reverse transcriptase (*e.g.*, Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequence shown in SEQ ID NO:1, 3, 10, or 11. A nucleic acid molecule of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so

amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to a B7-4 or PD-1 nucleotide sequence can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In another embodiment, an isolated nucleic acid molecule of the invention is at least 15, 20, 25, 30 or more nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11. In other embodiment, the nucleic acid molecule is at least 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, or 600 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 30%, 40%, 50%, or 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1, 3, 10, or 11 corresponds to a naturally-occurring nucleic acid molecule.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein). In addition to the B7-4 or PD-1 nucleotide sequences shown in SEQ ID NO:1, 3, 10, and 11, it should be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to minor changes in the nucleotide or amino acid sequences of a B7-4 or PD-1 may exist within a population. Such genetic polymorphism in a B7-4 or PD-1 gene may exist among individuals within a population due to natural allelic variation. Such natural allelic variations can typically result in 1-2 % variance in the nucleotide sequence of the gene. Such nucleotide variations and

resulting amino acid polymorphisms in a B7-4 or PD-1 that are the result of natural allelic variation and that do not alter the functional activity of a B7-4 or PD-1 polypeptide are within the scope of the invention.

In addition to naturally-occurring allelic variants of B7-4 or PD-1 sequences that may exist in the population, the skilled artisan will further appreciate that minor changes may be introduced by mutation into nucleotide sequences, *e.g.*, of SEQ ID NO:1, 3, 10, or 11, thereby leading to changes in the amino acid sequence of the encoded protein, without altering the functional activity of a B7-4 or PD-1 protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made in the sequence of SEQ ID NO:1, 3, 10, or 11. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a B7-4 nucleic acid molecule (*e.g.*, the sequence of SEQ ID NO:1, 3, 10, or 11) without altering the functional activity of a B7-4 or PD-1 molecule. Preferably, residues in the extracellular domain of B7-4 or PD-1 which are found to be required for binding of B7-4 to a receptor or PD-1 to a natural ligand (*e.g.*, identified using an alanine scanning mutagenesis screen or other art recognized screening assay) are not altered. For B7-4 molecules, exemplary residues which are non-essential and, therefore, amenable to substitution, can be identified by one of ordinary skill in the art by performing an amino acid alignment of B7 family members (or of B7-4 family members) and determining residues that are not conserved. Such residues, because they have not been conserved, are more likely amenable to substitution.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding B7-4 or PD-1 proteins that contain changes in amino acid residues that are not essential for a B7-4 or PD-1 activity. Such B7-4 or PD-1 proteins differ in amino acid sequence from SEQ ID NO:2, 4, or 12 yet retain an inherent B7-4 activity or, in the case of PD-1, retain the ability to bind to B7-4. An isolated nucleic acid molecule encoding a non-natural variant of a B7-4 or PD-1 protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into

SEQ ID NO:1, 3, 10, or 11 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in a B7-4 or PD-1 is preferably replaced with another amino acid residue from the same side chain family.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a B7-4 or PD-1 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for their ability to bind to DNA and/or activate transcription, to identify mutants that retain functional activity. Following mutagenesis, the encoded B7-4 or PD-1 mutant protein can be expressed recombinantly in a host cell and the functional activity of the mutant protein can be determined using assays available in the art for assessing a B7-4 or PD-1 activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding B7-4 or PD-1 proteins that contain changes in amino acid residues that are not essential for activity.

Yet another aspect of the invention pertains to isolated nucleic acid molecules encoding a B7-4 or PD-1 fusion proteins. Such nucleic acid molecules, comprising at least a first nucleotide sequence encoding a B7-4 or PD-1 protein, polypeptide or peptide operatively linked to a second nucleotide sequence encoding a non- a B7-4 or PD-1 protein, polypeptide or peptide, can be prepared by standard recombinant DNA techniques.

In a preferred embodiment, a mutant B7-4 protein can be assayed for the ability to: 1) costimulate (or inhibit the costimulation of, *e.g.*, in soluble form) the proliferation and/or effector function of activated immune cells; 2) bind to an anti-B7 family- or anti B7-4-antibody; and/or 3) bind to a natural receptor(s) of B7-4 (*e.g.*, PD-1).

In a preferred embodiment, a mutant PD-1 protein can be assayed for the ability to: 1) inhibit the costimulation of (*e.g.*, in soluble form) the proliferation and/or effector function of activated immune cells; 2) bind to an anti-PD-1 antibody; and/or 3) bind to a natural ligand(s) of PD-1 (*e.g.*, B7-4).

In addition to the nucleic acid molecules encoding B7-4 or PD-1 proteins described above, isolated nucleic acid molecules which are antisense thereto can be used as modulating agents. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire B7-4 or PD-1 coding strand, or only to a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding B7-4 or PD-1 . The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding B7-4 or PD-1 . The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences encoding B7-4 or PD-1 disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of B7-4 or PD-1 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of B7-4 or PD-1 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of B7-4 or PD-1

mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid molecule (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid is of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a B7-4 or PD-1 protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in

the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid molecule of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid molecule, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haseloff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave B7-4 or PD-1 mRNA transcripts to thereby inhibit translation of B7-4 or PD-1 mRNA. A ribozyme having specificity for a B7-4 or PD-1-encoding nucleic acid can be designed based upon the nucleotide sequence of a B7-4 or PD-1 cDNA disclosed herein (*i.e.*, SEQ ID NO:1, 3, 10, or 11). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a B7-4 or PD-1-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and

Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, B7-4 or PD-1 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J. W. (1993) *Science* 261:1411-1418.

Alternatively, B7-4 or PD-1 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the B7-4 or PD-1 (*e.g.*, the B7-4 or PD-1 promoter and/or enhancers) to form triple helical structures that prevent transcription of the B7-4 or PD-1 gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L. J. (1992) *Bioessays* 14(12):807-15.

In yet another embodiment, the B7-4 or PD-1 nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety, or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup, B. and Nielsen, P. E. (1996) *Bioorg. Med. Chem.* 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup and Nielsen (1996) *supra* and Perry-O'Keefe *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14670-675.

PNAs of B7-4 or PD-1 nucleic acid molecules can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of B7-4 or PD-1 nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup and Nielsen (1996) *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup B. and Nielsen (1996) *supra*; Perry-O'Keefe *et al.* (1996) *supra*).

In another embodiment, PNAs of B7-4 or PD-1 can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of B7-4 or PD-1 nucleic acid molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, (*e.g.*, RNAse H and DNA polymerases), to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup B. and Nielsen (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup B. and Nielsen (1996) *supra* and Finn P. J. *et al.* (1996) *Nucleic Acids Res.* 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry. Modified nucleoside analogs, (*e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite), can be used as a linker between the PNA and the 5' end of DNA (Mag, M. *et al.* (1989) *Nucleic Acid Res.* 17:5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn P. J. *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K. H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5:1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.* (1988) *Biotechniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide can be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

III. Isolated B7-4 or PD-1 Proteins and Anti-B7-4 or PD-1 Antibodies

In addition, isolated B7-4 or PD-1 proteins, and biologically active portions thereof, as well as anti-B7-4 or PD-1 antibodies can be used as modulating agents. In one embodiment, native B7-4 or PD-1 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, B7-4 or PD-1 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a B7-4 or PD-1 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

Another aspect of the invention pertains to isolated B7-4 or PD-1 proteins. Preferably, the B7-4 or PD-1 proteins comprise the amino acid sequence encoded by SEQ ID NO:1, 3, 10 or 11. In another preferred embodiment, the protein comprises the amino acid sequence of SEQ ID NO:2, 4, or 12. In other embodiments, the protein has at least 50%, at least 60% amino acid identity, more preferably 70% amino acid identity, more preferably 80%, and even more preferably, 90% or 95% amino acid identity with the amino acid sequence shown in SEQ ID NO:2, 4, or 12.

In other embodiments, the invention provides isolated portions of a B7-4 or PD-1 protein. For example, B7-4 proteins comprise a signal sequence, and an IgV domain and an IgC domain. The signal sequence of SEQ ID NO:2 is shown from about amino acid 1 to about amino acid 18. The signal sequence of SEQ ID NO:4 is shown from about amino acid 1 to about amino acid 18. The IgV domain of SEQ ID NO:2 is shown from about amino acid 19 to about amino acid 134 and the IgV domain of SEQ ID NO:4 is shown from about amino acid 19 to about amino acid 134. The IgC domain of SEQ ID NO:2 is shown from about amino acid 135 to about amino acid 227 and the IgC domain of SEQ ID NO:4 is shown from about amino acid 135 to about amino acid 227. The hydrophilic tail of the B7-4 molecule exemplified in SEQ ID NO:2 comprises a hydrophilic tail shown from about amino acid 228 to about amino acid 245. The B7-4 polypeptide exemplified in SEQ ID NO:4 comprises a transmembrane domain shown from about amino acid 239 to about amino acid 259 of SEQ ID NO:4 and a cytoplasmic domain shown from about amino acid 260 to about amino acid 290 of SEQ ID NO:4.

The PD-1 polypeptide is 288 amino acids in length and its domain structure is known in the art (Shinohara *et al.* (1994) *Genomics* 23:704). The predicted mature form of the protein contains about 268 amino acids and comprises an extracellular domain (147 amino acids), a transmembrane domain (27 amino acids), a transmembrane region (27 amino acids) and a cytoplasmic domain (94 amino acids) Four potential N-glycosylation sites are found in the extracellular domain (U.S. Patent 5,698,520). The 68 amino acid residues between two cysteine residues (cys 54 and cys 123) bear resemblance to a disulfide-linked immunoglobulin domain of the V-set sequences (U.S. Patent 5,698,520).

The invention further pertains to soluble forms of B7-4 or PD-1 proteins. Such forms can be naturally occurring, *e.g.*, as shown in SEQ ID NO:2 or can be engineered and can comprise, *e.g.*, an extracellular domain of a B7-4 or PD-1 protein. Exemplary B7-4 extracellular domains comprise from about amino acids 19-238 of SEQ ID NO:4. Exemplary PD-1 extracellular domains comprise from about amino acids 21-288 of SEQ ID NO:12.

In one embodiment, the extracellular domain of a B7-4 polypeptide comprises the mature form of a B7-4 polypeptide, *e.g.*, the IgV and IgC domains, but not the transmembrane and cytoplasmic domains of a B7-4 polypeptide (*e.g.*, from about amino acid 19 to amino acid 238 of SEQ ID NO:4) or from about amino acid 19 to amino acid 245 of SEQ. ID. NO: 2.

In one embodiment, the extracellular domain of a PD-1 polypeptide comprises the mature form of a PD-1 polypeptide, *e.g.*, immunoglobulin superfamily domains (*e.g.*, V-set sequences), but not the transmembrane and cytoplasmic domains of a PD-1 polypeptide (*e.g.*, from about amino acid 21-288 of SEQ ID NO:12).

