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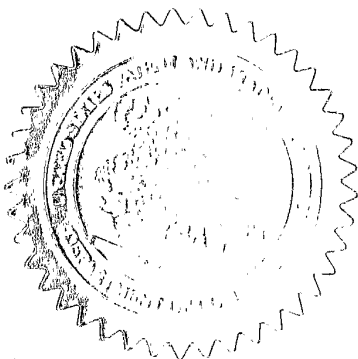
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APPLICATION NUMBER: 60/738,434

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).

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112105

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Alan J.	Korman	Piedmont, California
Mark J.	Selby	San Francisco, California
Josephine M.	Cardarelli	San Carlos, California
Bing	Chen	Milpitas, 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**

☒ The address corresponding to Customer Number: 07278

OR

☐ Firm or Individual Name **DARBY & DARBY P.C.**

Address **P.O. Box 5257**

City **New York** State **NY** Zip **10150-5257**

Country **US** Telephone **(206) 262-8900** Email _____

ENCLOSED APPLICATION PARTS (check all that apply)

☒ Application Data Sheet. See 37 CFR 1.76 ☐ CD(s), Number of CDs _____

☒ Specification Number of Pages **47** ☒ Other (specify) Fee Transmittal (1 page)
Certificate of Express Mailing (1 page)
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☒ Drawing(s) Number of Sheets **33**

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.

☒ A check or money order is enclosed to cover the filing fee and application size fee (if applicable). TOTAL FEE
AMOUNT (\$) **200.00**

☐ Payment by credit card. Form PTO-2038 is attached.

☒ 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.

☒ 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. PepeREGISTRATION
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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PTO/SB/17 (12-04v2)
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FEE TRANSMITTAL

For FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT		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)

☐ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, except for the filing fee

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES		SEARCH FEES		EXAMINATION FEES		Fees Paid (\$)
	Fee (\$)	Small Entity Fee (\$)	Fee (\$)	Small Entity Fee (\$)	Fee (\$)	Small Entity Fee (\$)	
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 _____ = _____

Multiple Dependent Claims

Fee (\$) **Fee Paid (\$)**

_____ - = _____ x _____ = _____

Indep. Claims **Extra Claims** **Fee (\$)** **Fee Paid (\$)**

_____ - = _____ x _____ = _____

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)

Non-English Specification, \$130 fee (no small entity)

Other (e.g., late filing surcharge): _____

Feas Paid (\$)

SUBMITTED BY

Signature		Registration No.	46,985
Name (Print/Type)	Jeffrey C. Pepe	Telephone	(206) 262-8911
		Date	November 21, 2005

{S:\04280\0203401-us0\80044016.DOC (REVISED 06/29/2006) }

Application No. (if known): Not Yet Assigned

Attorney Docket No.: 04280/0203401-US0

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P.O. Box 1450
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Signature

Jeffrey C. Pepe

Typed or printed name of person signing Certificate

46,985

Registration Number, if applicable

(206) 262-8911

Telephone Number

Note: Each paper must have its own certificate of mailing, or this certificate must identify each submitted paper.

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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)
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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

Applicant Information

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Alan
Middle Name:: J.
Family Name:: Korman
City of Residence:: Piedmont
State or Province of Residence:: CA
Country of Residence:: US
Street of mailing address:: 707 State Road
City of mailing address:: Princeton
State or Province of mailing address:: NJ
Postal or Zip Code of mailing address:: 08540

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Mark
Middle Name:: J.
Family Name:: Selby
City of Residence:: San Francisco
State or Province of Residence:: CA
Country of Residence:: US
Street of mailing address:: 136 Galewood Circle
City of mailing address:: San Francisco
State or Province of mailing address:: CA
Postal or Zip Code of mailing address:: 94131

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Josephine
Middle Name:: M.
Family Name:: Cardarelli
City of Residence:: San Carlos
State or Province of Residence:: CA
Country of Residence:: US
Street of mailing address:: 126 Leslie Drive
City of mailing address:: San Carlos
State or Province of mailing address:: CA
Postal or Zip Code of mailing address:: 94070

Applicant Authority Type:: Inventor
Status:: Full Capacity
Given Name:: Bing
Family Name:: Chen
Country of Residence:: US
Street of mailing address:: 521 Cottonwood Drive
City of mailing address:: Milpitas
State or Province of mailing address:: CA
Postal or Zip Code of mailing address:: 95035

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Tseng-Hui
Middle Name:: Timothy
Family Name:: Chen
City of Residence:: Burlingame
State or Province of Residence:: CA
Country of Residence:: US
Street of mailing address:: 8 Davis Court
City of mailing address:: Burlingame
State or Province of mailing address:: CA
Postal or Zip Code of mailing address:: 94010

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Changyu
Family Name:: Wang
City of Residence:: Fremont
State or Province of Residence:: CA
Country of Residence:: US
Street of mailing address:: 3803 Darwin Drive, Apt. 203
City of mailing address:: Fremont
State or Province of mailing address:: CA
Postal or Zip Code of mailing address:: 94555

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Haichun
Family Name:: Huang
City of Residence:: Fremont
State or Province of Residence:: CA
Country of Residence:: US
Street of mailing address:: 4329 Crestwood Street
City of mailing address:: Fremont
State or Province of mailing address:: CA
Postal or Zip Code of mailing address:: 94538

Correspondence Information

Correspondence Customer Number:: 07278

Representative Information

Representative Customer Number:: 07278

Assignee Information

Assignee name:: Medarex, Inc.
Street of mailing address:: 707 State Road
City of mailing address:: Princeton
State or Province of mailing address:: NJ
Postal or Zip Code of mailing address:: 08540

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

TECHNICAL FIELD

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

BACKGROUND

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

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

studies. U.S. Patent No. 5,855,887 discloses a method of increasing the response of a mammalian T cell to antigenic stimulation by combining a T cell with a CTLA-4 blocking agent. U.S. Patent No. 5,811,097 discloses a method of decreasing the growth of non-T cell tumors by administering a CTLA-4 blocking agent. U.S. Patent Application Nos. 09/644,668 and 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.

Figures 4A to 4D show the tumor volume over time in individual mice that were implanted with MC38 colon tumor cells (PD-L1⁻) and one week later treated with one of the following therapies: (A) mouse IgG (control), (B) anti-CTLA-4 antibody, (C) anti-PD-1 antibody, and (D) anti-CTLA-4 antibody and anti-PD-1 antibody. The tumor volume on the first day of treatment was about 315 mm³. The mice received subsequent antibody treatments on days 3, 6 and 10 as described in Example 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, X₂₀) (B) anti-PD-1 antibody (10 mg/kg) and mouse IgG (10 mg/kg) (P₁₀X₁₀), (C) anti-CTLA-4 antibody (10 mg/kg) and mouse IgG (10 mg/kg) (C₁₀X₁₀), (D) anti-CTLA-4 antibody and anti-PD-1 antibody (10 mg/kg each) (C₁₀P₁₀), (E) anti-CTLA-4 antibody and anti-PD-1 antibody (3 mg/kg each) (C₃P₃), and (F) anti-CTLA-4 antibody and anti-PD-1 antibody (1 mg/kg each) (C₁P₁). Two groups of mice were treated with each antibody sequentially as follows: (G) anti-CTLA-4 antibody (10 mg/kg, day 0), anti-CTLA-4 antibody (10 mg/kg, day 3), anti-PD-1 antibody (10 mg/kg, day 6), and anti-PD-1 antibody (10 mg/kg, day 10) (C₁₀C₁₀P₁₀P₁₀); and (H) anti-PD-1 antibody (10 mg/kg, day 0), anti-PD-1 antibody (10 mg/kg, day 3), anti-CTLA-4 antibody (10 mg/kg, day 6), and anti-CTLA-4 antibody (10 mg/kg, day 10) (P₁₀P₁₀C₁₀C₁₀).

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

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

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

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

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

Figures 13A to 13J show the tumor volume over time in individual mice that were implanted with SA1/N fibrosarcoma cells (PD-L1⁺) and then treated on days 7, 10, 13 and 17 post-implantation (as described in Example 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 mm³.

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

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

Figures 16A and 16B show the tumor volume over time in individual mice that were implanted with SA1/N fibrosarcoma cells (PD-L1⁺) and then treated on days 10, 13, 16 and 19 post-implantation (as described in Example 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 mm³.

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

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

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

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

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

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

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

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

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

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

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

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

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
20 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
25 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
30 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.

5 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
10 (*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
15 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
20 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).

25 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.

30 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.

5 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.

10 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.

15 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.

20 The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1} , C_{H2} and C_{H3} . Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L . The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding

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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 (C1q) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., PD-1). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L , V_H , C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H , are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

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

The term "human antibody", as used herein, is intended to include antibodies having 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.

5 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
10 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
15 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
20 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
25 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.

30 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 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

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.

5 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
10 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
15 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
20 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
25 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
30 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-MouseTM", 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

5 reduced expression of mouse IgM or κ , and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal (Lonberg *et al.* (1994), *supra*; reviewed in Lonberg (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg and Huszar (1995) *Intern. Rev. Immunol.* Vol. 13: 65-93, and Harding and Lonberg (1995) *Ann. N.Y. Acad. Sci.* 764:536-546).

10 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*

15 *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

20 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.

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

30 Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CTLA-4 or anti-PD-1 antibodies of the

instant disclosure. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomal animal systems expressing human
5 immunoglobulin genes are available in the art and can be used to raise anti-CTLA-4 or anti-PD-1 antibodies of the instant disclosure. For example, mice carrying both a human heavy chain transchromosome and a human light chain transchromosome, referred to as "TC mice" can be used; such mice are described in Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been
10 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
15 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
20 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
25 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.
30 WO 01/14424 and WO 00/37504; and in U.S. Patent Publication No. 2002/0039581. Each of these references is specifically incorporated herein by reference for purposes of description of

anti-CTLA-4 antibodies. An exemplary clinical anti-CTLA-4 antibody is human monoclonal antibody 10D1 as disclosed in WO 01/14424 and U.S. Patent Application No. 09/644,668.

Antibody 10D1 has been administered in single and multiple doses, alone or in combination with a vaccine, chemotherapy, or interleukin-2 to more than 500 patients diagnosed with metastatic melanoma, prostate cancer, lymphoma, renal cell cancer, breast cancer, ovarian cancer, and HIV.

Other anti-CTLA-4 antibodies encompassed by the methods of the present invention include, for example, those disclosed in: WO 98/42752; WO 00/37504; U.S. Patent No. 6,207,156; Hurwitz *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95(17):10067-10071; Camacho *et al.* (2004) *J. Clin. Oncology* 22(145): Abstract No. 2505 (antibody CP-675206); and Mokyr *et al.* (1998) *Cancer Res.* 58:5301-5304. In certain embodiments, the methods of the instant invention comprise use

of an anti-CTLA-4 antibody that is a human sequence antibody, preferably a monoclonal antibody and in another embodiment is monoclonal antibody 10D1.

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

Anti-PD-1 antibodies useful in the instant invention may be characterized by particular functional features or properties. For example, the antibodies may bind specifically to PD-1 (e.g., bind to human PD-1 or may cross-react with PD-1 from other species). In certain

embodiments, an antibody that binds to PD-1 may be by high affinity, for example, with a K_D of 1×10^{-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.

5 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
10 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,
15 antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin),
anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*,
20 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
25 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
30 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, Iodine¹³¹, Indium¹¹¹, Yttrium⁹⁰ and Lutetium¹⁷⁷. Methods for preparing radioimmunoconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including ZevalinTM (IDEC Pharmaceuticals) and BexxarTM (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

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.

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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
10 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.

15 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.

20 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.*,
25 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
30 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
5 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
10 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,
15 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
20 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
25 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
30 ensured both by sterilization procedures, *supra*, and by the inclusion of various antibacterial and

antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

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

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

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion 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, 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
5 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
10 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
15 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
20 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.

25 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
30 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 $\mu\text{g/ml}$ and in some methods about 25-300 $\mu\text{g/ml}$.

A composition of the present invention can be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. Preferred routes of administration for antibodies of the invention include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of administration, for example by injection or infusion. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal,

transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Alternatively, an antibody composition of the instant disclosure can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for
5 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,
10 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
15 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
20 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
25 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
30 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.

10 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

cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced cancers including those induced by asbestos, and combinations of said cancers. The present invention is also useful for treatment of metastatic cancers.

In certain embodiments, the combination of therapeutic antibodies discussed herein may be administered concurrently as a single composition in a pharmaceutically acceptable carrier, or concurrently as separate compositions with each antibody in a pharmaceutically acceptable carrier. In another embodiment, the combination of therapeutic antibodies can be administered sequentially. For example, an anti-CTLA-4 antibody and an anti-PD-1 antibody can be administered sequentially, such as anti-CTLA-4 being administered first and anti-PD-1 second, or anti-PD-1 being administered first and anti-CTLA-4 second. Furthermore, if more than one dose of the combination therapy is administered sequentially, the order of the sequential administration can be reversed or kept in the same order at each time point of administration, sequential administrations may be combined with concurrent administrations, or any combination thereof. For example, the first administration of a combination anti-CTLA-4 antibody and anti-PD-1 antibody may be concurrent, the second administration may be sequential with anti-CTLA-4 first and anti-PD-1 second, and the third administration may be sequential with anti-PD-1 first and anti-CTLA-4 second, etc. Another representative dosing scheme may involve a first administration that is sequential with anti-PD-1 first and anti-CTLA-4 second, and subsequent administrations may be concurrent.

Optionally, the combination of anti-PD-1 and anti-CTLA-4 antibodies can be further combined with an immunogenic agent, such as cancerous cells, purified tumor antigens

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

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

The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg (1999) *Immunity* 10:281-7). In many cases, these tumor specific antigens are differentiation antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE antigens, and Trp-2. More importantly, many of these antigens can be shown to be the targets of tumor specific T cells found in the host. In certain embodiments, a combined PD-1 and CTLA-4 blockade using the antibody compositions described herein may be used in conjunction with a collection of recombinant proteins and/or peptides expressed in a tumor in order to generate an immune response to these proteins. These proteins are normally viewed by the immune system as self-antigens and are, therefore, tolerant to them. The tumor antigen may also include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim *et al.* (1994) *Science* 266: 2011-2013). (These somatic tissues may be protected from immune attack by various means). Tumor antigen may also be "neo-antigens" expressed in cancer cells because of somatic mutations that alter protein sequence

or create fusion proteins between two unrelated sequences (*i.e.*, bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

Other tumor vaccines may include the proteins from viruses implicated in human cancers such a Human Papilloma Viruses (HPV), Hepatitis Viruses (HBV and HCV) and Kaposi's
5 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*
10 278:117-120).