Biologically active portions of a B7-4 or PD-1 protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the B7-4 or PD-1 protein, which include less amino acids than the full length B7-4 or PD-1 proteins, and exhibit at least one activity of a B7-4 or PD-1 protein, preferably the ability to bind to a natural binding partner. Typically, biologically active portions comprise a domain or motif with at least one activity of the B7-4 or PD-1

protein. A biologically active portion of a B7-4 or PD-1 protein can be a polypeptide which is, for example, at least 10, 25, 50, 100, 150, 200 or more amino acids in length.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The residues at corresponding positions are then compared and when a position in one sequence is occupied by the same residue as the corresponding position in the other sequence, then the molecules are identical at that position. The percent identity between two sequences, therefore, is a function of the number of identical positions shared by two sequences (*i.e.*, % identity = # of identical positions/total # of positions x 100). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. As used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology".

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to B7-4 or PD-1 nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to B7-4 or PD-1 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. For example, the nucleotide sequences of the invention were analyzed using the default Blastn matrix 1-3 with gap penalties set at: existence 11 and extension 1. The amino acid sequences of the invention were analyzed using the default settings: the Blosum 62 matrix with gap penalties set at existence 11 and extension 1. See http://www.ncbi.nlm.nih.gov.

The invention also provides B7-4 or PD-1 chimeric or fusion proteins. As used herein, a B7-4 or PD-1 "chimeric protein" or "fusion protein" comprises a B7-4 or PD-1 polypeptide operatively linked to a non-B7-4 or PD-1 polypeptide. A "B7-4 or PD-1 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to B7-4 or PD-1 polypeptide, whereas a "non-B7-4 or PD-1 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the B7-4 or PD-1 protein, *e.g.*, a protein which is different from the B7-4 or PD-1 protein and which is derived from the same or a different organism. Within a B7-4 or PD-1 fusion protein the B7-4 or PD-1 polypeptide can correspond to all or a portion of a B7-4 or PD-1 protein. In a preferred embodiment, a B7-4 or PD-1 fusion protein comprises at least one biologically active portion of a B7-4 or PD-1 protein, *e.g.*, an extracellular domain of a B7-4 or PD-1 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the B7-4 or PD-1 polypeptide and the non-B7-4 or PD-1 polypeptide are fused in-frame to each other.

The non-B7-4 or PD-1 polypeptide can be fused to the N-terminus or C-terminus of the B7-4 or PD-1 polypeptide.

For example, in one embodiment, the fusion protein is a GST-B7-4 or GST-PD-1 fusion protein in which the B7-4 or PD-1 sequences are fused to the C-terminus of the GST sequences. In another embodiment, the fusion protein is a B7-4 or PD-1-HA fusion protein in which the B7-4 or PD-1 nucleotide sequence is inserted in a vector such as pCEP4-HA vector (Herrscher, R. F. *et al.* (1995) *Genes Dev.* 9:3067-3082) such that the B7-4 or PD-1 sequences are fused in frame to an influenza hemagglutinin epitope tag. Such fusion proteins can facilitate the purification of a recombinant B7-4 or PD-1 protein.

A B7-4 or PD-1 fusion protein can be produced by recombinant expression of a nucleotide sequence encoding a first peptide having B74 activity and a nucleotide sequence encoding second peptide corresponding to a moiety that alters the solubility, affinity, stability or valency of the first peptide, for example, an immunoglobulin constant region. Preferably, the first peptide consists of a portion of the B7-4 polypeptide (*e.g.*, a portion of amino acid residues 1-238 or 19-238 (after cleavage of the signal sequence) of the sequence shown in SEQ ID NO:4 that is sufficient to modulate costimulation or inhibition of activated immune cells). In another preferred embodiment, the first peptide consists of a portion of a PD-1 polypeptide (*e.g.*, a portion of amino acid residues 1-288 (or 21-288 after cleavage of the signal peptide) of the sequence shown in SEQ ID NO:12 that is sufficient to modulate costimulation or inhibition of activated immune cells) The second peptide can include an immunoglobulin constant region, for example, a human Cγ1 domain or Cγ4 domain (*e.g.*, the hinge, CH2 and CH3 regions of human IgCγ1, or human IgCγ4, see *e.g.*, Capon *et al.* US patent 5,116,964; 5,580,756; 5,844,095 and the like, incorporated herein by reference). A resulting fusion protein may have altered B7-4 or PD-1 solubility, binding affinity, stability and/or valency (*i.e.*, the number of binding sites available per molecule) and may increase the efficiency of protein purification. Fusion proteins and peptides produced by recombinant techniques can be secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the protein or peptide can be retained cytoplasmically and the cells harvested, lysed and the

protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. Protein and peptides can be isolated from cell culture media, host cells, or both using techniques known in the art for purifying proteins and peptides. Techniques for transfecting host cells and purifying proteins and peptides are known in the art.

Particularly preferred B7-4 or PD-1 Ig fusion proteins include the extracellular domain portion or variable region-like domain of a human B7-4 or PD-1 coupled to an immunoglobulin constant region (*e.g.*, the Fc region). The immunoglobulin constant region may contain genetic modifications which reduce or eliminate effector activity inherent in the immunoglobulin structure. For example, DNA encoding the extracellular portion of a B7-4 or PD-1 polypeptide can be joined to DNA encoding the hinge, CH2 and CH3 regions of human IgGγ1 and/or IgGγ4 modified by site directed mutagenesis, *e.g.*, as taught in WO 97/28267.

Preferably, a B7-4 or PD-1 fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide or an HA epitope tag). A B7-4 or PD-1 encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the B7-4 or PD-1 protein.

In another embodiment, the fusion protein is a B7-4 or PD-1 protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of B7-4 or PD-1 can be increased through use of a heterologous signal sequence.

The B7-4 or PD-1 fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. Use of B7-4 or PD-1 fusion proteins is useful therapeutically for the treatment of immunological disorders, *e.g.*, autoimmune diseases, or in the case of inhibiting rejection of transplants. Moreover, the B7-4 or PD-1-fusion proteins of the invention can be used as immunogens to produce anti-B7-4 or PD-1 antibodies in a subject, to purify B7-4 or PD-1 and in screening assays to identify molecules which inhibit the interaction of B7-4 with a B7-4 receptor, *e.g.*, PD-1.

Preferably, a B7-4 or PD-1 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A B7-4 or PD-1-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the B7-4 or PD-1 protein.

The present invention also pertains to variants of the B7-4 or PD-1 proteins which function as either B7-4 or PD-1 agonists (mimetics) or as B7-4 or PD-1 antagonists. Variants of the B7-4 or PD-1 proteins can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a B7-4 or PD-1 protein. An agonist of the B7-4 or PD-1 proteins can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a B7-4 or PD-1 protein. An antagonist of a B7-4 or PD-1 protein can inhibit one or more of the activities of the naturally occurring form of the B7-4 or PD-1 protein by, for example, competitively modulating a cellular activity of a B7-4 or PD-1 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the B7-4 or PD-1 protein.

In one embodiment, variants of a B7-4 or PD-1 protein which function as either B7-4 or PD-1 agonists (mimetics) or as B7-4 or PD-1 antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of a B7-4 or PD-1 protein for B7-4 or PD-1 protein agonist or antagonist activity. In one embodiment, a variegated library of B7-4 or PD-1 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of B7-4 or PD-1 variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential B7-4 or PD-1 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of B7-4 or PD-1 sequences therein. There are a variety of methods which can be used to produce libraries of potential B7-4 or PD-1 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential B7-4 or PD-1 sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S. A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev.*

Biochem. 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of a B7-4 or PD-1 protein coding sequence can be used to generate a variegated population of B7-4 or PD-1 fragments for screening and subsequent selection of variants of a B7-4 or PD-1 protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a B7-4 or PD-1 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the B7-4 or PD-1 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of B7-4 or PD-1 proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify B7-4 or PD-1 variants (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrave *et al.* (1993) *Protein Eng.* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated B7-4 or PD-1 library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes and secretes B7-4 or PD-1. The transfected

cells are then cultured such that B7-4 or PD-1 and a particular mutant B7-4 or PD-1 are secreted and the effect of expression of the mutant on B7-4 or PD-1 activity in cell supernatants can be detected, *e.g.*, by any of a number of functional assays. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of B7-4 or PD-1 activity, and the individual clones further characterized.

In addition to B7-4 or PD-1 polypeptides consisting only of naturally-occurring amino acids, B7-4 or PD-1 peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) *Adv. Drug Res.* 15:29; Veber and Freidinger (1985) TINS p.392; and Evans *et al.* (1987) *J. Med. Chem.* 30:1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.*, a polypeptide that has a biological or pharmacological activity), such as human B7-4 or PD-1, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-, by methods known in the art and further described in the following references: Spatola, A. F. in "*Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*" Weinstein, B., ed., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J. S. (1980) *Trends Pharm. Sci.* pp. 463-468 (general review); Hudson, D. *et al.* (1979) *Int. J. Pept. Prot. Res.* 14:177-185 (-CH2NH-, CH2CH2-); Spatola, A. F. *et al.* (1986) *Life Sci.* 38:1243-1249 (-CH2-S); Hann, M. M. (1982) *J. Chem. Soc. Perkin Trans. I.* 307-314 (-CH-CH-, cis and trans); Almquist, R. G. *et al.* (190) *J. Med. Chem.* 23:1392-1398 (-COCH2-); Jennings-White, C. *et al.* (1982) *Tetrahedron Lett.* 23:2533 (-COCH2-); Szelke, M. *et al.* European Appln. EP 45665 (1982) CA: 97:39405 (1982)(-CH(OH)CH2-); Holladay, M. W. *et al.* (1983) *Tetrahedron Lett.* (1983) 24:4401-4404 (-C(OH)CH2-); and Hruby, V. J. (1982) *Life*

Sci. (1982) 31:189-199 (-CH2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (*e.g.*, a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (*e.g.*, an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (*e.g.*, labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Systematic substitution of one or more amino acids of a B7-4 or PD-1 amino acid sequence with a D-amino acid of the same type (*e.g.*, D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides comprising a B7-4 or PD-1 amino acid sequence or a substantially identical sequence variation can be generated by methods known in the art (Rizo and Gierasch (1992) *Annu. Rev. Biochem.* 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of B7-4 or PD-1 polypeptides identified herein will enable those of skill in the art to produce polypeptides corresponding to B7-4 or PD-1 peptide sequences and sequence variants thereof. Such polypeptides can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a B7-4 or PD-1 peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides can be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis *et al.*

Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, Calif.; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91:501; Chaiken I. M. (1981) *CRC Crit. Rev. Biochem.* 11: 255; Kaiser *et al.* (1989) *Science* 243:187; Merrifield, B. (1986) *Science* 232:342; Kent, S. B. H. (1988) *Annu. Rev. Biochem.* 57:957; and Offord, R. E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

Peptides can be produced, typically by direct chemical synthesis, and used *e.g.*, as agonists or antagonists of a B7-4/PD-1 interaction. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred embodiments, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (*e.g.*, acetylation) or alkylation (*e.g.*, methylation) and carboxy-terminal-modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various embodiments of the invention. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others. Peptides can be used therapeutically to treat disease, *e.g.*, by altering costimulation in a patient.