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
15 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
20 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
25 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
30 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
5 into host antigen presentation pathways.

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

15 In another example, a combination of anti-PD-1 and anti-CTLA-4 antibodies can be used in conjunction with anti-neoplastic antibodies, such as Rituxan® (rituximab), Herceptin® (trastuzumab), Bexxar® (tositumomab), Zevalin® (ibritumomab), Campath® (alemtuzumab), Lymphocide® (epratuzumab), Avastin® (bevacizumab), and Tarceva® (erlotinib), and the like. By way of example and not wishing to be bound by theory, treatment with an anti-cancer
20 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
25 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
30 another example, antibodies to each of these entities may be further combined with an anti-PD-1

and anti-CTLA-4 combination to counteract the effects of immunosuppressive agents and favor anti-tumor immune responses by the host.

Other antibodies that may be used to activate host immune responsiveness can be further used in combination with an anti-PD-1 and anti-CTLA-4 combination. These include molecules 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

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

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

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

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

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

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

EXAMPLES**Example 1** *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 (2×10^6 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 mm³

(85246), and one mouse was tumor-free (86057) (Figure 1B). In the anti-PD-1 antibody alone group, six mice reached the tumor end-point by about day 60, one mouse had an ulcerated tumor (86055), and three mice were tumor-free (84955, 85239 and 86750) (Figure 1C). In the anti-CTLA-4 antibody and anti-PD-1 antibody combination group, four mice reached the tumor end-point by about Day 40, and six mice were tumor-free (84596, 85240, 86056, 86071, 86082 and 86761) (Figure 1D).

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

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

Example 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 x 10⁶ cells/mouse) for a time sufficient (about 6 to 7 days) to permit the formation of tumors. On day 6 post-implantation (day -1), tumor measurements were taken and mice were randomized based on mean tumor volume (about 250 mm³) into 11 groups for subsequent antibody therapy. At day 0 (*i.e.*, one week after the MC38 cells were implanted), mice were injected IP with (1) mouse IgG (control), (2) anti-CTLA-4 monoclonal antibody 9D9, (3) anti-PD-1 monoclonal antibody 4H2, or (4) anti-CTLA-4 monoclonal antibody 9D9 and anti-PD-1 antibody monoclonal antibody 4H2, at a concentration of 10 mg/kg per mouse. Antibody injections were also

administered on days 3, 6 and 10. The monoclonal antibody compositions used had low levels of endotoxin and did not significantly aggregate. Using an electronic caliper, the tumors were measured three dimensionally (height x width x length) and tumor volume was calculated. Tumor measurements were taken on day 0 (tumors at the beginning of treatment had a volume of about 125 mm³), and on days 3, 6, 10, 13, 17 and 20 post-antibody injection. Mice were euthanized when the tumors reached a designated tumor end-point (a particular tumor volume such as 1500 mm³ and/or when the mice showed greater than about 15% weight loss).

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

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

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

Example 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 10⁶ 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:

Group (A) mouse IgG (control, 20 mg/kg), Group (B) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg) and mouse IgG (10 mg/kg), Group (C) anti-CTLA-4 monoclonal antibody 9D9 (10 mg/kg) and mouse IgG (10 mg/kg), Group (D) anti-CTLA-4 monoclonal antibody 9D9 (10 mg/kg) and anti-PD-1 antibody monoclonal antibody 4H2 (10 mg/kg), Group (E) anti-CTLA-4 monoclonal antibody 9D9 (3 mg/kg) and anti-PD-1 antibody monoclonal antibody 4H2 (3 mg/kg), or Group (F) anti-CTLA-4 monoclonal antibody 9D9 (1 mg/kg) and anti-PD-1 antibody monoclonal antibody 4H2 (1 mg/kg). Using an electronic caliper, the tumors were measured three dimensionally (height x width x length) and tumor volume was calculated. Tumor measurements were taken at the beginning of treatment (*i.e.*, on day 0 tumors had an average volume of about 90 mm³), and on days 3, 6, 10, 13, 17 and 20 post-antibody treatment. Mice were euthanized when the tumors reached a designated tumor end-point (a particular tumor volume such as 1500 mm³ and/or when the mice showed greater than about 15% weight loss).

Figure 7A shows that all 10 control mice had reached a tumor end-point. Figure 7B shows that the group treated with 10 mg/kg anti-PD-1 antibody (Group B) had 4 mice that reached the tumor end-point and 6 mice with tumors having a volume of about 750 mm³ or less. Figure 7C shows that the group treated with 10 mg/kg anti-CTLA-4 antibody (Group C) had 3 mice that reached the tumor end-point and 7 mice with tumors having a volume of about 1000 mm³ or less. Figure 7D shows that the group treated with a combination of 10 mg/kg anti-PD-1 antibody with 10 mg/kg anti-CTLA-4 antibody (Group D) had 2 mice with tumors having a volume of about 1000 mm³ or less, and 8 mice that were tumor free. Figure 7E shows that the group treated with a combination of 3 mg/kg anti-PD-1 antibody with 3 mg/kg anti-CTLA-4 antibody (Group E) had one mouse that had reached the tumor end-point, 7 mice with tumors having a volume of about 500 mm³ or less, and 2 mice that were tumor free. Figure 7F shows that the group treated with a combination of 1 mg/kg anti-PD-1 antibody with 1 mg/kg anti-CTLA-4 antibody (Group F) had 4 mice that had reached the tumor end-point, 5 mice with tumors having a volume of about 1100 mm³ or less, and one mouse that was tumor free.

Figures 7G and 7H show the tumor volumes in mice treated sequentially with anti-PD-1 antibody first and anti-CTLA-4 antibody second, and vice versa. The mice of Figure 7G first received 10 mg/kg anti-CTLA-4 on each of days 0 and 3, and then received 10 mg/kg anti-PD-1 antibody on each of days 6 and 10. The mice of Figure 7H first received 10 mg/kg anti-PD-1

antibody on each of days 0 and 3, and then received 10 mg/kg anti-CTLA-4 antibody on each of days 6 and 10. 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 mm³ for the IgG control group; about 470 mm³ for the PD-1 antibody with the IgG control; about 290 mm³ for the CTLA-4 antibody with the IgG control (measured at day 6); about 40 mm³ for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 165 mm³ for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 400 mm³ for the anti-CTLA-4 antibody (1 mg/kg) and anti-PD-1 antibody (1 mg/kg) combination group. Figure 9 shows that the median tumor volume measured at day 13 was about 1680 mm³ for the IgG control group; about 400 mm³ for the PD-1 antibody with the IgG control; about 660 mm³ for the CTLA-4 antibody with the IgG control; 0 mm³ for the anti-CTLA-4 antibody (10 mg/kg) and anti-PD-1 antibody (10 mg/kg) combination group; about 90 mm³ for the anti-CTLA-4 antibody (3 mg/kg) and anti-PD-1 antibody (3 mg/kg) combination group; and about 650 mm³ for the anti-CTLA-4 antibody (1 mg/kg) and anti-PD-1 antibody (1 mg/kg) combination group. For the combination treatment of the anti-PD-1 antibody with the anti-CTLA-4 antibody, the number of mice per group that were tumor free at 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 10⁶ cells/mouse) on day 0. On days 1, 4, 7 and 11 post-implantation, mice were injected IP as follows: Group (A) PBS alone (referred to as the "vehicle"); Group (B) mouse IgG (control, 10 mg/kg per mouse), Group (C) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse), Group (D) anti-CTLA-4 monoclonal antibody

9D9 (10 mg/kg or 0.2 mg/kg per mouse), and Group (E) anti-PD-1 monoclonal antibody 4H2 (10 mg/kg per mouse) in combination with anti-CTLA-4 monoclonal antibody 9D9 (0.2 mg/kg per mouse). The study lasted 41 days and tumor measurements were taken on various days throughout the course of the study (*see* Figure 9). Tumor volume was calculated by measuring tumors in three dimensions (height x width x length) using an electronic caliper. Mice were euthanized when the tumors reached a designated tumor end-point – a volume of 1500 mm³ and/or an ulcerated tumor.

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

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

Table 2. Tumor Growth Inhibition and Tumor Free Mice 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

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 10⁶ 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 mm³ were injected IP as follows: Group (A) PBS alone (referred to as the "vehicle"); Group (B) mouse IgG (control, 10 mg/kg per mouse); Group (C) anti-CTLA-4 monoclonal antibody 9D9 (0.25 mg/kg); Group (D) anti-CTLA-4 monoclonal antibody 9D9 (0.5 mg/kg per mouse); Group (E) anti-CTLA-4 monoclonal antibody 9D9 (5 mg/kg); Group (F) anti-PD-1 monoclonal antibody 4H2 (3 mg/kg per mouse); Group (G) anti-PD-1 monoclonal

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

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

Figure 13 shows the response to immunostimulatory antibody treatment in mice with tumors having an initial volume of about 110 mm³ (*i.e.*, at the time of the first antibody treatment. Figures 13A and 13B show that all 16 control mice (Groups A and B) reached a tumor end-point (15 with a tumor volume greater than 1500 mm³ and 1 with an ulcerated tumor). Figures 13C-13E show that tumor bearing mice respond to treatment with anti-CTLA-4 antibody in a dose-dependent manner (*e.g.*, Group C receiving 0.25 mg/kg had 7/8 mice reach the tumor end-point and one mouse with a tumor volume less than 200 mm³, whereas Group E receiving 5 mg/kg had 6/8 mice reach the tumor end-point and two mice were tumor free). Figures 13F and 13G show that mice responded about the same regardless of the anti-PD-1 antibody dose (Group F received 3 mg/kg and Group G received 10 mg/kg). In contrast, the mice receiving a combination treatment of 10 or 3 mg/kg anti-PD-1 antibody with 0.25 or 0.5 mg/kg anti-CTLA-4 antibody (Groups H, I and J) showed a significant reduction in tumor growth. For example, Figure 13J shows that the group treated with a combination of 3 mg/kg anti-PD-1 antibody with 0.5 mg/kg anti-CTLA-4 antibody (Group J) had 2 mice that had ulcerated tumors, 2 mice with a tumor volume less than 500 mm³, and 4 mice that were tumor free. The unexpected synergistic effect of an anti-PD-1 antibody combined with an anti-CTLA-4 antibody,

along with the surprising effectiveness of subtherapeutic levels of anti-CTLA-4 antibody in the combination, are shown in Figures 14 (mean tumor volume) and 15 (median tumor volume).

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

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

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

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

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

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

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

Example 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 EC₅₀ (50% effective concentration) of about 0.38 µg (Figure 23) and a K_D of 4.7 x 10⁻⁹ M. To examine the inhibition of PD-L1 binding to PD-1, the same assay was performed except that the cells were also 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

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 EC₅₀ of about 0.72 µg (Figure 24).

5 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
10 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-
15 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
20 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.

CLAIMS

We claim:

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

composition comprising the anti-CTLA-4 antibody and a pharmaceutically acceptable carrier.

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

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

34. The method of any one of claims 20 to 27 wherein the anti-CTLA-4 antibody is a human sequence antibody.
35. The method of claim 34 wherein the anti-CTLA-4 human sequence antibody is a monoclonal antibody.
36. The method of claim 35 wherein the anti-CTLA-4 human sequence monoclonal antibody is 10D1 (MDX-010).

ABSTRACT

5 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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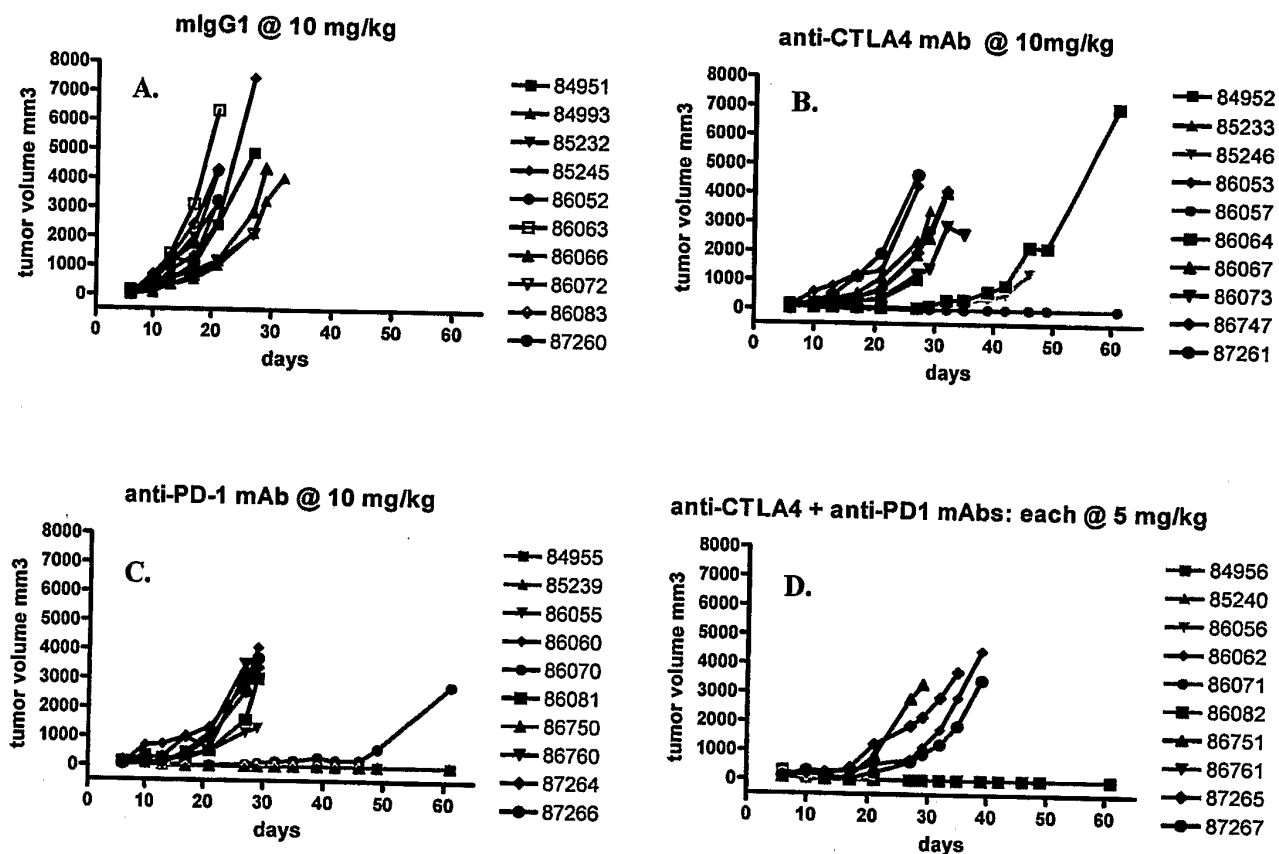


Figure 1.

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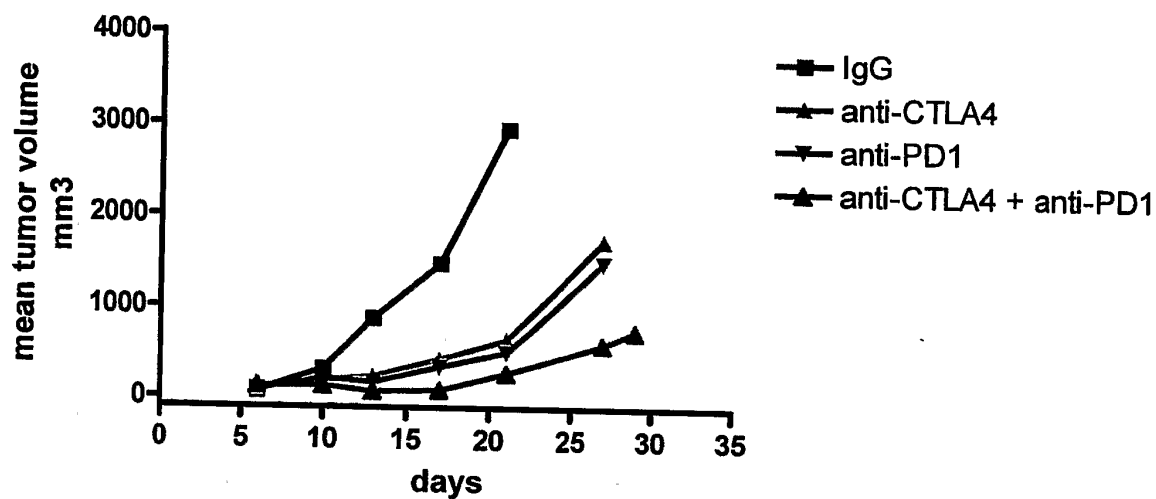


Figure 2.

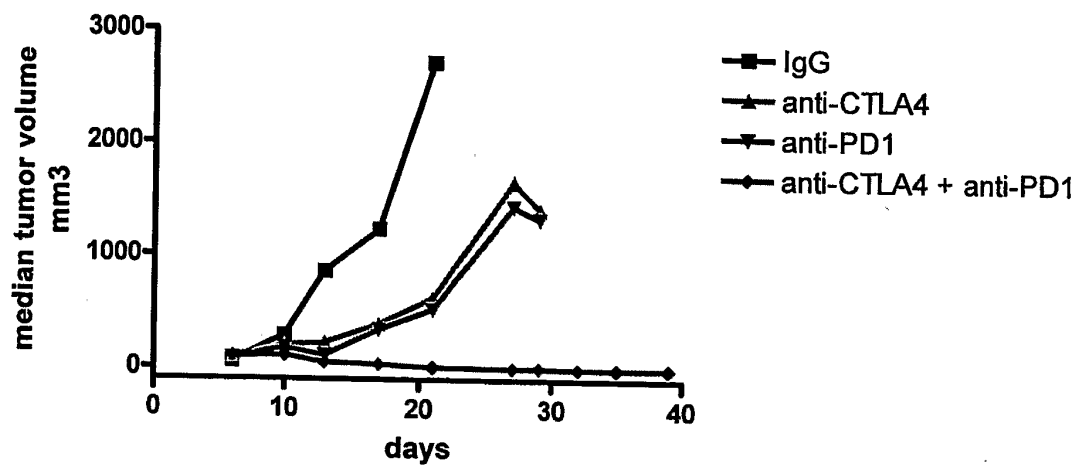


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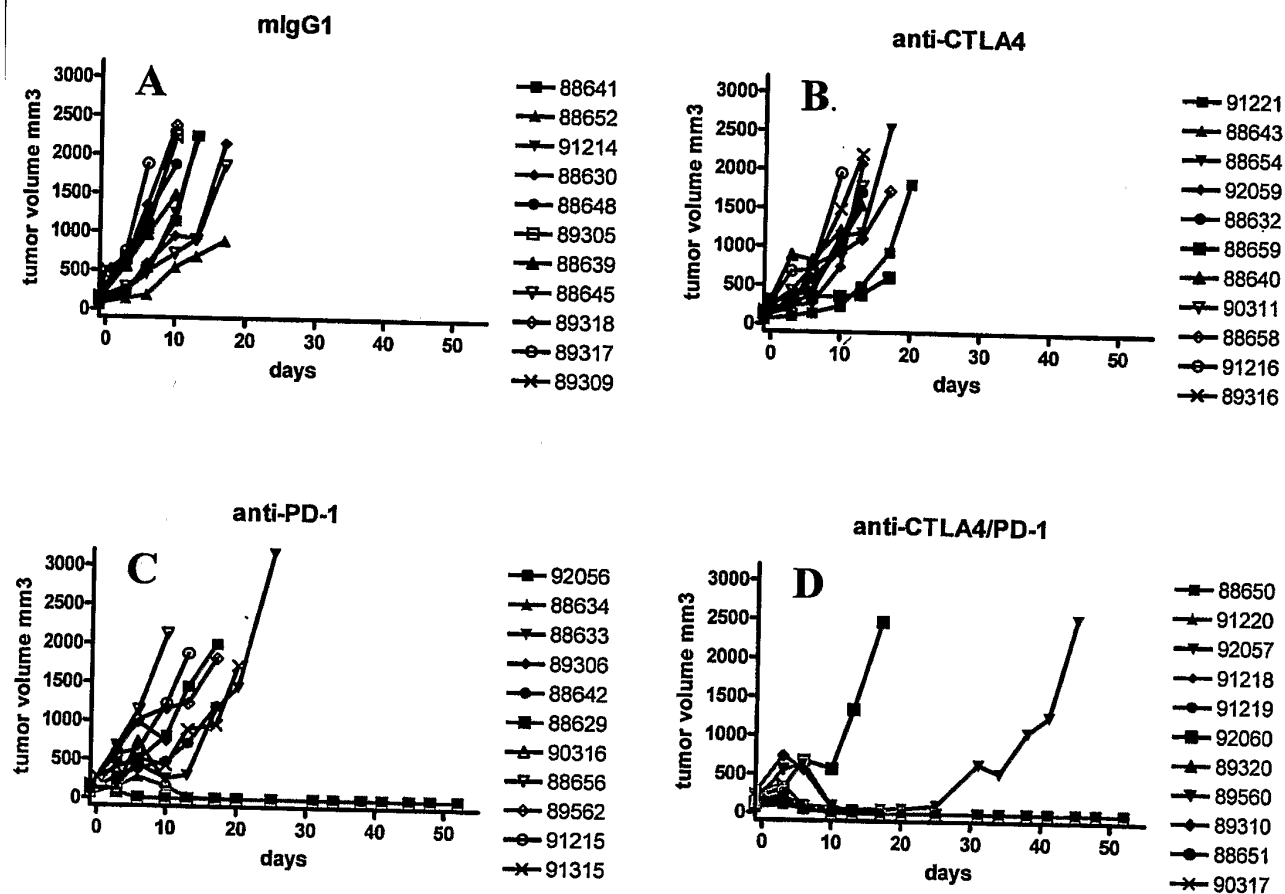


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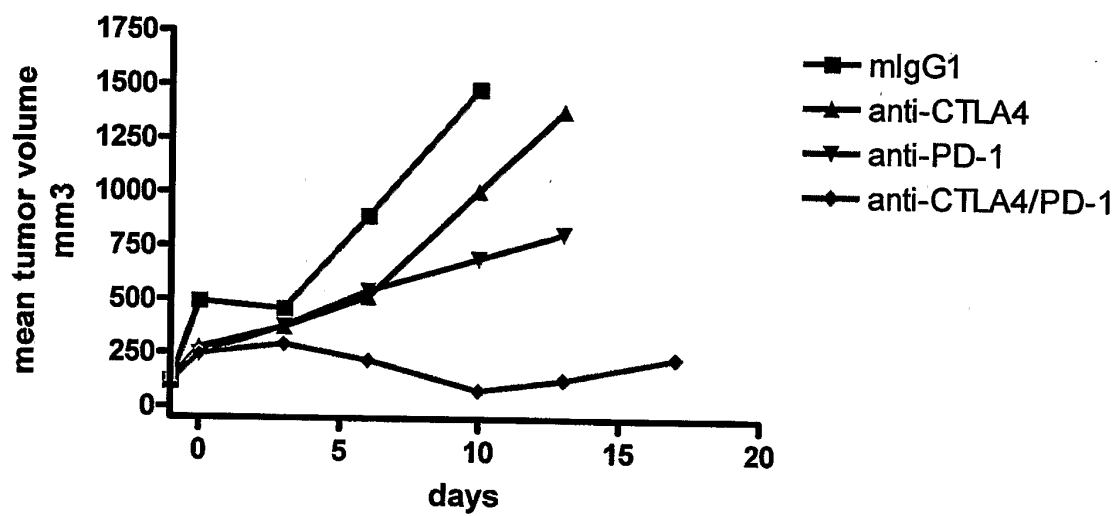


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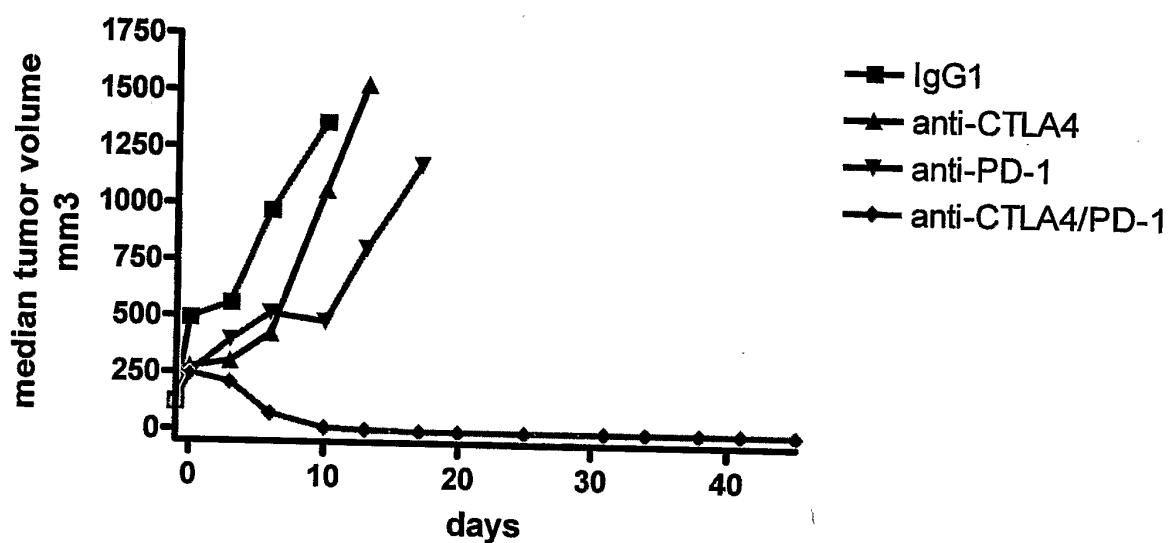


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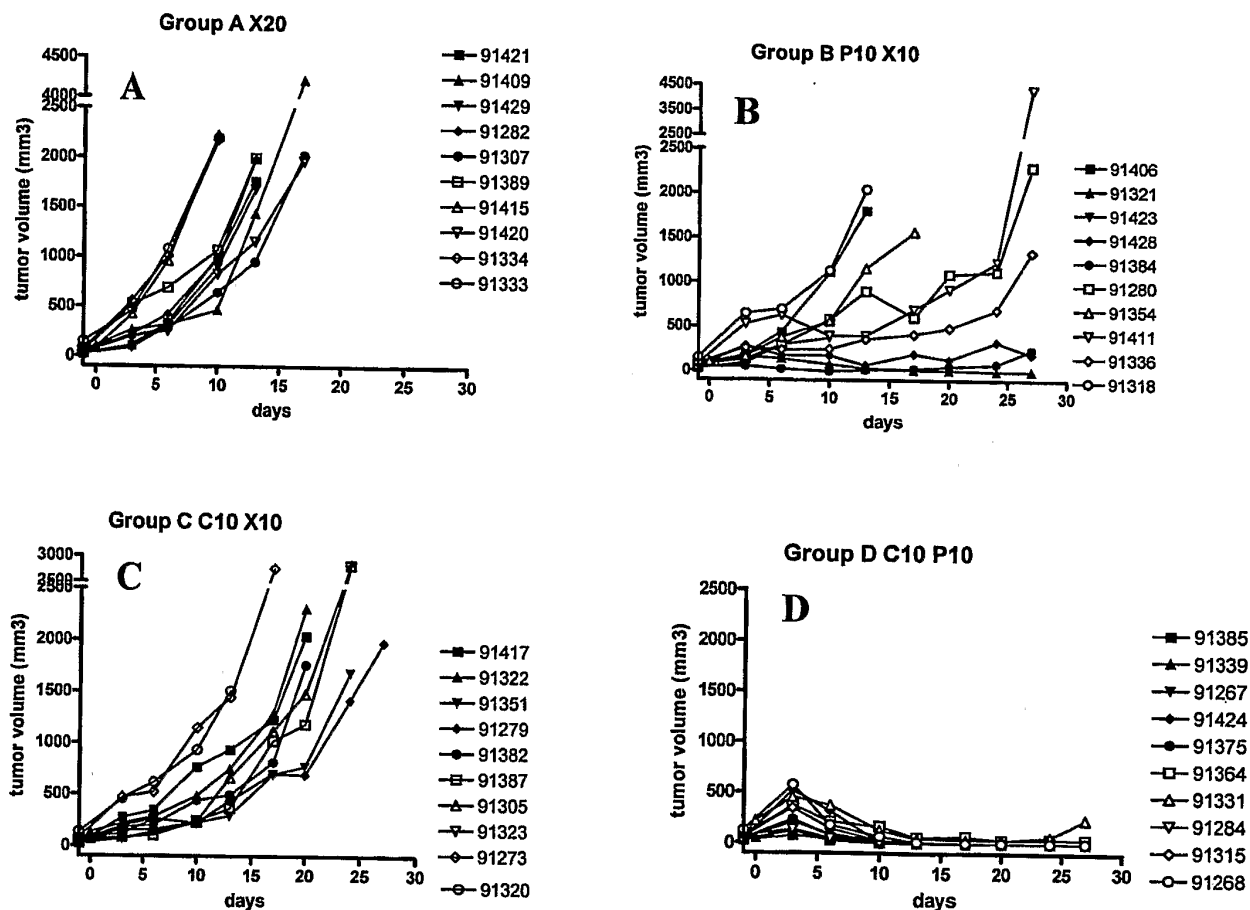


Figure 7.