An isolated B7-4 or PD-1 protein, or a portion or fragment thereof (or a nucleic acid molecule encoding such a polypeptide), can be used as an immunogen to generate antibodies that bind B7-4 or PD-1 using standard techniques for polyclonal and monoclonal antibody preparation. A full-length B7-4 or PD-1 protein can be used, or alternatively, the invention provides antigenic peptide fragments of B7-4 or PD-1 for use as immunogens. The antigenic peptide of B7-4 or PD-1 comprises at least 8 amino acid residues and encompasses an epitope of B7-4 or PD-1 such that an antibody raised against the peptide forms a specific immune complex with B7-4 or PD-1. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least

15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Alternatively, an antigenic peptide fragment of a B7-4 or PD-1 polypeptide can be used as the immunogen. An antigenic peptide fragment of a B7-4 or PD-1 polypeptide typically comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2, 4, or 12 and encompasses an epitope of a B7-4 or PD-1 polypeptide such that an antibody raised against the peptide forms an immune complex with a B7-4 or PD-1 molecule. Preferred epitopes encompassed by the antigenic peptide are regions of B7-4 or PD-1 that are located on the surface of the protein, *e.g.*, hydrophilic regions. In one embodiment, an antibody binds substantially specifically to a B7-4 or PD-1 molecule. In another embodiment, an antibody binds specifically to a B7-4 or PD-1 polypeptide.

Preferably, the antigenic peptide comprises at least about 10 amino acid residues, more preferably at least about 15 amino acid residues, even more preferably at least 20 about amino acid residues, and most preferably at least about 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of a B7-4 or PD-1 polypeptide that are located on the surface of the protein, *e.g.*, hydrophilic regions, and that are unique to a B7-4 or PD-1 polypeptide. In one embodiment such epitopes can be specific for a B7-4 or PD-1 proteins from one species, such as mouse or human (*i.e.*, an antigenic peptide that spans a region of a B7-4 or PD-1 polypeptide that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein). A standard hydrophobicity analysis of the B7-4 or PD-1 protein can be performed to identify hydrophilic regions.

A B7-4 or PD-1 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, a recombinantly expressed B7-4 or PD-1 protein or a chemically synthesized B7-4 or PD-1 peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic B7-4 or PD-1 preparation induces a polyclonal anti- B7-4 or PD-1 antibody response.

In another embodiment, nucleic acid vaccines can be administered by a variety of means, for example, by injection (e.g., intramuscular, intradermal, or the biolistic injection of DNA-coated gold particles into the epidermis with a gene gun that uses a particle accelerator or a compressed gas to inject the particles into the skin (Haynes et al. 1996. *J. Biotechnol.* 44:37)). Alternatively, nucleic acid vaccines can be administered by non-invasive means. For example, pure or lipid-formulated DNA can be delivered to the respiratory system or targeted elsewhere, e.g., Peyers patches by oral delivery of DNA (Schubbert. 1997. *Proc. Natl. Acad. Sci. USA* 94:961). Attenuated microorganisms can be used for delivery to mucosal surfaces. (Sizemore et al. 1995. *Science.* 270:29)

Polyclonal anti-B7-4 or PD-1 antibodies can be prepared as described above by immunizing a suitable subject with a B7-4 or PD-1 immunogen. The anti-B7-4 or PD-1 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized a B7-4 or PD-1 polypeptide. If desired, the antibody molecules directed against a B7-4 or PD-1 polypeptide can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti- B7-4 or PD-1 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci.* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an

immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a B7-4 or PD-1 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to a B7-4 or PD-1 polypeptide, preferably specifically.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-B7-4 or PD-1 monoclonal antibody (see, *e.g.*, Galfre, G. *et al.* (1977) *Nature* 266:55052; Gefter *et al.* (1977) *supra*; Lerner (1981) *supra*; Kenneth (1980) *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind a B7-4 or PD-1 molecule, *e.g.*, using a standard ELISA assay.

As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-B7-4 or PD-1 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with a B7-4 or PD-1 to thereby isolate immunoglobulin library members that bind a B7-4 or PD-1 polypeptide. Kits for generating and screening phage display

libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP*™ *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Biotechnology (NY)* 9:1369-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Biotechnology (NY)* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Additionally, recombinant anti-B7-4 or PD-1 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira *et al.* European Patent Application 184,187; Taniguchi, M. European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.*

(1986) *Biotechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In addition, humanized antibodies can be made according to standard protocols such as those disclosed in US patent 5,565,332. In another embodiment, antibody chains or specific binding pair members can be produced by recombination between vectors comprising nucleic acid molecules encoding a fusion of a polypeptide chain of a specific binding pair member and a component of a replicable geneic display package and vectors containing nucleic acid molecules encoding a second polypeptide chain of a single binding pair member using techniques known in the art, *e.g.*, as described in US patents 5,565,332, 5,871,907, or 5,733,743. The use of intracellular antibodies to inhibit protein function in a cell is also known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T. M. *et al.* (1990) *FEBS Lett.* 274:193-198; Carlson, J. R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W. A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Biotechnology (NY)* 12:396-399; Chen, S-Y. *et al.* (1994) *Hum. Gene Ther.* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R. R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R. R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J. H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

In one embodiment, an antibody for use in the instant invention is a bispecific antibody. A bispecific antibody has binding sites for two different antigens within a single antibody molecule. Antigen binding may be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Examples of bispecific antibodies produced by a hybrid hybridoma or a trioma are disclosed in U.S. Pat. 4,474,893. Bispecific antibodies have been constructed by chemical means (Staerz *et al.* (1985) *Nature* 314:628, and Perez *et al.* (1985) *Nature* 316:354) and hybridoma technology (Staerz and Bevan (1986) *Proc.*

Natl. Acad. Sci. USA, 83:1453, and Staerz and Bevan (1986) *Immunol. Today* 7:241). Bispecific antibodies are also described in U.S. patent 5,959,084. Fragments of bispecific antibodies are described in US patent 5,798,229.

Bispecific agents can also be generated by making heterohybridomas by fusing hybridomas or other cells making different antibodies, followed by identification of clones producing and co-assembling both antibodies. They can also be generated by chemical or genetic conjugation of complete immunoglobulin chains or portions thereof such as Fab and Fv sequences. The antibody component can bind to PD-1 or B7-4.

An anti-B7-4 or PD-1 antibody (*e.g.*, monoclonal antibody) can be used to isolate a B7-4 or PD-1 polypeptide by standard techniques, such as affinity chromatography or immunoprecipitation. Anti-B7-4 or PD-1 antibodies can facilitate the purification of natural B7-4 or PD-1 polypeptides from cells and of recombinantly produced B7-4 or PD-1 polypeptides expressed in host cells. Moreover, an anti-B7-4 or PD-1 antibody can be used to detect a B7-4 or PD-1 protein (*e.g.*, in a cellular lysate or cell supernatant). Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Accordingly, in one embodiment, an anti-B7-4 or PD-1 antibody of the invention is labeled with a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S, and ^{3}H.

Yet another aspect of the invention pertains to anti-B7-4 or PD-1 antibodies that are obtainable by a process comprising:

(a) immunizing an animal with an immunogenic B7-4 or PD-1 protein, or an immunogenic portion thereof unique to a B7-4 or PD-1 polypeptide; and

(b) isolating from the animal antibodies that specifically bind to a B7-4 or PD-1 protein.

IV. Recombinant Expression Vectors and Host Cells

Nucleic acid molecules encoding a B7-4 or PD-1 family protein (or a portion thereof) can be contained in vectors, preferably expression vectors. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

Recombinant expression vectors can comprise a nucleic acid molecule of the invention in a form suitable for expression, *e.g.*, constitutive or inducible expression, of a PD-1 or B7-4 molecule in the indicator cell(s) of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*,

polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel (1990) *Methods Enzymol.* 185:3-7. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It should be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, B7-4 or PD-1 family proteins, mutant forms of B7-4 or PD-1 proteins, fusion proteins, and the like).

The recombinant expression vectors of the invention can be designed for expression of B7-4 or PD-1 proteins in prokaryotic or eukaryotic cells. For example, B7-4 or PD-1 proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel (1990) *supra*. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D. B. and Johnson, K. S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly,

MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Purified fusion proteins can be utilized in B7-4 or PD-1 activity assays, (*e.g.*, direct assays or competitive assays described in detail below), or to generate antibodies specific for B7-4 or PD-1 proteins, for example.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.* (1990) *Methods Enzymol.* 185:60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S. (1990) *Methods Enzymol.*185:119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.* (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the B7-4 or PD-1 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.* (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.* (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (Invitrogen Corp, San Diego, CA).

Alternatively, a B7-4 or PD-1 polypeptide can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf9 cells) include the pAc series (Smith *et al.* (1983) *Mol.*

Cell Biol. 3:2156-2165) and the pVL series (Lucklow, V. A. and Summers, M. D. (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pMex-NeoI, pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J. *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Moreover, inducible regulatory systems for use in mammalian cells are known in the art, for example systems in which gene expression is regulated by heavy metal ions (see *e.g.*, Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42;

Searle *et al.* (1985) *Mol. Cell. Biol.* 5:1480-1489), heat shock (see *e.g.*, Nouer *et al.* (1991) in *Heat Shock Response*, ed. Nouer, L. , CRC, Boca Raton , FL, pp167-220), hormones (see *e.g.*, Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel and Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see *e.g.*, PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313). Accordingly, in another embodiment, the invention provides a recombinant expression vector in which a B7-4 or PD-1 DNA is operatively linked to an inducible eukaryotic promoter, thereby allowing for inducible expression of a B7-4 or PD-1 protein in eukaryotic cells.

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to B7-4 or PD-1 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.* (1986) "Antisense RNA as a molecular tool for genetic analysis" *Reviews - Trends in Genetics*, Vol. 1(1).

The invention further pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not

only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a B7-4 or PD-1 protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a B7-4 or PD-1 protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a B7-4 or PD-1 protein. Accordingly, the invention further provides methods for producing a B7-4 or PD-1 protein using the host

cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding a B7-4 or PD-1 protein has been introduced) in a suitable medium such that a B7-4 or PD-1 protein is produced. In another embodiment, the method further comprises isolating a B7-4 or PD-1 protein from the medium or the host cell.