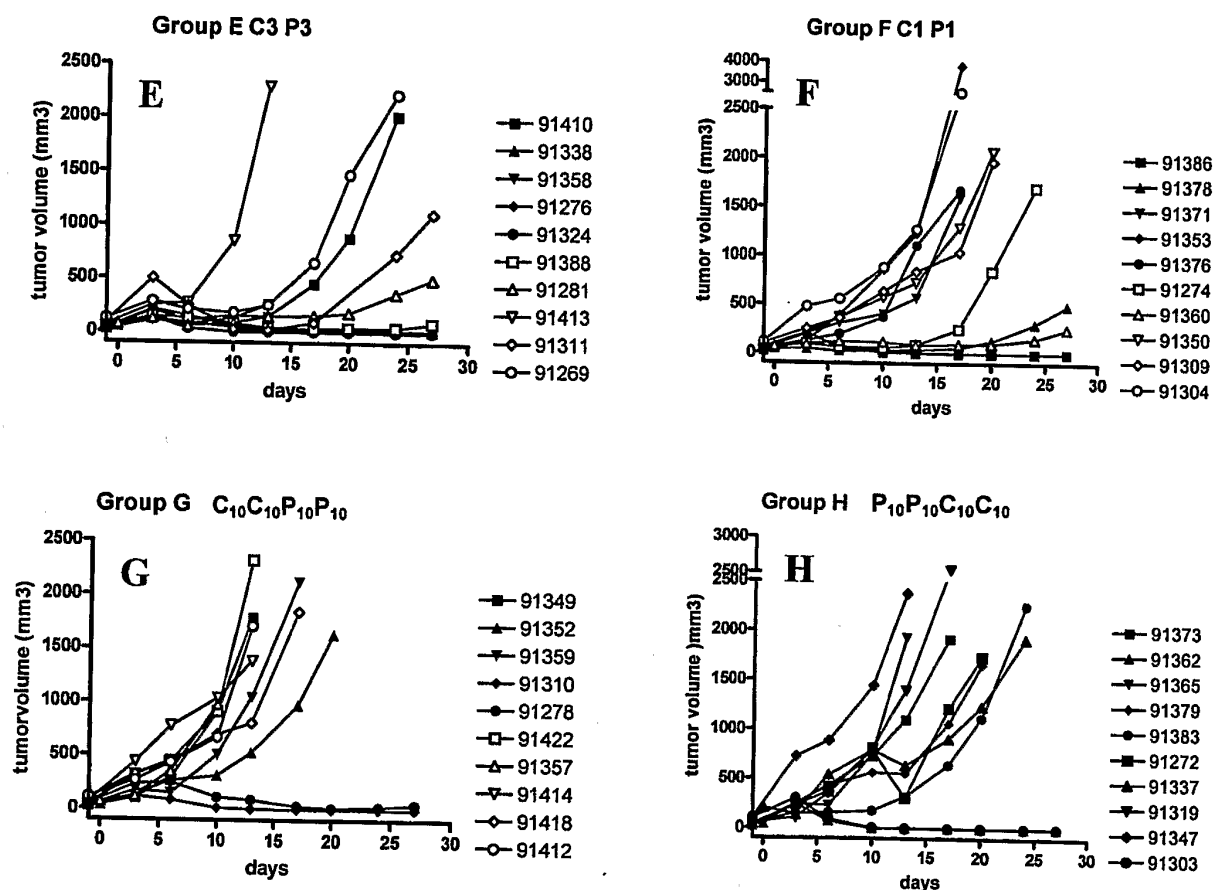


Figure 7. (cont.)

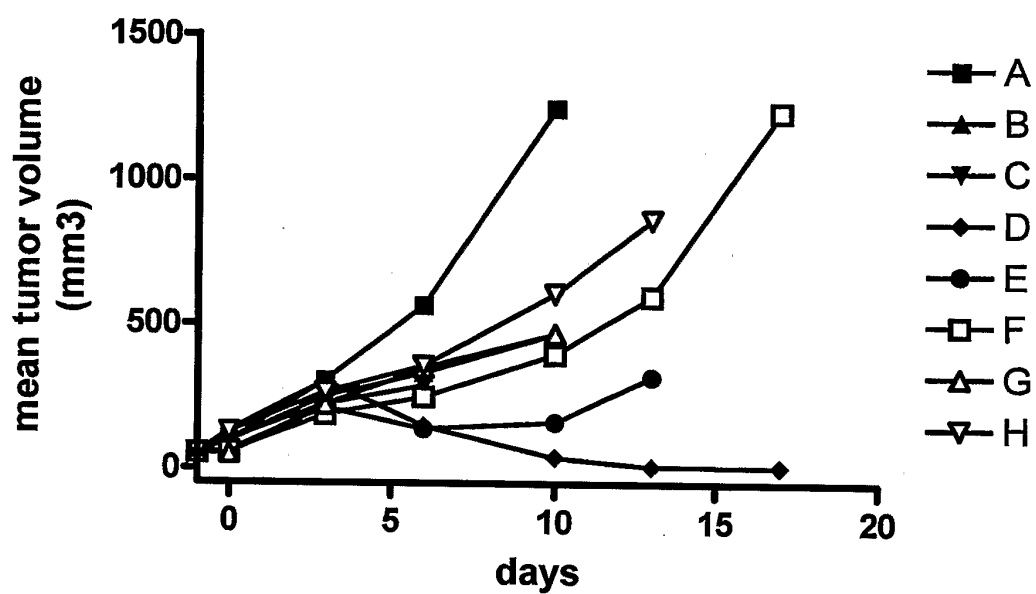


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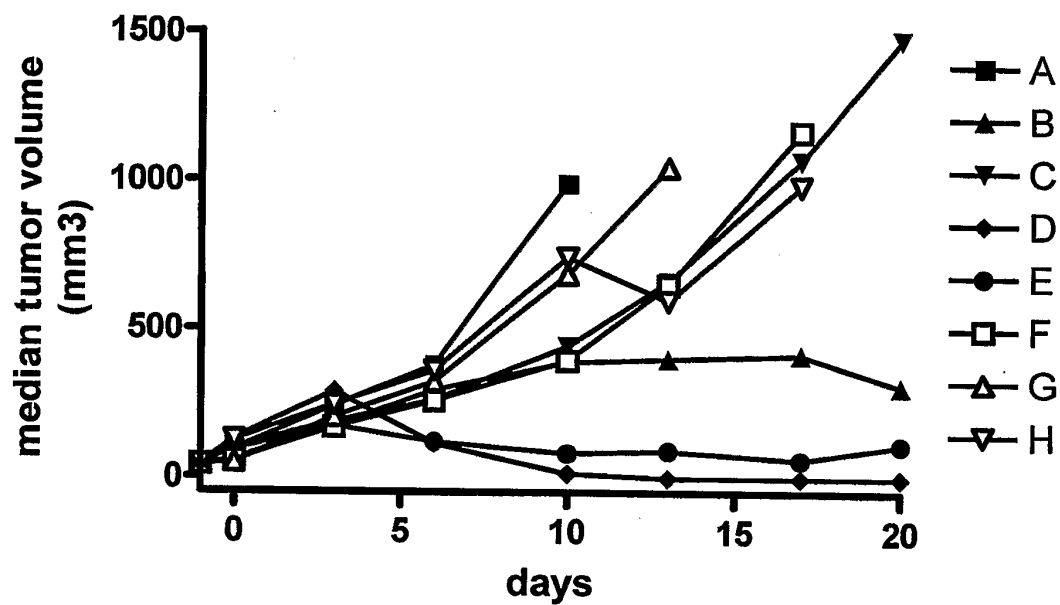


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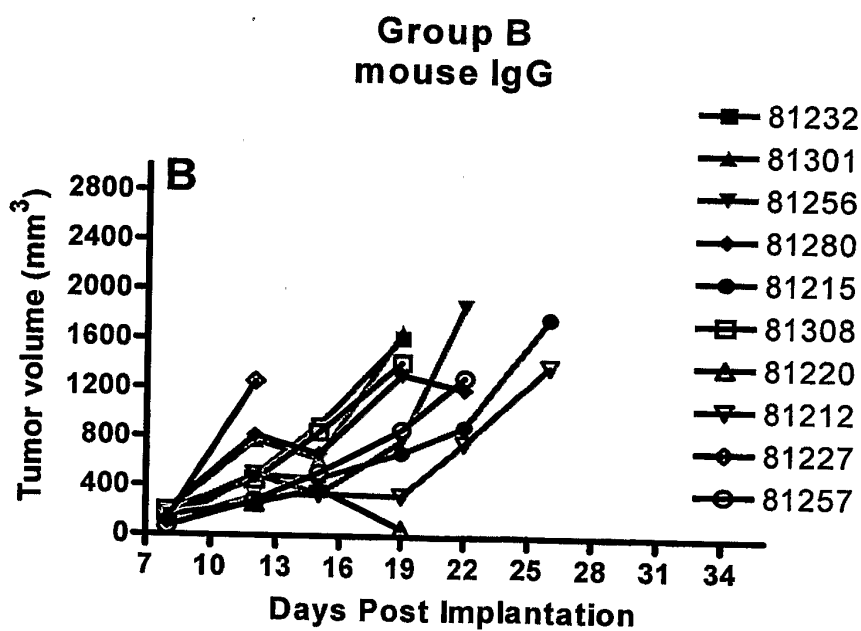
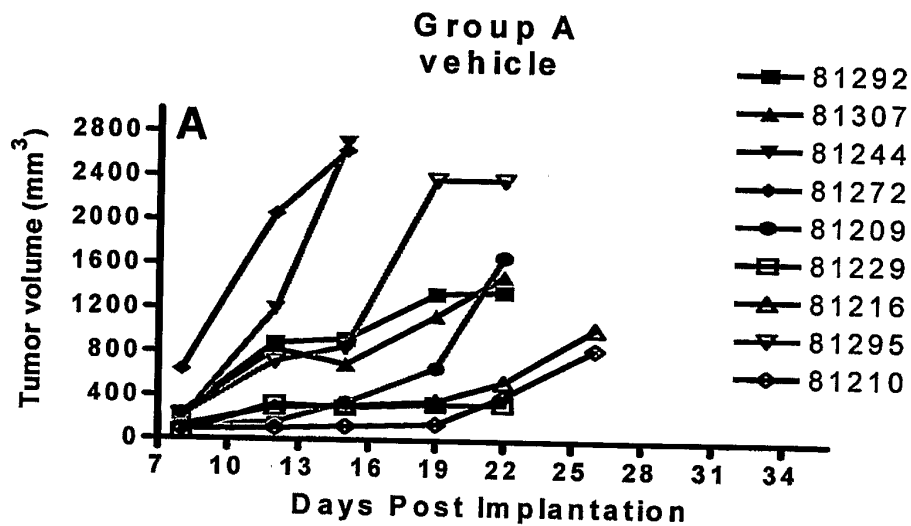