Certain host cells can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell is a fertilized oocyte or an embryonic stem cell into which B7-4 or PD-1-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous B7-4 or PD-1 sequences have been introduced into their genome or homologous recombinant animals in which endogenous B7-4 or PD-1 sequences have been altered. Such animals are useful for studying the function and/or activity of a B7-4 or PD-1 polypeptide and for identifying and/or evaluating modulators of B7-4 or PD-1 activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, and the like. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous B7-4 or PD-1 gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal can be created by introducing a B7-4 or PD-1-encoding nucleic acid molecule into the male pronucleus of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The B7-4 or PD-1 cDNA sequence of SEQ ID NO:1, 3, 10, or 11 can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homologue of a human B7-4 or PD-1 gene, such as a

mouse or rat B7-4 or PD-1 gene, can be used as a transgene. Alternatively, a B7-4 or PD-1 gene homologue, such as another B7-4 or PD-1 family member, can be isolated based on hybridization to the B7-4 or PD-1 family cDNA sequences of SEQ ID NO:1, 3, 10, or 11 (described further in subsection I above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a B7-4 or PD-1 transgene to direct expression of a B7-4 or PD-1 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of a B7-4 or PD-1 transgene in its genome and/or expression of B7-4 or PD-1 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding a B7-4 or PD-1 protein can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a B7-4 or PD-1 gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the B7-4 or PD-1 gene. The B7-4 or PD-1 gene can be a human gene (*e.g.*, the SEQ ID NO:1, 3, 10, or 11), but more preferably, is a non-human homologue of a human B7-4 or PD-1 gene (*e.g.*, a cDNA isolated by stringent hybridization with the nucleotide sequence of SEQ ID NO:1, 3, 10, or 11). For example, a mouse B7-4 or PD-1 gene can be used to construct a homologous recombination vector suitable for altering an endogenous B7-4 or PD-1 gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous B7-4 or PD-1 gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous

recombination, the endogenous B7-4 or PD-1 gene is mutated or otherwise altered but still encodes a functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous B7-4 or PD-1 protein). In the homologous recombination vector, the altered portion of the B7-4 or PD-1 gene is flanked at its 5' and 3' ends by additional nucleic acid sequence of the B7-4 or PD-1 gene to allow for homologous recombination to occur between the exogenous B7-4 or PD-1 gene carried by the vector and an endogenous B7-4 or PD-1 gene in an embryonic stem cell. The additional flanking B7-4 or PD-1 nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K. R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced B7-4 or PD-1 gene has homologously recombined with the endogenous B7-4 or PD-1 gene are selected (see, *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, E. J., ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Curr. Opin. Biotechnol.* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In addition to the foregoing, the skilled artisan will appreciate that other approaches known in the art for homologous recombination can be applied to the instant invention. Enzyme-assisted site-specific integration systems are known in the art and can be applied to integrate a DNA molecule at a predetermined location in a second target DNA molecule. Examples of such enzyme-assisted integration systems include

the Cre recombinase-lox target system (*e.g.*, as described in Baubonis, W. and Sauer, B. (1993) *Nucl. Acids Res.* 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* 89:7905-7909) and the FLP recombinase-FRT target system (*e.g.*, as described in Dang, D. T. and Perrimon, N. (1992) *Dev. Genet.* 13:367-375; and Fiering, S. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:8469-8473). Tetracycline-regulated inducible homologous recombination systems, such as described in PCT Publication No. WO 94/29442 and PCT Publication No. WO 96/01313, also can be used.

For example, in another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. *et al.* (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_0 phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

V. Pharmaceutical Compositions

B7-4 or PD-1 modulators (*e.g.*, B7-4 or PD-1 inhibitory or stimulatory agents, including B7-4 or PD-1 nucleic acid molecules, proteins, antibodies described above, or compounds identified as modulators of a B7-4 or PD-1 activity and/or expression or modulators of the interaction between B7-4 and PD-1) can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For

intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene 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. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a B7-4 or PD-1 protein or anti-B7-4 or PD-1 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion 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 which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared

using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, modulatory agents are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations should be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova

Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or 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 subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as

determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

VI. Uses and Methods of the Invention

The B7-4 and/or PD-1 modulatory agents, *e.g.*, the nucleic acid molecules, proteins, protein homologues, and antibodies described herein, can be used in one or more of the following methods: a) methods of treatment, *e.g.*, by up- or down-modulating the immune response; b) screening assays; c) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics). The isolated nucleic acid molecules of the invention can be used, for example, to express B7-4 or PD-1 protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect B7-4 or PD-1 mRNA (*e.g.*, in a biological sample) or a genetic alteration in a B7-4 or PD-1 gene, and to modulate B7-4 or PD-1 activity, as described further below. The B7-4 or PD-1 proteins can be used to treat disorders characterized by insufficient or excessive production of B7-4 or PD-1 inhibitors. In addition, the B7-4 or PD-1 proteins can be used to screen for naturally occurring B7-4 or PD-1 binding partners, to screen for drugs or compounds which modulate B7-4 or PD-1 activity, as well as to treat disorders characterized by insufficient or excessive

production of B7-4 or PD-1 protein or production of B7-4 or PD-1 protein forms which have decreased or aberrant activity compared to B7-4 or PD-1 wild type protein. Moreover, the anti-B7-4 or PD-1 antibodies of the invention can be used to detect and isolate B7-4 or PD-1 proteins, regulate the bioavailability of B7-4 or PD-1 proteins, and modulate B7-4 or PD-1 activity *e.g.*, by modulating the interaction of B7-4 and PD-1.

A. Methods of Treatment:

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant B7-4 or PD-1 expression or activity.

1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant B7-4 or PD-1 expression or activity, by administering to the subject a B7-4 or PD-1 polypeptide or an agent which modulates B7-4 or PD-1 polypeptide expression or at least one B7-4 or PD-1 activity. Subjects at risk for a disease which is caused or contributed to by aberrant B7-4 or PD-1 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of B7-4 or PD-1 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of B7-4 or PD-1 aberrancy or condition, for example, a B7-4 or PD-1 polypeptide, B7-4 or PD-1 agonist or B7-4 or PD-1 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on clinical indications and can be identified, *e.g.*, using screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating B7-4 or PD-1 expression or activity for therapeutic purposes. B7-4 has been demonstrated to inhibit the costimulation and proliferation of activated immune cells and to transmit an inhibitory signal to immune cells via PD-1. Accordingly, the activity and/or expression

of B7-4 or PD-1 as well as the interaction between B7-4 and PD-1 can be modulated in order to modulate the immune response. It should be understood that in embodiments where B7-4 binds to a costimulatory receptor, upregulation of B7-4 activity results in upregulation of immune responses, whereas downregulation of B7-4 activity results in downregulation of immune responses. In embodiments where B7-4 binds to inhibitory receptors, upregulation of B7-4 activity results in downregulation of immune responses, whereas downregulation of B7-4 activity results in upregulation of immune responses. In a preferred embodiment, B7-4 binds to inhibitory receptors. In a particularly preferred embodiment, B7-4 binds to PD-1.

Modulatory methods of the invention involve contacting a cell with a modulator of a B7-4 or a PD-1 polypeptide, *e.g.*, an agent that modulates expression or activity of B7-4 and/or PD-1, or an agent that modulates the interaction of B7-4 and PD-1.

An agent that modulates B7-4 or PD-1 protein activity is an agent as described herein, such as a nucleic acid or a protein molecule, a naturally-occurring target molecule of a B7-4 or PD-1 protein (*e.g.*, PD-1 in the case of B7-4 or B7-4 in the case of PD-1), a B7-4 or PD-1 antibody, a B7-4 or PD-1 agonist or antagonist, a peptidomimetic of a B7-4 or PD-1 agonist or antagonist, or other small molecule.

An agent that modulates the expression of B7-4 or PD-1 is, *e.g.*, an antisense nucleic acid molecule, triplex oligonucleotide, or a ribozyme or a recombinant vector for expression of a B7-4 or PD-1 protein. For example, an oligonucleotide complementary to the area around a B7-4 or PD-1 polypeptide translation initiation site, can be synthesized. One or more antisense oligonucleotides can be added to cell media, typically at 200 μg/ml, or administered to a patient to prevent the synthesis of a B7-4 or PD-1 polypeptide. The antisense oligonucleotide is taken up by cells and hybridizes to a B7-4 or PD-1 mRNA to prevent translation. Alternatively, an oligonucleotide which binds double-stranded DNA to form a triplex construct to prevent DNA unwinding and transcription can be used. As a result of either, synthesis of a B7-4 or PD-1 polypeptide is blocked. When PD-1 expression is modulated, preferably, such modulation occurs by a means other than by knocking out the PD-1 gene.

Agents which modulate expression, by virtue of the fact that they control the amount of PD-1 or B7-4 in a cell, also modulate the total amount of PD-1 or B7-4 activity in a cell.

In one embodiment, an agent that stimulates an inhibitory or activity of B7-4 or an inhibitory activity of PD-1 is an agonist of B7-4 or PD-1. Examples of such agents include active B7-4 or PD-1 protein and a nucleic acid molecule encoding B7-4 or PD-1 polypeptide that has been introduced into the cell.

In another embodiment, the agent inhibits the costimulatory or inhibitory activity of B7-4 or inhibitory activity of PD-1 and is an antagonist of B7-4 or PD-1. Examples of such agents include antisense B7-4 or PD-1 nucleic acid molecules, anti-B7-4 or PD-1 antibodies, soluble, nonactivating forms of B7-4 or PD-1 molecules, and B7-4 or PD-1 inhibitors.

These modulatory agents can be administered *in vitro* (*e.g.*, by contacting the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder that would benefit from modulation of a B7-4 or PD-1 protein, *e.g.*, a disorder which would benefit from up or downmodulation of the immune response, or which is characterized by aberrant expression or activity of a B7-4 or PD-1 protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) B7-4 or PD-1 expression or activity. In another embodiment, the method involves administering a B7-4 or PD-1 protein or nucleic acid molecule as therapy to compensate for reduced or aberrant B7-4 or PD-1 expression or activity.

Stimulation of B7-4 or PD-1 activity is desirable in situations in which B7-4 or PD-1 is abnormally downregulated and/or in which increased B7-4 or PD-1 activity is likely to have a beneficial effect. Likewise, inhibition of B7-4 or PD-1 activity is desirable in situations in which B7-4 or PD-1 is abnormally upregulated and/or in which decreased B7-4 or PD-1 activity is likely to have a beneficial effect. One of ordinary skill in the art should recognize that in embodiments where B7-4 binds to a costimulatory, stimulation of B7-4 and stimulation of PD-1 have opposite effects on

immune cell costimulation, and therefore, on the immune response. In such an instance, when stimulation of the activity of one molecule is desirable, suppression of the activity of the other molecule is desirable.

Exemplary agents for use in downmodulating B7-4 (B7-4 antagonists) include (for example): antisense molecules, antibodies that recognize B7-4, compounds that block interaction of B7-4 and one of its naturally occurring receptors on a immune cell (*e.g.*, soluble, monovalent B7-4 molecules, and soluble forms of B7-4 ligands or compounds identified in the subject screening assays). Exemplary agents for use in downmodulating PD-1 (PD-1 antagonists) include (for example): antisense molecules, antibodies that bind to PD-1, but do not transduce an inhibitory signal to the immune cell ("non-activating antibodies"), and soluble forms of PD-1.

Exemplary agents for use in upmodulating B7-4 (B7-4 agonists) include (for example): nucleic acid molecules encoding B7-4 polypeptides, multivalent forms of B7-4, compounds that increase the expression of B7-4, and cells that express B7-4, etc. Exemplary agents for use in upmodulating PD-1 (PD-1 agonists) include (for example): antibodies that transmit an inhibitory signal via PD-1, compounds that enhance the expression of PD-1, nucleic acid molecules encoding PD-1, and forms of B7-4 that transduce a signal via PD-1.

3. Downregulation of Immune Responses by Modulation of B7-4 or PD-1

There are numerous embodiments of the invention for upregulating the inhibitory function or downregulating the costimulatory function of a B7-4 polypeptide to thereby downregulate immune responses. Downregulation can be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response.

The functions of activated immune cells can be inhibited by down-regulating immune cell responses or by inducing specific anergy in immune cells, or both.

For example, anti-B7-4 antibodies or B7-4 polypeptides (e.g., soluble, monomeric forms of a B7-4 polypeptide such as B7-4-Ig), and/or anti-B7-4 antibodies that block the interaction of B7-4 with a costimulatory receptor can be used to inhibit a costimulatory signal and, thus, downmodulate the immune response.

In addition, in embodiments where B7-4 binds to an inhibitory receptor, forms of B7-4 that bind to the inhibitory receptor, e.g., multivalent B7-4 on a cell surface, can be used to downmodulate the immune response.

Likewise, the PD-1 pathway can also be stimulated by the use of an agent to thereby downmodulate the immune response. Inhibition of the interaction of B7-4 with a stimulatory receptor on an immune cell (e.g., by using a soluble form of PD-1 and/or CTLA4) or activation of PD-1 (e.g., using an activating antibody which cross-links PD-1) may provide negative signals to immune cells.

In one embodiment of the invention, an activating antibody used to stimulate PD-1 activity is a bispecific antibody. For example, such an antibody can comprise a PD-1 binding site and another binding site which targets a cell surface receptor on an immune cell, e.g., on a T cell, a B cell, or a myeloid cell. In one embodiment, such an antibody, in addition to comprising a PD-1 binding site can further comprise a binding site which binds to a molecule which is in proximity to an activating or inhibitory receptor, e.g., B-cell antigen receptor, a T-cell antigen receptor, or an Fc receptor in order to target the molecule to a specific cell population. For example, a CD3 antigen, a T-cell receptor chain, LFA-1, CD2, CTLA-4, immunoglobulin, B cell receptor, Ig alpha, Ig beta, CD22, or Fc receptor could be used. Such antibodies (or other bispecific agents) are art recognized and can be produced, e.g., as described herein. Selection of this second antigen for the bispecific antibody provides flexibility in selection of cell population to be targeted for inhibition.

In another embodiment, the co-ligation of PD-1 and an activating or inhibitory receptor on a cell can enhance the generation of a negative signal via PD-1. Such co-ligation can be accomplished e.g., by use of a bispecific agent, e.g., a bispecific antibody as described herein having specificity for both PD-1 and a molecule associated with a receptor. In another embodiment, the use of a multivalent form of an agent that transmits a negative signal via PD-1 can be used to enhance the transmission of a negative signal via PD-1, e.g., an agent presented on a bead or on a surface. In another embodiment, a such a multivalent agent can comprise two specificities to achieve co-ligation of PD-1 and a receptor or a receptor associated molecule (e.g., a bead comprising anti CD3 and B7-4).

Agents that block or inhibit interaction of B7-4 with a costimulatory receptor (e.g., soluble forms of B7-4 or blocking antibodies to B7-4) as well as agents that promote a B7-4-mediated inhibitory signal or agonists of PD-1 which activate PD-1 (e.g., PD-1 activating antibodies or PD-1 activating small molecules) can be identified by their ability to inhibit immune cell proliferation and/or effector function or to induce anergy when added to an *in vitro* assay. For example, cells can cultured in the presence of an agent that stimulates signal transduction via an activating receptor. A number of art recognized readouts of cell activation can be employed to measure, e.g., cell proliferation or effector function (e.g., antibody production, cytokine production, phagocytosis) in the presence of the activating agent. The ability of a test agent to block this activation can be readily determined by measuring the ability of the agent to effect a decrease in proliferation or effector function being measured.

In one embodiment of the invention, tolerance is induced against specific antigens by co-administering an antigen with a PD-1 agonist. For example, tolerance can be induced to specific proteins. In one embodiment, immune responses to allergens or foreign proteins to which an immune response is undesirable can be inhibited. For example, patients that receive Factor VIII frequently generate antibodies against this clotting factor. Co-administration of an agent that blocks a B7-4-mediated costimulatory signal or an agent that stimulates a PD-1 mediated inhibitory signal in combination with recombinant factor VIII (or by physically linked to Factor VIII, e.g., by cross-linking) can result in downmodulation.

In one embodiment, fusion proteins comprising a B7-4 first peptide fused to a second peptide having an activity of another B lymphocyte antigen (e.g., B7-1 or B7-2), can be used to block interaction of B7-4 with a costimulatory receptor on a immune cell to downmodulate immune responses. Alternatively, two separate peptides (for example, a B7-4 polypeptide with B7-2 and/or B7-1), or a combination of blocking antibodies (e.g., antibodies against a B7-4 polypeptide with anti-B7-2 and/or anti-B7-1 monoclonal antibodies) can be combined as a single composition or administered separately (simultaneously or sequentially) to downregulate immune cell mediated immune responses in a subject. Furthermore, a therapeutically active amount of one or more peptides having a B7-4 polypeptide activity, with B7-1 and/or B7-1 activity can be used

in conjunction with other downmodulating reagents to influence immune responses. Examples of other immunomodulating reagents include antibodies that block a costimulatory signal, (e.g., against CD28, ICOS), antibodies that activate an inhibitory signal via CTLA4, and/or antibodies against other immune cell markers (e.g., against CD40, against CD40 ligand, or against cytokines), fusion proteins (e.g., CTLA4-Fc, PD-1-Fc), and immunosuppressive drugs, (e.g., rapamycin, cyclosporine A or FK506).

The B7-4 and/or PD-1 peptides may also be useful in the construction of therapeutic agents which block immune cell function by destruction of cells. For example, portions of a B7-4 or PD-1 polypeptide can be linked to a toxin to make a cytotoxic agent capable of triggering the destruction of cells to which it binds.

For making cytotoxic agents, polypeptides of the invention may be linked, or operatively attached, to toxins using techniques that are known in the art, e.g., crosslinking or via recombinant DNA techniques. The preparation of immunotoxins is, in general, well known in the art (see, e.g., U.S. Pat. Nos. 4,340,535, and EP 44167, both incorporated herein by reference). Numerous types of disulfide-bond containing linkers are known which can successfully be employed to conjugate the toxin moiety with a polypeptide. In one embodiment, linkers that contain a disulfide bond that is sterically "hindered" are to be preferred, due to their greater stability in vivo, thus preventing release of the toxin moiety prior to binding at the site of action.

A wide variety of toxins are known that may be conjugated to polypeptides or antibodies of the invention. Examples include: numerous useful plant-, fungus- or even bacteria-derived toxins, which, by way of example, include various A chain toxins, particularly ricin A chain, ribosome inactivating proteins such as saporin or gelonin, .alpha.-sarcin, aspergillin, restrictocin, ribonucleases such as placental ribonuclease, angiogenic, diphtheria toxin, and pseudomonas exotoxin, etc. A preferred toxin moiety for use in connection with the invention is toxin A chain which has been treated to modify or remove carbohydrate residues, deglycosylated A chain. (U.S. patent 5,776,427).

Infusion of one or a combination of such cytotoxic agents, (e.g., B7-4 ricin (alone or in combination with B7-2-ricin or B7-1-ricin), into a patient may result in the death of immune cells, particularly in light of the fact that activated immune cells that

express higher amounts of B7-4 ligands. For example, because PD-1 is induced on the surface of activated lymphocytes, an antibody against PD-1 can be used to target the depletion of these specific cells by Fc-R dependent mechanisms or by ablation by conjugating a cytotoxic drug (e.g., ricin, saporin, or calicheamicin) to the antibody. In one embodiment, the antibody toxin can be a bispecific antibody. Such bispecific antibodies are useful for targeting a specific cell population, e.g., using a marker found only on a certain type of cell, e.g., a TCR, BCR, or FcR molecule.

Downregulating or preventing B7-4 polypeptide costimulatory functions or activating a B7-4 or a PD-1 inhibitory function (e.g., by stimulation of the negative signaling function of PD-1) is useful to downmodulate the immune response, e.g., in situations of tissue, skin and organ transplantation, in graft-versus-host disease (GVHD), or in autoimmune diseases such as systemic lupus erythematosus, and multiple sclerosis. For example, blockage of immune cell function results in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by immune cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits or blocks interaction of a B7 molecule with a costimulatory receptor(s) on immune cells (such as a soluble, monomeric form of a B7-4 or PD-1 polypeptide) alone or in conjunction with another downmodulatory agent prior to or at the time of transplantation can inhibit the generation of a costimulatory signal. Moreover, inhibition of B7-4 costimulatory signals, or promotion of a B7-4 or PD-1 inhibitory signal may also be sufficient to anergize the immune cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by blocking a B7-4 mediated costimulatory signal may avoid the necessity of repeated administration of these blocking reagents.

To achieve sufficient immunosuppression or tolerance in a subject, it may also be desirable to block the costimulatory function of other molecules. For example, it may be desirable to block the function of B7-1 and B7-4, B7-2 and B7-4, or B7-1 and B7-2 and B7-4 by administering a soluble form of a combination of peptides having an activity of each of these antigens or blocking antibodies against these antigens (separately or together in a single composition) prior to or at the time of transplantation. Alternatively, it may be desirable to promote inhibitory activity of B7-4 or PD-1 and

inhibit a costimulatory activity of B7-1 and/or B7-2. Other downmodulatory agents that can be used in connection with the downmodulatory methods of the invention include, for example, agents that transmit an inhibitory signal via CTLA4, soluble forms of CTLA4, antibodies that activate an inhibitory signal via CTLA4, blocking antibodies against other immune cell markers or soluble forms of other receptor ligand pairs (e.g., agents that disrupt the interaction between CD40 and CD40 ligand (e.g., anti CD40 ligand antibodies)), antibodies against cytokines, or immunosuppressive drugs. In another embodiment, a combination of at least two different B7-4 antibodies can be administered to achieve optimal blocking activity.