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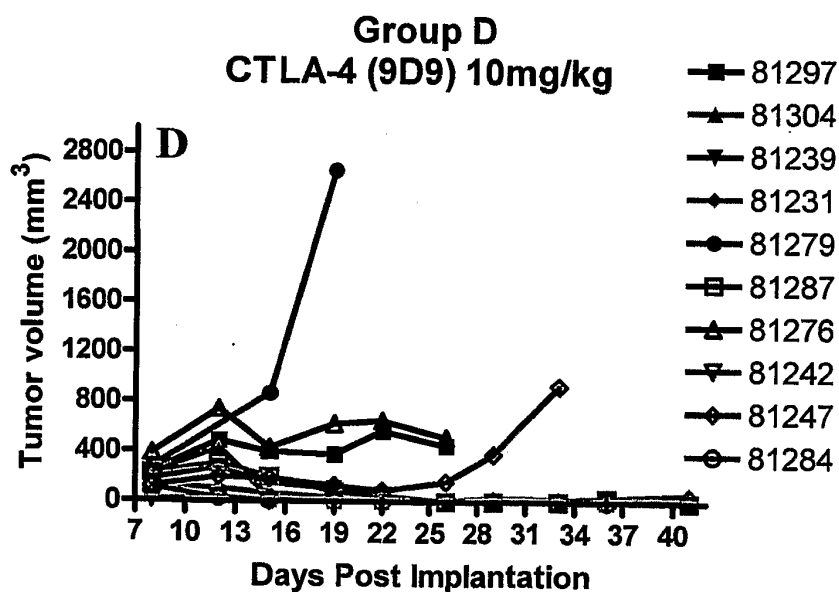
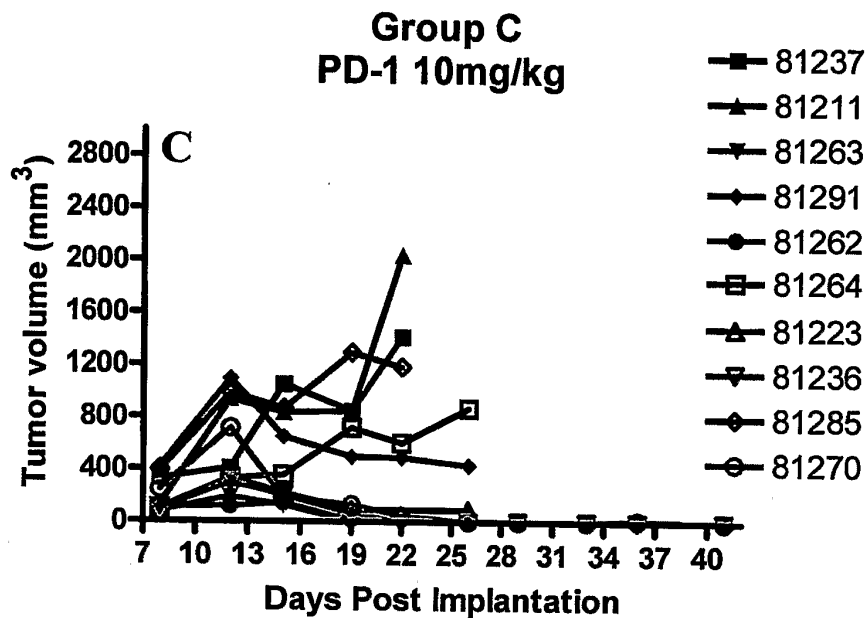
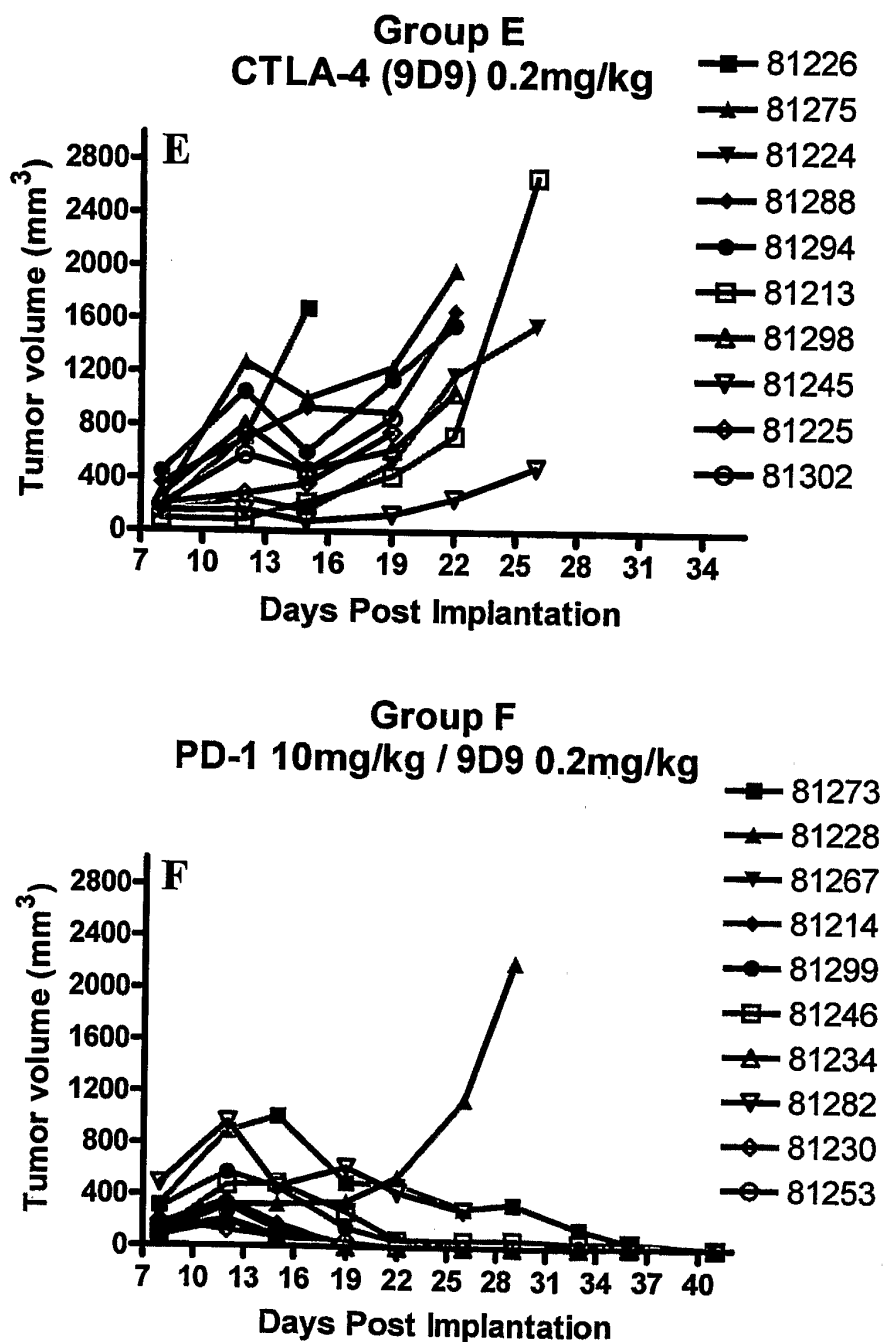


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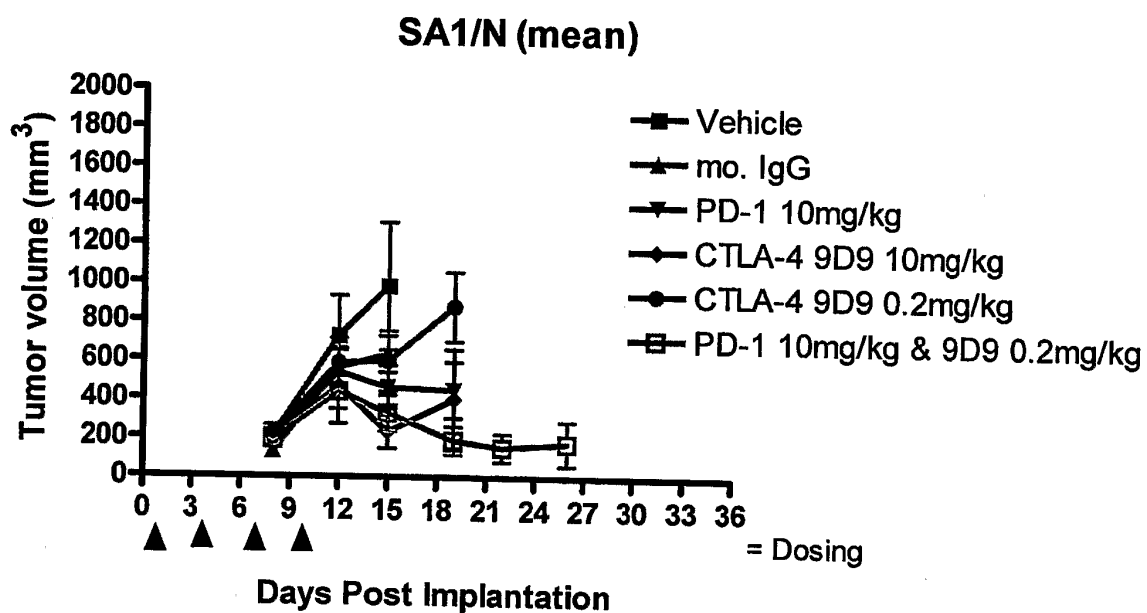


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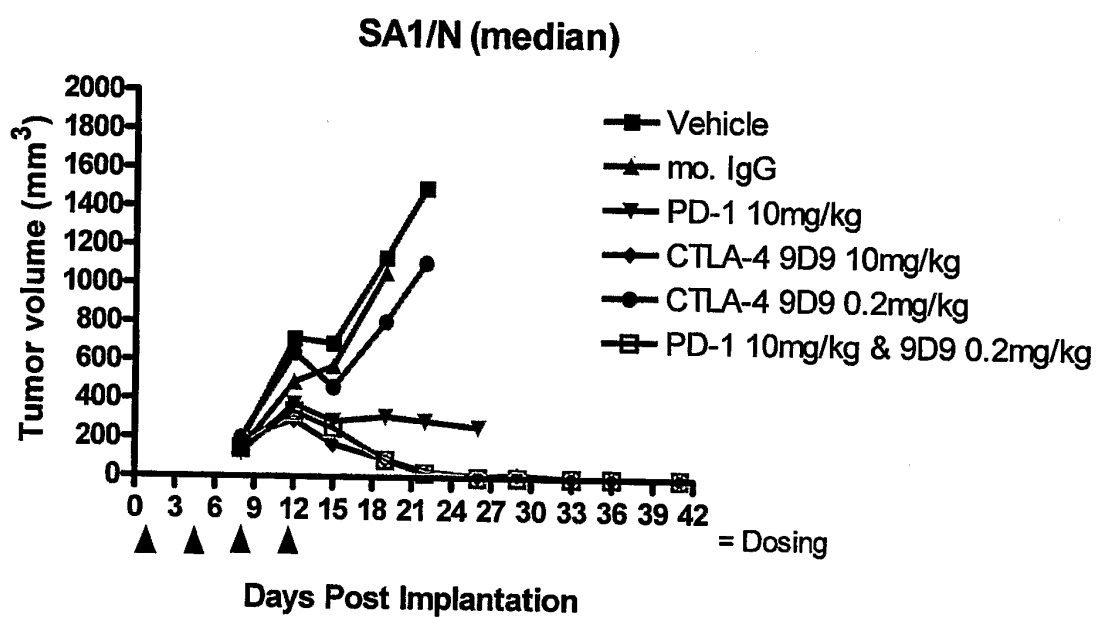


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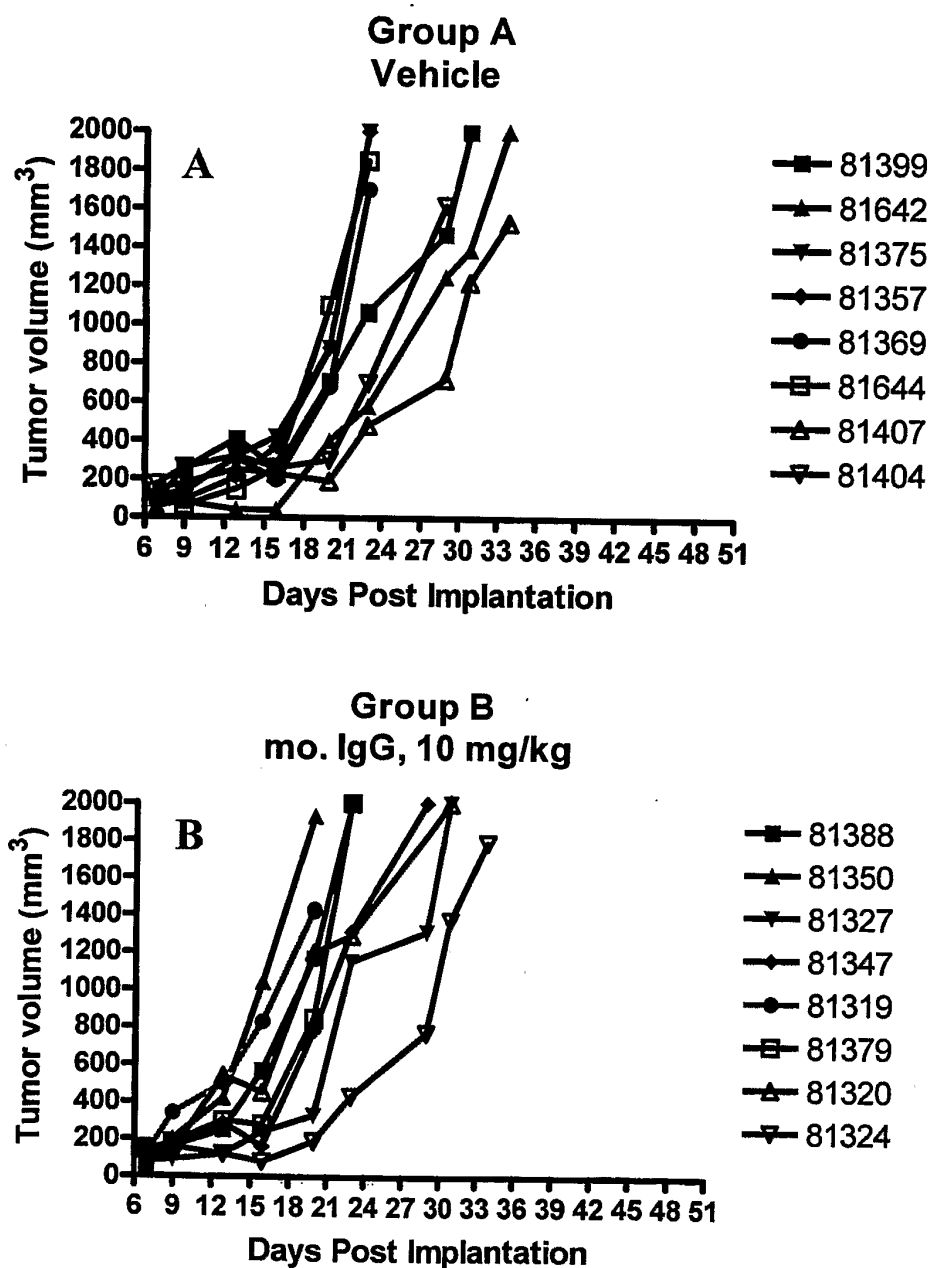


Figure 13.

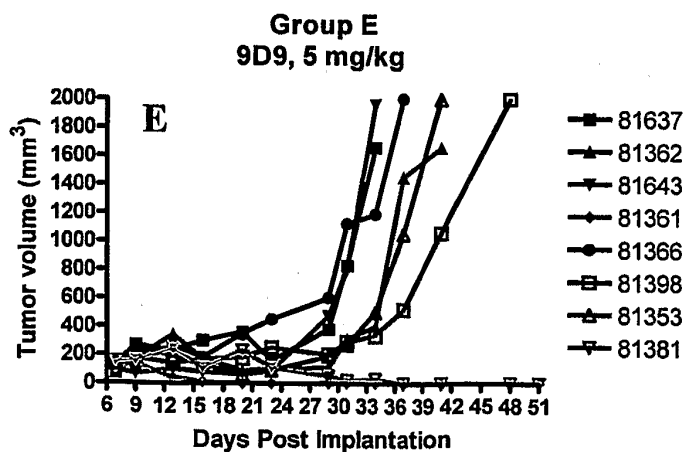
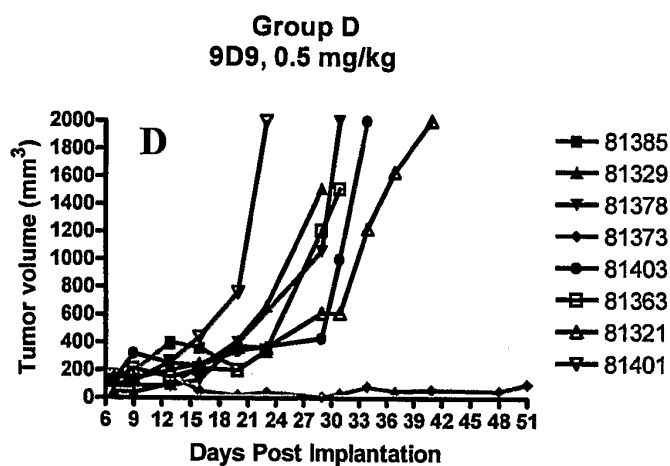
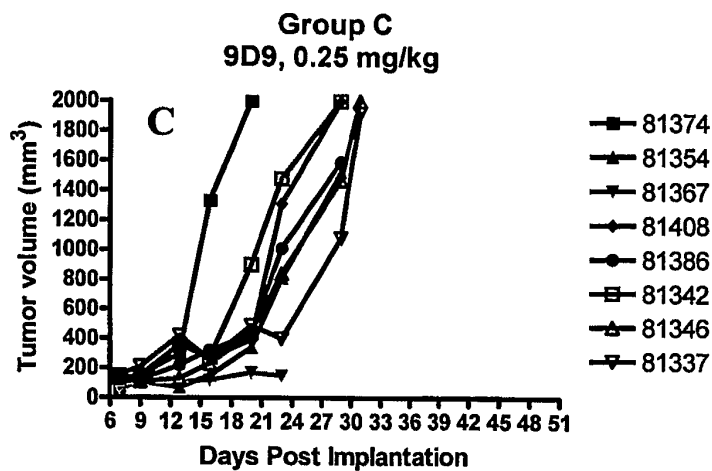


Figure 13. (cont.)

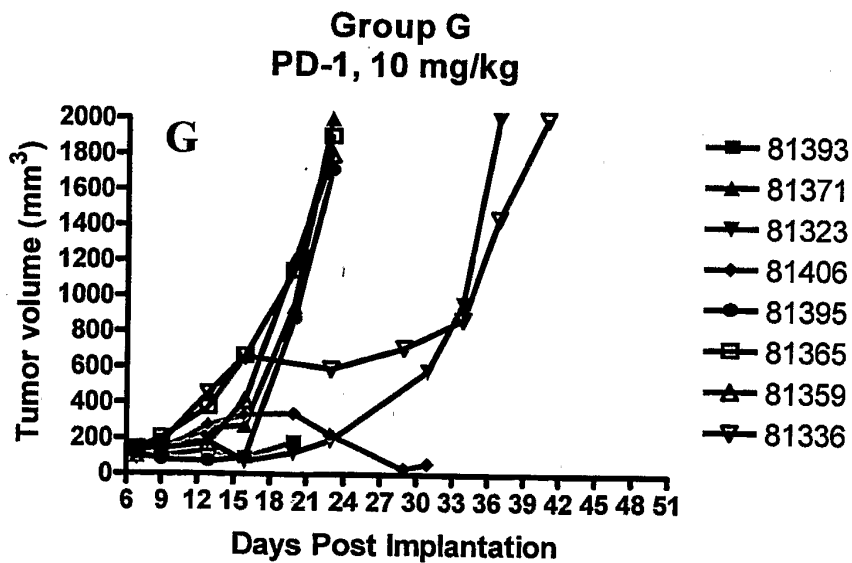
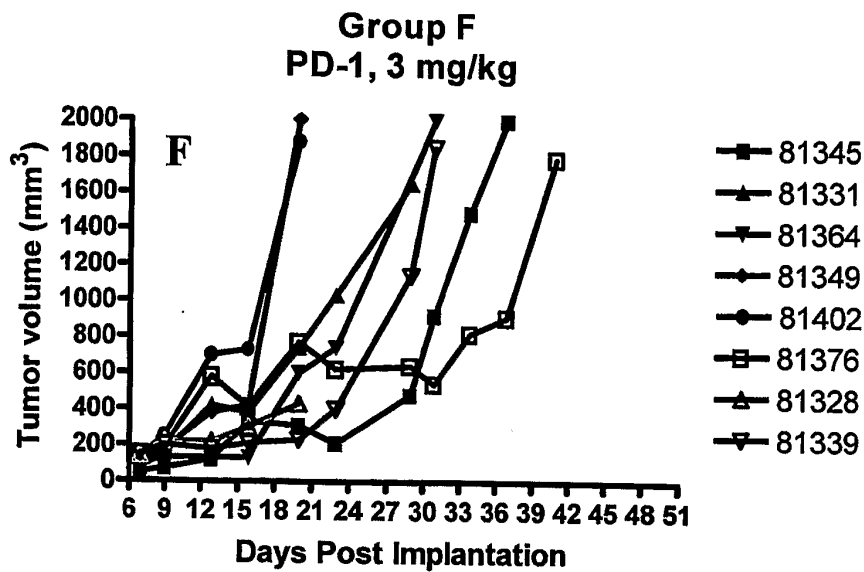


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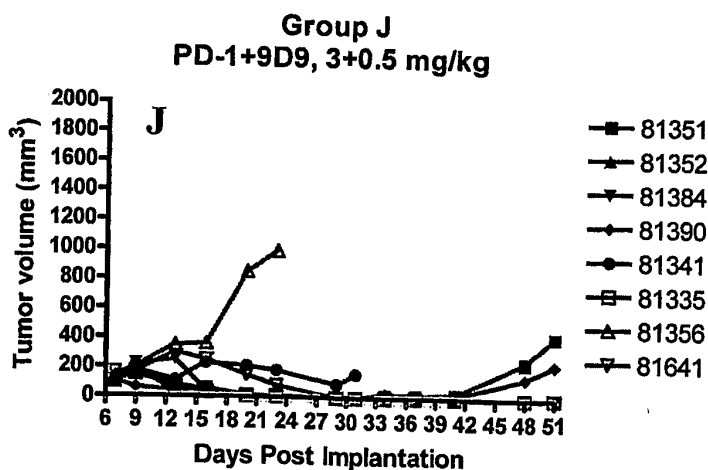
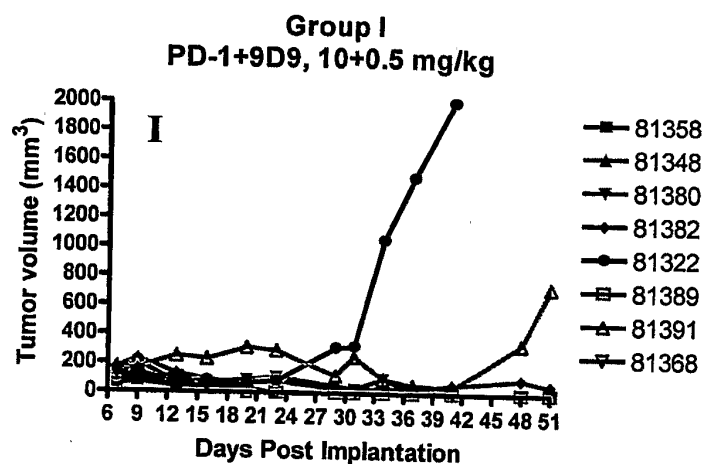
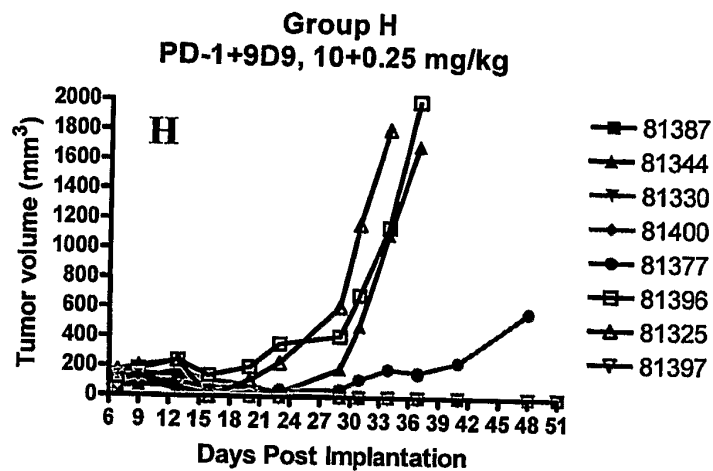


Figure 13. (cont.)

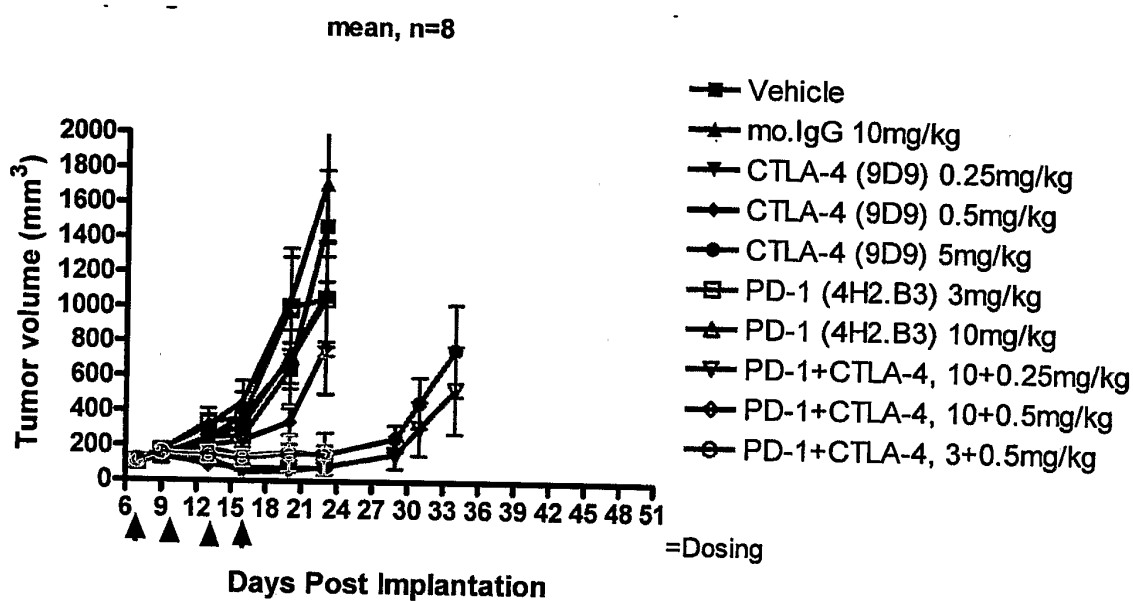


Figure 14.

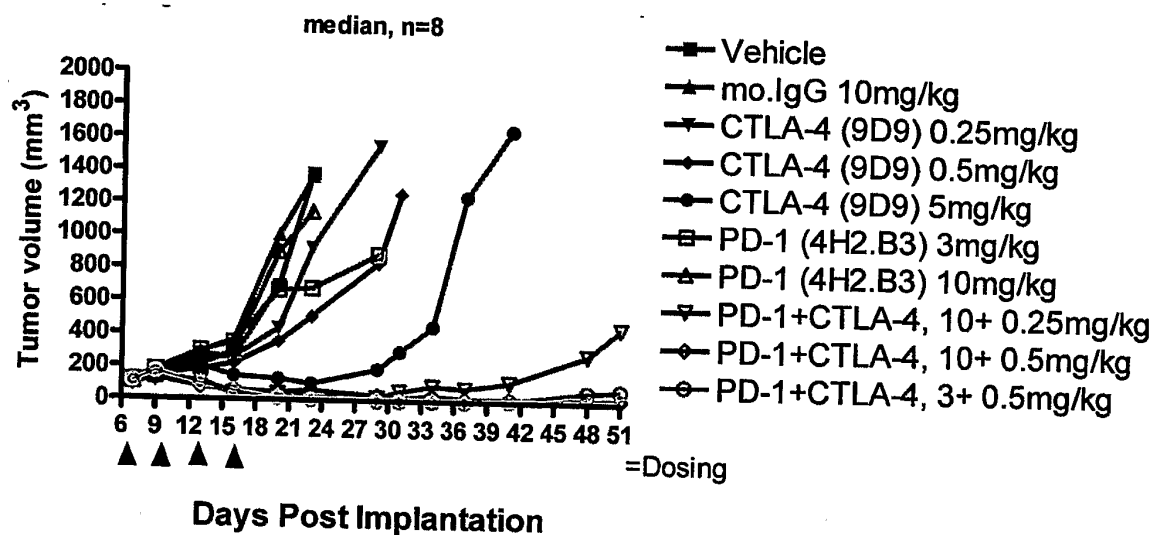


Figure 15.

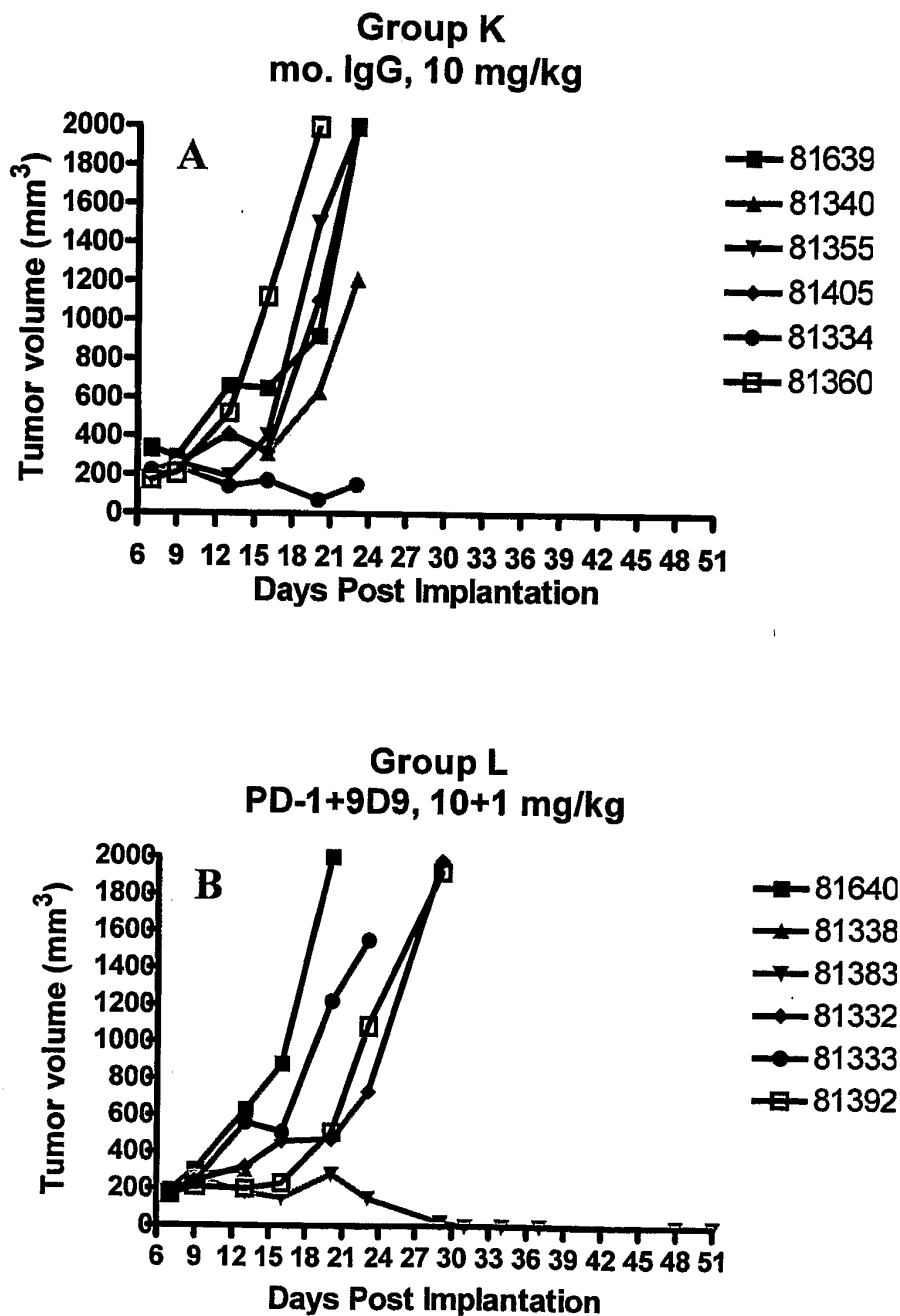


Figure 16.

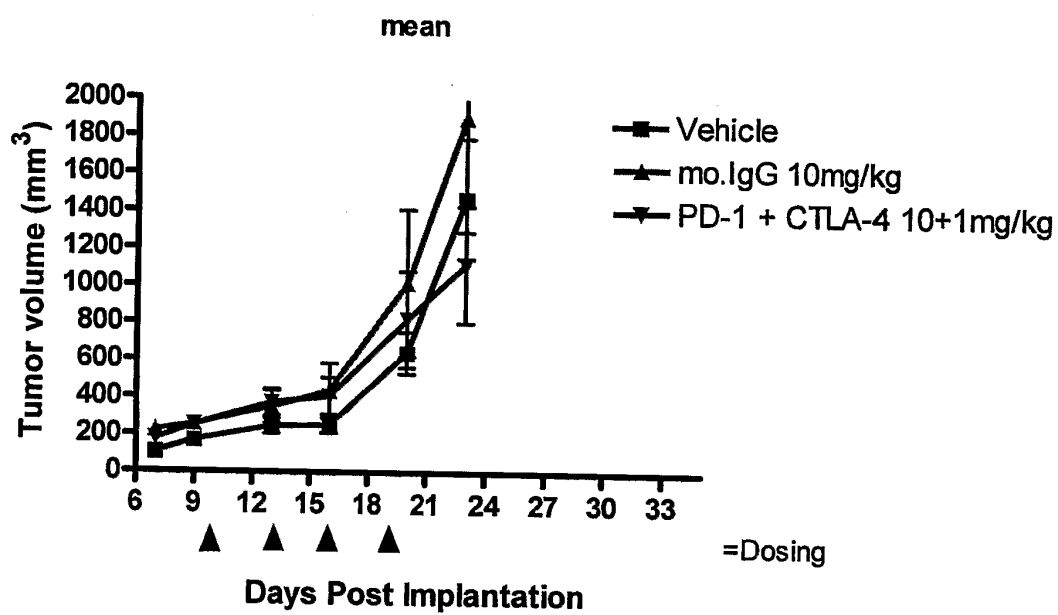


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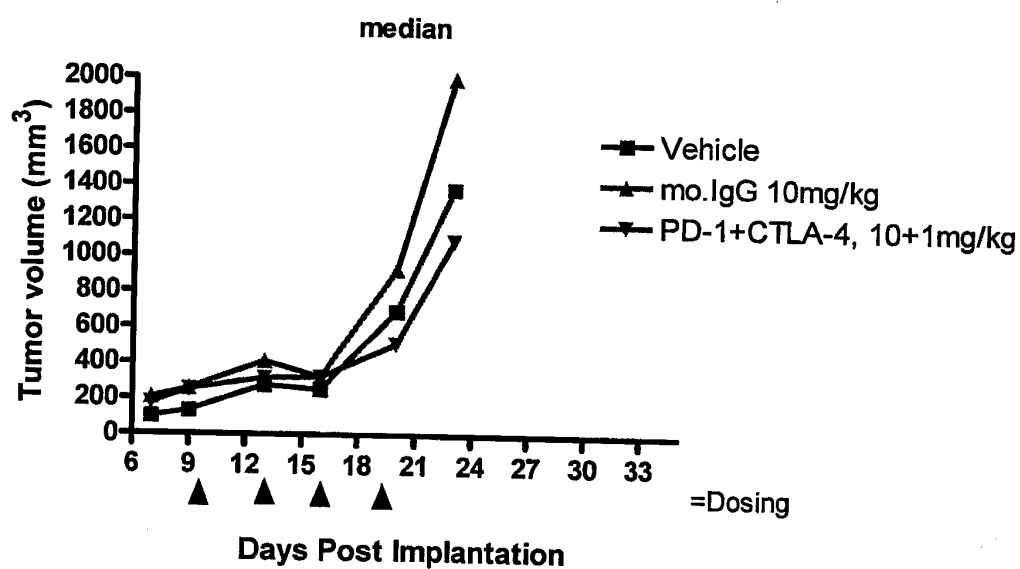


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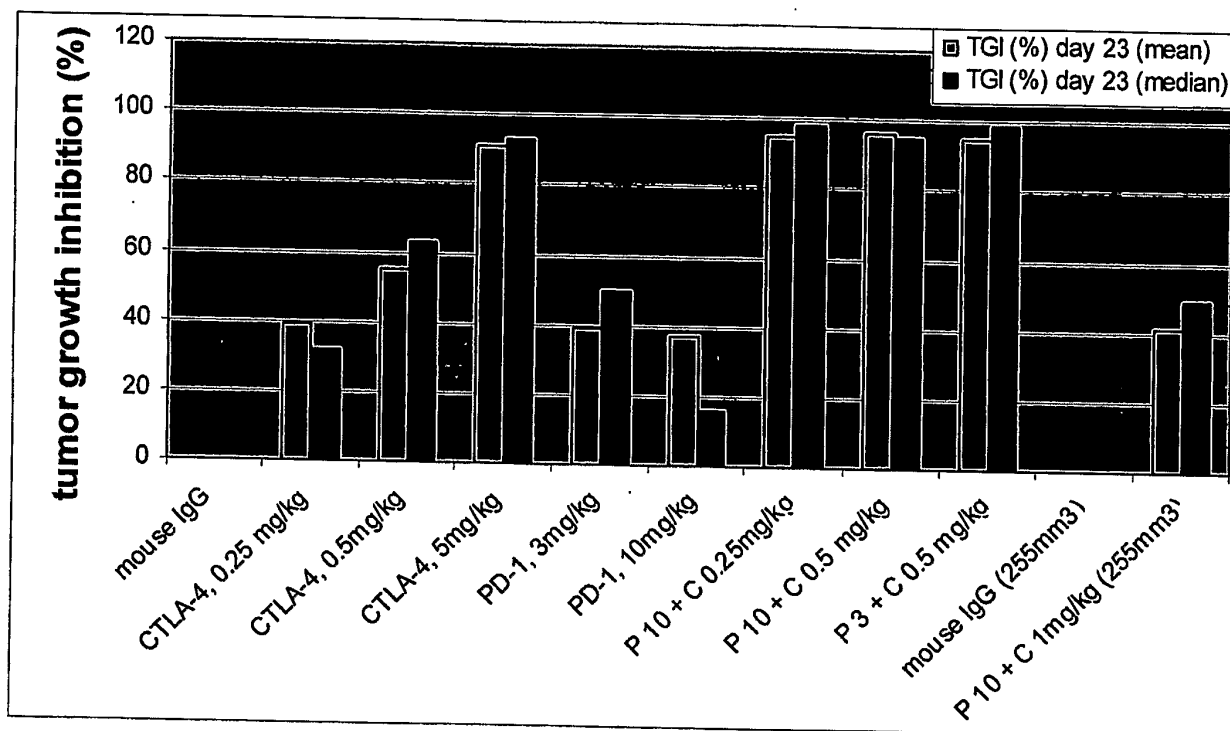


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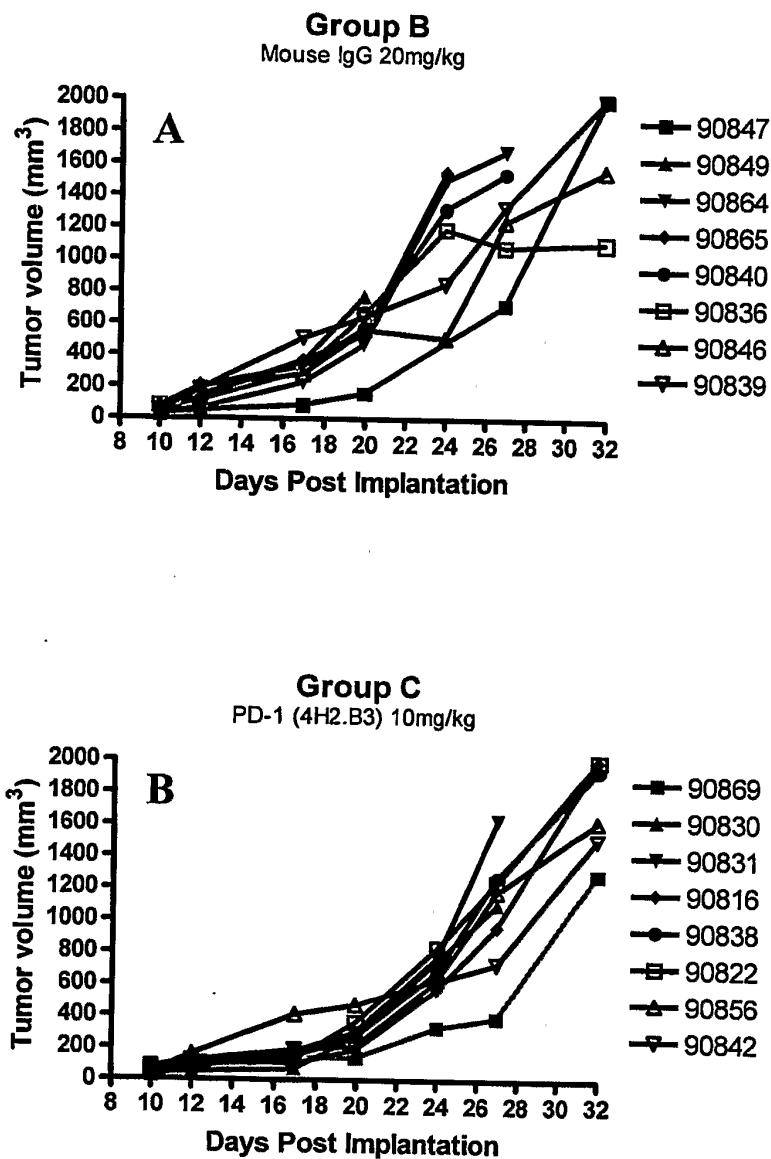


Figure 20.

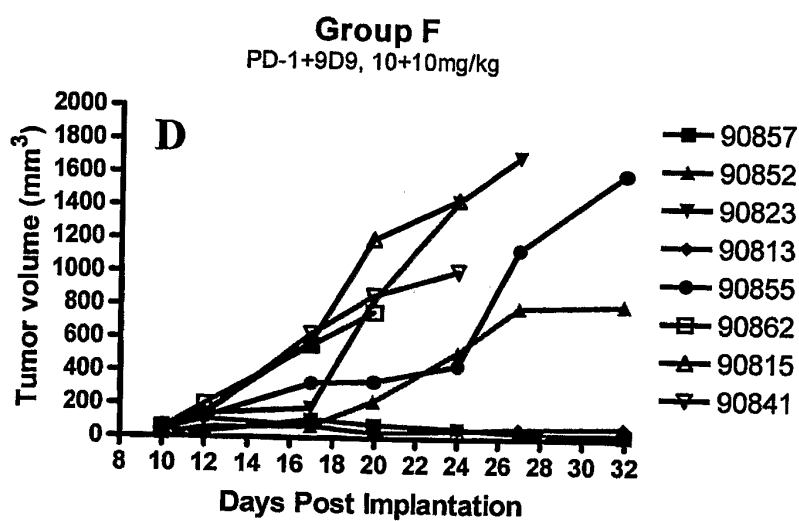
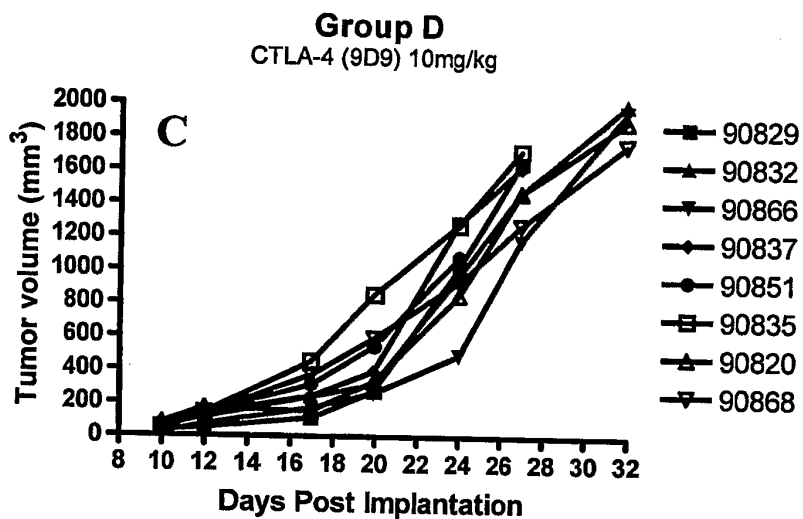


Figure 20. (cont.)

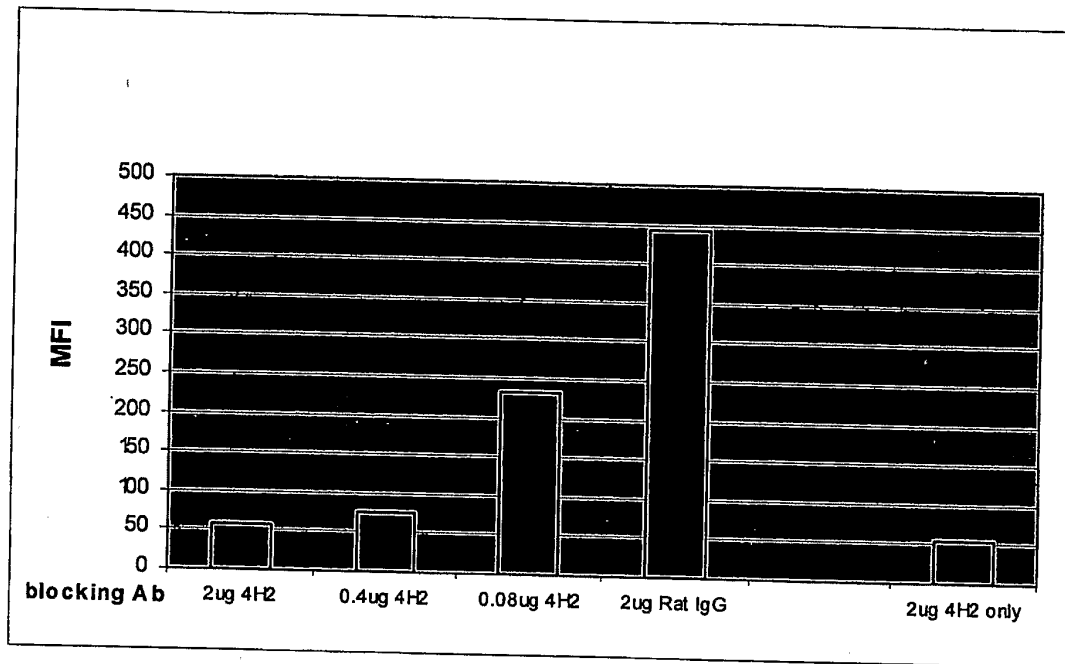


Figure 21.

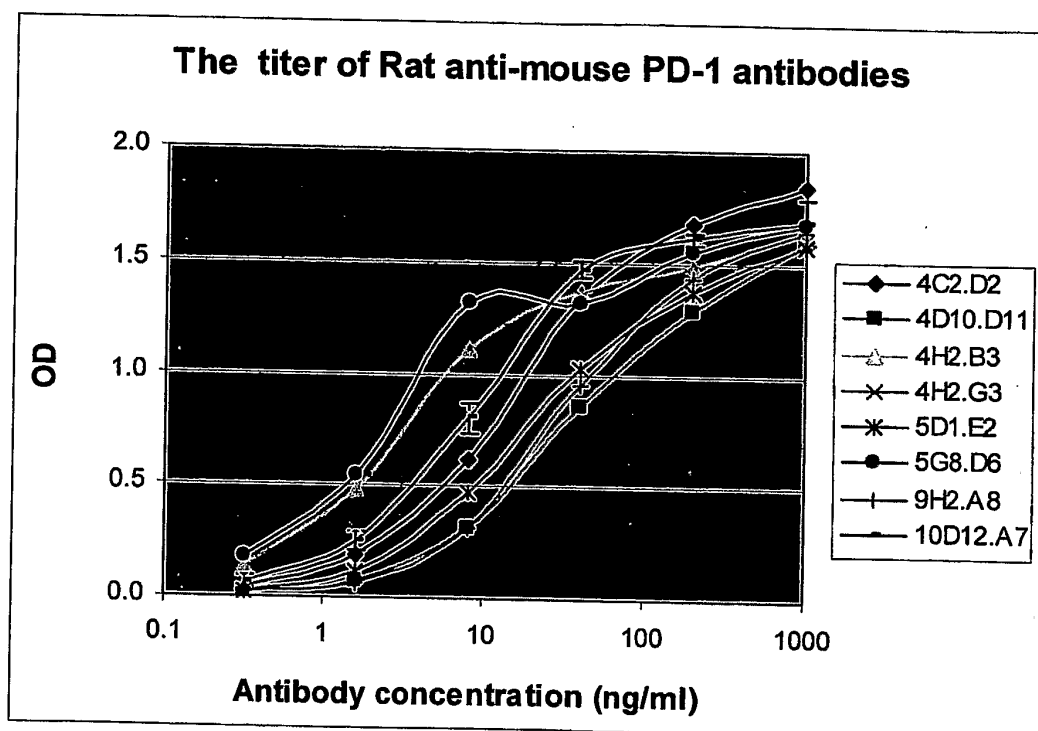


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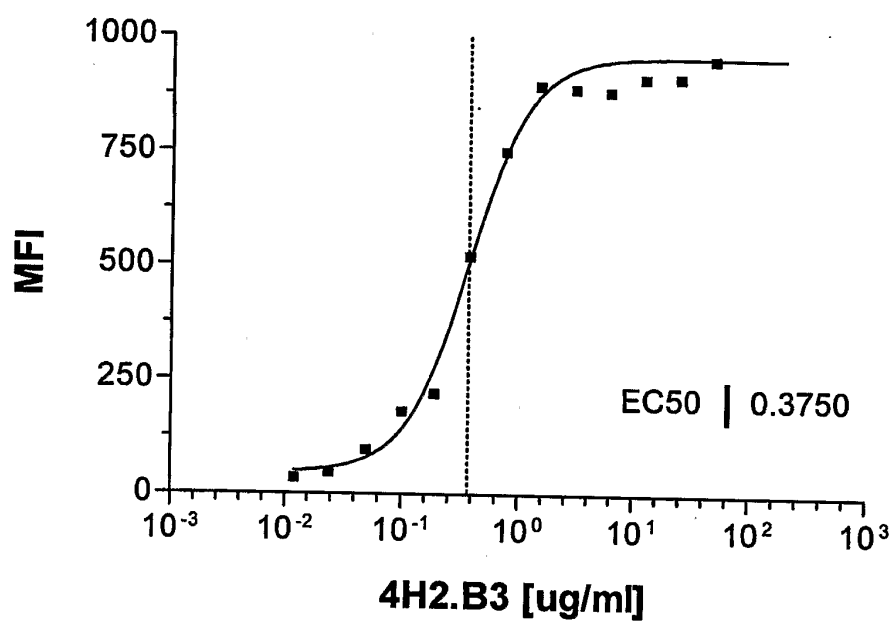


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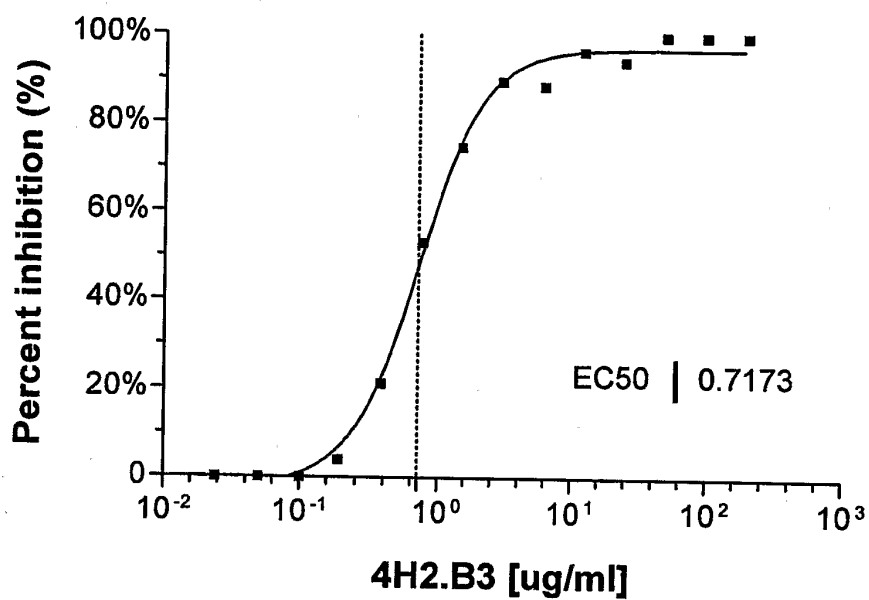


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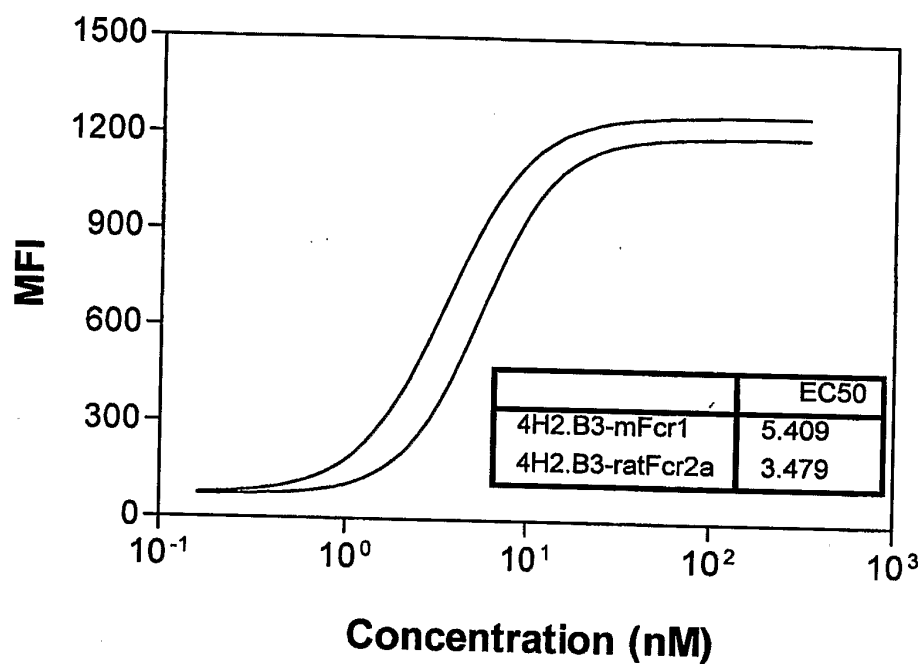


Figure 25.

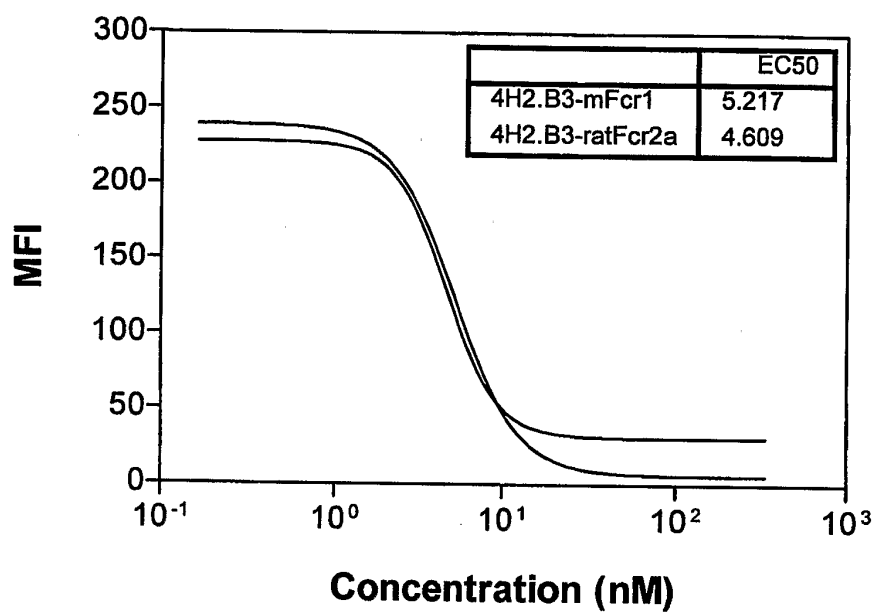


Figure 26.