For example, blocking B7-4 polypeptide costimulation or activating a B7-4 or PD-1 inhibitory function is also be useful in treating autoimmune disease. Many autoimmune disorders are the result of inappropriate activation of immune cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive immune cells may reduce or eliminate disease symptoms. Administration of reagents which block costimulation of immune cells by disrupting receptor:ligand interactions of B7 molecules with costimulatory receptors is useful to inhibit immune cell activation and prevent production of autoantibodies or cytokines which may be involved in the disease process. Additionally, agents that promote an inhibitory function of B7-4 or PD-1 may induce antigen-specific tolerance of autoreactive immune cells which could lead to long-term relief from the disease. The efficacy of reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythematosus in MRL/*lpr/lpr* mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856).

Inhibition of immune cell activation is useful therapeutically in the treatment of allergy and allergic reactions, e.g., by inhibiting IgE production. An agent that promotes a B7-4 or PD-1 inhibitory function can be administered to an allergic subject to inhibit immune cell mediated allergic responses in the subject.

Activating a PD-1 polypeptide may also be useful in treating allergies. Inhibition of B7-4 costimulation of immune cells or stimulation of a B7-4 or PD-1 inhibitory pathway can be accompanied by exposure to allergen in conjunction with appropriate MHC molecules. Allergic reactions can be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, inhibition of immune cell mediated allergic responses locally or systemically by administration of an inhibitory form of an agent that inhibits the interaction of B7-4 with a costimulatory receptor or an agent that promotes an inhibitory function of B7-4 or PD-1.

Inhibition of immune cell activation through blockage of a B7-4 costimulatory activity or stimulation of PD-1 inhibitory activity may also be important therapeutically in viral infections of immune cells. For example, in the acquired immune deficiency syndrome (AIDS), viral replication is stimulated by immune cell activation. Blocking a B7-4/costimulatory receptor interaction or stimulation of B7-4 or PD-1 inhibitory function may result in inhibition of viral replication and thereby ameliorate the course of AIDS. Downregulation of an immune response via stimulation of B7-4 activity or B7-4 interaction with its natural binding partner(s), *e.g*, PD-1, may also be useful in promoting the maintenance of pregnancy. B7-4 is normally highly expressed in placental trophoblasts, the layer of cells that forms the interface between mother and fetus and may play a role in preventing maternal rejection of the fetus. Females at risk for spontaneous abortion (*e.g.*, those identified by screening for B7-4 activity, as described in the "Prognostic Assays" section, those who have previously had a spontaneous abortion or those who have had difficulty conceiving) because of immunologic rejection of the embryo or fetus can be treated with agents that stimulate the activity of B7-4 or its interaction with its natural binding partner(s), *e.g.*, PD-1.

Downregulation of an immune response via stimulation of B7-4 activity or B7-4 interaction with its natural binding partner(s), *e.g.*, PD-1, may also be useful in treating an autoimmune attack of autologous tissues For example, B7-4 is normally highly expressed in the heart and protects the heart from autoimmune attack. This is evidenced by the fact that the Balb/c PD-1 knockout mouse exhibits massive autoimmune attack on the heart with thrombosis. Thus, conditions that are caused or exacerbated by

autoimmune attack (e.g., in this example, heart disease, myocardial infarction or atherosclerosis) may be ameliorated or improved by increasing B7-4 activity or B7-4 biding to its natural binding partner, *e.g.*, PD-1. It is therefore within the scope of the invention to modulate conditions exacerbated by autoimmune attack, such as autoimmune disorders (as well as conditions such as heart disease, myocardial infarction, and atherosclerosi) by stimulating B7-4 activity or B7-4 interaction with B7-4.

4. Upregulation of Immune Responses

Upregulation of B7-4 costimulatory activity or inhibit an inhibitory activity of PD-1 or B7-4 as a means of upregulating immune responses is also useful in therapy. Upregulation of immune responses can be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response through stimulating B7-4 costimulatory activity or inhibition of B7-4 or PD-1 inhibitory activity is useful in cases of infections with microbes, e.g., bacteria, viruses, or parasites. For example, in one embodiment, a form of B7-4 that promotes a costimulatory signal in an immune cell (e.g., a B7-4 peptide in a multi-valent form (e.g., a soluble multivalent form or a form expressed on a cell surface)) or an agent that inhibits the interaction of B7-4 with an inhibitory receptor or an agent that inhibits transduction of an inhibitory signal via PD-1, e.g., a non-activating antibody against PD-1, is therapeutically useful in situations where upregulation of antibody and cell-mediated responses, resulting in more rapid or thorough clearance of virus, would be beneficial. These would include viral skin diseases such as Herpes or shingles, in which case such an agent can be delivered topically to the skin. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the administration of such agents systemically.

In certain instances, it may be desirable to further administer other agents that upregulate immune responses, for example, forms other B7 family members that transduce signals via costimulatory receptors, in order further augment the immune response.

Alternatively, immune responses can be enhanced in an infected patient by removing immune cells from the patient, contacting immune cells *in vitro* with a form of B7-4 that promotes a costimulatory signal in an immune cell or an agent that inhibits the interaction of B7-4 with an inhibitory receptor, or an agent that inhibits transduction of an inhibitory signal via PD-1, and reintroducing the *in vitro* stimulated immune cells into the patient. In another embodiment, a method of enhancing immune responses involves isolating infected cells from a patient, e.g., virally infected cells, transfecting them with a nucleic acid molecule encoding a form of B7-4 that binds to a costimulatory receptor such that the cells express all or a portion of the B7-4 molecule on their surface, and reintroducing the transfected cells into the patient. The transfected cells are capable of delivering a costimulatory signal to, and thereby activate, immune cells *in vivo*.

Forms of B7-4 that promote a costimulatory signal in an immune cell, or an agent that inhibits the interaction of B7-4 with an inhibitory receptor, or an agent that inhibits transduction of an inhibitory signal via PD-1 can be used prophylactically in vaccines against various polypeptides, e.g., polypeptides derived from pathogens. Immunity against a pathogen, e.g., a virus, can be induced by vaccinating with a viral protein along with a form of B7-4 that promotes a costimulatory signal in an immune cell, or an agent that inhibits the interaction of B7-4 with an inhibitory receptor, or an agent that inhibits transduction of an inhibitory signal via PD-1 in an appropriate adjuvant. Alternately, a vector comprising genes which encode for both a pathogenic antigen and a form of B7-4 that binds to costimulatory receptors can be used for vaccination. Nucleic acid vaccines can be administered by a variety of means, for example, by injection (e.g., intramuscular, intradermal, or the biolistic injection of DNA-coated gold particles into the epidermis with a gene gun that uses a particle accelerator or a compressed gas to inject the particles into the skin (Haynes et al. 1996. *J. Biotechnol.* 44:37)). Alternatively, nucleic acid vaccines can be administered by non-invasive means. For example, pure or lipid-formulated DNA can be delivered to the respiratory system or targeted elsewhere, e.g., Peyers patches by oral delivery of DNA (Schubbert. 1997. *Proc. Natl. Acad. Sci. USA* 94:961). Attenuated microorganisms can be used for delivery to mucosal surfaces. (Sizemore et al. 1995. *Science.* 270:29)

In one embodiment, a form of a B7-4 polypeptide which transmits a costimulatory signal can be administered with class I MHC proteins by, for example, a cell transfected to coexpress a B7-4 polypeptide and MHC class I α chain protein and β_2 microglobulin to result in activation of T cells and provide immunity from infection. For example, pathogens for which vaccines are useful include hepatitis B, hepatitis C, Epstein-Barr virus, cytomegalovirus, HIV-1, HIV-2, tuberculosis, malaria and schistosomiasis.

In another application, upregulation or enhancement of a B7-4 costimulatory function is useful in the induction of tumor immunity. Tumor cells (e.g., sarcoma, melanoma, lymphoma, leukemia, neuroblastoma, carcinoma) transfected with a nucleic acid molecule encoding a B7-4 antigen can be administered to a subject to overcome tumor-specific tolerance in the subject. If desired, the tumor cell can be transfected to express a combination of B7 polypeptides (e.g., B7-1, B7-2, B7-4). For example, tumor cells obtained from a patient can be transfected *ex vivo* with an expression vector directing the expression of a B7-4 polypeptide alone, or in conjunction with a peptide having B7-1 activity and/or B7-2 activity. The transfected tumor cells are returned to the patient to result in expression of the peptides on the surface of the transfected cell. Alternatively, gene therapy techniques can be used to target a tumor cell for transfection *in vivo*.

In addition, tumor cells which lack MHC class I or MHC class II molecules, or which fail to express sufficient amounts of MHC class I or MHC class II molecules, can be transfected with nucleic acid encoding all or a portion of (e.g., a cytoplasmic-domain truncated portion) of an MHC class I α chain protein and β_2 microglobulin protein or an MHC class II α chain protein and an MHC class II β chain protein to thereby express MHC class I or MHC class II proteins on the cell surface. Expression of the appropriate class I or class II MHC in conjunction with a peptide having the activity of a B lymphocyte antigen (e.g., B7-1, B7-2, B7-4) induces a T cell mediated immune response against the transfected tumor cell. Optionally, a gene encoding an antisense construct which blocks expression of an MHC class II associated protein, such as the invariant chain, can also be cotransfected with a DNA encoding a B7-4 polypeptide to promote

presentation of tumor associated antigens and induce tumor specific immunity. Expression of B7-1 by B7 negative murine tumor cells has been shown to induce T cell mediated specific immunity accompanied by tumor rejection and prolonged protection to tumor challenge in mice (Chen, L., et al. (1992) *Cell* 71, 1093-1102; Townsend, S. E. and Allison, J. P. (1993) *Science* 259, 368-370; Baskar, S., et al. (1993) *Proc. Natl. Acad. Sci.* 90, 5687-5690). Thus, the induction of a immune cell mediated immune response in a human subject can be sufficient to overcome tumor-specific tolerance in the subject.

In another embodiment, the immune response can be stimulated by the transmission of a signal via a costimulatory receptor that binds to B7-4 or by the inhibiting signaling via an inhibitory receptor that binds to B7-4, e.g.,PD-1, such that preexisting tolerance is overcome. For example, immune responses against antigens to which a subject cannot mount a significant immune response, e.g., to an autologous antigen, such as a tumor specific antigens can be induced by administering an agent that inhibits the inhibitory activity of PD-1 or the ability of B7-4 to bind to an inhibitory ligand. For example, in one embodiment, soluble PD-1 or soluble B7-4 can be used (e.g., PD-1Fc or B7-4 Fc) to enhance an immune response, e.g., to a tumor cell. In one embodiment, an autologous antigen, such as a tumor-specific antigen can be coadministered with an agent that inhibits the inhibitory activity of PD-1 or the ability of B7-4 to bind to an inhibitory ligand. In another embodiment, an immune response can be stimulated against an antigen (e.g., an autologous antigen) to treat a neurological disorder. In another embodiment, PD-1 antagonists can be used as adjuvants to boost responses to foreign antigens in the process of active immunization.

In yet another embodiment, the production of a form of B7-4 that binds to an inhibitory receptor or that competes with the binding of B7-4 to a costimulatory receptor (e.g., a form of B7-4 that binds to PD-1 or a naturally occurring soluble molecule) can be inhibited, e.g., using antisense RNA, in order to upregulate the immune response. For example, in one embodiment, the production of inhibitory B7-4 molecules by a tumor cell can be inhibited in order to increase anti-tumor immunity.

In one embodiment, immune cells are obtained from a subject and cultured *ex vivo* to in the presence of a form of B7-4 that binds a costimulatory molecule or in the

presence of an agent that that inhibits a B7-4 or PD-1 inhibitory signal, to expand the population of immune cells. In a further embodiment the immune cells are then administered to a subject. Immune cells can be stimulated to proliferate *in vitro* by, for example, providing to the immune cells a primary activation signal and a costimulatory signal, as is known in the art. Various forms of B7-4 proteins or agents that bind a costimulatory receptor or that inhibit signaling via PD-1 can also be used to costimulate proliferation of immune cells. In one embodiment immune cells are cultured *ex vivo* according to the method described in PCT Application No. WO 94/29436. The costimulatory molecule can be soluble, attached to a cell membrane or attached to a solid surface, such as a bead.

B. Identification of Cytokines Modulated by Modulation of B7-4 and/or PD-1

The B7-4 and PD-1 molecules described herein can be used to identify cytokines which are produced by or whose production is enhanced or inhibited in immune cells in response to modulation of B7-4 and/or PD-1 activity. Immune cells expressing PD-1 can be suboptimally stimulated *in vitro* with a primary activation signal, for example, T cells can be stimulated with phorbol ester, anti-CD3 antibody or preferably antigen in association with an MHC class II molecule, and given a costimulatory signal, e.g., by a stimulatory form of B7 family antigen, for instance by a cell transfected with nucleic acid encoding a B7 polypeptide and expressing the peptide on its surface or by a soluble, stimulatory form of the peptide. Known cytokines released into the media can be identified by ELISA or by the ability of an antibody which blocks the cytokine to inhibit immune cell proliferation or proliferation of other cell types that is induced by the cytokine. For example, an IL-4 ELISA kit is available from Genzyme (Cambridge MA), as is an IL-7 blocking antibody. Blocking antibodies against IL-9 and IL-12 are available from Genetics Institute (Cambridge, MA). The effect of stimulating or blocking the interaction of B7-4 with PD-1 on the cytokine profile can then be determined.

An *in vitro* immune cell costimulation assay as described above can also be used in a method for identifying novel cytokines which can be modulated by modulation of B7-4 and or PD-1. For example, where stimulation of the CD28/CTLA4 pathway seems

to enhance IL-2 secretion, stimulation of the ICOS pathway seems to enhance IL-10 secretion (Hutloff et al. 199. Nature 397:263). If a particular activity induced upon costimulation, e.g., immune cell proliferation, cannot be inhibited by addition of blocking antibodies to known cytokines, the activity may result from the action of an unknown cytokine. Following costimulation, this cytokine can be purified from the media by conventional methods and its activity measured by its ability to induce immune cell proliferation.

To identify cytokines which may play a role the induction of tolerance, an *in vitro* T cell costimulation assay as described above can be used. In this case, T cells would be given the primary activation signal and contacted with a selected cytokine, but would not be given the costimulatory signal. After washing and resting the immune cells, the cells would be rechallenged with both a primary activation signal and a costimulatory signal. If the immune cells do not respond (e.g., proliferate or produce cytokines) they have become tolerized and the cytokine has not prevented the induction of tolerance. However, if the immune cells respond, induction of tolerance has been prevented by the cytokine. Those cytokines which are capable of preventing the induction of tolerance can be targeted for blockage *in vivo* in conjunction with reagents which block B lymphocyte antigens as a more efficient means to induce tolerance in transplant recipients or subjects with autoimmune diseases. For example, one could administer a cytokine blocking antibody to a subject along with an agent that promotes a B7-4 or a PD-1 inhibitory activity.

C. Identification of Molecules which Modulate Expression of a B7-4 or PD-1 Polypeptide

The antibodies produced using the proteins and peptides of the current invention can be used in a screening assay for molecules which modulate the expression of B7-4 or PD-1 polypeptide on cells. For example, molecules which modulate intracellular signaling pathways that culminate in changes in expression of B7-4 or PD-1 polypeptides (*e.g.*, in response to activation signals), can be identified by assaying expression of one or more B7-4 or PD-1 polypeptides on the cell surface. Reduced immunofluorescent staining by an appropriate antibody in the presence of the molecule

would indicate that the molecule inhibits intracellular signals. Molecules which upregulate B7-4 or PD-1 polypeptide expression result in an increased immunofluorescent staining. Alternatively, the effect of a molecule on expression of a polypeptide can be determined by detecting cellular mRNA levels using a probe of the invention. For example, a cell which expresses a B7-4 or PD-1 polypeptide can be contacted with a molecule to be tested, and an increase or decrease in mRNA levels in the cell detected by standard techniques, such as Northern hybridization analysis or conventional dot blot of mRNA or total poly(A^+)RNAs using a cDNA probe labeled with a detectable marker. Molecules which modulate expression of a B7-4 or PD-1 polypeptide are useful therapeutically for either upregulating or downregulating immune responses alone or in conjunction with soluble blocking or stimulating reagents as described above. For instance, a molecule which inhibits expression of B7-4 can be administered together with a second agent, *e.g.*, an immunosuppressant or a molecule which inhibits expression of PD-1 can be given with an immunostimulant, *e.g.*, an adjuvant. Exemplary molecules which can be tested for their ability to modulate B7-4 or PD-1 include cytokines such as IL-4, γINF, IL-10, IL-12, GM-CSF and prostagladins.

D. Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) which bind to B7-4 or PD-1 proteins, have a stimulatory or inhibitory effect on, for example, B7-4 or PD-1 expression or B7-4 or PD-1 activity.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a B7-4 or PD-1 protein or polypeptide or biologically active portion thereof, *e.g.*, modulate the ability of B7-4 or PD-1 polypeptide to interact with its cognate binding partner or an interactor molecule (*e.g.*, an intracellular interactor molecule). The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the

'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K. S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a B7-4 target molecule (an intracellular interactor molecule or a PD-1 receptor) or PD-1 target molecule (*e.g.*, a B7-4 ligand or intracellular interactor molecule) with a test compound and determining the ability of the test compound to modulate (*e.g.* stimulate or inhibit) the activity of the B7-4 or PD-1 target molecule. Determining the ability of the test compound to modulate the activity of a B7-4 or PD-1 target molecule can be accomplished, for example, by determining the ability of the B7-4 or PD-1 protein to bind to or interact with the B7-4 or PD-1 target molecule. Determining the ability of the B7-4 or PD-1 protein to bind to or interact with its binding partner can be accomplished, *e.g.*, by measuring direct binding.

In a direct binding assay, the B7-4 or PD-1 protein (or their respective target molecules) can be coupled with a radioisotope or enzymatic label such that binding of the B7-4 or PD-1 protein to a B7-4 or PD-1 target molecule can be determined by

detecting the labeled protein in a complex. For example, B7-4 or PD-1 molecules, *e.g.*, B7-4 or PD-1 proteins, can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, B7-4 or PD-1 molecules can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a compound to modulate the interaction between B7-4 or PD-1 and its target molecule, without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of B7-4 or PD-1 with its target molecule without the labeling of either B7-4 or PD-1 or the target molecule (McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between compound and receptor.

In a preferred embodiment, determining the ability of the B7-4 or PD-1 protein to bind to or interact with a B7-4 or PD-1 target molecule can be accomplished by determining the activity of the B7-4, PD-1 or the appropriate target molecule. For example, the activity of B7-4, PD-1 or the appropriate target molecule can be determined by detecting induction of a cellular second messenger (*e.g.*, tyrosine kinase activity), detecting catalytic/enzymatic activity of an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, chloramphenicol acetyl transferase), or detecting a cellular response regulated by B7-4, PD-1 or the appropriate target molecule. For example, determining the ability of the B7-4 or PD-1 protein to bind to or interact with a B7-4 or PD-1 target molecule can be accomplished, for example, by measuring the ability of a compound to modulate immune cell costimulation or inhibition in a proliferation assay, or by interfering with the ability of a B7-4 or PD-1 polypeptide to bind to antibodies that recognize a portion of the B7-4 or PD-1 polypeptide.

In yet another embodiment, an assay of the present invention is a cell-free assay in which a B7-4 or PD-1 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the B7-4 or PD-1 protein or biologically active portion thereof is determined. Binding of the test compound to the B7-4 or PD-1 protein can be determined either directly or indirectly as described above. In a preferred embodiment, the assay includes contacting the B7-4 or PD-1 protein or biologically active portion thereof with a known compound which binds B7-4 or PD-1 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a B7-4 or PD-1 protein, wherein determining the ability of the test compound to interact with a B7-4 or PD-1 protein comprises determining the ability of the test compound to preferentially bind to B7-4 or PD-1 polypeptide or biologically active portion thereof as compared to the known compound.

In another embodiment, the assay is a cell-free assay in which a B7-4 or PD-1 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the B7-4 or PD-1 protein or biologically active portion thereof is determined. Determining the ability of the test compound to modulate the activity of a B7-4 or PD-1 protein can be accomplished, for example, by determining the ability of the B7-4 or PD-1 protein to bind to a B7-4 or PD-1 target molecule by one of the methods described above for determining direct binding. Determining the ability of the B7-4 or PD-1 protein to bind to a B7-4 or PD-1 target molecule can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another embodiment, the cell-free assay involves contacting a B7-4 or PD-1 protein or biologically active portion thereof with a known compound which binds the B7-4 or PD-1 protein to form an assay mixture, contacting the assay mixture with a test

compound, and determining the ability of the test compound to interact with the B7-4 or PD-1 protein, wherein determining the ability of the test compound to interact with the B7-4 or PD-1 protein comprises determining the ability of the B7-4 or PD-1 protein to preferentially bind to or modulate the activity of a B7-4 or PD-1 target molecule.

The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of proteins (*e.g.*, B7-4 or PD-1 proteins or biologically active portions thereof, or binding partners to which B7-4 or PD-1 binds). In the case of cell-free assays in which a membrane-bound form a protein is used (*e.g.*, a cell surface B7-4 or PD-1 receptor) it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either B7-4or PD-1 or an appropriate target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a B7-4 or PD-1 protein, or interaction of a B7-4 or PD-1 protein with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/B7-4 or PD-1 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or B7-4 or PD-1 protein, and the mixture incubated under conditions conducive to complex

formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of B7-4 or PD-1 binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either a B7-4 or PD-1 protein or a B7-4 or PD-1 target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated B7-4 or PD-1 protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with B7-4 or PD-1 protein or target molecules but which do not interfere with binding of the B7-4 or PD-1 protein to its target molecule can be derivatized to the wells of the plate, and unbound target or B7-4 or PD-1 protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the B7-4 or PD-1 protein or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the B7-4 or PD-1 protein or target molecule.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of a B7-4 or PD-1 protein can be accomplished by determining the ability of the test compound to modulate the activity of a molecule that functions downstream of B7-4, *e.g.*, a molecule that interacts with B7-4, or a molecule that functions downstream of PD-1, *e.g.*, by interacting with the cytoplasmic domain of PD-1. For example, levels of second messengers can be determined, the activity of the interactor molecule on an appropriate target can be determined, or the binding of the interactor to an appropriate target can be determined as previously described.

In another embodiment, modulators of B7-4 or PD-1 expression are identified in a method wherein a cell is contacted with a candidate compound and the expression of

B7-4 or PD-1 mRNA or protein in the cell is determined. The level of expression of B7-4 or PD-1 mRNA or protein in the presence of the candidate compound is compared to the level of expression of B7-4 or PD-1 mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of B7-4 or PD-1 expression based on this comparison. For example, when expression of B7-4 or PD-1 mRNA or protein is greater (*e.g.*, statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of B7-4 or PD-1 mRNA or protein expression. Alternatively, when expression of B7-4 or PD-1 mRNA or protein is less (*e.g.*, statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of B7-4 or PD-1 mRNA or protein expression. The level of B7-4 or PD-1 mRNA or protein expression in the cells can be determined by methods described herein for detecting B7-4 or PD-1 mRNA or protein.

In yet another aspect of the invention, the B7-4 or PD-1 proteins, preferably in membrane bound form, can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins ("B7-4or PD-1 binding proteins" or "B7-4 or PD-1 bp"), which bind to or interact with B7-4 or PD-1 and are involved in B7-4 or PD-1 activity. Such B7-4-or PD-1 binding proteins are also likely to be involved in the propagation of signals by the B7-4 or PD-1 proteins or B7-4 or PD-1 targets as, for example, upstream or downstream elements of a B7-4 or PD-1 mediated signaling pathway. Alternatively, such B7-4 or PD-1 binding proteins may be B7-4 or PD-1 inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a B7-4 or PD-1 protein is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is

fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming a B7-4-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the B7-4 or PD-1 protein.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a B7-4 or PD-1 modulating agent, an antisense B7-4 or PD-1 nucleic acid molecule, a B7-4-or PD-1 specific antibody, or a B7-4 or PD-1 binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

F. Detection Assays

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

1. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is

called chromosome mapping. Accordingly, portions or fragments of B7-4 nucleotide sequences, described herein, can be used to map the location of B7-4 genes on a chromosome. The mapping of B7-4 sequences to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, B7-4 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from B7-4 nucleotide sequences. Computer analysis of B7-4 sequences can be used to predict primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to B7-4 sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (*e.g.*, human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio, P. *et al.* (1983) *Science* 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using B7-4 nucleotide sequences to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a sequence to its chromosome include *in situ* hybridization (described in Fan, Y. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6223-27), pre-screening with

labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical such as colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in McKusick, V., Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between a gene and a disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. *et al.* (1987) *Nature* 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the B7-4 gene can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected

individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

2. Tissue Typing

The B7-4 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the B7-4 nucleotide sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The B7-4 nucleotide sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of

about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1 or 3 can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences are used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from B7-4 nucleotide sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

3. Use of Partial B7-4 Sequences in Forensic Biology

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, *e.g.*, hair or skin, or body fluids, *e.g.*, blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (*i.e.* another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding

regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the B7-4 nucleotide sequences or portions thereof having a length of at least 20 bases, preferably at least 30 bases.

The B7-4 nucleotide sequences described herein can further be used to provide polynucleotide reagents, *e.g.*, labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such B7-4 probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, *e.g.*, B7-4 primers or probes can be used to screen tissue culture for contamination (*i.e.* screen for the presence of a mixture of different types of cells in a culture).

G. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining B7-4 or PD-1 protein and/or nucleic acid expression as well as B7-4 or PD-1 activity, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant B7-4 or PD-1 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with B7-4 or PD-1 protein, nucleic acid expression or activity. For example, mutations in a B7-4 or PD-1 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with B7-4 or PD-1 protein, nucleic acid expression or activity. The assays described herein, such as the preceding diagnostic assays or the following assays, can also be used to detect a tendency to have spontaneous abortions.

Another aspect of the invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of B7-4 or PD-1 in clinical trials.

These and other agents are described in further detail in the following sections.

1. Diagnostic Assays

An exemplary method for detecting the presence or absence of B7-4 or PD-1 protein or nucleic acid in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting B7-4 or PD-1 protein or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes B7-4 or PD-1 protein such that the presence of B7-4 or PD-1 protein or nucleic acid is detected in the biological sample. A preferred agent for detecting B7-4 or PD-1 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to B7-4 or PD-1 mRNA or genomic DNA. The nucleic acid probe can be, for example, a human B7-4 or PD-1 nucleic acid, such as the nucleic acid of SEQ ID NO:1, 3, 10, or 11 or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to B7-4 or PD-1 mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

A preferred agent for detecting B7-4 or PD-1 protein is an antibody capable of binding to B7-4 or PD-1 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or $F(ab')_2$) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect B7-4 or PD-1 mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example,

in vitro techniques for detection of B7-4 or PD-1 mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of B7-4 or PD-1 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of B7-4 or PD-1 genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of B7-4 or PD-1 protein include introducing into a subject a labeled anti-B7-4 or PD-1 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is a serum sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting B7-4 or PD-1 protein, mRNA, or genomic DNA, such that the presence of B7-4 or PD-1 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of B7-4 or PD-1 protein, mRNA or genomic DNA in the control sample with the presence of B7-4 or PD-1 protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of B7-4 or PD-1 in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting B7-4 or PD-1 protein or mRNA in a biological sample; means for determining the amount of B7-4 or PD-1 in the sample; and means for comparing the amount of B7-4 or PD-1 in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect B7-4 or PD-1 protein or nucleic acid.

2. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant

B7-4 or PD-1 expression or activity. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with B7-4 or PD-1 protein, expression or activity. Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity in which a test sample is obtained from a subject and B7-4 or PD-1 protein or nucleic acid (*e.g.*, mRNA, genomic DNA) is detected, wherein the presence of B7-4 or PD-1 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant B7-4 or PD-1 expression or activity. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant B7-4 or PD-1 expression or activity in which a test sample is obtained and B7-4 or PD-1 protein or nucleic acid expression or activity is detected (*e.g.*, wherein the abundance of B7-4 or PD-1 protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant B7-4 or PD-1 expression or activity).

The methods of the invention can also be used to detect genetic alterations in a B7-4 or PD-1 gene, thereby determining if a subject with the altered gene is at risk for a disorder associated with the B7-4 or PD-1 gene. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding a B7-4 or PD-1 protein, or the mis-expression of the B7-4 or PD-1 gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a B7-4 or PD-1 gene; 2) an addition of one or more nucleotides to a B7-4 or PD-1 gene; 3) a substitution of one or

more nucleotides of a B7-4 or PD-1 gene, 4) a chromosomal rearrangement of a B7-4 or PD-1 gene; 5) an alteration in the level of a messenger RNA transcript of a B7-4 or PD-1 gene, 6) aberrant modification of a B7-4 or PD-1 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a B7-4 or PD-1 gene, 8) a non-wild type level of a B7-4 or PD-1 protein, 9) allelic loss of a B7-4 or PD-1 gene, and 10) inappropriate post-translational modification of a B7-4 or PD-1 protein. As described herein, there are a large number of assay techniques known in the art which can be used for detecting alterations in a B7-4 or PD-1 gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject, *e.g.*, a cardiac tissue sample.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the B7-4 or PD-1 gene (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a B7-4 or PD-1 gene under conditions such that hybridization and amplification of the B7-4 or PD-1 gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. *et al.* (1988) *Biotechnology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified

molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a B7-4 or PD-1 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in B7-4 or PD-1 can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M. T. *et al.* (1996) *Hum. Mutat.* 7:244-255; Kozal, M. J. *et al.* (1996) *Nat. Med.* 2:753-759). For example, genetic mutations in B7-4 or PD-1 can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M. T. *et al.* (1996) *supra.* Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the B7-4 or PD-1 gene and detect mutations by comparing the sequence of the sample B7-4 or PD-1 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) *Proc. Natl. Acad. Sci. USA* 74:560)

or Sanger ((1977) *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the B7-4 or PD-1 gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type B7-4 or PD-1 sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in B7-4 or PD-1 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a B7-4 sequence, *e.g.*, a wild-type B7-4 or PD-1 sequence, is hybridized to a cDNA

or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility can be used to identify mutations in B7-4 or PD-1 genes. For example, single strand conformation polymorphism (SSCP) can be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci. USA*: 86:2766, see also Cotton (1993) *Mutat. Res.* 285:125-144; and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control B7-4 or PD-1 nucleic acids can be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments can be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet.* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA can be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys. Chem.* 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature*

324:163); Saiki *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification can be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner *et al.* (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci. USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein can be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which can be conveniently used, *e.g.,* in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a B7-4 or PD-1 gene.

Furthermore, any cell type or tissue in which B7-4 or PD-1 is expressed can be utilized in the prognostic assays described herein.

VII. Administration of B7-4 or PD-1 Modulating Agents

B7-4 or PD-1 modulating agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to either enhance or suppress immune cell mediated immune responses. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be

administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of an agent as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a B7-4 or PD-1 polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered daily or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The B7-4 or PD-1 modulating agent (*e.g.*, a peptide, a nucleic acid molecule, antibody, peptidomimetic, or small molecule) can be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound can be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. For example, to administer B7-4 or PD-1 modulating agent by other than parenteral administration, it may be desirable to coat the peptide with, or co-administer the peptide with, a material to prevent its inactivation.

A B7-4 or PD-1 modulating agent can be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor,