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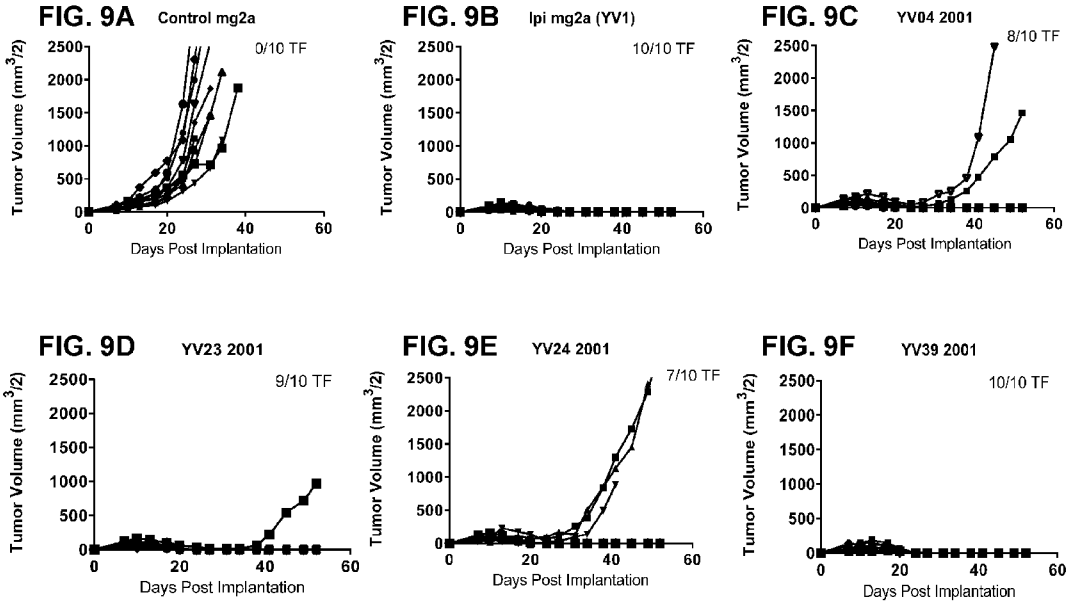
(54) Title: ACTIVATABLE ANTI-CTLA-4 ANTIBODIES AND USES THEREOF



(57) Abstract: Provided herein are activatable anti-human CTLA-4 antibodies comprising a heavy chain comprising a VH domain and a light chain comprising a masking moiety (MM), a cleavable moiety (CM), and a VL domain. Such activatable anti-human CTLA-4 antibodies have CTLA-4 binding activity in the tumor microenvironment, where the masking moiety is removed by proteolytic cleavage of the cleavable moiety by tumor-specific proteases, but exhibit greatly reduced binding to CTLA-4 outside the tumor. In this way, the activatable anti-human CTLA-4 antibodies of the present invention retain anti-tumor activity while reducing the side effects associated with anti-CTLA-4 activity outside the tumor.

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[Continued on next page]

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ACTIVATABLE ANTI-CTLA-4 ANTIBODIES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application No. 62/417,212, filed November 3, 2016, which is hereby incorporated by reference in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

[0002] The content of the electronically submitted sequence listing (Name: 3338_059PC02_SeqListing.txt; Size: 527,968 bytes; and Date of Creation: October 27, 2017) is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0003] The immune system is capable of controlling tumor development and mediating tumor regression. This requires the generation and activation of tumor antigen–specific T cells. Multiple T-cell co-stimulatory receptors and T-cell negative regulators, or co-inhibitory receptors, act in concert to control T-cell activation, proliferation, and gain or loss of effector function. Among the earliest and best-characterized T-cell co-stimulatory and co-inhibitory molecules are CD28 and CTLA-4. Rudd *et al.* (2009) *Immunol. Rev.* 229: 12. CD28 provides co-stimulatory signals to T-cell receptor engagement by binding to B7-1 and B7-2 ligands on antigen-presenting cells, while CTLA-4 provides a negative signal down-regulating T-cell proliferation and function. CTLA-4, which also binds the B7-1 (CD80) and B7-2 (CD86) ligands but with higher affinity than CD28, acts as a negative regulator of T-cell function through both cell autonomous (or intrinsic) and cell non-autonomous (or extrinsic) pathways. Intrinsic control of CD8 and CD4 T effector (T_{eff}) function is mediated by the inducible surface expression of CTLA-4 as a result of T-cell activation, and inhibition of T-cell proliferation and cytokine proliferation by multivalent engagement of B7 ligands on opposing cells. Peggs *et al.* (2008) *Immunol. Rev.* 224:141.

[0004] Anti-CTLA-4 antibodies, when cross-linked, suppress T cell function *in vitro*. *Krummel & Allison (1995) J. Exp. Med.* 182:459; Walunas *et al.* (1994) *Immunity* 1:405. Regulatory T cells (T_{regs}), which express CTLA-4 constitutively, control effector T cell (T_{eff}) function in a non-cell autonomous fashion. T_{regs} that are deficient for CTLA-4 have impaired suppressive ability (Wing *et al.* (2008) *Science* 322:271) and antibodies that block CTLA-4 interaction with B7 can inhibit T_{reg} function (Read *et al.* (2000) *J. Exp. Med.* 192:295; Quezada *et al.* (2006) *J. Clin. Invest.* 116:1935). More recently, T_{effs} have also been shown to control T cell function through extrinsic pathways (Corse & Allison (2012) *J. Immunol.* 189:1123; Wang *et al.* (2012) *J. Immunol.* 189:1118). Extrinsic control of T cell function by T_{regs} and T_{effs} occurs through the ability of CTLA-4-positive cells to remove B7 ligands on antigen-presenting cells, thereby limiting their co-stimulatory potential. Qureshi *et al.* (2011) *Science* 332: 600; Onishi *et al.* (2008) *Proc. Nat'l Acad. Sci. (USA)* 105:10113. Antibody blockade of CTLA-4/B7 interactions is thought to promote T_{eff} activation by interfering with negative signals transmitted by CTLA-4 engagement; this intrinsic control of T-cell activation and proliferation can promote both T_{eff} and T_{reg} proliferation (*Krummel & Allison (1995) J. Exp. Med.* 182:459; Quezada *et al.* (2006) *J. Clin. Invest.* 116:1935). In early studies with animal models, antibody blockade of CTLA-4 was shown to exacerbate autoimmunity. Perrin *et al.* (1996) *J. Immunol.* 157:1333; Hurwitz *et al.* (1997) *J. Neuroimmunol.* 73:57. By extension to tumor immunity, the ability of anti-CTLA-4 to cause regression of established tumors provided a dramatic example of the therapeutic potential of CTLA-4 blockade. Leach *et al.* (1996) *Science* 271:1734.

[0005] Human antibodies to human CTLA-4, ipilimumab and tremelimumab, were selected to inhibit CTLA-4-B7 interactions (Keler *et al.* (2003) *J. Immunol.* 171:6251; Ribas *et al.* (2007) *Oncologist* 12:873) and have been tested in a variety of clinical trials for multiple malignancies. Hoos *et al.* (2010) *Semin. Oncol.* 37:533; Ascierto *et al.* (2011) *J. Transl. Med.* 9:196. Tumor regressions and disease stabilization were frequently observed, and treatment with these antibodies has been accompanied by adverse events with inflammatory infiltrates capable of affecting a variety of organ systems. In 2011, ipilimumab, which has an IgG1 constant region, was approved in the US and EU for the treatment of unresectable or metastatic melanoma based on an improvement in overall

survival in a phase III trial of previously treated patients with advanced melanoma. Hodi *et al.* (2010) *N. Engl. J. Med.* 363:711.

[0006] Treatment with ipilimumab has, however, been hampered by dose limiting toxicities, such as colitis. Di Giacomo *et al.* (2010) *Seminars in Oncology* 37:499. Accordingly, the need exists for improved anti-CTLA-4 antibodies, such as modified forms of ipilimumab, with reduced toxicity but with comparable anti-tumor efficacy. Such improved anti-CTLA-4 antibodies may be more effective anti-tumor agents than current antibodies.

SUMMARY OF THE INVENTION

[0007] Provided herein are activatable anti-human CTLA-4 antibodies comprising a heavy chain comprising a VH domain and a light chain comprising a masking moiety (MM), a cleavable moiety (CM), and a VL domain. Such activatable anti-human CTLA-4 antibodies have CTLA-4 binding activity in the tumor microenvironment, where the masking moiety is removed by proteolytic cleavage of the cleavable moiety by tumor-specific proteases, but exhibit greatly reduced binding to CTLA-4 outside the tumor. In this way, the activatable anti-human CTLA-4 antibodies of the present invention retain anti-tumor activity while reducing the side effects associated with anti-CTLA-4 activity outside the tumor.

[0008] Provided herein are improved anti-CTLA-4 antibodies, such as an improved ipilimumab, in particular an activatable antibody that when activated binds Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). In some embodiments, the activatable anti-human CTLA-4 antibody comprises:

(i) a heavy chain comprising a heavy chain variable domain (VH) comprising complementarity determining regions (CDRs) CDRH1: SYTMH (SEQ ID NO: 557); CDRH2: FISYDGNNKYYADSVKG (SEQ ID NO: 558); and CDRH3: TGWLGPFDY (SEQ ID NO: 559); and

(ii) a light chain comprising:

(a) a light chain variable domain (VL) comprising CDRL1: RASQSVGSSYLA (SEQ ID NO: 560); CDRL2: GAFSRAT (SEQ ID NO: 561); and CDRL3: QQYGSSPWT (SEQ ID NO: 562);

(b) a cleavable moiety (CM); and

(c) a masking moiety (MM),

wherein the light chain has the structural arrangement from N-terminus to C-terminus as follows: MM-CM-VL.

[0009] In some embodiments, an activatable anti-human CTLA-4 antibody comprises:

(i) a heavy chain comprising a heavy chain variable domain (VH) comprising CDRH1: SYTMH (SEQ ID NO: 557); CDRH2: FISYDGNNKYYADSVKG (SEQ ID NO: 558); and CDRH3: TGWLGPFDY (SEQ ID NO: 559); and

(ii) a light chain comprising, from N-terminus to C-Terminus:

(a) a masking moiety (MM);

(b) a cleavable moiety (CM); and

(c) a light chain variable domain (VL) comprising CDRL1: RASQSVGSSYLA (SEQ ID NO: 560); CDRL2: GAFSRAT (SEQ ID NO: 561); and CDRL3: QQYGSSPWT (SEQ ID NO: 562).

[0010] In some embodiments, the activatable antibody comprises a heavy chain and a light chain such that the light chain has the structural arrangement, from N-terminus to C-terminus of the light chain, MM-CM-VL. As used herein, the N-terminal fragment that is joined to the VL domain is referred to as the prodomain and comprises MM and CM.

[0011] In some embodiments, the activatable antibody comprises a complete antibody, i.e., an antibody comprising two mature full-length heavy chains and two mature full-length light chains. In some embodiments, the activatable antibody comprises a Fab fragment, a $F(ab')_2$ fragment, an scFv, or a scAb. In some embodiments, the activatable antibody comprises a monoclonal antibody.

[0012] In some embodiments, the CM functions as a substrate for a protease. In some embodiments, the CM is selected from the group of CMs provided in Table 3. In some embodiments, the CM is selected from the group consisting of 2001 (SEQ ID NO: 297), 2003 (SEQ ID NO: 298), 2005 (SEQ ID NO: 299), 2006 (SEQ ID NO: 300), 2007 (SEQ ID NO: 301), 2008 (SEQ ID NO: 302), 2009 (SEQ ID NO: 303), 2011 (SEQ ID NO: 304), 2012 (SEQ ID NO: 305), 3001 (SEQ ID NO: 306), 3006 (SEQ ID NO: 307), 3007 (SEQ ID NO: 308), 3008 (SEQ ID NO: 309), 3009 (SEQ ID NO: 310), 3011 (SEQ ID NO: 311), and 3012 (SEQ ID NO: 312). In some embodiments, the CM is 2001 (SEQ ID NO: 297). In some embodiments, the CM is 2011 (SEQ ID NO: 304). In some embodiments, the CM is 2012 (SEQ ID NO: 305).

[0013] In some embodiments, the MM is selected from the group consisting of the MMs provided in Tables 4-6. In some embodiments, the MM is selected from the group consisting of YV01 (SEQ ID NO: 1), YV02 (SEQ ID NO: 2), YV03, (SEQ ID NO: 3), YV04 (SEQ ID NO: 4), YV09, (SEQ ID NO: 9), YV23 (SEQ ID NO: 23), YV24 (SEQ ID NO: 24), YV35 (SEQ ID NO: 35), YV39 (SEQ ID NO: 39), YV51 (SEQ ID NO: 51), YV61 (SEQ ID NO: 60), YV62 (SEQ IDNO: 61), YV63 (SEQ ID NO: 62), YV64 (SEQ ID NO: 63), YV65 (SEQ ID NO: 64), and YV66 (SEQ ID NO: 65); and the CM is selected from the group consisting of 2001, 2006, 2007, 2008, 2009, 2011, and 2012. In some embodiments, the MM is YV39 and the CM is 2011. In some embodiments, the MM is YV39 and the CM is 2012. In some embodiments, the MM is YV39 and the CM is 2001.

[0014] In some embodiments, the activatable antibody comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 353 and a light chain comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 356 to 529.
In some embodiments, the activatable anti-CTLA-4 antibodies comprise a light chain having a prodomain and VL corresponding to the prodomain and VL of SEQ ID NOs: 356 to 529. In some embodiments, the activatable anti-CTLA-4 antibodies comprise a light chain having a prodomain and VL of SEQ ID NOs: 564, 565, or 563. In one embodiment, the activatable anti-CTLA-4 antibody comprises a light chain having a prodomain and VL of SEQ ID NO: 564.

[0015] In some embodiments, the activatable anti-CTLA-4 antibodies comprise a heavy chain variable domain amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 345. In some embodiments, the activatable anti-CTLA-4 antibodies comprise a light chain variable domain amino acid that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 564, 565, and 563.

[0016] In some embodiments, the activatable antibody comprises a combination of heavy chain sequence SEQ ID NO: 353 and light chain sequence SEQ ID NO: 449, 473, or 383. In some embodiments, the activatable antibody comprises a combination of heavy chain sequence SEQ ID NO: 349 and light chain sequence SEQ ID NO: 448, 472, or 382.

[0017] Provided herein is an activatable anti-CTLA-4 antibody that, when activated, specifically binds to human CTLA-4 and is referred to as an activated activatable anti-CTLA-4 antibody. In some embodiments, the activated activatable anti-CTLA-4 antibody binds to CTLA-4 with the same binding affinity as ipilimumab. Also provided herein is an activatable anti-CTLA-4 antibody that does not bind to CTLA-4 as effectively as ipilimumab since the activatable anti-CTLA-4 antibody comprises a heavy chain and a light chain comprising a prodomain comprising a MM and CM linked to the ipilimumab light chain such that the prodomain reduces the ability of the ipilimumab to bind to CTLA-4

[0018] In some embodiments, the activatable antibody binds to human CTLA-4 with an EC_{50} of 1 μg/mL or higher as measured by flow cytometry. In some embodiments, the activatable anti-CTLA-4 antibodies bind to CTLA-4 with an EC_{50} of 5 μg/mL or higher, 10 μg/mL or higher, 20 μg/mL or higher, or 40 μg/mL or higher.

[0019] In some embodiments, the MM is a polypeptide of no more than 40 amino acids in length. In some embodiments, the MM is a polypeptide that is no more than 50% identical to any natural binding partner of the antibody. In some embodiments, the MM does not comprise more than 25% amino acid sequence identity to CTLA-4. In some embodiments, the MM does not comprise more than 10% amino acid sequence identity to CTLA-4.

[0020] Activatable anti-CTLA-4 antibodies of the disclosure are activated when the cleavable moiety is cleaved by a protease. In some embodiments, the protease is produced by a tumor that is in proximity to T cells that express CTLA-4. In some embodiments, the protease is produced by a tumor that is co-localized with T cells that express CTLA-4. In some embodiments, the protease is selected from the group of proteases provided in Table 1 provided below. In some embodiments, the protease is selected from the group consisting of a matrix metalloprotease (MMP), a thrombin, a neutrophil elastase, a cysteine protease, a legumain, and a serine protease, such as a matriptase or a urokinase (uPA). In some embodiments, the protease is selected from the group consisting of MMP1, MMP2, MMP3, MMP8, MMP9, MMP11, MMP13, MMP14, MMP17, legumain, matriptase, and uPA, or a combination of one or more of such proteases. In some embodiments, the CM is cleaved by a matrix metalloprotease (MMP) and a serine

protease. In some embodiments, the CM is cleaved by a matrix metalloprotease (MMP), a serine protease and a legumain.

Table 1: Exemplary Proteases and/or Enzymes

ADAMS, ADAMTS, *e.g.*	Cysteine proteinases, *e.g.*,	Serine proteases, *e.g.*,
ADAM8	Cruzipain	activated protein C
ADAM9	Legumain	Cathepsin A
ADAM10	Otubain-2	Cathepsin G
ADAM12		Chymase
ADAM15	KLKs, *e.g.*,	coagulation factor proteases
ADAM17/TACE	KLK4	(*e.g.*, FVIIa, FIXa, FXa, FXIa, FXIIa)
ADAMDEC1	KLK5	
ADAMTS1	KLK6	Elastase
ADAMTS4	KLK7	Granzyme B
ADAMTS5	KLK8	Guanidinobenzoatase
	KLK10	HtrA1
Aspartate proteases, *e.g.*,	KLK11	Human Neutrophil Elastase
BACE	KLK13	Lactoferrin
Renin	KLK14	Marapsin
		NS3/4A
Aspartic cathepsins, *e.g.*,	Metallo proteinases, *e.g.*,	PACE4
Cathepsin D	Meprin	Plasmin
Cathepsin E	Neprilysin	PSA
	PSMA	tPA
Caspases, *e.g.*,	BMP-1	Thrombin
Caspase 1		Tryptase
Caspase 2	MMPs, *e.g.*,	uPA
Caspase 3	MMP1	
Caspase 4	MMP2	Type II Transmembrane
Caspase 5	MMP3	Serine Proteases (TTSPs), *e.g.*,
Caspase 6	MMP7	DESC1
Caspase 7	MMP8	DPP-4
Caspase 8	MMP9	FAP
Caspase 9	MMP10	Hepsin
Caspase 10	MMP11	Matriptase-2
Caspase 14	MMP12	MT-SP1/Matriptase
	MMP13	TMPRSS2
Cysteine cathepsins, *e.g.*,	MMP14	TMPRSS3
Cathepsin B	MMP15	TMPRSS4
Cathepsin C	MMP16	
Cathepsin K	MMP17	
Cathepsin L	MMP19	
Cathepsin S	MMP20	
Cathepsin V/L2	MMP23	

Cathepsin X/Z/P	MMP24
	MMP26
	MMP27

[0021] Provided herein are activatable anti-CTLA-4 antibodies that further comprise one or more linker peptides. In some embodiments, the linker peptide is between the MM and the CM. In some embodiments, the linker peptide is between the CM and the VL. In some embodiments, the activatable antibody comprises a first linker peptide (LP1) and a second linker peptide (LP2). In some embodiments, the activatable antibody comprises a heavy chain and a light chain such that the light chain has the structural arrangement, from N-terminus to C-terminus of the light chain, MM-LP1-CM-LP2-VL. In some embodiments, the LP1 and the LP2 are not identical to each other. In some embodiments, the LP1 and the LP2 are identical to each other. In some embodiments, the prodomain comprises MM-LP1-CM-LP2.

[0022] In some embodiments, the LP1 and/or the LP2 comprise a glycine-serine polymer. In some embodiments, the LP1 and/or the LP2 comprise an amino acid sequence selected from the group consisting of $(GS)_n$ (SEQ ID NO: 532), $(GGS)_n$ (SEQ ID NO: 533), $(GSGGS)_n$ (SEQ ID NO: 534), and $(GGGS)_n$ (SEQ ID NO: 535), where n is an integer of at least one. In some embodiments, the LP1 comprises the amino acid sequence GGGSSGGS (SEQ ID NO: 542). In some embodiments, the LP2 comprises the amino acid sequence GGGS (SEQ ID NO: 543).

[0023] Provided herein are activatable anti-CTLA-4 antibodies that also comprise a spacer. In some embodiments, the spacer is joined directly to the MM and has the structural arrangement from N-terminus to C-terminus as follows: spacer-MM-CM-VL. In some embodiments, the spacer comprises an amino acid sequence selected from the group consisting of QGQSGQG (SEQ ID NO: 544), GQSGQG (SEQ ID NO: 545), QGQSGS (SEQ ID NO: 546), QGQSGQ (SEQ ID NO: 547), QSGQG (SEQ ID NO: 548), GQSGS (SEQ ID NO: SEQ ID NO: 549), QGQSG (SEQ ID NO: 550), SGQG (SEQ ID NO: 551), QSGS (SEQ ID NO: 552), QGQS (SEQ ID NO: 553), GQG, SGS, QGQ, QG, GS, G, S, and Q. In some embodiments, the spacer and the MM comprise the amino acid sequence QGQSGSCRTQLYGYNLCPY (SEQ ID NO: 556).

[0024] Also provided herein are activatable antibodies that comprise a toxic agent, such as a dolastatin, an auristatin, an auristatin E, a monomethyl auristatin E (MMAE), a maytansinoid, a duocarmycin, a calicheamicin, a pyrrolobenzodiazepine, or a derivative

thereof. In some embodiments, the toxic agent is conjugated to the activatable antibody via a linker. In some embodiments, the linker is a cleavable linker. In some embodiments, the linker is a non-cleavable linker.

[0025] Provided herein are activatable anti-CTLA-4 antibodies that comprises a detectable moiety. In some embodiments, the detectable moiety is a diagnostic agent.

[0026] Provided herein are pharmaceutical compositions comprising an activatable anti-CTLA-4 antibody described herein. In some embodiments, the pharmaceutical composition comprises an additional therapeutic agent.

[0027] Also provided herein are isolated nucleic acid molecules encoding the heavy and/or light chains of the activatable anti-CTLA-4 antibodies described herein, vectors that comprise one or more of the isolated nucleic acid molecules, and methods of producing an activatable antibody by culturing a cell comprising the vector or vectors under conditions that lead to expression of the activatable antibody.

[0028] Provided herein are methods of manufacturing an activatable antibody, the methods comprising: (a) culturing a cell comprising a nucleic acid construct that encodes the activatable antibody described herein under conditions that lead to expression of the activatable antibody, and (b) recovering the activatable antibody.

[0029] Provided herein are methods of reducing CTLA-4 activity comprising administering an effective amount of the activatable antibody described herein or pharmaceutical compositions comprising an activatable anti-CTLA-4 antibody described herein to a subject in need thereof.

[0030] Provided herein are methods of blocking binding of a natural ligand to CTLA-4 comprising administering an effective amount of the activatable antibodies described herein or pharmaceutical compositions comprising an activatable anti-CTLA-4 antibody described herein to a subject in need thereof.

[0031] Provided herein are methods of treating, alleviating a symptom of, or delaying the progression of a CTLA-4-related disorder comprising administering a therapeutically effective amount of the activatable antibodies described herein or the pharmaceutical compositions comprising an activatable anti-CTLA-4 antibody described herein to a subject in need thereof. In some embodiments, the CTLA-4 related disorder is a cancer. In some embodiments, the cancer is a melanoma, such as unresectable or metastatic melanoma, breast cancer, colorectal cancer, gastric cancer, glioblastoma, head and neck

cancer, lung cancer, ovarian cancer, endometrial cancer, pancreatic cancer, prostate cancer, renal cancer, sarcoma, or skin cancer. In some embodiments, the CTLA-4 related disorder is a disorder known to be treatable with ipilimumab.

[0032] Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but also each member of the group individually and all possible subgroups of the main group, and also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0033] FIGs. 1A to 1C show tumor volumes as a function of days post tumor implantation in mice (n = 10) treated with (i) an unrelated mouse IgG2a antibody (FIG. 1A), (ii) a mouse anti-CTLA-4 (9D9) IgG2a antibody (FIG. 2B), or (iii) an activatable 9D9 antibody (FIG. 1C). All antibodies and activatable antibodies were dosed at 25 µg/mouse. The activatable 9D9 antibody comprises MY11 (SEQ ID NO: 294) as the masking moiety and 2001 (SEQ ID NO: 297) as the cleavable moiety. "TF" indicates the number of tumor free mice at the end of each experiment. The unrelated mouse IgG2a antibody and the mouse anti-CTLA-4 (9D9) IgG2a antibody were used as controls.

[0034] FIGs. 2A to 2C show the frequency of regulatory T cells in the tumor (FIG. 2A) and proliferation and activation of regulatory T cells in the spleen (FIGs. 2B and 2C) of mice treated with different activatable mouse anti-CTLA-4 (9D9) IgG2a antibodies. The different activatable 9D9 antibodies comprise (i) either MY03 (SEQ ID NO: 293) or MY11 (SEQ ID NO: 294) as the masking moiety and (2) 0003 (SEQ ID NO: 320), 1004 (SEQ ID NO: 323), or 2001 (SEQ ID NO: 297) as the cleavable moiety. The unrelated mouse IgG2a antibody ("DT 1D12 mg2a") and the mouse anti-CTLA-4 (9D9) IgG2a antibody ("9D9 mg2a") were used as controls. In FIG. 2A, the frequency of regulatory T cells is shown as a percentage of total CD4+ T cells that are Foxp3+ in the tumor. FIGs. 2B and 2C show the frequency of proliferating (Ki-67+) and activated (ICOS+) regulatory T cells, as a percentage of Foxp3+ T cells, in the spleen, respectively.

[0035] FIGs. 3A to 3E show the ability of different anti-CTLA-4 activatable antibodies (human IgG1 isotype) to bind to human CTLA-4, as measured *in vitro* with an ELISA

binding assay. Ipilimumab ("YV1") was used as a control in all experiments. In FIG. 3A, the anti-CTLA-4 activatable antibodies comprise YV04 (SEQ ID NO: 4), YV06 (SEQ ID NO: 6), YV09 (SEQ ID NO: 9), or YV23 (SEQ ID NO: 23) as the masking moiety. In FIG. 3B, the anti-CTLA-4 activatable antibodies comprise YV27 (SEQ ID NO: 27), YV29 (SEQ ID NO: 29), YV32 (SEQ ID NO: 32), or YV33 (SEQ ID NO: 33) as the masking moiety. In FIG. 3C, the anti-CTLA-4 activatable antibodies comprise YV35 (SEQ ID NO: 35) or YV41 (SEQ ID NO: 41) as the masking moiety. In FIG. 3D, the anti-CTLA-4 activatable antibodies comprise YV24 (SEQ ID NO: 24), YV39 (SEQ ID NO: 39), YV51 (SEQ ID NO: 51), YV52 (SEQ ID NO: 52), or YV53 (SEQ ID NO: 53) as the masking moiety. In FIG. 3E, the anti-CTLA-4 activatable antibodies comprise YV54 (SEQ ID NO: 54), YV55 (SEQ ID NO: 55), YV56 (SEQ ID NO: 56), YV57 (SEQ ID NO: 57), or YV58 (SEQ ID NO: 58) as the masking moiety. In FIGs. 3A to 3E, all the anti-CTLA-4 activatable antibodies comprise 2001 (SEQ ID NO: 297) as the cleavable moiety.

[0036] FIGs. 4A to 4D show the ability of additional anti-CTLA-4 activatable antibodies (human IgG1 isotype) to bind to human CTLA-4, as measured *in vitro* with an ELISA binding assay. Ipilimumab ("YV1") was used as a control in all experiments. In FIG. 4A, the anti-CTLA-4 activatable antibodies comprise YV04, YV06, YV09, YV23, YV27, or YV29 as the masking moiety. In FIG. 4B, the anti-CTLA-4 activatable antibodies comprise YV32, YV33, YV35, or YV41 as the masking moiety. In FIG. 4C, the anti-CTLA-4 activatable antibodies comprise YV24, YV39, YV51, YV52, or YV53 as the masking moiety. In FIG. 4D, the anti-CTLA-4 activatable antibodies comprise YV54, YV55, YV56, YV57, or YV58 as the masking moiety. In FIGs. 4A to 4D, all the anti-CTLA-4 activatable antibodies comprise 3001 as the cleavable moiety.

[0037] FIGs. 5A to 5F show the ability of several anti-CTLA-4 activatable antibodies (mouse IgG2a isotype) to bind to human CTLA-4, as measured *in vitro* with an ELISA binding assay. Ipilimumab ("YV1") was used as a control. In FIG. 5A, the anti-CTLA-4 activatable antibodies comprise YV04 as the masking moiety and 2001 (SEQ ID NO: 297), 2006 (SEQ ID NO: 300), 2007 (SEQ ID NO: 301), 2008 (SEQ ID NO: 302), or 2009 (SEQ ID NO: 303) as the cleavable moiety. In FIG. 5B, the anti-CTLA-4 activatable antibodies comprise YV04 or YV23 as the masking moiety, and 2001, 2006, 2007, 2008, or 2009 as the cleavable moiety. In FIG. 5C, the anti-CTLA-4 activatable

antibodies comprise YV39 as the masking moiety and 2001, 2006, 2008, or 2009 as the cleavable moiety. In FIG. 5D, the anti-CTLA-4 activatable antibodies comprise YV61 (SEQ ID NO: 60), YV62 (SEQ ID NO: 61), YV63 (SEQ ID NO: 62), YV64 (SEQ ID NO: 63), or YV39 (SEQ ID NO: 39) as the masking moiety and 2001 or 2012 as the cleavable moiety. In FIG. 5E, the anti-CTLA-4 activatable antibodies comprise YV65 (SEQ ID NO: 64), YV66 (SEQ ID NO: 65), YV01 (SEQ ID NO: 1), YV02 (SEQ ID NO: 2), or YV39 (SEQ ID NO: 39) as the masking moiety and 2001 or 2012 as the cleavable moiety. In FIG. 5F, the anti-CTLA-4 activatable antibodies comprise YV39 or YV03 (SEQ ID NO: 3) as the masking moiety and 2001 or 2012 as the cleavable moiety.

[0038] FIGs. 6A and 6B compares the ability of anti-CTLA-4 activatable antibodies having either a mouse IgG2a isotype (FIG. 6A) or human IgG1 isotype (FIG. 6B) to bind to human CTLA-4, as measured *in vitro* with an ELISA binding assay. Ipilimumab ("YV1") was used as a control. In both FIGs. 6A and 6B, the anti-CTLA-4 activatable antibodies comprise YV39 as the masking moiety and 2001, 2008, 2011, or 2012 as the cleavable moiety. In a modified antibody of the disclosure (YV39-NSUB), the cleavable moiety was replaced with a protease resistant linker ("NSUB") comprising the amino acid sequence GGSGGSGGGSGGGS (SEQ ID NO: 570).

[0039] FIGs. 7A to 7D show the ability of different anti-CTLA-4 activatable antibodies to bind 58 $\alpha^-\beta^-$ cells overexpressing human CTLA-4, as measured via flow cytometry. Binding is presented as arbitrary fluorescence units (mean fluorescence intensity, MFI, or geometric mean fluorescence intensity, gMFI) as a function of the concentration of anti-CTLA-4 antibody added. In FIG. 7A, the anti-CTLA-4 activatable antibodies comprise YV04, YV23, YV24, or YV39 as the masking moiety and 2001 as the cleavable moiety. In FIG. 7B, the anti-CTLA-4 activatable antibodies comprise YV61, YV62, YV64, or YV39 as the masking moiety and 2001 or 2011 as the cleavable moiety. In FIG. 7C, the anti-CTLA-4 activatable antibodies comprise YV39 as the masking moiety and for the cleavable moiety, 2011 ("Ipi YV39 2011") or three variants of Ipi YV39 2011: (i) mono-clipped ("Ipi YV39 MMP monoclipped"), (ii) fully clipped by MMP ("Ipi YV39 MMP"), or (iii) fully clipped by uPA ("Ipi YV39 2011 uPA"). FIG. 7D provides the EC50 values for the different activatable antibodies shown in FIG. 7C. Ipilimumab was used as a control for FIGs. 7A to 7D.

[0040] FIG. 8 shows the activity of the anti-CTLA-4 activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011") (square) at different concentrations, as measured *in vitro* with an SEB (Staphylococcal enterotoxin B) assay. Antibody activity is shown via IL-2 production by the human PBMCs after SEB stimulation. An unrelated human IgG1 isotype (triangle), ipilimumab (circle), and SEB only stimulation (x-mark) were used as controls.

[0041] FIGs. 9A to 9F show tumor volume as a function of days post tumor implantation in human CTLA-4 knock-in mice (n = 10) treated with different anti-human CTLA-4 activatable antibodies (mouse IgG2a isotype) dosed once at 10 mg/kg. An unrelated mouse IgG2a antibody (FIG. 9A) and ipilimumab with a mouse IgG2a isotype (FIG. 9B) were used as controls. In FIGs. 9C to 9F, the activatable antibodies comprise YV04, YV23, YV24, and YV39, respectively, as the masking moiety and 2001 as the cleavable moiety.

[0042] FIGs. 10A to 10F show tumor volume as a function of days post tumor implantation in human CTLA-4 knock-in mice (n = 10) treated with different anti-human CTLA-4 activatable antibodies (human IgG1 isotype). The antibodies were dosed once at 200 μg/mouse on day 7 post-implantation. An unrelated human IgG1 antibody (FIG. 10A) and ipilimumab with a human IgG1 isotype (FIG. 10B) were used as controls. In FIGs. 10C to 10F, the activatable antibodies comprise YV39 as the masking moiety and 2001, 2012, 2011, or 2008 as the cleavable moiety. Cleavable moieties 2012, 2011, and 2008 have been modified to overcome a deamidation site in 2001.

[0043] FIGs. 11A to 11G show tumor volume as a function of days post tumor implantation in human CTLA-4 knock-in mice (n = 16) treated with different doses of an anti-CTLA activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011") (FIGs. 11E to 11G). The antibody was dosed once at 10 mg/kg (FIG. 11E), 3 mg/kg (FIG. 11F), or 1 mg/kg (FIG. 11G) on day 7 post tumor implantation. Control animals were treated with ipilimumab (10 mg/kg, 3 mg/kg, or 1 mg/kg; FIGs. 11B to 11D, respectively) or an unrelated human IgG1 antibody (FIG. 11A).

[0044] FIGs. 12A to 12D show the frequency of regulatory T cells in the tumor (FIGs. 12A and 12B) or the spleen (FIGs. 12C and 12D) in human CTLA-4 knock-in mice (n = 10) treated with different anti-human CTLA-4 activatable antibodies with a mouse IgG2a

isotype. All antibodies were dosed once at 10 mg/kg. The activatable antibodies comprise YV04, YV23, YV24, or YV39 as the masking moiety and 2001 as the cleavable moiety. The labels on the abscissas of FIGs. 12C and 12D also apply to FIGs. 12A and 12B, respectively. An unrelated human IgG1 antibody and ipilimumab with a mouse IgG2a isotype were used as controls. In FIGs. 12A and 12C, the frequency of regulatory T cells is shown as a percentage of total CD4+ T cells that are Foxp3+. In FIGs. 12B and 12D, the frequency of regulatory T cells is shown as a percentage of total CD45+ T cells that are Foxp3+. FIGs. 12E and 12F show the frequency of activated (ICOS+) cells and proliferating (Ki-67+) cells is shown as a percentage of regulatory T cells in the spleen.

[0045] FIGs. 13A to 13C show the frequency of regulatory T cells in the tumor (FIGs. 13A and 13B) or the spleen (FIG. 13C) in human CTLA-4 knock-in mice treated with anti-CTLA-4 activatable antibody. The activatable antibody used comprises YV39 as the masking moiety and were either a mouse IgG2a isotype or human IgG1 isotype. An unrelated human IgG1 antibody and ipilimumab with a human IgG1 isotype were used as controls. In FIGs. 13A and 13C, the frequency of regulatory T cells is shown as a percentage of total CD4+ T cells that are Foxp3+. In FIG. 13B, the frequency of regulatory T cells is shown as a percentage of total CD45+ T cells that are Foxp3+. FIGs. 13D and 13E show the frequency of proliferating (Ki-67+) and activated (ICOS+) cells as a percentage of regulatory T cells in the spleen.

[0046] FIGs. 14A to 14C show the frequency of regulatory T cells (FIGs. 14A and 14B) or CD4+ effector T cells (FIG. 14C) in the tumors of mice treated with different anti-CTLA-4 activatable antibodies. FIGs. 14D and 14E show the regulatory T cells in the spleen. The anti-CTLA-4 activatable antibodies comprise YV39 as the masking moiety and 2012, 2011, 2008, or 2001 as the cleavable moiety. An unrelated human IgG1 antibody and ipilimumab with a human IgG1 isotype were used as controls. In FIGs. 14A and 14D, the frequency of regulatory T cells is shown as a percentage of total CD4+ T cells that are Foxp3+. In FIGs. 14B and 14E, the frequency of regulatory T cells is shown as a percentage of total CD45+ T cells that are Foxp3+. FIG. 14C shows the frequency of CD4+ effector T cells as a percentage of the total CD45+ T cells in the tumor. FIGs. 14F and 14G show the percentages of proliferating (Ki-67+) and activated (ICOS+) regulatory T cells in the spleen

[0047] FIG. 15 shows the frequency of regulatory T cells in the tumors of human CTLA-4 knock-in mice (n = 8) treated with different doses of either ipilimumab or an anti-CTLA-4 activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011"). The antibodies were dosed once at 10 mg/kg, 3 mg/kg, or 1 mg/kg on day 7 post tumor implantation. An unrelated human IgG1 antibody was used as a control.

[0048] FIGs. 16A and 16B show the percentages of activated (ICOS+) and proliferating (Ki-67+) regulatory T cells in the spleen of human CTLA-4 knock-in mice (n = 8) treated with different doses of either ipilimumab or an anti-CTLA-4 activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011"). The antibodies were dosed once at 10 mg/kg, 3 mg/kg, or 1 mg/kg on day 7 post tumor implantation. An unrelated human IgG1 antibody was used as a control.

[0049] FIGs. 17A to 17D show tumor volume as a function of days post tumor implantation in human CTLA-4 knock-in mice (n = 10) treated with different doses of ipilimumab ("Ipi") (FIG. 17B), a nonfucosylated version of ipilimumab ("Ipi NF") (FIG. 17C), or a nonfucosylated version of an anti-CTLA-4 activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011 NF") (FIG. 17D). The antibodies were dosed once at 10 mg/kg, 3 mg/kg, or 1 mg/kg (left panel, middle panel, and right panel, respectively, in FIGs. 17B to 17D). Control animals received an unrelated human IgG1 antibody (FIG. 17A).

[0050] FIG. 18 shows the frequency of regulatory T cells in the tumors of human CTLA-4 knock-in mice (n = 5) treated with either the nonfucosylated version of ipilimumab ("Ipi NF") or a nonfucosylated version of the anti-CTLA-4 activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("NF Ipi YV39 2011"). The antibodies were dosed once at 200 μg/mouse on day 7 post tumor implantation. An unrelated human IgG1 antibody was used as a control.

[0051] FIG. 19 shows the binding affinities (Kd) for both ipilimumab ("Ipi") and a nonfucosylated version of ipilimumab ("Ipi NF") to various human, cyno, and mouse Fc receptors.

[0052] FIG. 20 shows the median percentage of Ki67+ CD4+ T cells in the blood of cynomolgus monkeys after treatment with an anti-CTLA-4 activatable antibody. The anti-

CTLA-4 activatable antibody comprises YV39 as the masking moiety and 2001 as the cleavable moiety. Vehicle and ipilimumab were used as controls.

DETAILED DESCRIPTION OF INVENTION

[0053] In order that the present description can be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[0054] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a nucleotide sequence," is understood to represent one or more nucleotide sequences. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0055] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0056] It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0057] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0058] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleotide sequences are written left to right in 5' to 3' orientation. Amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects of the

disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0059] The term "cytotoxic T-lymphocyte antigen 4" or "CTLA-4" as used herein refers to a receptor that is a member of the immunoglobulin superfamily that is expressed by activated T cells and transmits an inhibitory signal to T cells. CTLA-4 is homologous to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CTLA4 is also found in regulatory T cells and contributes to its inhibitory function. CTLA-4 is also referred to as cytotoxic T-lymphocyte-associated protein 4, CD152, Insulin-dependent Diabetes Mellitus 12 (IDDM12), Celiac Disease 3 (CELIAC3), GRD4, and GSE. The term "CTLA-4" includes any variants or isoforms of CTLA-4 which are naturally expressed by cells.

[0060] The term "T cell" as used herein is defined as a thymus-derived lymphocyte that participates in a variety of cell-mediated immune reactions. The term "regulatory T cell" as used herein refers to a $CD4^+CD25^+FoxP3^+$ T cell with suppressive properties. "Treg" is the abbreviation used herein for a regulatory T cell.

[0061] The term "helper T cell" as used herein refers to a $CD4^+$ T cell; helper T cells recognize antigen bound to MHC Class II molecules. There are at least two types of helper T cells, Th1 and Th2, which produce different cytokines. Helper T cells become $CD25^+$ when activated, but only transiently become $FoxP3^+$.

[0062] The term "cytotoxic T cell" as used herein refers to a $CD8^+$ T cell; cytotoxic T cells recognize antigen bound to MHC Class I molecules.

[0063] The term "antibody" refers to immunoglobulin molecules and immunologically active portions of immunoglobulin (Ig) molecules, i.e., molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. By "specifically bind" or "immunoreacts with" or "immunospecifically bind" is meant that the antibody reacts with one or more antigenic determinants of the desired antigen and does not react with other polypeptides or binds at much lower affinity ($Kd > 10^{-6}$). Antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, domain antibody, single chain, Fab, and F(ab′)2 fragments, scFvs, and a Fab expression library.

[0064] The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. In general, antibody molecules obtained from humans relate to any of the classes IgG, IgM, IgA, IgE and IgD, which differ from one another by the nature of the heavy chain present in the molecule. Certain classes have subclasses as well, such as IgG1, IgG2, and others. Furthermore, in humans, the light chain may be a kappa chain or a lambda chain.

[0065] As used herein, the term “activatable antibody” refers to an antibody that also comprises a masking moiety (MM) and a cleavable moiety (CM), wherein the MM is joined to the VL of the antibody via the CM, which is cleavable by a protease. As used herein, a "prodomain" comprises the N-terminal fragment that is joined to the VL domain of the anti-human CTLA-4 activatable antibodies and, as such, comprises the MM and CM. In some embodiments, the light chain of the activatable antibody has the structural arrangement from N-terminus to C-terminus as follows: MM-CM-VL. In some embodiments, the prodomain is joined to the VH domain of the anti-human CTLA-4 antibody. An activatable antibody is designed to be cleaved by upregulated proteolytic activity present in most if not all cancers. Such proteolytic cleavage, or activation, removes the prodomain and releases an active antibody, i.e., an activated activatable antibody. Protease activation of activatable antibodies in normal tissue is significantly reduced due to the tight control of proteolytic activity in normal tissues. As such, activatable antibodies remain largely inert in circulation and in normal tissues.

[0066] An activatable antibody, in view of its prodomain masking the antigen binding domain thereby inhibiting the ability of the antigen binding domain to bind to its target, has a lower affinity for binding to the target than does an activated activatable antibody, in which the MM has been removed by proteolytic cleavage of the CM thereby releasing an active antibody. Such released antibody exhibits higher affinity for binding to its target. In some embodiments, the MM interacts specifically with the antigen binding domain of ipilimumab to reduce the antibody’s ability to bind to its target. When the MM

is removed by proteolytic cleavage of the activatable antibody, the released antibody binds to its target with an affinity similar to the parental ipilimumab.

[0067] Schematic representations of activatable antibodies of the present invention, *e.g.* MM-CM-VL, are not intended to be exclusive. Other sequence elements, such as linkers, spacers and signal sequences, may be present before, after, or between the listed sequence elements in such schematic representations. It is also to be appreciated that a prodomain comprising a MM and a CM can be joined to a VH of an antibody instead of to a VL of an antibody such that the heavy chain has the structural arrangement from N-terminus to C-terminus as follows: MM-CM-VH.

[0068] The term "monoclonal antibody" (mAb) or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one molecular species of antibody molecule consisting of a unique light chain gene product and a unique heavy chain gene product. In particular, the complementarity determining regions (CDRs) of the monoclonal antibody are identical in all the molecules of the population. MAbs contain an antigen binding site, or domain, capable of immunoreacting with a particular epitope of the antigen characterized by a unique binding affinity for it. Monoclonal antibody molecules will typically comprise two heavy chains and two light chains.

[0069] The term "antigen binding domain" refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains, referred to as "hypervariable regions," are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus, the term "FR" refers to amino acid sequences which are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs." The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of Proteins of

Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk *J. Mol. Biol.* 196:901-917 (1987), Chothia *et al. Nature* 342:878-883 (1989).

[0070] As used herein, the term "epitope" includes any protein determinant capable of specific binding to an immunoglobulin, an scFv, or a T-cell receptor. The term "epitope" includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. For example, antibodies may be raised against N-terminal or C-terminal peptides of a polypeptide. An antibody is said to specifically bind an antigen when the dissociation constant is $\leqq$1 μM; preferably $\leqq$100 nM and most preferably $\leqq$10 nM.

[0071] As used herein, the terms "specific binding," "immunological binding," and "immunological binding properties" refer to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (k_{on}) and the "off rate constant" (k_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. (*See Nature* 361:186-87 (1993)). The ratio of k_{off}/k_{on} enables the cancellation of all parameters not related to affinity, and is equal to the dissociation constant K_d. (*See*, generally, Davies *et al.* (1990) *Annual Rev Biochem* 59:439-473). An antibody of the present invention is said to specifically bind to CTLA-4, when the equilibrium binding constant (K_d) is $\leqq$1 μM, preferably $\leqq$100 nM, more preferably $\leqq$10 nM, and most preferably $\leqq$100 pM to about 1 pM, as measured by assays such as radioligand binding assays or similar assays known to those skilled in the art.

[0072] The term "isolated polynucleotide" as used herein refers to a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated polynucleotide" (1) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotide" is found in nature, (2) is operably linked to a polynucleotide which it is not linked to in nature, or (3) does not occur in nature as part of a larger sequence. Polynucleotides in accordance with the invention include the nucleic acid molecules encoding the heavy chain immunoglobulin molecules shown herein, and nucleic acid molecules encoding the light chain immunoglobulin molecules shown herein.

[0073] The term "isolated protein" referred to herein means a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin, or source of derivation, the "isolated protein" (1) is not associated with proteins found in nature, (2) is free of other proteins from the same source, e.g., free of murine proteins, (3) is expressed by a cell from a different species, or (4) does not occur in nature.

[0074] The term "polypeptide" is used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein fragments, and analogs are species of the polypeptide genus. Polypeptides in accordance with the invention comprise the heavy chain immunoglobulin molecules shown herein, and the light chain immunoglobulin molecules shown herein, as well as antibody molecules formed by combinations comprising the heavy chain immunoglobulin molecules with light chain immunoglobulin molecules, such as kappa light chain immunoglobulin molecules, and vice versa, as well as fragments and analogs thereof.

[0075] The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory or otherwise is naturally-occurring.

[0076] The term "operably linked" as used herein refers to positions of components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that

expression of the coding sequence is achieved under conditions compatible with the control sequences.

[0077] The term "control sequence" as used herein refers to polynucleotide sequences which are necessary to effect the expression and processing of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. The term "polynucleotide" as referred to herein means nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

[0078] The term “oligonucleotide” referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset generally comprising a length of 200 bases or fewer. Preferably oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, e.g., for probes, although oligonucleotides may be double stranded, e.g., for use in the construction of a gene mutant. Oligonucleotides of the invention are either sense or antisense oligonucleotides.

[0079] The term "naturally occurring nucleotides" referred to herein includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" referred to herein includes nucleotides with modified or substituted sugar groups and the like. The term "oligonucleotide linkages" referred to herein includes oligonucleotide linkages such as phosphorothioate, phosphorodithioate, phosphoroselerloate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate, phosphoronmidate, and the like. *See e.g.*, LaPlanche *et al. Nucl. Acids Res.* 14:9081 (1986); Stec *et al. J. Am. Chem. Soc.* 106:6077 (1984), Stein *et al. Nucl. Acids Res.* 16:3209 (1988), Zon *et al. Anti Cancer Drug Design* 6:539 (1991); Zon *et al.* Oligonucleotides and Analogues: A Practical Approach, pp. 87-108 (F. Eckstein, Ed., Oxford University Press, Oxford England (1991)); Stec *et al.* U.S.

Pat. No. 5,151,510; Uhlmann and Peyman *Chemical Reviews* 90:543 (1990). An oligonucleotide can include a label for detection, if desired.

[0080] As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. See Immunology—A Synthesis (2nd Edition, E. S. Golub and D. R. Gren, Eds., Sinauer Associates, Sunderland Mass. (1991)). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α-, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4 hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

[0081] As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity, and most preferably at least 99 percent sequence identity.

[0082] As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present invention, providing that the variations in the amino acid sequence maintain at least 75%, more preferably at least 80%, 90%, 95%, and most preferably 99% sequence identity. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic amino acids are aspartate, glutamate; (2) basic amino acids are lysine, arginine, histidine; (3) non-polar amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and (4) uncharged polar amino acids are glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. The hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and

threonine. The hydrophobic amino acids include alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine. Other families of amino acids include (i) serine and threonine, which are the aliphatic-hydroxy family; (ii) asparagine and glutamine, which are the amide containing family; (iii) alanine, valine, leucine and isoleucine, which are the aliphatic family; and (iv) phenylalanine, tryptophan, and tyrosine, which are the aromatic family. In the case of an antibody, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a CDR or framework region. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art. Preferred amino- and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie *et al. Science* 253:164 (1991). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in accordance with the invention.

[0083] Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis in regions of the activatable antibody other than in the cleavable linker comprising the CM, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring sequence (preferably in the portion

of the polypeptide outside the domain(s) forming intermolecular contacts). A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton *et al. Nature* 354:105 (1991).

[0084] The term "polypeptide fragment" as used herein refers to a polypeptide that has an amino terminal and/or carboxy-terminal deletion and/or one or more internal deletion(s), but where the remaining amino acid sequence is identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full length cDNA sequence. Fragments typically are at least 5, 6, 8 or 10 amino acids long, preferably at least 14 amino acids long′ more preferably at least 20 amino acids long, usually at least 50 amino acids long, and even more preferably at least 70 amino acids long. The term "analog" as used herein refers to polypeptides which comprise a segment of at least 25 amino acids that has substantial identity to a portion of a deduced amino acid sequence and which has specific binding to CTLA-4, under suitable binding conditions. Typically, polypeptide analogs comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally-occurring sequence. Analogs typically are at least 20 amino acids long, preferably at least 50 amino acids long or longer, and can often be as long as a full-length naturally-occurring polypeptide.

[0085] The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials.

[0086] As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods). In certain situations, the label or marker can also be therapeutic. Various methods of labeling polypeptides and glycoproteins are known in the art and may

be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (e.g., ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I) fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, p-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

[0087] Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (Parker, S., Ed., McGraw-Hill, San Francisco (1985)).

[0088] As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, more preferably more than about 85%, 90%, 95%, and 99%. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

[0089] As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. Beneficial or desired clinical results may include, but are not limited to, any one or more of: alleviation of one or more symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing or delaying spread (e.g., metastasis) of disease, preventing or delaying occurrence or recurrence of disease, delay or slowing of disease progression, amelioration of the disease state, and remission (whether partial or total). Also encompassed by "treatment" is a reduction of pathological consequence of a proliferative disease such as cancer. The methods provided herein contemplate any one or more of these aspects of treatment.

[0090] The term "effective amount" used herein refers to an amount of a compound or composition, when used alone or in combination with a second therapy, is sufficient to

treat a specified disorder, condition or disease such as ameliorate, palliate, lessen, and/or delay one or more of its symptoms. In reference to cancers or other unwanted cell proliferation, an effective amount comprises an amount sufficient to cause a tumor to shrink and/or to decrease the growth rate of the tumor (such as to suppress tumor growth) or to prevent or delay other unwanted cell proliferation. An effective amount can be administered in one or more administrations.

[0091] As used herein, by "combination therapy" is meant that a first agent be administered in conjunction with another agent. "In conjunction with" refers to administration of one treatment modality in addition to another treatment modality. As such, "in conjunction with" refers to administration of one treatment modality before, during, or after delivery of the other treatment modality to the individual.

[0092] The term "pharmaceutical agent or drug" as used herein refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a subject.

[0093] As used herein, by "pharmaceutically acceptable" or "pharmacologically compatible" is meant a material that is not biologically or otherwise undesirable, e.g., the material may be incorporated into a pharmaceutical composition administered to an individual or subject without causing any significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained. Pharmaceutically acceptable carriers or excipients have for example met the required standards of toxicological and manufacturing testing and/or are included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug administration.

[0094] The terms "cancer", "cancerous", or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, for example, melanoma, such as unresectable or metastatic melanoma, leukemia, lymphoma, blastoma, carcinoma and sarcoma. More particular examples of such cancers include chronic myeloid leukemia, acute lymphoblastic leukemia, Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL), squamous cell carcinoma, small-cell lung cancer, non-small cell lung cancer, glioma, gastrointestinal cancer, renal cancer, ovarian cancer, liver cancer, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer,

neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer, gastric cancer, germ cell tumor, pediatric sarcoma, sinonasal natural killer, multiple myeloma, acute myelogenous leukemia (AML), and chronic lymphocytic leukemia (CML).

[0095] "Leukemia" refers to progressive, malignant diseases of the blood-forming organs and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Leukemia is generally clinically classified on the basis of (1) the duration and character of the disease--acute or chronic; (2) the type of cell involved; myeloid (myelogenous), lymphoid (lymphogenous), or monocytic; and (3) the increase or non-increase in the number of abnormal cells in the blood--leukemic or aleukemic (subleukemic). Leukemia includes, for example, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemic leukemia, basophylic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, promyelocytic leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, and undifferentiated cell leukemia. In certain aspects, the present invention provides treatment for chronic myeloid leukemia, acute lymphoblastic leukemia, and/or Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL).

I. Anti-CTLA-4 Activatable Antibodies

[0096] The present invention provides improved anti-CTLA-4 antibodies that are as efficacious as the traditional anti-CTLA-4 antibodies (e.g., ipilimumab) but with a

greater, i.e., improved, safety profile. Specifically, the improved anti-CTLA-4 antibodies are activatable monoclonal antibodies (mAbs) that specifically bind human CTLA-4 when activated. These improved anti-CTLA-4 antibodies, also referred to herein as activatable anti-CTLA-4 antibodies or CTLA-4 activatable antibodies, are used in methods of treating, preventing, delaying the progression of, ameliorating and/or alleviating a symptom of a disease or disorder, including but not limited to, a disease or disorder associated with aberrant CTLA-4 expression and/or activity. For example, the activatable anti-CTLA-4 antibodies are used in methods of treating, preventing, delaying the progression of, ameliorating and/or alleviating a symptom of a cancer or other neoplastic condition. Activatable antibodies are described in, for example, US Pat. Nos. 8,513,390, 8,518,404; 9,120,853; 9,127,053 and International Publ. No. WO 2016/149201.

[0097] In some embodiments, the activatable anti-CTLA-4 antibodies provided herein comprise (i) ipilimumab or antigen binding domain thereof (AB), such as an ipilimumab variable light chain (VL), (ii) a cleavable moiety (CM), and (iii) a masking moiety (MM). In some embodiments, the VL is coupled to the MM, such that coupling of the MM reduces the ability of the ipilimumab to bind to CTLA-4. In some embodiments, the MM is coupled to the VL via a cleavable moiety (CM) (also known as a substrate linker) that includes a substrate for a protease, for example, a protease that is over-expressed in the tumor microenvironment.

Antibody or Antigen Binding Fragment Thereof

[0098] In some embodiments, the antibody or antigen binding domain thereof (AB) comprises the complementarity determining regions (CDRs) of the anti-CTLA-4 antibody ipilimumab, identified as 10D1 in U.S. Patent Nos. 6,984,720 and 7,605,238, which are hereby incorporated by reference in their entireties. Ipilimumab (also formerly known as MDX-010 and BMS-734016) is marketed as YERVOY® and has been approved for the treatment of metastatic melanoma and is in clinical testing in other cancers. *See* Hoos *et al.* (2010) *Semin. Oncol.* 37:533; Hodi *et al.* (2010) *N. Engl. J. Med.* 363:711; Pardoll (2012) *Nat. Immunol.* 13(12): 1129.

[0099] Ipilimumab has a human IgG1 isotype, which binds best to most human Fc receptors (Bruhns *et al.* (2009) *Blood* 113: 3716) and is considered equivalent to murine

IgG2a with respect to the types of activating Fc receptors that it binds. Since IgG1 binds to the activating receptor CD16 (FcγRIIIa) expressed by human NK cells and monocytes, ipilimumab can mediate ADCC. The IgG1-isotype ipilimumab was originally isolated directly from a hybridoma but was subsequently cloned and expressed in Chinese hamster ovary (CHO) cells. Notwithstanding the consideration that an isotype that mediates ADCC and/or CDC might be undesirable in an antibody targeting a receptor on T cells that seeks to upregulate an immune response, the IgG1 isotype of the antibody was retained, in part, because it enhanced vaccine response in cynomolgus monkey and was considered functional. Ipilimumab has been shown to increase the numbers of activated T cells in the blood, as evidenced, for example, by a significant increase in the expression of HLA-DR on the surface of post-treatment $CD4^+$ and $CD8^+$ cells as well as increases in absolute lymphocyte count (Ku *et al.* (2010) *Cancer* 116:1767; Attia *et al.* (2005) *J. Clin. Oncol.* 23:6043; Maker *et al.* (2005) *J. Immunol.* 175:7746; Berman *et al.* (2009) *J. Clin. Oncol.* 27(suppl):15s.3020; Hamid *et al.* (2009) *J. Clin. Oncol.* 27(suppl): 15s.9008), indicating that depletion of T cells does not occur in the periphery in man. Ipilimumab demonstrated only modest levels of ADCC of activated T cells using IL-2-activated PBMCs as effector cells; however, use of T_{regs} as targets was not tested. Minor changes in peripheral T_{reg} frequency in the blood of patients treated with ipilimumab have been observed (Maker *et al.* (2005) *J. Immunol.* 175:7746), but little information of the effect of ipilimumab on intratumoral T_{regs} is available. However, a positive correlation between a high $CD8^+$ to T_{reg} ratio and tumor necrosis in biopsies from metastatic melanoma lesions from patients treated with ipilimumab have been described. Hodi *et al.* (2008) *Proc. Nat'l Acad. Sci. (USA)* 105:3005. In addition, tumor tissue from ipilimumab-treated bladder cancer patients had lower percentages of $CD4^+$ $Foxp3^+$ T cells than tumors from untreated bladder cancer patients. Liakou *et al.* (2008) *Proc. Nat'l Acad. Sci. (USA)* 105:14987.

[0100] In some embodiments, the activatable anti-CTLA-4 antibody comprises a combination of a variable heavy chain CDR1 (VH CDR1, also referred to herein as CDRH1), CDR2 (VH CDR2, also referred to herein as CDRH2), and CDR3 (VH CDR3, also referred to herein as CDRH3), and a variable light chain CDR1 (VL CDR1, also referred to herein as CDRL1), CDR2 (VL CDR2, also referred to herein as CDRL2), and

CDR3 (VL CDR3, also referred to herein as CDRL3). These CDR sequences are provided at Table 2.

Table 2: CDR Sequences of heavy and light chains for Ipilimumab

CHAIN	CDR1	CDR2	CDR3
LIGHT	RASQSVGSSYLA (SEQ ID NO: 560)	GAFSRAT (SEQ ID NO: 561)	QQYGSSPWT (SEQ ID NO: 562)
HEAVY	SYTMH (SEQ ID NO: 557)	FISYDGNNKYYADSVKG (SEQ ID NO: 558)	TGWLGPFDY (SEQ ID NO: 559)

[0101] Ipilimumab-VL chain
EIVLTQSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIK (SEQ ID NO: 344)

[0102] Ipilimumab-VH chain
QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYD GNNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDY WGQGTLVTVSS (SEQ ID NO: 345)

[0103] Various other sequences, as indicated, are provided below.

[0104] Human Kappa constant LC
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES VTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 346)

[0105] Mouse Kappa constant light chain
RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNS WTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 347)

[0106] Ipilimumab—Human Kappa LC
EIVLTQSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTVA

APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 348)

[0107] Ipilimumab—Mouse Kappa LC

EIVLTQSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRA TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRADA APTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQ DSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 349)

[0108] Human IgG1 constant HC

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPP CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP G (SEQ ID NO: 350)

[0109] Mouse IgG1 constant HC

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PG (SEQ ID NO: 351)

[0110] Mouse IgG2a constant HC

AKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPA VLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKC PAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVH TAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPK

GSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKN
TEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK
(SEQ ID NO: 352)

[0111] Ipilimumab-VH—Human IgG1 constant HC
QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYD
GNNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDY
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEP
KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPG (SEQ ID NO: 353)

[0112] Ipilimumab-VH—Mouse IgG1 constant HC
QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYD
GNNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDY
WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPG (SEQ ID NO: 354)

[0113] Ipilimumab-VH—Mouse IgG2a constant HC
QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYD
GNNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDY
WGQGTLVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNS
GSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEP
RGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDV
QISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNK

DLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEW TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHN HHTTKSFSRTPGK (SEQ ID NO: 355)

[0114] In some embodiments, the antibody comprises a combination of a VH CDR1 sequence, a VH CDR2 sequence, a VH CDR3 sequence, a VL CDR1 sequence, a VL CDR2 sequence, and a VL CDR3 sequence, wherein at least one CDR sequence comprises 1, 2, 3, 4 or more amino acid sequence differences compared with the CDR sequences shown in Table 2, including conservative amino acid differences.

[0115] In some embodiments, the activatable anti-CTLA-4 antibody comprises a heavy chain variable domain that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the group consisting of SEQ ID NO: 345. In some embodiments, the activatable anti-CTLA-4 antibody comprises a light chain variable domain, not including any MM, CM, linker, spacer or other sequence added in creation of the activatable form of the antibody, that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the group consisting of SEQ ID NOs: 563 to 565.

[0116] In some embodiments, the antibody or antigen-binding fragment thereof that binds CTLA-4 in the activatable antibodies can include modifications, particularly in the Fc region of the antibody or antigen-binding fragment thereof. For example, the interaction of antibodies with FcγRs can be enhanced by modifying the glycan moiety attached to each Fc fragment at the N297 residue. In particular, the absence of core fucose residues strongly enhances ADCC via improved binding of IgG to activating FcγRIIIA without altering antigen binding or CDC. Natsume *et al.* (2009) *Drug Des. Devel. Ther.* 3:7. There is convincing evidence that afucosylated tumor-specific antibodies translate into enhanced therapeutic activity in mouse models *in vivo*. Nimmerjahn & Ravetch (2005) *Science* 310:1510; Mossner *et al.* (2010) *Blood* 115:4393.

[0117] Modification of antibody glycosylation can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of this disclosure to thereby produce an antibody with altered glycosylation. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (α-(1,6) fucosyltransferase) (see U.S. Pat.

App. Publication No. 20040110704; Yamane-Ohnuki *et al.* (2004) *Biotechnol. Bioeng.* 87: 614), such that antibodies expressed in these cell lines lack fucose on their carbohydrates. As another example, EP 1176195 also describes a cell line with a functionally disrupted FUT8 gene as well as cell lines that have little or no activity for adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody, for example, the rat myeloma cell line YB2/0 (ATCC CRL 1662). PCT Publication WO 03/035835 describes a variant CHO cell line, Lec13, with reduced ability to attach fucose to Asn (297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell. See also Shields *et al.* (2002) *J. Biol. Chem.* 277:26733. Antibodies with a modified glycosylation profile can also be produced in chicken eggs, as described in PCT Publication No. WO 2006/089231. Alternatively, antibodies with a modified glycosylation profile can be produced in plant cells, such as Lemna. *See e.g.* U.S. Publication No. 2012/0276086. PCT Publication No. WO 99/54342 describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (e.g., beta(1,4)-N-acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies. *See also* Umaña *et al.* (1999) *Nat. Biotech.* 17:176. Alternatively, the fucose residues of the antibody may be cleaved off using a fucosidase enzyme. For example, the enzyme alpha-L-fucosidase removes fucosyl residues from antibodies. Tarentino *et al.* (1975) *Biochem.* 14:5516. Core fucosylation may also be reduced by culturing antibody-producing cells in the presence of small molecule fucose analogs, such as those described at EP2282773B1, or in the presence of castanospermine, as described at WO 08/052030.

Cleavable Moiety

[0118] In some embodiments, the CM is specific for a protease, which is useful in leveraging the dysregulated protease activity in tumor cells for targeted activatable antibody activation at the site of treatment and/or diagnosis. Numerous studies have demonstrated the correlation of aberrant protease levels, e.g., uPA, legumain, MT-SP1, matrix metalloproteases (MMPs), in solid tumors. (*See e.g.*, Murthy R V, *et al.* "Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer." *Clin Cancer Res.* 11 (2005): 2293-2299; Nielsen B S, *et al.*

"Urokinase plasminogen activator is localized in stromal cells in ductal breast cancer." *Lab Invest* 81 (2001): 1485-1501; Look O R, *et al.* "In situ localization of gelatinolytic activity in the extracellular matrix of metastases of colon cancer in rat liver using quenched fluorogenic DQ-gelatin." *J Histochem Cytochem*. 51 (2003): 821-829).

[0119] A general overview of this process is discussed in US Pat. Nos. 7,666,817, 8,513,390, and 9,120,853 and International Publication Nos. WO 2016/118629 and WO 2016/149201, which are hereby incorporated by reference in their entireties. The cleavable moiety selection process is used to identify cleavable moieties that have a number of desirable characteristics. For example, the selected cleavable moieties are systemically stable (i.e., stable in the systemic circulation of a subject), are generally not susceptible to cleavage by circulating proteases such as plasmin, thrombin, tissue plasminogen activator (tPA) or a kallikrein (KLK) such as KLK-5 and/or KLK-7, are non-toxic, are generally not susceptible to cleavage at potential sites of toxicity such as the skin by proteases such as ADAM 9, ADAM 10, ADAM 17 and/or kallikreins, such as KLK-5 and KLK-7, and are active at an intended site of treatment and/or diagnosis. In some embodiments, the identified cleavable moieties are selected for proteases that are overexpressed at an intended site of therapy and/or diagnosis but are not typically expressed at or in normal, healthy or otherwise non-diseased or non-damaged tissue, and then the selected substrates are subsequently counter-screened against proteases expressed in normal, e.g., non-diseased, tissue. Exemplary proteases and/or enzymes are provided in Table 1 as indicated earlier.

[0120] In some embodiments, the cleavable moiety is selected from the group consisting of 2001 and 3001, and derivatives thereof. In some embodiments, the cleavable moiety is selected from the group consisting of 2001 (SEQ ID NO: 297), 2006 (SEQ ID NO: 300), 2007 (SEQ ID NO: 301), 2008 (SEQ ID NO: 302), 2009 (SEQ ID NO: 303), 2012 (SEQ ID NO: 305), 2011 (SEQ ID NO: 304), 2003 (SEQ ID NO: 298), 3001 (SEQ ID NO: 306), 3006 (SEQ ID NO: 313), 3007 (SEQ ID NO: 308), 3008 (SEQ ID NO: 309), 3009 (SEQ ID NO: 310), 3012 (SEQ ID NO: 312), 3011 (SEQ ID NO: 311), and 2005 (SEQ ID NO: 299). Table 3 provides additional cleavable moieties that may be used with the activatable anti-CTLA-4 antibodies disclosed herein.

Table 3. Anti-CTLA-4 Activatable Cleavable Moieties

SEQUENCE IDENTIFIER	CM Sequence
313	LSGRSDNH
314	LSGRSANPRG
315	TGRGPSWV
316	PLTGRSGG
317	TARGPSFK
318	NTLSGRSENHSG
319	NTLSGRSGNHGS
320	TSTSGRSANPRG
321	TSGRSANP
322	VHMPLGFLGP
306	AVGLLAPPGGLSGRSDNH
307	AVGLLAPPGGLSGRSDDH
308	AVGLLAPPGGLSGRSDIH
309	AVGLLAPPGGLSGRSDQH
310	AVGLLAPPGGLSGRSDTH
338	AVGLLAPPGGLSGRSDYH
339	AVGLLAPPGGLSGRSANI
340	AVGLLAPPGGLSGRSDNI
312	AVGLLAPPGGLSGRSANP
311	AVGLLAPPGGLSGRSDNP
299	AVGLLAPPSGRSANPRG
323	AVGLLAPP
324	AQNLLGMV
325	QNQALRMA
326	LAAPLGLL
327	STFPFGMF
328	ISSGLLSS
329	PAGLWLDP
330	VAGRSMRP
331	VVPEGRRS
332	ILPRSPAF
333	MVLGRSLL
334	VAGRSMRP
335	QGRAITFI
336	SPRSIMLA
337	SMLRSMPL
297	ISSGLLSGRSDNH
300	ISSGLLSGRSDDH
301	ISSGLLSGRSDIH
302	ISSGLLSGRSDQH
303	ISSGLLSGRSDTH
341	ISSGLLSGRSDYH

342	ISSGLLSGRSANI
343	ISSGLLSGRSDNI
305	ISSGLLSGRSANP
304	ISSGLLSGRSDNP
298	ISSGLLSGRSANPRG

Masking Moiety

[0121] The activatable anti-CTLA-4 antibodies provided herein comprise a masking moiety (MM). In some embodiments, the MM is an amino acid sequence that is coupled, or otherwise attached, to the anti-CTLA-4 antibody and is positioned within the activatable anti-CTLA-4 antibody construct such that the MM reduces the ability of the anti-CTLA-4 antibody to specifically bind CTLA-4. In some embodiments, the MM binds specifically to the antigen binding domain. Suitable MMs are identified using any of a variety of known techniques. For example, peptide MMs are identified using the methods described in U.S. Patent Application Publication Nos. 2009/0062142 by Daugherty *et al.* and 2012/0244154 by Daugherty *et al.*, the contents of which are hereby incorporated by reference in their entirety.

[0122] In some embodiments, the MM is selected from the group consisting of YV01 to YV66 and comprises an amino acid sequence selected from Table 4 below.

Table 4: Anti-CTLA4 Masking Moieties (MM)

SEQUENCE IDENTIFIER	MM SEQUENCE	SEQUENCE IDENTIFIER	MM SEQUENCE
1	DFSCLHSMYNVCLDP	147	EHCDVWMFGFNLCPY
2	QPCAQMYGYSMCPHT	148	EPCDYWMFGVNLCPY
3	LHCRTQMYGYNLCPY	149	EQCTMWMYGFNLCPY
4	LHCRTQLYGYNLCPY	150	ESACSLRMYEVCLQP
5	CTYSFFNVC	151	ESCASMYGYSMCPRT
6	CAQMYGYSMC	152	ESCSYWMFGYNLCPY
7	CPNHPMC	153	FSNTCPHHPMCYDYR
8	GTACTYSFFNVCLDP	154	FWNTCPHHPMCHDYK
9	FGTACPNHPMCHDWQ	155	FYQNCYPPTWCSMFS
10	SACAYWMFGVNLCPY	156	GECSYWMFGYNLCPY
11	CRTQLYGYNLC	157	GGSCMYSFFNICLDP
12	CRTQIYGYNLC	158	GGSCVYVMYNVCLDP
13	LHCRTQIYGYNLCPY	159	GHCLMHMYGYNLCPK
14	CPNHPMCHDWQ	160	GHCRMSMYEMTLCPR
15	GTACPNHPMCHDWQ	161	GISCVHIMFNFCLDP
16	CAYWMFGVNLCPY	162	GLCVMYMFGVNLCPY
17	QECHLYMYGVNLCPY	163	GSCDYWMFGYNLCPY

18	CHLYMYGVNLCPY	164	GSYCMYVMYNVCLDP
19	GQCQFYMFGYNLCPY	165	GTKCIYSFYNVCLDP
20	LSTCMYSFFNVCLDP	166	GTSTCPYHPMCHDYR
21	CLHSMYNVCLDP	167	GTTCTYSFFNVCLDP
22	CLHSMYNVC	168	GVCHFFMYGVSMCPA
23	CLHSLYNVCLDP	169	GVPCWYSMYNVCLDP
24	CLHSAYNVCLDP	170	GVSCMYSMFNICLDP
25	CMYSFFNVCLDP	171	HAKCVYSFFNVCLDP
26	CMYSFFNVC	172	HDSCMYSMYNFCLDP
27	QPCAQMYGYSMC	173	HGNTCPNHPMCHDYQ
28	CAQLYGYSMCPHT	174	HKGCLYSFYNICLDP
29	CAQMYGYSMCAHT	175	HKGCLYSFYNVCLDP
30	CAQMYGYSMCPAT	176	HLSCMYIMYNVCLDP
31	CAQMYGYSMCPHT	177	HSSCIYSMFNVCLDP
32	CPNHPLCHDWQ	178	HTNMCPYHPMCYDYK
33	CPNHPMCADWQ	179	HTPCTYSFFNVCLDP
34	CPNHPMCHAWQ	180	IMNTCPYHPMCHDYQ
35	CPNHPMCHDAQ	181	IVPCTYMMFGVCLQP
36	CPNHPMCHDWA	182	KKCDYWFYGVNLCPY
37	GTACPNHPMC	183	KNTCVYSFFNVCLDP
38	LHCRTQLYGYNLC	184	KPCAQMYGYSMCPHP
39	CRTQLYGYNLCPY	185	KPSCMYSFFNVCLDP
40	CRTQLYGYNLCAY	186	KRPCMYSFYNVCLDP
41	CRTQLYGYNLCPA	187	KTSCMYSFYNICLDP
42	FGTACPNHPLCHDWQ	188	KTTCTYSFFNVCLDP
43	CPNHPLCHDFQ	189	LDCQMYWWFGACGDM
44	CPNHPLCHDYQ	190	LHCAIYMYGYNLCPF
45	CPNHPLCPY	191	LHCPFQMYGYNLCPH
46	CPNHPLCPA	192	LHCSMYMYGFNLCPN
47	CMYSFFNVCYP	193	RECMAYMYGYNLCPY
48	CMYSFFNVCYA	194	RHCQMHMFGYDLCPY
49	CLYSFFNVCYP	195	LIHCRYVMYGMCLEP
50	CLYSFFNVCYA	196	LLPCEVMGPSRCKHD
51	FGAACPNHPICHDWQ	197	LPCHAYMYGYSLCPY
52	FGAACPNHPLCHDWQ	198	LPCLAYMYGVNLCPN
53	FGAACPNHPMCHDAQ	199	LPCMAYMFGFNLCPH
54	CLHSAYNACLDP	200	LPCNFHMFGFNLCPY
55	CAHSAYNVCLDP	201	LQCAMYMYGYNLCPY
56	CLHSAYNVCADP	202	LSSCTYSFFNVCLDP
57	CLHSAYNVCLAP	203	LTCPFQMYGYNLCPY
58	CLHSAYNVCLDA	204	LTSQCSPWYWCQIYD
59	KNTCTYVMYNVCLDP	205	LYCPYMMYGYNLCPY
60	YISDCPYHPMCHDYQ	206	LYHCTYSFYNVCLDP
61	FRNTCPYHPMCHDYR	207	LYRCIYSFYNVCLDP
62	RECHMWMFGVNLCPY	208	MGCSMRMWGMELCPE
63	AVCHMYMYGYNLCPF	209	MKCDYWLYGYNLCPY
64	RSCPQMYGYSMCPHT	210	MNHCTLHMYNICMDP
65	QPCAQMFGYSMCPHT	211	MNPECPHHPMCHNSN
66	TAKCTYSFFNVCLDP	212	MPACTYSFFNICLDP
67	DFSCLYSMYNVCLDP	213	MPQCHVIMYNLCLDP
68	DVSCMYMMYNFCLDP	214	MSTCTYSFFNVCLDP

69	CPNHPMC	215	MTCNYWFYGVNLCPY
70	CMYSFFNVCPY	216	MYCHQSMFGFRMCPD
71	CMYSFFNVCPA	217	NACAQMYGYSMCPHT
72	CTYSFFNVCPY	218	NDCDISMFDQSLCPY
73	CTYSFFNVCPA	219	NFSCVYVMFNVCLDP
74	GFPCMYSMFNVCLDP	220	NFTCALTMYEVCLDP
75	GLSCMYSMYGYCLDP	221	NLCHAFMFGFNLCPY
76	IPCDYWMFGVNLCPY	222	NLNNCPHHPMCHDYQ
77	QVCHAYMYGYNLCPY	223	NPPCMYSFFNICLDP
78	RMYCTYSFYNVCLDP	224	NSACTYSFFNVCLDP
79	ALSCMYIMYNVCLDP	225	NVCTVSMFGVMLCPS
80	DFSCMYVMFNVCLDP	226	PACATLMYSVPLCPA
81	DFSCVYSMFNVCLDP	227	PAPCMYSFYNVCLDP
82	DMNTCPNHPMCYDYR	228	PLCAEMYGYSMCPHN
83	DMNTCPRHPMCHDYH	229	PQCHLYMYGYNLCPY
84	DSRCMYVMYNVCLDP	230	PRPCMYSFYNVCLDP
85	EHLCTYSFYNVCLDP	231	QHCPFQMYGYNLCPY
86	ELSCVYSMFGFCLDP	232	QHCQMHMFGYNLCPY
87	FTNNCPYHPMCHDYL	233	QHSCMYSFFNVCLDP
88	GFSCTYIMYDVCLDP	234	QKCHSYLYGVNLCPY
89	GSSCMYSMYNVCLDP	235	QKCNMFMFGYNLCPY
90	HFSCMYIMYNVCLDP	236	QMNDCPNHPMCHDYH
91	LHCGMWMFGVNLCPK	237	QPCAQMYGYSMCPAT
92	LPCQMWMFGHNLCPH	238	QPCAQMYGYSMCPRT
93	LPCTMYMYGYNLCPY	239	RECHFFFYGVNLCPY
94	LTCHHWMFGVNLCPY	240	LNCGMFMYGYNLCPY
95	NFSCMYSMFNVCLDP	241	RLCTSYMFGYNLCPQ
96	NNHCMYSFFNICLDP	242	RLSCMYSMFNVCLDP
97	NRSCMYIMYNVCLDP	243	RNCPFVMFGVNLCPY
98	NSCTMFMFGVNLCPY	244	RNGCMYSFFNVCLDP
99	NTCELYMFGVNLCPY	245	RNGCVYSFFNVCLDP
100	QHCDMWMFGYNLCPY	246	RPCHLYMFGYNLCPD
101	QHCPMYMFGYNLCPF	247	RPCHSYMYGINLCPY
102	QVCHIQMYGFDLCPH	248	RSCDMIMFGFNLCPY
103	RACDYWMYGVNLCPY	249	RSCPMWFYGVNLCPY
104	RQCHMQMFGYDLCPF	250	RSTVCFYDFCGPWER
105	SGSCLYSFYNVCLDP	251	RTCHFYMYGVNLCPY
106	SNGCTYSFFNVCLDP	252	RTCSMVMFGVNLCPY
107	STCAQMYGYSMCPH	253	SGKCTYSFFNVCLDP
108	SYKCLYSFYNVCLDP	254	SIVCDLYWEATCLRP
109	VLYCTYVMYNVCLDP	255	SLSCTYSFFNICLDP
110	VNCGMWMFGYNLCPK	256	SMNTCPYHPMCFDYK
111	YGSCLYSFYNICLDP	257	SQCWMWMYGYNLCPK
112	YPCAQMYGYSMCPHT	258	SSSCMYSFFNVCLDP
113	AACDLWMFGVNLCPY	259	STACTYSFYNVCLDP
114	AFCTLAPYNQACIAN	260	STCAQMYGYSMCPHT
115	AGSCLYSMYNVCLDP	261	STRCVYSFYNVCLDP
116	ALCENTMYGYHLCPW	262	TACGAWMFGVNLCPY
117	ALSCMYIMYGVCLDP	263	TGACMYSFYNVCLDP
118	APVCDVLMFGFCMQP	264	TLSCMYSMYNVCLDP
119	AQVCSIMMYGTCLMP	265	TSCTVTMYQISMCPY

120	ASTCMYSFYNVCLDP	266	VGGCRHSFYNVCLDP
121	AVCEFWMFGFNLCPY	267	VHCQMYMYGYNLCPY
122	DANTCPNHPMCYDYH	268	VHNCMYSFFNVCLDP
123	DFSCIYIMFDVCLDP	269	VMCKLHMYGIPVCPK
124	DFSCMYVMYGFCLDP	270	VNFCNYSMYGICLLP
125	DFTCMYSMYNVCLDP	271	VNFCYACYCMSCVFS
126	DFTCTYSMYNVCLDP	272	VNQCTYSFFNVCLDP
127	DHYCTYIMYSICLDP	273	VPCPFHMFGYNLCPY
128	DICTNFMFGVNLCPY	274	VRCQMWMYGFNLCPH
129	DINTCPYHPMCHDYH	275	VRPCTYSFFNVCLDP
130	DKNTCPLHPMCHDYR	276	VSGCTYSFFNICLDP
131	DMNMCPNHPMCHDWH	277	YCSSWDTMTIPACNN
132	DMNSCPNHPMCHDYH	278	YDCDLSMFGIEMCPQ
133	DMNSCPNHPMCYDYR	279	YGNTCPFHPMCHDYK
134	DMNTCPNHPMCFDYR	280	YGYCMYSFFNVCLDP
135	DMNTCPNHPMCHDFQ	281	YHCTMHMFGYNLCPF
136	DMNTCPNHPMCHDYR	282	YMNTCPNHPMCFDYQ
137	DMNTCPNHPMCYDYH	283	YMNTCPYHPMCHDYL
138	DMNTCPNHPMCYDYK	284	YMNTCPYHPMCHDYR
139	DMSTCPNHPMCHDYM	285	YNNCTYSFFNVCLDP
140	DRNMCPYHPMCYDYR	286	YPGCQYSFFNVCLDP
141	DSCAFMMFGVNLCPY	287	YRSCTHIMYNVCLDP
142	DSCRSVFDMVWNCWN	288	YSFCDMLMYDVCLVP
143	DTPNCPHHPMCHNHM	289	YSIDCGLSWWCGGMT
144	DVSCLYVMYSVCLDP	290	YSTTCPYHPMCHDYH
145	DWCASMMFGYNLCPY	291	YVNTCPHHPMCHDYH
146	EFSCMYSMFNVCLDP	292	YVNTCPYHPMCHDYN

[0123] In some embodiments, the K_d of the activatable anti-CTLA-4 antibody, comprising a MM disclosed herein, towards the target is at least 2, 3, 4, 5, 10, 25, 50, 100, 250, 500, 1,000 times greater than, or between 5-10, 10-100, 10-200, 10-500, 10-1,000 times greater than the K_d of the AB not modified with a MM or of the parental AB towards the target.

[0124] In some embodiments, the MM is not a natural binding partner of the activatable antibody. In some embodiments, the MM contains no or substantially no homology to any natural binding partner of the activatable antibody. In some embodiments, the MM is no more than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80% identical to any natural binding partner of the activatable antibody. In some embodiments, the MM is no more than 50%, 25%, 20%, or 10% identical to any natural binding partner of the activatable antibody. In some embodiments, the MM is no more than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80% identical to human CTLA-4. In some embodiments, the MM is no more than 50%, 25%, 20%, or 10% identical to human CTLA-4.

Exemplary Activatable anti-CTLA-4 Antibodies

[0125] Particular antibodies described herein are activatable anti-CTLA-4 antibodies comprising any combination of the masking moieties, cleavable moieties, light chain variable domains (VL) (or the corresponding CDRs), and heavy chain variable domains (VH) (or the corresponding CDRs) provided in Tables 2-6. In some embodiments, the activatable anti-CTLA-4 antibody comprises a light chain comprising YV01 (SEQ ID NO: 1) as the masking moiety, LSGRSDNH (SEQ ID NO: 313) as the cleavable moiety, and the light chain variable domain (VL) of ipilimumab (SEQ ID NO: 344). In some embodiments, the activatable anti-CTLA-4 antibody comprises a light chain comprising YV01 (SEQ ID NO: 1) as the masking moiety, ISSGLLSGRSDNH (2001) (SEQ ID NO: 297) as the cleavable moiety, and the CDRs of the light chain variable domain (VL) of ipilimumab (SEQ ID NOs: 560, 561, and 562, respectively). In some embodiments, the activatable anti-CTLA-4 antibody comprises the heavy chain variable domain (VH) of ipilimumab (SEQ ID NO: 345) or just the corresponding CDRs (SEQ ID NOs: 557, 558, and 559).

[0126] In some embodiments, the activatable anti-CTLA-4 comprises YV39 (SEQ ID NO: 39) as the masking moiety, and ISSGLLSGRSDNP ("2011") (SEQ ID NO: 304) as the cleavable moiety, and the heavy and light chain variable domains of ipilimumab ((SEQ ID NOs: 345 and 344, respectively), wherein the MM and CM are linked to the VL in the arrangement MM-CM-VL.

[0127] In some embodiments, the activatable anti-CTLA-4 antibody includes a signal peptide. The signal peptide can be linked to the activatable anti-CTLA-4 antibody by a spacer. In some embodiments, the spacer is conjugated to the activatable antibody in the absence of a signal peptide. In some embodiments, the spacer is joined directly to the MM of the activatable antibody. In some embodiments, the spacer has amino acid sequence QGQSGS (SEQ ID NO: 546). In some embodiments, an activatable antibody comprises a spacer of sequence QGQSGS (SEQ ID NO: 546) joined directly to a MM sequence CRTQLYGYNLCPY (YV39) (SEQ ID NO: 39) in the structural arrangement from N-terminus to C-terminus of "spacer-MM-CM-VL" or "spacer-MM-CM-AB."

[0128] In some embodiments, the activatable anti-CTLA-4 antibody comprises a linker peptide (LP) between the MM and the CM. In some embodiments, the activatable anti-CTLA-4 antibody comprises a linker peptide between the CM and the antibody or antigen

binding domain thereof (AB). In some embodiments, the activatable anti-CTLA-4 antibody comprises a first linker peptide (LP1) and a second linker peptide (LP2), and wherein the activatable anti-CTLA-4 antibody has the structural arrangement from N-terminus to C-terminus as follows: MM-LP1-CM-LP2-AB. In some embodiments, the light chain of the activatable anti-CTLA-4 antibody has the structural arrangement from N-terminus to C-terminus as follows: MM-LP1-CM-LP2-VL. In some embodiments, the two linker peptides need not be identical to each other. Examples of linker peptides that may be used with the activatable anti-CTLA-4 antibodies as disclosed herein are provided in U.S. Patent Publication No. 2016/0193332 and International Publication No. WO 2016/149201, *ibid.*

[0129] The disclosure also comprises a modified anti-CTLA-4 antibody that comprises a MM that is joined to the light chain of the antibody via a non-protease cleavable linker. In some embodiments, the non-protease cleavable linker comprises the amino acid sequence set forth in SEQ ID NO: 570. In some embodiments, such a modified anti-CTLA-4 antibody has a light chain comprising YV39 and a non-protease cleavable linker. In some embodiments, the light chain of the modified anti-CTLA-4 antibody comprises the amino acid sequence:

QGQSGSCRTQLYGYNLCPYGGGSSGGSGGSGGSGGGSGGGSGGSEIVLT
QSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSR
ATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKV
EIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNA
LQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLS
SPVTKSFNRGEC (SEQ ID NO: 530) or

CRTQLYGYNLCPYGGGSSGGSGGSGGSGGGSGGGSGGSEIVLTQSPGTL
SLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPD
RFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF
NRGEC (SEQ ID NO: 531).

[0130] Linkers suitable for use in compositions described herein are generally ones that provide flexibility of the activatable anti-CTLA-4 antibody to facilitate the inhibition of the binding of the activatable antibody to the target. Such linkers are generally referred to as flexible linkers (also referred to as linker peptides herein). Suitable linkers can be readily selected and can be of any of a suitable of different lengths, such as from 1 amino acid (e.g. , Gly) to 20 amino acids, from 2 amino acids to 15 amino acids, from 3 amino acids to 12 amino acids, including 4 amino acids to 10 amino acids, 5 amino acids to 9 amino acids, 6 amino acids to 8 amino acids, or 7 amino acids to 8 amino acids, and may

be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids in length.

[0131] Exemplary flexible linkers include glycine polymers (G)n, glycine-serine polymers (including, for example, (GS)n, (GSGGS)n (GSGGS is SEQ ID NO: 534) and (GGGS)n (GGGS is SEQ ID NO: 535), where n is an integer of at least one), glycine-alanine polymers, alanine- serine polymers, and other flexible linkers known in the art. Glycine and glycine-serine polymers are relatively unstructured, and therefore may be able to serve as a neutral tether between components. Glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (*see* Scheraga, *Rev. Computational Chem.* 11173-142 (1992)). Exemplary flexible linkers include, but are not limited to Gly-Gly-Ser-Gly (SEQ ID NO: 536), Gly-Gly-Ser-Gly-Gly (SEQ ID NO: 537), Gly-Ser-Gly-Ser-Gly (SEQ ID NO: 538), Gly-Ser-Gly-Gly-Gly (SEQ ID NO: 539), Gly- Gly-Gly-Ser-Gly (SEQ ID NO: 540), Gly-Ser-Ser-Ser-Gly (SEQ ID NO: 541), and the like. The ordinarily skilled artisan will recognize that design of an activatable antibodies can include linkers that are all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired activatable antibodies structure.

[0132] In some embodiments, the activatable anti-CTLA-4 antibodies comprise the VL and VH (or the corresponding CDRs) of ipilimumab and a combination of MMs and CMs provided in Table 5 below, such that any MM in column 2 can be combined with any CM in column 4.

Table 5. Activatable anti-CTLA-4 Antibody Combinations

SEQ ID NO.	Masking Moiety (MM)	SEQ ID NO.	Cleavable Moiety (CM)
1	(YV01) DFSCLHSMYNVCLDP	313	LSGRSDNH
2	(YV02) QPCAQMYGYSMCPHT	314	LSGRSANPRG
3	(YV03) LHCRTQMYGYNLCPY	315	TGRGPSWV
4	(YV04) LHCRTQLYGYNLCPY	316	PLTGRSGG
5	(YV05) CTYSFFNVC	317	TARGPSFK
6	(YV06) CAQMYGYSMC	318	NTLSGRSENHSG
7	(YV07) CPNHPMC	319	NTLSGRSGNHGS
8	(YV08) GTACTYSFFNVCLDP	320	TSTSGRSANPRG
9	(YV09) FGTACPNHPMCHDWQ	321	TSGRSANP
10	(YV10) SACAYWMFGVNLCPY	322	VHMPLGFLGP
11	(YV11) CRTQLYGYNLC	323	AVGLLAPP
12	(YV12) CRTQIYGYNLC		
13	(YV13) LHCRTQIYGYNLCPY		

14	(YV14) CPNHPMCHDWQ	324	AQNLLGMV
15	(YV15) GTACPNHPMCHDWQ	325	QNQALRMA
16	(YV16) CAYWMFGVNLCPY	326	LAAPLGLL
17	(YV17) QECHLYMYGVNLCPY	327	STFPFGMF
18	(YV18) CHLYMYGVNLCPY	328	ISSGLLSS
19	(YV19) GQCQFYMFGYNLCPY	329	PAGLWLDP
20	(YV20) LSTCMYSFFNVCLDP	330	VAGRSMRP
21	(YV21) CLHSMYNVCLDP	331	VVPEGRRS
22	(YV22) CLHSMYNVC	332	ILPRSPAF
23	(YV23) CLHSLYNVCLDP	333	MVLGRSLL
24	(YV24) CLHSAYNVCLDP	334	VAGRSMRP
25	(YV25) CMYSFFNVCLDP	335	QGRAITFI
26	(YV26) CMYSFFNVC	336	SPRSIMLA
27	(YV27) QPCAQMYGYSMC	337	SMLRSMPL
28	(YV28) CAQLYGYSMCPHT	297	ISSGLLSGRSDNH
29	(YV29) CAQMYGYSMCAHT	300	ISSGLLSGRSDDH
30	(YV30) CAQMYGYSMCPAT	301	ISSGLLSGRSDIH
31	(YV31) CAQMYGYSMCPHT	302	ISSGLLSGRSDQH
32	(YV32) CPNHPLCHDWQ	303	ISSGLLSGRSDTH
33	(YV33) CPNHPMCADWQ	341	ISSGLLSGRSDYH
35	(YV34) CPNHPMCHAWQ	342	ISSGLLSGRSANI
35	(YV35) CPNHPMCHDAQ	343	ISSGLLSGRSDNI
36	(YV36) CPNHPMCHDWA	305	ISSGLLSGRSANP
37	(YV37) GTACPNHPMC	304	ISSGLLSGRSDNP
38	(YV38) LHCRTQLYGYNLC	298	ISSGLLSGRSANPRG
39	(YV39) CRTQLYGYNLCPY	306	AVGLLAPPGGLSGRSDNH
40	(YV40) CRTQLYGYNLCAY	307	AVGLLAPPGGLSGRSDDH
41	(YV41) CRTQLYGYNLCPA	308	AVGLLAPPGGLSGRSDIH
42	(YV42) FGTACPNHPLCHDWQ	309	AVGLLAPPGGLSGRSDQH
43	(YV43) CPNHPLCHDFQ	310	AVGLLAPPGGLSGRSDTH
44	(YV44) CPNHPLCHDYQ	338	AVGLLAPPGGLSGRSDYH
45	(YV45) CPNHPLCPY	339	AVGLLAPPGGLSGRSANI
46	(YV46) CPNHPLCPA	340	AVGLLAPPGGLSGRSDNI
47	(YV47) CMYSFFNVCYP	312	AVGLLAPPGGLSGRSANP
48	(YV48) CMYSFFNVCYA	311	AVGLLAPPGGLSGRSDNP
49	(YV49) CLYSFFNVCYP	299	AVGLLAPPSGRSANPRG
50	(YV50) CLYSFFNVCYA		
51	(YV51) FGAACPNHPICHDWQ		
52	(YV52) FGAACPNHPLCHDWQ		
53	(YV53) FGAACPNHPMCHDAQ		
54	(YV54) CLHSAYNACLDP		
55	(YV55) CAHSAYNVCLDP		
56	(YV56) CLHSAYNVCADP		
57	(YV57) CLHSAYNVCLAP		
58	(YV58) CLHSAYNVCLDA		
59	(YV60) KNTCTYVMYNVCLDP		
60	(YV61) YISDCPYHPMCHDYQ		
61	(YV62) FRNTCPYHPMCHDYR		
62	(YV63) RECHMWMFGVNLCPY		
63	(YV64) AVCHMYMYGYNLCPF		
64	(YV65) RSCPQMYGYSMCPHT		
65	(YV66) QPCAQMFGYSMCPHT		

[0133] In some embodiments, the activatable anti-CTLA-4 antibodies comprise the specific combination of MMs and CMs provided in Table 6 below.

Table 6. Exemplary Activatable Anti-CTLA-4 Antibody Combination

Comb. No.	Masking Moiety (MM)	Cleavable Moiety (CM)
1	CRTQLYGYNLCPY (SEQ ID NO: 39)	ISSGLLSGRSDNH (SEQ ID NO: 297)
2	CRTQLYGYNLCPY (SEQ ID NO: 39)	ISSGLLSGRSDNP (SEQ ID NO: 304)
3	CRTQLYGYNLCPY (SEQ ID NO: 39)	ISSGLLSGRSANP (SEQ ID NO: 305)
4	CRTQLYGYNLCPY (SEQ ID NO: 39)	ISSGLLSGRSDQH (SEQ ID NO: 302)
5	CRTQLYGYNLCPY (SEQ ID NO: 39)	ISSGLLSGRSDDH (SEQ ID NO: 300)
6	CRTQLYGYNLCPY (SEQ ID NO: 39)	ISSGLLSGRSDTH (SEQ ID NO: 303)
7	LHCRTQMYGYNLCPY (SEQ ID NO: 3)	ISSGLLSGRSDNH (SEQ ID NO: 297)
8	LHCRTQMYGYNLCPY (SEQ ID NO: 3)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
9	LHCRTQMYGYNLCPY (SEQ ID NO: 3)	ISSGLLSGRSDDH (SEQ ID NO: 300)
10	LHCRTQMYGYNLCPY (SEQ ID NO: 3)	ISSGLLSGRSDIH (SEQ ID NO: 301)
11	LHCRTQMYGYNLCPY (SEQ ID NO: 3)	ISSGLLSGRSDQH (SEQ ID NO: 302)
12	LHCRTQMYGYNLCPY (SEQ ID NO: 3)	ISSGLLSGRSDTH (SEQ ID NO: 303)
13	CAQMYGYSMC (SEQ ID NO: 06)	ISSGLLSGRSDNH (SEQ ID NO: 297)
14	CAQMYGYSMC (SEQ ID NO: 06)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
15	FGTACPNHPMCHDWQ (SEQ ID NO: 09)	ISSGLLSGRSDNH (SEQ ID NO: 297)
16	FGTACPNHPMCHDWQ (SEQ ID NO: 09)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
17	CLHSLYNVCLDP (SEQ ID NO: 23)	ISSGLLSGRSDNH (SEQ ID NO: 297)
18	CLHSLYNVCLDP (SEQ ID NO: 23)	ISSGLLSGRSDDH (SEQ ID NO: 300)
19	CLHSLYNVCLDP (SEQ ID NO: 23)	ISSGLLSGRSDIH (SEQ ID NO: 301)
20	CLHSLYNVCLDP (SEQ ID NO: 23)	ISSGLLSGRSDQH (SEQ ID NO: 302)

21	CLHSLYNVCLDP (SEQ ID NO: 23)	ISSGLLSGRSDTH (SEQ ID NO: 303)
22	CLHSLYNVCLDP (SEQ ID NO: 23)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
23	CLHSAYNVCLDP (SEQ ID NO: 24)	ISSGLLSGRSDNH (SEQ ID NO: 297)
24	CLHSAYNVCLDP (SEQ ID NO: 24)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
25	QPCAQMYGYSMC (SEQ ID NO: 27)	ISSGLLSGRSDNH (SEQ ID NO: 297)
26	QPCAQMYGYSMC (SEQ ID NO: 27)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
27	CAQMYGYSMCAHT (SEQ ID NO: 29)	ISSGLLSGRSDNH (SEQ ID NO: 297)
28	CAQMYGYSMCAHT (SEQ ID NO: 29)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
29	CPNHPLCHDWQ (SEQ ID NO: 32)	ISSGLLSGRSDNH (SEQ ID NO: 297)
30	CPNHPLCHDWQ (SEQ ID NO: 32)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
31	CPNHPMCADWQ (SEQ ID NO: 33)	ISSGLLSGRSDNH (SEQ ID NO: 297)
32	CPNHPMCADWQ (SEQ ID NO: 33)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
33	CPNHPMCHDAQ (SEQ ID NO: 35)	ISSGLLSGRSDNH (SEQ ID NO: 297)
34	CPNHPMCHDAQ (SEQ ID NO: 35)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
35	CRTQLYGYNLCPY (SEQ ID NO: 39)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
36	CRTQLYGYNLCPA (SEQ ID NO: 41)	ISSGLLSGRSDNH (SEQ ID NO: 297)
37	CRTQLYGYNLCPA (SEQ ID NO: 41)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
38	FGAACPNHPICHDWQ (SEQ ID NO: 51)	ISSGLLSGRSDNH (SEQ ID NO: 297)
39	FGAACPNHPICHDWQ (SEQ ID NO: 51)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
40	FGAACPNHPLCHDWQ (SEQ ID NO: 52)	ISSGLLSGRSDNH (SEQ ID NO: 297)
41	FGAACPNHPLCHDWQ (SEQ ID NO: 52)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
42	FGAACPNHPMCHDAQ (SEQ ID NO: 53)	ISSGLLSGRSDNH (SEQ ID NO: 297)
43	FGAACPNHPMCHDAQ (SEQ ID NO: 53)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
44	CLHSAYNACLDP (SEQ ID NO: 54)	ISSGLLSGRSDNH (SEQ ID NO: 297)

45	CLHSAYNACLDP (SEQ ID NO: 54)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
46	CAHSAYNVCLDP (SEQ ID NO: 55)	ISSGLLSGRSDNH (SEQ ID NO: 297)
47	CAHSAYNVCLDP (SEQ ID NO: 55)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
48	CLHSAYNVCADP (SEQ ID NO: 56)	ISSGLLSGRSDNH (SEQ ID NO: 297)
49	CLHSAYNVCADP (SEQ ID NO: 56)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
50	CLHSAYNVCLAP (SEQ ID NO: 57)	ISSGLLSGRSDNH (SEQ ID NO: 297)
51	CLHSAYNVCLAP (SEQ ID NO: 57)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
52	CLHSAYNVCLDA (SEQ ID NO: 58)	ISSGLLSGRSDNH (SEQ ID NO: 297)
53	CLHSAYNVCLDA (SEQ ID NO: 58)	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306)
54	YISDCPYHPMCHDYQ (SEQ ID NO: 60)	ISSGLLSGRSDNH (SEQ ID NO: 297)
55	FRNTCPYHPMCHDYR (SEQ ID NO: 61)	ISSGLLSGRSDNH (SEQ ID NO: 297)
56	AVCHMYMYGYNLCPF (SEQ ID NO: 63)	ISSGLLSGRSDNH (SEQ ID NO: 297)
57	RSCPQMYGYSMCPHT (SEQ ID NO: 64)	ISSGLLSGRSANP (SEQ ID NO: 305)
58	QPCAQMFGYSMCPHT (SEQ ID NO: 65)	ISSGLLSGRSANP (SEQ ID NO: 305)

[0134] In some embodiments, the activatable anti-CTLA-4 antibodies described herein also include an agent conjugated to the activatable antibody. In some embodiments, the conjugated agent is a therapeutic agent, such as an anti-neoplastic agent. In some embodiments, the agent is conjugated to a carbohydrate moiety of the activatable antibody, preferably where the carbohydrate moiety is located outside the antigen-binding region of the antibody or antigen-binding fragment in the activatable antibody. In some embodiments, the agent is conjugated to a sulfhydryl group of the antibody or antigen-binding fragment in the activatable antibody. In some embodiments, the agent is conjugated to an amino group of the antibody or antigen-binding fragment of the activatable antibody. In some embodiments, the agent is conjugated to a carboxylic acid group of the antibody or antigen-binding fragment of the activatable antibody.

[0135] In some embodiments, the agent is a cytotoxic agent such as a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

[0136] In some embodiments, the conjugated activatable antibody can be modified for site-specific conjugation through modified amino acid sequences inserted or otherwise included in the activatable antibody sequence. These modified amino acid sequences are designed to allow for controlled placement and/or dosage of the conjugated agent within a conjugated activatable anti-CTLA-4 antibody. For example, the activatable antibody can be engineered to include cysteine substitutions at positions on light and heavy chains that provide reactive thiol groups and do not negatively impact protein folding and assembly, nor alter antigen binding. In some embodiments, the activatable antibody can be engineered to include or otherwise introduce one or more non-natural amino acid residues within the activatable antibody to provide suitable sites for conjugation. In some embodiments, the activatable antibody can be engineered to include or otherwise introduce enzymatically activatable peptide sequences within the activatable antibody sequence.

[0137] In some embodiments, the agent is a detectable moiety such as, for example, a label or other marker. For example, the agent is or includes a radiolabeled amino acid, one or more biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), one or more radioisotopes or radionuclides, one or more fluorescent labels, one or more enzymatic labels, and/or one or more chemiluminescent agents. In some embodiments, detectable moieties are attached by linker molecules.

[0138] Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238: 1098 (1987).

Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. (See WO94/11026).

[0139] Those of ordinary skill in the art will recognize that a large variety of possible moieties can be coupled to the resultant antibodies of the invention. (*See, e.g.*, "Conjugate Vaccines", Contributions to Microbiology and Immunology, J. M. Cruse and R. E. Lewis, Jr (eds), Carger Press, New York, (1989), the entire contents of which are incorporated herein by reference).

II. Uses of Anti-CTLA-4 Activatable Antibodies

[0140] Therapeutic formulations of the invention, which include an activatable anti-CTLA-4 antibody, are used to prevent, treat or otherwise ameliorate a disease or disorder, including but not limited to, a disease or disorder associated with aberrant CTLA-4 expression and/or activity. For example, therapeutic formulations of the invention, which include an activatable anti-CTLA-4 antibody, are used as cancer immunotherapy, e.g., potentiating an endogenous immune response in a subject afflicted with a cancer so as to thereby treat the subject, which method comprises administering to the subject therapeutically effective amount of any of the activatable anti-CTLA-4 antibodies described herein.

[0141] Examples of cancers that may be treated using the immunotherapeutic methods of the disclosure include bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, breast cancer, lung cancer, cutaneous or intraocular malignant melanoma, unresectable or metastatic melanoma, renal cancer, uterine cancer, ovarian cancer, colorectal cancer, colon cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, 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, a hematological malignancy, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor,

brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, environmentally induced cancers including those induced by asbestos, metastatic cancers, and any combinations of said cancers. In some embodiments, the cancer is selected from MEL, RCC, squamous NSCLC, non-squamous NSCLC, CRC, CRPC, squamous cell carcinoma of the head and neck, and carcinomas of the esophagus, ovary, gastrointestinal tract and breast. The present methods are also applicable to treatment of metastatic cancers.

[0142] Other cancers include hematologic malignancies including, for example, multiple myeloma, B-cell lymphoma, Hodgkin lymphoma/primary mediastinal B-cell lymphoma, non-Hodgkin's lymphomas, acute myeloid lymphoma, chronic myelogenous leukemia, chronic lymphoid leukemia, follicular lymphoma, diffuse large B-cell lymphoma, Burkitt's lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, acute lymphoblastic leukemia, mycosis fungoides, anaplastic large cell lymphoma, T-cell lymphoma, and precursor T-lymphoblastic lymphoma, and any combinations of said cancers.

[0143] Increased proteolysis is known to be a hallmark of cancer. (*See e.g.*, Affara N I, *et al.* "Delineating protease functions during cancer development." *Methods Mol Biol.* 539 (2009): 1-32). Progression, invasion and metastasis of tumors result from several interdependent processes in which proteases are implicated. This process is described generally in U.S. Publication No. 2016/0193332 A1, which is incorporated in its entirety.

[0144] In some embodiments of these methods for treating a cancer subject, the activatable antibodies of the present invention, e.g. activatable ipilimumab, is administered to the subject as monotherapy. In some embodiments, stimulation or blockade of immunomodulatory targets may be effectively combined with standard cancer treatments, including chemotherapeutic regimes, radiation, surgery, hormone deprivation and angiogenesis inhibitors. The activatable anti-CTLA-4 antibody can be linked to an anti-neoplastic agent (as an immunoconjugate) or can be administered separately from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapeutic agents. Chemotherapeutic drugs include, among others, doxorubicin (ADRIAMYCIN®), cisplatin, carboplatin, bleomycin sulfate, carmustine, chlorambucil (LEUKERAN®), cyclophosphamide (CYTOXAN®; NEOSAR®),

lenalidomide (REVLIMID®), bortezomib (VELCADE®), dexamethasone, mitoxantrone, etoposide, cytarabine, bendamustine (TREANDA®), rituximab (RITUXAN®), ifosfamide, vincristine (ONCOVIN®), fludarabine (FLUDARA®), thalidomide (THALOMID®), alemtuzumab (CAMPATH®, ofatumumab (ARZERRA®), everolimus (AFINITOR®, ZORTRESS®), and carfilzomib (KYPROLISTM). Co-administration of anti-cancer agents that operate via different mechanisms can help overcome the development of resistance to drugs or changes in the antigenicity of tumor cells.

[0145] Activatable anti-CTLA-4 antibodies of the present invention, such as the activatable ipilimumab, may also be used in combination with other immunomodulatory agents, such as antibodies against other immunomodulatory receptors or their ligands. Several other co-stimulatory and inhibitory receptors and ligands that regulate T cell responses have been identified. Examples of stimulatory receptors include Inducible T cell Co-Stimulator (ICOS), CD137 (4-1BB), CD134 (OX40), CD27, Glucocorticoid-Induced TNFR-Related protein (GITR), and Herpes Virus Entry Mediator (HVEM), whereas examples of inhibitory receptors include Programmed Death-1 (PD-1), Programmed Death Ligand-1 (PD-L1), B and T Lymphocyte Attenuator (BTLA), T cell Immunoglobulin and Mucin domain-3 (TIM-3), Lymphocyte Activation Gene-3 (LAG-3), adenosine A2a receptor (A2aR), Killer cell Lectin-like Receptor G1 (KLRG-1), Natural Killer Cell Receptor 2B4 (CD244), CD160, T cell Immunoreceptor with Ig and ITIM domains (TIGIT), and the receptor for V-domain Ig Suppressor of T cell Activation (VISTA). Mellman *et al.* (2011) *Nature* 480:480; Pardoll (2012) *Nat. Rev. Cancer* 12: 252; Baitsch *et al.* (2012) *PloS One* 7:e30852.

[0146] Anti-PD-1 antibodies OPDIVO® (nivolumab) and KEYTRUDA® (pembrolizumab), as well as anti-PD-L1 antibody TECENTRIQ® (atezolizumab), have been approved for use in treating cancer, and may be combined with the activatable anti-CLTA-4 antibodies of the present invention, e.g. activatable ipilimumab. These receptors and their ligands provide targets for therapeutics designed to stimulate, or prevent the suppression, of an immune response so as to thereby attack tumor cells. Weber (2010) *Semin. Oncol.* 37:430; Flies *et al.* (2011) *Yale J. Biol. Med.* 84:409; Mellman *et al.* (2011) *Nature* 480:480; Pardoll (2012) Nat. Rev. Cancer 12:252. Stimulatory receptors or receptor ligands are targeted by agonist agents, whereas inhibitory receptors or receptor ligands are targeted by blocking agents. Among the most promising approaches to

enhancing immunotherapeutic anti-tumor activity is the blockade of so-called "immune checkpoints," which refer to the plethora of inhibitory signaling pathways that regulate the immune system and are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize collateral tissue damage. *See e.g.* Weber (2010) *Semin. Oncol.* 37:430; Pardoll (2012) *Nat. Rev. Cancer* 12:252. Because many of the immune checkpoints are initiated by ligand-receptor interactions, they can be readily blocked by antibodies or modulated by recombinant forms of ligands or receptors.

Anti-PD-1 Antibodies Useful for the Invention

[0147] Any anti-PD-1 antibody that is known in the art can be used in the presently described methods. In particular, various human monoclonal antibodies that bind specifically to PD-1 with high affinity have been disclosed in U.S. Patent No. 8,008,449. Each of the anti-PD-1 humanized antibodies disclosed in U.S. Patent No. 8,008,449 has been demonstrated to exhibit one or more of the following characteristics: (a) binds to human PD-1 with a K_D of 1 x 10^{-7} M or less, as determined by surface plasmon resonance using a Biacore biosensor system; (b) does not substantially bind to human CD28, CTLA-4 or ICOS; (c) increases T-cell proliferation in a Mixed Lymphocyte Reaction (MLR) assay; (d) increases interferon-γ production in an MLR assay; (e) increases IL-2 secretion in an MLR assay; (f) binds to human PD-1 and cynomolgus monkey PD-1; (g) inhibits the binding of PD-L1 and/or PD-L2 to PD-1; (h) stimulates antigen-specific memory responses; (i) stimulates antibody responses; and (j) inhibits tumor cell growth *in vivo*. Anti-PD-1 antibodies usable in the present invention include monoclonal antibodies that bind specifically to human PD-1 and exhibit at least one, in some embodiments, at least five, of the preceding characteristics.

[0148] Other anti-PD-1 monoclonal antibodies have been described in, for example, U.S. Patent Nos. 6,808,710, 7,488,802, 8,168,757 and 8,354,509, US Publication No. 2016/0272708, and PCT Publication Nos. WO 2012/145493, WO 2008/156712, WO 2015/112900, WO 2012/145493, WO 2015/112800, WO 2014/206107, WO 2015/35606, WO 2015/085847, WO 2014/179664, WO 2017/020291, WO 2017/020858, WO 2016/197367, WO 2017/024515, WO 2017/025051, WO 2017/123557, WO 2016/106159, WO 2014/194302, WO 2017/040790, WO 2017/133540, WO

2017/132827, WO 2017/024465, WO 2017/025016, WO 2017/106061, each of which is incorporated by reference in its entirety.

[0149] In some embodiments, the anti-PD-1 antibody is selected from the group consisting of nivolumab (also known as "OPDIVO®"; formerly designated 5C4, BMS-936558, MDX-1106, or ONO-4538), pembrolizumab (Merck, also known as "KEYTRUDA®", lambrolizumab, and MK-3475. *See* WO2008156712A1), PDR001 (Novartis; *see* WO 2015/112900), MEDI-0680 (AstraZeneca; AMP-514; *see* WO 2012/145493), REGN-2810 (Regeneron; *see* WO 2015/112800), JS001 (TAIZHOU JUNSHI PHARMA; *see* Si-Yang Liu et al., *J. Hematol. Oncol. 10*:136 (2017)), BGB-A317 (Beigene; *see* WO 2015/35606 and US 2015/0079109), INCSHR1210 (SHR-1210; Jiangsu Hengrui Medicine; *see* WO 2015/085847; Si-Yang Liu et al., *J. Hematol. Oncol. 10*:136 (2017)), TSR-042 (ANB011; Tesaro Biopharmaceutical; *see* WO2014/179664), GLS-010 (WBP3055; Wuxi/Harbin Gloria Pharmaceuticals; *see* Si-Yang Liu et al., *J. Hematol. Oncol. 10*:136 (2017)), AM-0001 (Armo), STI-1110 (Sorrento Therapeutics; *see* WO 2014/194302), AGEN2034 (Agenus; *see* WO 2017/040790), and MGD013 (Macrogenics).

[0150] In one embodiment, the anti-PD-1 antibody is nivolumab. Nivolumab is a fully human IgG4 (S228P) PD-1 immune checkpoint inhibitor antibody that selectively prevents interaction with PD-1 ligands (PD-L1 and PD-L2), thereby blocking the down-regulation of antitumor T-cell functions (U.S. Patent No. 8,008,449; Wang et al., 2014 *Cancer Immunol Res. 2(9)*:846-56).

[0151] In another embodiment, the anti-PD-1 antibody is pembrolizumab. Pembrolizumab is a humanized monoclonal IgG4 antibody directed against human cell surface receptor PD-1 (programmed death-1 or programmed cell death-1). Pembrolizumab is described, for example, in U.S. Patent Nos. 8,354,509 and 8,900,587; see also www.cancer.gov/drugdictionary?cdrid=695789 (last accessed: December 14, 2014). Pembrolizumab has been approved by the FDA for the treatment of relapsed or refractory melanoma.

[0152] Anti-PD-1 antibodies usable in the disclosed methods also include isolated antibodies that bind specifically to human PD-1 and cross-compete for binding to human PD-1 with any anti-PD-1 antibody disclosed herein, e.g., nivolumab (*see, e.g.*, U.S. Patent No. 8,008,449 and 8,779,105; WO 2013/173223). In some embodiments, the anti-PD-1

antibody binds the same epitope as any of the anti-PD-1 antibodies described herein, e.g., nivolumab. The ability of antibodies to cross-compete for binding to an antigen indicates that these monoclonal antibodies bind to the same epitope region of the antigen and sterically hinder the binding of other cross-competing antibodies to that particular epitope region. These cross-competing antibodies are expected to have functional properties very similar those of the reference antibody, *e.g.*, nivolumab, by virtue of their binding to the same epitope region of PD-1. Cross-competing antibodies can be readily identified based on their ability to cross-compete with nivolumab in standard PD-1 binding assays such as Biacore analysis, ELISA assays or flow cytometry (*see, e.g.*, WO 2013/173223).

[0153] In certain embodiments, the antibodies that cross-compete for binding to human PD-1 with, or bind to the same epitope region of human PD-1 antibody, nivolumab, are monoclonal antibodies. For administration to human subjects, these cross-competing antibodies are chimeric antibodies, engineered antibodies, or humanized or human antibodies. Such chimeric, engineered, humanized or human monoclonal antibodies can be prepared and isolated by methods well known in the art.

[0154] Anti-PD-1 antibodies usable in the methods of the disclosed invention also include antigen-binding portions of the above antibodies. It has been amply demonstrated that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody.

[0155] Anti-PD-1 antibodies suitable for use in the disclosed methods or compositions are antibodies that bind to PD-1 with high specificity and affinity, block the binding of PD-L1 and or PD-L2, and inhibit the immunosuppressive effect of the PD-1 signaling pathway. In any of the compositions or methods disclosed herein, an anti-PD-1 "antibody" includes an antigen-binding portion or fragment that binds to the PD-1 receptor and exhibits the functional properties similar to those of whole antibodies in inhibiting ligand binding and up-regulating the immune system. In certain embodiments, the anti-PD-1 antibody or antigen-binding portion thereof cross-competes with nivolumab for binding to human PD-1.

Anti-PD-L1 Antibodies Useful for the Invention

[0156] Any anti-PD-L1 antibody can be used in the methods of the present disclosure. Examples of anti-PD-L1 antibodies useful in the methods of the present disclosure include the antibodies disclosed in US Patent No. 9,580,507. Each of the anti-PD-L1

human monoclonal antibodies disclosed in U.S. Patent No. 9,580,507 have been demonstrated to exhibit one or more of the following characteristics: (a) binds to human PD-L1 with a K_D of 1 x 10^{-7} M or less, as determined by surface plasmon resonance using a Biacore biosensor system; (b) increases T-cell proliferation in a Mixed Lymphocyte Reaction (MLR) assay; (c) increases interferon-γ production in an MLR assay; (d) increases IL-2 secretion in an MLR assay; (e) stimulates antibody responses; and (f) reverses the effect of T regulatory cells on T cell effector cells and/or dendritic cells. Anti-PD-L1 antibodies usable in the present invention include monoclonal antibodies that bind specifically to human PD-L1 and exhibit at least one, in some embodiments, at least five, of the preceding characteristics.

[0157] In certain embodiments, the anti-PD-L1 antibody is selected from the group consisting of BMS-936559 (formerly 12A4 or MDX-1105; *see, e.g.,* U.S. Patent No. 7,943,743 and WO 2013/173223), MPDL3280A (also known as RG7446, atezolizumab, and TECENTRIQ®; US 8,217,149; *see, also*, Herbst et al. (2013) J Clin Oncol 31(suppl):3000), durvalumab (IMFINZI™; MEDI-4736; AstraZeneca; *see* WO 2011/066389), avelumab (Pfizer; MSB-0010718C; BAVENCIO®; *see* WO 2013/079174), STI-1014 (Sorrento; *see* WO2013/181634), CX-072 (CytomX; *see* WO2016/149201), KN035 (3D Med/Alphamab; *see* Zhang et al., *Cell Discov. 7*:3 (March 2017), LY3300054 (Eli Lilly Co.; *see, e.g.*, WO 2017/034916), and CK-301 (Checkpoint Therapeutics; *see* Gorelik et al., AACR:Abstract 4606 (Apr 2016)).

[0158] In certain embodiments, the PD-L1 antibody is atezolizumab (TECENTRIQ®). Atezolizumab is a fully humanized IgG1 monoclonal anti-PD-L1 antibody.

[0159] In certain embodiments, the PD-L1 antibody is durvalumab (IMFINZI™). Durvalumab is a human IgG1 kappa monoclonal anti-PD-L1 antibody.

[0160] In certain embodiments, the PD-L1 antibody is avelumab (BAVENCIO®). Avelumab is a human IgG1 lambda monoclonal anti-PD-L1 antibody.

[0161] In other embodiments, the anti-PD-L1 monoclonal antibody is selected from the group consisting of 28-8, 28-1, 28-12, 29-8, 5H1, and any combination thereof.

[0162] Anti-PD-L1 antibodies usable in the disclosed methods also include isolated antibodies that bind specifically to human PD-L1 and cross-compete for binding to human PD-L1 with any anti-PD-L1 antibody disclosed herein, *e.g.*, atezolizumab and/or avelumab. In some embodiments, the anti-PD-L1 antibody binds the same epitope as any

of the anti-PD-L1 antibodies described herein, *e.g.*, atezolizumab and/or avelumab. The ability of antibodies to cross-compete for binding to an antigen indicates that these antibodies bind to the same epitope region of the antigen and sterically hinder the binding of other cross-competing antibodies to that particular epitope region. These cross-competing antibodies are expected to have functional properties very similar those of the reference antibody, *e.g.*, atezolizumab and/or avelumab, by virtue of their binding to the same epitope region of PD-L1. Cross-competing antibodies can be readily identified based on their ability to cross-compete with atezolizumab and/or avelumab in standard PD-L1 binding assays such as Biacore analysis, ELISA assays or flow cytometry (*see, e.g.*, WO 2013/173223).

[0163] In certain embodiments, the antibodies that cross-compete for binding to human PD-L1 with, or bind to the same epitope region of human PD-L1 antibody as, atezolizumab and/or avelumab, are monoclonal antibodies. For administration to human subjects, these cross-competing antibodies are chimeric antibodies, engineered antibodies, or humanized or human antibodies. Such chimeric, engineered, humanized or human monoclonal antibodies can be prepared and isolated by methods well known in the art.

[0164] Anti-PD-L1 antibodies usable in the methods of the disclosed invention also include antigen-binding portions of the above antibodies. It has been amply demonstrated that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody.

[0165] Anti-PD-L1 antibodies suitable for use in the disclosed methods or compositions are antibodies that bind to PD-L1 with high specificity and affinity, block the binding of PD-1, and inhibit the immunosuppressive effect of the PD-1 signaling pathway. In any of the compositions or methods disclosed herein, an anti-PD-L1 "antibody" includes an antigen-binding portion or fragment that binds to PD-L1 and exhibits the functional properties similar to those of whole antibodies in inhibiting receptor binding and up-regulating the immune system. In certain embodiments, the anti-PD-L1 antibody or antigen-binding portion thereof cross-competes with atezolizumab and/or avelumab for binding to human PD-L1.

[0166] Efficaciousness of prevention, amelioration or treatment is determined in association with any known method for diagnosing or treating the disease or disorder, including but not limited to, a disease or disorder associated with aberrant CTLA-4

expression and/or activity. Prolonging the survival of a subject or otherwise delaying the progression of the disease or disorder, including but not limited to, a disease or disorder associated with aberrant CTLA-4 expression and/or activity in a subject, indicates that the activatable antibody confers a clinical benefit.

[0167] It will be appreciated that therapeutic entities in accordance with the invention will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, Pa. (1975)), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as Lipofectin™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in treatments and therapies in accordance with the present invention, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. *See also* Baldrick P. "Pharmaceutical excipient development: the need for preclinical guidance." *Regul. Toxicol Pharmacol.* 32(2):210-8 (2000), Wang W. "Lyophilization and development of solid protein pharmaceuticals." *Int. J. Pharm.* 203(1-2):1-60 (2000), Charman W N "Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts." *J Pharm Sci.* 89(8):967-78 (2000), Powell *et al.* "Compendium of excipients for parenteral formulations" *PDA J Pharm Sci Technol.* 52:238-311 (1998) and the citations therein for additional information related to formulations, excipients and carriers well known to pharmaceutical chemists.

[0168] Activatable anti-CTLA-4 antibodies can be administered in the form of pharmaceutical compositions. Principles and considerations involved in preparing such compositions, as well as guidance in the choice of components are provided, for example, in Remington: The Science And Practice Of Pharmacy 19th ed. (Alfonso R. Gennaro, *et al.*, editors) Mack Pub. Co., Easton, Pa.: 1995; Drug Absorption Enhancement: Concepts, Possibilities, Limitations, And Trends, Harwood Academic Publishers, Langhorne, Pa.,

1994; and Peptide And Protein Drug Delivery (Advances In Parenteral Sciences, Vol. 4), 1991, M. Dekker, New York.

[0169] The formulation can also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[0170] The active ingredients can also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions.

[0171] The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

[0172] Sustained-release preparations can be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

[0173] In some embodiments, the activatable antibody contains a detectable label. An intact antibody, or a fragment thereof (e.g., Fab, scFv, or F(ab)2) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity

with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently-labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently-labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. Included within the usage of the term "biological sample", therefore, is blood and a fraction or component of blood including blood serum, blood plasma, or lymph. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

III. Pharmaceutical Compositions

[0174] The activatable anti-CTLA-4 antibodies of the invention (also referred to herein as "active compounds"), and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the activatable antibody and a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0175] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (i.e., topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the

following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0176] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringe ability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid 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. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0177] 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 filtered sterilization. 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, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0178] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[0179] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[0180] Activatable antibodies of the present invention may also be administered subcutaneously in conjunction with agents to facilitate injection of large volumes at a single site (insterstitial drug dispersion agents) such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

[0181] The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[0182] In some embodiments, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[0183] It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0184] The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

[0185] Embodiments of the present disclosure can be further defined by reference to the following non-limiting examples, which describe in detail preparation of certain antibodies of the present disclosure and methods for using antibodies of the present disclosure. It will be apparent to those skilled in the art that many modifications, both to materials and methods, may be practiced without departing from the scope of the present disclosure.

[0186] The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1: Identification of Masking Moieties for the Activatable anti-CTLA-4 Antibody

[0187] In order to identify masking moieties (MM) that reduce the binding of anti-CTLA-4 antibodies to their target protein, anti-CTLA-4 antibody (i.e., ipilimumab) was used to

screen peptide libraries using methods similar to that described in PCT International Publication Nos. WO 2009/025846, WO 2010/081173, and WO 2016/149201, the contents of which are hereby incorporated by reference in their entireties. The screening consisted of two rounds of magnetic-activated cell sorting (MACS) purification followed by three rounds of fluorescence-activated cell sorting (FACS).

[0188] The initial MACS purification was done with protein-A Dynabeads® (Invitrogen) and anti-CTLA-4 antibody at a concentration of 100 nM. Approximately 10^{11} cells were screened for binding, and $6x10^{6}$ cells were collected. The second MACS purification was done with streptavidin DYNABEADS® (Thermo Fisher Scientific) and biotinylated anti-CTLA-4 antibody at a concentration of 100 nM. The eluate from the initial MACS purification was expanded, approximately 10^{11} cells were screened for binding, and approximately 10^{7} cells were collected. The output of the previously described MACS purification was subjected to serial rounds of FACS sorting with decreasing concentrations of anti-CTLA-4 labeled with Alexa Fluor® 488 (Thermo Fisher Scientific). Labeled anti-CTLA4 antibody was used at concentrations of 10 nM, 1 nM, and 200 pM for the first, second, and third sorts, respectively. Individual peptide clones, from the third sort were identified by sequence analysis and subsequently verified for their ability to bind the anti-CTLA4 antibody. Two peptide consensus sequences were selected for affinity maturation: XXCXXXMYGYNLCPY (SEQ ID NO: 554) and XXXCXHSMYNVCLDP (SEQ ID NO: 555).

[0189] Affinity maturation libraries were built on these consensus sequences as described in Table 7. Rows 1 and 3 represent the consensus sequence and rows 2 and 4 represent the nucleotide sequences encoding the peptide libraries that were inserted into the display system using a method similar to that described in PCT International Publication Number WO 2010/081173, *ibid.*

Table 7: Maturation Libraries

1	X	X	C	X	X	X	M	Y	G	Y	N	L	C	P	Y
2	NNK	NNK	TGC	NNK	NNK	NNK	NTT	TWT	GGG	KWT	AAT	CTG	TGC	CCG	TAT
3	X	X	X	C	X	H	S	M	Y	N	V	C	L	D	P
4	NNK	NNK	NNK	TGC	NNK	NWT	AGT	NTT	TWT	AAT	NTT	TGC	CTT	GAT	CCT

[0190] The maturation libraries were screened in a manner similar to that described for the naïve libraries described above. The screening consisted of one round of MACS and

subsequent rounds of FACS sorting. The MACS was done with protein-A DYNABEADS® (Thermo Fisher Scientific) and the anti-CTLA-4 antibody at a concentration of 100 nM. For MACS, 10^{11} cells were screened for binding, and approximately 10^{8} cells were selected. The eluate from the MACS was expanded, and approximately 10^{11} cells were subjected to serial rounds of FACS sorting with decreasing concentrations of Alexa Fluor® 488-labeled anti-CTLA4 antibody. Labeled anti-CTLA4 antibody was used at concentrations of 100 nM, 20 nM, 5 nM, 1 nM, and 1 nM for the first, second, third, fourth and fifth sorts, respectively. Individual peptide clones from the fourth and fifth sorts were identified by sequence analysis and subsequently verified for their ability to bind the anti-CTLA4 antibody. The sequences of the anti-CTLA-4 masking moieties identified through the methods described above are provided in Tables 4 and 5. Four consensus sequences can be derived from the mask sequences listed in Tables 4 and 5:

Consensus 1. C(L/M/V/T)Y(S/V/I)(F/L/M/A)(Y/F)N(V/I)CLDP (SEQ ID NO: 566)

Consensus 2. CAQMYGYSMC(P/A)(H/R/A)T (SEQ ID NO: 567)

Consensus 3. CX(M/I/Y/L/N/F)(Y/W/F/Q/T)(M/Y)YG(Y/V/F)(N/D)LCP(Y/F) (SEQ ID NO: 568)

Consensus 4. (N/T)(S/T/M/A)CP(N/Y)HP(M/L)C(H/F/Y)D(Y/F/W) (SEQ ID NO: 569)

Example 2:
Construction and Characterization of Activatable Anti-muCTLA-4 Antibodies

[0191] In order to show a proof-of-concept that the activatable anti-CTLA-4 antibodies can be used to treat tumors, six activatable anti-mouse CTLA-4 antibodies (based on clone 9D9) were constructed using techniques similar to those disclosed in Examples 1 and 3 herein. These antibodies comprise either MY11 or MY03 as the masking moiety, and cleavable moiety “0003” having amino acid sequence TSTSGRSANPRG (SEQ ID NO: 320), “1004” having amino acid sequence AVGLLAPP (SEQ ID NO: 323), or “2001” having amino acid sequence ISSGLLSGRSDNH (SEQ ID NO: 297). The antibodies were all mouse IgG2a isotype. As controls, anti-mouse CTLA-4 monoclonal antibody (9D9) ("9D9 mg2a") and a human anti-diptheria toxin antibody with a mIgG2a isotype ("mg2a") were used.

[0192] On day 0, BALB/c mice were subcutaneously injected with 1 x 10^6 CT26 tumor cells. Administration of the different antibodies began on day 7 post tumor implantation. Prior to administration, tumor size was measured and the mice were randomized into different treatment groups, so as to have comparable mean tumor volumes (e.g., 39-44 mm^3). Tumors were measured with calipers two-dimensionally, and tumor volume was calculated as $L\times(W^2/2)$, L = length (the longer of the 2 measurements), W = width. The mice were then treated intraperitoneally (i.p.) with the designated antibody (e.g., 25 μg/dose). Tumor volume was measured twice weekly. At day 12 post tumor implantation, some of the mice from each group were sacrificed, and tumor and spleen were harvested for immunomonitoring to investigate the effects of the antibodies on the T cell populations. Some or all of the remaining mice from the different groups were used for subsequent pharmacokinetic (PK) and/or pharmacodynamics (PD) analysis.

[0193] As shown in FIG. 1A, mice that received the unrelated mouse IgG2a antibody (i.e., the human anti-diptheria toxin antibody) failed to control the tumor. In contrast, as shown in FIG. 1C, mice that received the activatable anti-mouse CTLA-4 antibody (comprising MY11 as the masking moiety and 2001 as the cleavable moiety) controlled tumor size almost as well as those mice that received the anti-mouse CTLA-4 mAb (9D9) (FIG. 1B). These data demonstrate that tumor-specific protease can cleave the cleavable moiety, resulting in the removal of the masking moiety and the binding of the released antibody to its target protein.

[0194] To determine whether or not activatable anti-mouse CTLA-4 antibodies are active in the periphery, proliferation and activity of Foxp3+ regulatory T cells were determined in the spleen, and regulatory T cell abundance was determined in tumor samples for comparison, as described in Example 5, *infra*. In agreement with the data from FIGs. 1B and 1C, all the activatable anti-CTLA-4 antibodies behaved similarly to the anti-mouse CTLA-4 mAb (9D9) in the tumor (FIG. 2A). In contrast, the activatable antibodies resembled the unrelated mouse IgG2a antibody in the spleen (FIGs. 2B and 2C). Such data suggest that the masking moiety-containing prodomain of the activatable anti-mouse CTLA-4 antibodies remains intact and attached to the antibody in the spleen, blocking the activity of the antibody, whereas the prodomain is cleaved off by tumor specific proteases to generate fully active anti-CTLA-4 antibody in the tumor.

Example 3:
Construction of Activatable Anti-human CTLA-4 Antibodies

[0195] Activatable anti-CTLA4 antibodies comprising an anti-CTLA4 masking moiety, a cleavable moiety, and an anti-CTLA4 antibody (e.g., ipilimumab) of the disclosure were produced according to methods similar to those described in PCT Publication Nos. WO 2009/025846 *ibid.* and WO 2010/081173 *ibid*, and WO 2016/118629, *ibid.* Activatable anti-CTLA4 antibodies were expressed in EXPI293™ cells (Thermo Fisher Scientific) and purified by protein A chromatography (MabSelect SuRe, GE Healthcare) as per manufacturers' protocols. Quality control of the resultant activatable antibodies indicated that most comprise at least 95% monomer.

[0196] To assess the feasibility of using the activatable anti-CTLA-4 antibodies disclosed herein in a human setting, the antibodies were produced as human IgG1 (hIgG1) heavy chain (Hc) and human kappa (hK) light chain (Lc) format. The activatable antibodies all comprise the antibody or antigen binding domain thereof of ipilimumab. The cleavable moiety was selected from the group consisting of a cleavable moiety referred to herein as "2001" and comprising the sequence ISSGLLSGRSDNH (SEQ ID NO: 297) and derivatives thereof and a cleavable moiety referred to herein as "3001" and comprising the sequence AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306) and derivatives thereof. In some embodiments, the cleavable moiety was selected from the group consisting of ISSGLLSGRSDNH (SEQ ID NO: 297), also referred to herein as "2001"; ISSGLLSGRSDDH (SEQ ID NO: 300), also referred to herein as "2006"; ISSGLLSGRSDIH (SEQ ID NO: 301), also referred to herein as "2007"; ISSGLLSGRSDQH (SEQ ID NO: 302), also referred to herein as "2008"; ISSGLLSGRSDTH (SEQ ID NO: 303), also referred to herein as "2009"; ISSGLLSGRSANP (SEQ ID NO: 305), also referred to herein as "2012"; ISSGLLSGRSDNP (SEQ ID NO: 304), also referred to herein as "2011"; ISSGLLSGRSANPRG (SEQ ID NO: 298), also referred to herein as "2003"; AVGLLAPPGGLSGRSDNH (SEQ ID NO: 306), also referred to herein as "3001"; AVGLLAPPGGLSGRSDDH (SEQ ID NO: 307), also referred to herein as "3006"; AVGLLAPPGGLSGRSDIH (SEQ ID NO: 308), also referred to herein as "3007"; AVGLLAPPGGLSGRSDQH (SEQ ID NO: 309), also referred to herein as "3008"; AVGLLAPPGGLSGRSDTH (SEQ ID NO: 310), also referred to herein as "3009";

AVGLLAPPGGLSGRSANP (SEQ ID NO: 312), also referred to herein as "3012"; AVGLLAPPGGLSGRSDNP (SEQ ID NO: 311), also referred to herein as "3011"; and AVGLLAPPSGRSANPRG (SEQ ID NO: 299), also referred to herein as "2005". The masking moiety was selected from the group of masking moieties provided in Tables 4 and 5. In some embodiments, the masking moiety was CRTQLYGYNLCPY (SEQ ID NO: 39), referred to herein as YV39. Some of the activatable anti-CTLA-4 antibodies also included spacer sequences and/or linker peptides.

Example 4:
In vitro Characterization of Activatable Anti-Human CTLA-4 Antibodies

[0197] In order to assess the ability of the activatable antibodies to bind to CTLA-4 in the absence of protease activity, an enzyme-linked immunosorbent assay (ELISA) was used to measure binding affinity. Briefly, Nunc MaxiSorp® plates were coated overnight at 40°C with 100 µL/well of a 1 µg/mL solution of human CTLA-4 protein (Sino Biological) in PBS, pH 7.4. Plates were then washed three times with PBST (PBS, pH 7.4, 0.05% Tween-20), and the wells were blocked with 200 µL/well, 10 mg/mL bovine serum albumin (BSA) in PBST for 2 hours at room temperature. Afterwards, the plates were washed three more times with PBST. The activatable antibodies were then serially diluted, as shown below in Table 8.

Table 8. Serial Dilution of Activatable Anti-CTLA-4 Antibodies for Binding Analysis

	[Antibody] = nM Columns 1-3	[activatable antibody 1] = nM Columns 4-6	[activatable antibody 2] = nM Columns 7-9	[activatable antibody 3] = nM Columns 10-12
A	10	1000	1000	1000
B	3.33	333	333	333
C	1.11	111	111	111
D	0.37	37	37	37
E	0.123	12.3	12.3	12.3
F	0.041	4.1	4.1	4.1
G	0.0137	1.34	1.34	1.34
H	.0046	0.45	0.45	Blank

[0198] In the current Example, the highest concentration used for the parental antibody and the activatable antibodies were 10 nM and 100 nM, respectively. However, the

concentrations can be increased or decreased to give full saturation binding curves for activatable antibodies with stronger or weaker masking.

[0199] The diluted antibodies were added to the plates and incubated for 1 hour at room temperature. Afterwards, the plates were washed three times with PBST. Then, 100 µL of goat-anti-human IgG (Fab specific, Sigma cat # A0293; diluted at 1:4,000 in 10 mg/mL BSA in PBST) was added to each well, and the plate was incubated for an additional 1 hour at room temperature. Next, the plates were developed with tetramethylbenzidine (TMB) and 1N HCl. Absorbance at 450 nm was then measured and reported as optical density (OD 450 nm).

[0200] As shown in FIGs. 3A to 3E, anti-CTLA-4 activatable antibodies typically had reduced binding to CTLA-4 as compared to ipilimumab ("YV1"). *See also* FIGs. 4A to 4D, FIGs. 5A to 5F, and FIGs. 6A to 6B. Such data demonstrate that the masking moieties effectively conceal the antigen binding domain on the anti-CTLA-4 activatable antibodies.

[0201] To further assess the binding ability, the activatable human anti-CTLA-4 antibodies were serially diluted (e.g., 60 µg/mL to 0.0003 µg/mL) and added to 58 α-β-CTLA-4/CD3ζ cells, which stably express human CTLA-4. After 30 minutes of incubation at 4°C, an allophycocyanin (APC)-labeled anti-human secondary antibody was added and binding of the activatable anti-human CTLA-4 antibodies to human CTLA-4 was assessed using a Canto flow cytometer. The geometric mean fluorescence intensity (GMFI) was determined using FlowJo® analysis software. Ipilimumab was used as a control. As shown in FIGs. 7A and 7B, the activatable human anti-CTLA-4 antibodies did not bind to human CTLA-4 as effectively as ipilimumab. These data further demonstrate that in the absence of specific proteases, the masking moiety of the activatable antibodies inhibits binding of such activatable antibodies to human CTLA-4.

[0202] To confirm that the reduced binding observed with the activatable anti-CTLA-4 antibodies was due to the masking moiety, studies were performed on mono-clippped, MMP fully-clipped and uPA fully-clipped forms of the activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety. The mono-clipped form of the antibody was produced by expressing a construct producing one intact light chain (including the mask moiety) and a second light chain truncated at the same position as if it had been cleaved by MMP14. The MMP or uPA fully clipped forms were expressed

from constructs with both light chains truncated as if they had been cleaved by MMP or μPA, respectively. As shown in FIGs. 7C and 7D, the mono-clipped activatable antibody had intermediate binding (EC50 = 2.8 nM) as compared to the non-clipped activatable antibody (EC50 = 22 nM) and ipilimumab (EC50 = 0.54 nM). In contrast, the MMP or uPA fully-clipped activatable antibodies behaved similarly to ipilimumab (MMP clipped: EC50 = 0.65 nM; μPA clipped: EC50 = 0.76). Such data confirm that the reduced binding observed with the activatable anti-CTLA-4 antibody is due to the masking moiety.

[0203] Next, to determine whether the observed reduced binding to CTLA-4 correlated with reduced activity, the activity of an activatable human anti-CTLA-4 antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011") was characterized in an *in vitro* functional assay using staphylococcal enterotoxin B (SEB). SEB is a superantigen that strongly activates T cells and stimulates cytokine secretion. Whole fresh peripheral blood mononuclear cells (PBMC) were isolated from healthy human donors using a standard Ficoll-Paque separation method. Serial dilution of the antibodies (e.g., 40 μg/mL to 0.01 μg/mL) were performed and plated in triplicate in a 96-well flat-bottom tissue culture plate. The antibodies used included (i) Ipi YV39 2011, (ii) ipilimumab, and (iii) an unrelated isotype control. Next, the isolated PBMC were resuspended in T-cell assay media (RPMI media + 10% heat-inactivated fetal bovine serum (HI-FBS) + 1% HEPES buffer + 1% MEM non-essential amino acid + 1% Na-pyruvate) and added to the plate at $1x10^5$ cells/well. The cells were stimulated with a suboptimal concentration (e.g., 85 ng/mL – determined by titrating SEB and observing the stimulation on T-cell proliferation) of SEB. The cells were incubated at 37°C for 3 days. Then, the IL-2 concentration in the supernatants was measured by homogeneous time-resolved fluorescence (HTRF). The HTRF data were analyzed using Softmax Pro and graphed using GraphPad Prism.

[0204] As shown in FIG. 8, ipilimumab enhanced the SEB-mediated IL-2 production by the PBMC in a dose-dependent manner. In contrast, the Ipi YV39 2011 activatable antibody had activity similar to that of the isotype control, suggesting that the masking moiety (YV39) is effective in blocking the functional activity of ipilimumab. These data are in agreement with the binding data described above and demonstrate that in the absence of specific proteases, the activatable anti-human CTLA-4 antibodies exhibit reduced activity.

Example 5:
In vivo Characterization of Activatable Anti-Human CTLA-4 Antibodies

[0205] In order to characterize the antibodies disclosed herein *in vivo*, four activatable human anti-human CTLA-4 antibodies (based on ipilimumab) were prepared using mouse IgG2a. The antibodies comprise YV04, YV23, YV24, or YV39 as the masking moiety, and 2001 as the cleavable moiety ("Ipi YV04 2001", "Ipi YV23 2001", "Ipi YV24 2001", and "Ipi YV39 2001", respectively). As controls, ipilimumab ("Ipi mg2a") and an unrelated human anti-diphtheria toxin ("control mg2a") were used. The activity of these activatable anti-CTLA-4 antibodies was assessed using the MC38 tumor model as described below.

[0206] Briefly, on day 0, human CTLA-4 knock-in C57BL/6 mice were subcutaneously injected with 2 x 10^6 MC38 colon adenocarcinoma cells into their left lower abdominal quadrant. Tumors were measured with calipers two-dimensionally, and tumor volume was calculated as $L\times(W^2/2)$, L = length (the longer of the 2 measurements), W = width. Next, the mice were randomized into different groups, so as to have similar mean tumor volumes (e.g., 37 mm^3). Administration of the antibodies began on day 7 post tumor implantation with the mice receiving a single dose (e.g., 200 μg/mouse) of the relevant antibody via intraperitoneal (i.p.) injection. At day 12 post tumor implantation, several of the mice from each group were sacrificed, and tumor and spleen were harvested for immunomonitoring to investigate the effect of the antibodies on the T cell populations. Some or all of the remaining mice from the different groups were used for subsequent pharmacokinetic (PK) and/or pharmacodynamics (PD) analysis.

Immunomonitoring of T Cell Populations

[0207] The harvested tumor and spleen were processed on a gentleMACS Octo Dissociator™ (Miltenyi, San Diego, CA). Single cell suspensions were stained with the following T cell markers: CD4, CD8, CD19, ICOS, CD45, FoxP3, CTLA-4, CD3, Ki-67, PD-1, Granzyme B, and LIVE/DEAD®.

PK/PD Analysis

[0208] The mice were checked daily for postural, grooming, and respiratory changes, as well as lethargy. Tumors and group body weights were recorded twice a week until death,

euthanasia, or end of the study period. The response to the treatments was measured as a function of tumor growth inhibition (TGI), which was calculated as follows: % TGI = {1-[(Tt-To)/(Ct-Co)]}×100, Tt = tumor volume of the treatment group on a given day, To = initial tumor volume, Ct = tumor volume of the control group on a given day, Co = initial tumor volume of the control group. Animals were euthanized if the tumor reached a volume greater than approximately 2500 mm^3 or appeared ulcerated.

Statistical Analysis

[0209] Microsoft Excel was used to calculate the mean, standard deviation (SD), and median values of tumor volumes and body weights. The mean and median values were calculated when 100% and at least 60% of the study animals remained in each treatment group, respectively. GraphPad Prism® v.4 software was used to plot data.

[0210] As expected, mice that received the unrelated control antibody failed to control tumor growth (FIG. 9A) whereas all the mice that received ipilimumab effectively controlled tumor growth (FIG. 9B). Mice that received the different activatable human anti-CTLA-4 antibodies controlled tumor growth comparably with ipilimumab (FIGs. 9C to 9F). Of the activatable antibodies, Ipi YV39 2001 most closely resembled the efficacy of ipilimumab in controlling tumor growth (FIG. 9F).

[0211] In regard to the frequency of regulatory T cells in the tumor and spleen of the treated mice, as observed earlier with the activatable anti-mouse CTLA-4 antibodies (*see* Example 2), activatable anti-human CTLA-4 antibodies (mouse IgG2a isotype) behaved similarly to ipilimumab in tumors (FIGs. 12A and 12B), but in the spleen, the activatable antibodies were more comparable to the unrelated control antibody (FIGs. 12C to 12F).

[0212] The data shown here collectively demonstrate that the activatable human anti-CTLA-4 antibodies disclosed herein can effectively control tumors like the traditional ipilimumab while exhibiting less risk of undesirable side effects.

Example 6:
In vivo Characterization of Activatable Anti-Human CTLA-4 Antibodies Comprising Modified Cleavable Moieties

[0213] To address a possible deamidation site in certain cleavable moiety sequences (see Example 10), activatable human anti-CTLA4 antibodies were prepared using a human

IgG1 and various CM sequences. The activatable antibodies comprise YV39 as the masking moiety and one of several variants of the 2001 cleavable moiety: WT (2001), ANP (2012), DNP (2011), or Q (2008) ("Ipi YV39 2001", "Ipi YV39 2012", "Ipi YV39 2011", and "Ipi YV39 2008", respectively). Ipilimumab and the unrelated human anti-diphtheria toxin were again used as controls.

[0214] To measure the activity of the activatable anti-CTLA-4 antibodies, the MC38 tumor model was used as described above in Example 5. For the dose titration study (FIGs. 11A to 11F), the mice were treated with ipilimumab or the activatable antibody comprising YV39 as the masking moiety and 2011 as the cleavable moiety ("Ipi YV39 2011") at doses of 200 μg/dose, 60 μg/dose, and 20 μg/dose.

[0215] As shown in FIGs. 10A and 10B, mice treated with the control antibody failed to control the tumor, whereas 6 out of 10 mice treated with ipilimumab were tumor-free at the end of the experiment. Mice treated with the different activatable antibodies were able to control tumor as observed with the traditional ipilimumab (FIGs. 10C to 10F). *See also* FIGs. 11B – 11G.

[0216] In regard to the frequency of regulatory T cells in the tumor and spleen of the treated mice, as observed earlier, tumor-specific protease was required to cleave the 2001 cleavable moiety variants. In the tumors, these activatable antibodies behaved like ipilimumab in reducing the frequency of Foxp3+ regulatory T cells (FIGs. 13A, 13B, 14A, and 14B). *See also* FIG. 15. In the spleen, the antibodies more closely mirrored the unrelated control antibody (FIGs. 13C to 13E, 14D to 14G, and 16A to 16B), demonstrating that the masking moiety remains coupled to the activatable antibody in the absence of the specific tumor-associated proteases.

Example 7:
In vivo Characterization of A Non-Fucosylated Version of Activatable Anti-Human CTLA-4 Antibodies

[0217] As described above, the absence of core fucose residues can strongly enhance ADCC via improved binding of IgG to activating FcγRIIIA without altering antigen binding or CDC. Natsume *et al.* (2009) *Drug Des. Devel. Ther.* 3:7. Non-fucosylated forms of ipilimumab ("Ipi NF") and ipi YV39 2011 ("Ipi YV39 2011 NF") were prepared. Binding of Ipi and Ipi NF were determined for various mouse, human and

cynomolgus monkey Fc receptors. Results are provided at FIG. 19. As expected, Ipi NF showed dramatically enhanced affinity (i.e., lower K_d) for activating receptors human CD16a (FcγRIIIa), cyno CD16 (FcγRIII) and mouse FcγRIV.

[0218] Ipi YV39 2011 NF and Ipi-NF were tested at various doses in the MC38 tumor model described in Example 5. Ipilimumab and an unrelated hIgG1 were used as controls. Results are provided at FIGs. 17A - D. Ipi NF was somewhat more effective at limiting or preventing tumor growth than ipilimumab (compare FIGs. 17B and 17C), and Ipi YV39 2011 NF was equivalent to Ipi NF (compare FIGs. 17C and 17D). In addition, FoxP3+ regulatory T cells were also similarly depleted in the tumors of mice treated with Ipi NF and Ipi YV39 2011 antibody (*see* FIG. 18). In both experiments, the Ipi YV39 2011 NF is shown to be fully activated in the tumor.

[0219] These results confirm that the methods of the present invention are equally applicable to non-fucosylated forms of ipilimumab, including non-fucosylated activatable CTLA-4 antibodies such as YV39 2011 NF.

Example 8:
In vivo Characterization of Activatable Anti-Human CTLA-4 Antibodies in Cynomolgus Monkeys

[0220] To assess the anti-CTLA-4 antibodies in a primate, cynomolgous monkeys were administered activatable antibody comprising YV39 as the masking moiety and 2001 as the cleavable moiety. Vehicle and ipilimumab were used as controls. Each monkey received 10 mg of antibody or anti-CTLA-4 activatable antibody, and blood was collected on days 0, 4, 8, 15, 22, 36, and 43 post-antibody administration. As shown in FIG. 20, in monkeys that received ipilimumab, there was a spike in CD4+ T cell proliferation as measured by Ki67-staining at around days 8-15 post antibody administration. In contrast, activatable anti-CTLA-4 antibody behaved similarly to the vehicle control and did not induce CD4+ T cell proliferation in the monkeys. These data demonstrate that even in primates, the activatable anti-CTLA-4 antibody shows little if any activation, indicating the absence of specific proteases.

[0221] Collectively, the data presented at FIGs. 1 - 20 demonstrate that the activatable anti-CTLA-4 antibodies described herein offer an improvement over ipilimumab. The

activatable antibodies control tumor growth just as effectively as ipilimumab while reducing the risk of serious adverse events often observed with ipilimumab treatment.

Example 9:
K_{app} and ME Values for Activatable CTLA-4 Antibodies

[0222] Table 9 provides the K_{app} and masking efficiency (ME) values for activatable antibodies, disclosed herein, comprising a variety of masking moieties and cleavable moieties in a human IgG1 format. The values provided in this Table were calculated from the data depicted in the Figures. K_{app} represents the binding affinity of the activatable antibody under the conditions of the measurement, in this example binding by ELISA; it is to be appreciated, however, that binding affinity can also be measured by binding to CTLA-4 expressed on primary or transfected cells or by other physical methods such as, but not limited to, surface plasmon resonance or equilibrium dialysis. Masking efficiency (ME) is calculated by dividing the K_{app} of the activatable antibody by the K_D of ipilimumab, measured under the same conditions.

Table 9: K_{app} and ME Values

	CM 2001		CM 3001		CM 2008		CM 2011		CM 2012		NSUB	
	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME
YV04-YV1	17.8	57										
YV06-YV1	0.6	2										
YV09-YV1	33.6	112	44.4	126								
YV23-YV1	11.4	38	13.8	39								
YV24-YV1			9.0	29								
YV27-YV1	0.7	2.3	0.8	2.3								
YV29-YV1	0.7	2.3	0.8	2.3								
YV32-YV1	0.9	3.0	1.2	3.4								
YV33-YV1	1.3	4.3	1.9	5								
YV35-YV1	3.7	12.3	5.3	15								
YV39-YV1	16.9	56	14.3	41	31.4	135	13.2	57	14.9	64	31.8	137
YV41-YV1	14.4	48	22.6	65								
YV51-YV1	4.4	15	4.9	14								
YV52-YV1	0.8	2.7	0.9	2.6								
YV53-YV1	4.1	14	5.3	15								
YV54-YV1	0.6	2	1.0	2.8								
YV55-YV1	4.8	16	6.0	18								
YV56-YV1	0.4	1.3	0.4	1								
YV57-YV1	0.4	1.3	1.6	4.6								
YV58-YV1	0.3	1	0.4	1								

[0223] Table 10 provides the K_{app} and ME values for the activatable antibodies disclosed herein, comprising a variety of masking moieties and cleavable moieties in a YV1 mouse Ig2a format. The values provided were calculated from the data depicted in the Figures.

Table 10: K_{app} and ME values

	CM 2001		CM 2006		CM 2007		CM 2008		CM 2009	
	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME
YV04-YV1	5.7	16.2	26.4	75	19.3	55	19.1	54	16.4	47
YV23-YV1			12.5	36	7.8	22	2.7	8	9.4	27
YV39-YV1	18.0	51	23.9	68			17.6	50	18.0	51

[0224] Table 11 provides K_{app} and ME values for the activatable antibodies comprising masking moieties having higher ME values and the 2012 cleavable moiety in aYV1 mouse IgG2a format. The values provided were calculated from the data depicted in the Figures.

Table 11: K_{app} and ME values

	CM 2001		CM 2011		CM 2012		NSUB	
	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME	K_{app} nM	ME
YV39-YV1	18.0	51	18.0	51	12.9	144	29.8	85
YV61-YV1					17.9	200		
YV62-YV1					15.5	173		
YV63-YV1					104	1170		
YV64-YV1					56.5	631		
YV65-YV1					12.3	156		
YV66-YV1					18.9	242		
YV01-YV1					38.6	493		
YV02-YV1					14.8	189		

Example 10:
Deamidation, Isomerization, and Stabilization Assessment for Activatable CTLA-4 Antibodies

[0225] As suggested in Example 6, to address a possible deamidation site in certain cleavable moiety (CM) sequences in certain activatable human anti-CTLA-4 antibodies, such activatable antibodies were prepared using various CM sequences (i.e., 2001, 2011, 2012, and 2008). In the cleavable moieties 2011, 2012, and 2008, the DNH sequence found in the 2001 cleavable moiety was replaced with DNP, ANP, and DQH, respectively.

[0226] These activatable CTLA-4 antibodies were produced by transient transfection of the relevant constructs in HEK 293 cells, and subjected to peptide mapping liquid chromatography - mass spectroscopy (LC-MS) to detect potential breakdown products. The 2001 (DNH) cleavable moiety, which was initially selected for use in the activatable

anti-CTLA-4 antibodies of the present invention, showed deamidation of the asparagine (N) residue (6.4%) after 7 days in PBS at 4°C. Forced stability studies showed an increase from 18.5% to 32.8% deamidation when stored at 25°C for 4 weeks, and to 36.5% and 66.6% when stored at 40°C for one week and four weeks, respectively.

[0227] Cleavable moieties 2008, 2011 and 2012 were selected to try to overcome the deamidation problem with 2001 in these activatable CTLA-4 antibodies. All of these had 0.1% or less deamidation when stored 40°C for one week in PBS, compared with 6.4% deamidation of 2001. However, further stability analysis (also by LC-MS) showed that while these activatable CTLA-4 antibodies comprising the 2008 (DQH) cleavable moiety exhibited minimal deamidation, it showed significant aspartate isomerization at the aspartate residue under various conditions (*see* Table 12). In contrast, 2011 (DNP) exhibited minimal aspartate isomerization. Aspartate isomerization was not relevant for 2012 (ANP), in which the aspartate residue is replaced with alanine.

Table 12: Isomerization values

Temperature	**Time**	**Cleavable Moiety – Isomerization Values**		
		2011 (DNP)	**2012 (ANP)**	**2008 (DQH)**
-80°C	0 days (T_0)	0.1%	N/A	1.8%
4°C	0 days (T_0)	0.1%	N/A	2.4%
25°C	3 months	0.2%	N/A	8.2%
40°C	3 months	0.2%	N/A	34.5%

[0228] However, *in vitro* stability studies in mouse, rat, and cynomolgus monkey serum showed substantial clipping between asparagine and proline residues for 2012 (ANP) (*see* Table 13) in these activatable CTLA-4 antibodies. 2011 (DNP) remained as the cleavable moiety with acceptably low levels of deamidation, aspartate isomerization, and light chain clipping.

Table 13: Degree of clipping observed between the asparagine and proline residues

Serum	**Cleavable Moiety – Clipping Between Asparagine and Proline Residues**	
	2011 (DNP)	**2012 (ANP)**
Mouse	-	++
Cyno	+/-	+++

[0229] All publications, patents, patent applications, internet sites, and accession numbers/database sequences (including both polynucleotide and polypeptide sequences)

cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, internet site, or accession number/database sequence were specifically and individually indicated to be so incorporated by reference.

What is claimed:

1. An activatable anti-human CTLA-4 antibody comprising:

(i) a heavy chain comprising a heavy chain variable domain (VH) comprising CDRH1: SYTMH (SEQ ID NO: 557); CDRH2: FISYDGNNKYYADSVKG (SEQ ID NO: 558); and CDRH3: TGWLGPFDY (SEQ ID NO: 559); and

(ii) a light chain comprising:

(a) a light chain variable domain (VL) comprising CDRL1: RASQSVGSSYLA (SEQ ID NO: 560); CDRL2: GAFSRAT (SEQ ID NO: 561); and CDRL3: QQYGSSPWT (SEQ ID NO: 562);

(b) a cleavable moiety (CM); and

(c) a masking moiety (MM),

wherein the light chain has the structural arrangement from N-terminus to C-terminus as follows: MM-CM-VL.

2. The activatable anti-human CTLA-4 antibody of Claim 1, wherein the MM is selected from the group consisting of YV01, YV02, YV03, YV04, YV09, YV23, YV24, YV35, YV39, YV51, YV61, YV62, YV63, YV64, YV65, and YV66.

3. The activatable anti-human CTLA-4 antibody of Claim 2, wherein the CM is a substrate for a protease selected from the group consisting of MMP1, MMP2, MMP3, MMP8, MMP9, MMP11, MMP13, MMP14, MMP17, legumain, matriptase, and uPA.

4. The activatable anti-human CTLA-4 antibody of Claim 3, wherein the CM is selected from a group consisting of 2001, 2003, 2005, 2006, 2007, 2008, 2009, 2011, 2012, 3001, 3006, 3007, 3008, 3009, 3011, and 3012.

5. The activatable anti-human CTLA-4 antibody of Claim 4, wherein the MM is YV04, YV23, YV24, YV39, YV61, YV62, YV63, or YV64.

6. The activatable anti-human CTLA-4 antibody of Claim 5, wherein the MM is YV39.

7. The activatable anti-human CTLA-4 antibody of Claim 6, wherein the CM is 2001, 2011, or 2012.

8. The activatable anti-human CTLA-4 antibody of claim 7, wherein the CM is 2011.

9. The activatable anti-human CTLA-4 antibody of any one of claims 1 to 8 comprising:

(i) a heavy chain comprising the amino acid sequence of SEQ ID NO: 345; and

(ii) a light chain comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 563, 564, and 565.

10. The activatable anti-human CTLA-4 antibody of claim 9 comprising:

(i) a heavy chain comprising the amino acid sequence of SEQ ID NO: 345; and

(ii) a light chain comprising the amino acid sequence of SEQ ID NO: 564.

11. The activatable anti-human CTLA-4 antibody of claim 10, wherein:

(i) the heavy chain further comprises the human IgG1 constant domain sequence of SEQ ID NO: 350; and

(ii) the light chain further comprises the human light chain kappa constant domain sequence of SEQ ID NO: 346.

12. The activatable anti-human CTLA-4 antibody of any one of claims 1 to 11 further comprising a second linker peptide (LP2) as disclosed herein, and wherein the activatable anti-human CTLA-4 antibody has the structural arrangement, from N-terminus to C-terminus, MM-LP1-CM-LP2-VL or MM-LP2-CM-LP1-VL.

13. The activatable anti-human CTLA-4 antibody of claim 12, wherein the LP1 and the LP2 are not identical to each other.

14. The activatable anti-human CTLA-4 antibody of any one of claims 1 to 13 further comprising a spacer, and having the structural arrangement, from N-terminus to C-terminus, spacer-MM-CM-VL.

15. The activatable anti-human CTLA-4 antibody of any one of claims 1 to 14 further comprising a toxic agent.

16. The activatable anti-human CTLA-4 antibody of claim 15, wherein the toxic agent is conjugated to the activatable antibody via a cleavable linker.

17. The activatable anti-human CTLA-4 antibody of any one of claims 1 to 14 further comprising a detectable moiety.

18. The activatable anti-human CTLA-4 antibody of claim 178, wherein the detectable moiety is a diagnostic agent.

19. A pharmaceutical composition comprising the activatable anti-human CTLA-4 antibody of any one of claims 1 to 18 and a carrier.

20. The pharmaceutical composition of claim 19 comprising an additional therapeutic agent.

21. An isolated nucleic acid molecule encoding the heavy chain and/or the light chain of the activatable anti-human CTLA-4 antibody of any one of claims 1 to 14.

22. A vector comprising the isolated nucleic acid molecule of claim 21.

23. A method of producing an activatable anti-human CTLA-4 antibody comprising:

(i) culturing a cell comprising the vector of claim 22 under conditions that lead to expression of the activatable antibody; and

(ii) recovering the activatable antibody.

24. A method of reducing CTLA-4 activity in a subject in need thereof comprising administering an effective amount of the pharmaceutical composition of claim 19 or 20 to the subject.

25. A method of treating, alleviating a symptom of, or delaying the progression of a cancer in a subject comprising administering a therapeutically effective amount of the pharmaceutical composition of claim 19 or 20 to the subject.

26. The method of claim 25, wherein the cancer is a bladder cancer, a bone cancer, a breast cancer, a carcinoid, a cervical cancer, a colon cancer, an endometrial cancer, a glioma, a

head and neck cancer, a liver cancer, a lung cancer, a lymphoma, a melanoma, an ovarian cancer, a pancreatic cancer, a prostate cancer, a renal cancer, a sarcoma, a skin cancer, a stomach cancer, a testis cancer, a thyroid cancer, a urogenital cancer, or a urothelial cancer.

27. The method of claim 26, wherein the cancer is melanoma.

FIG. 1A

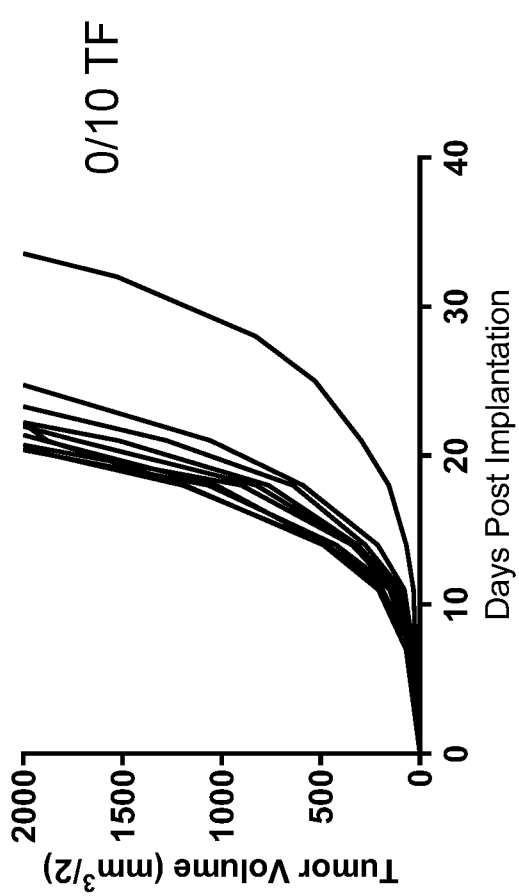


FIG. 1B

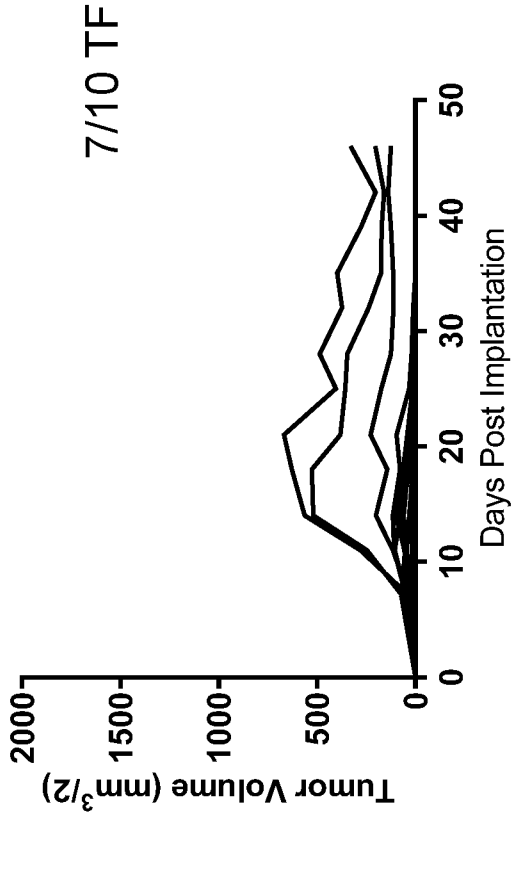
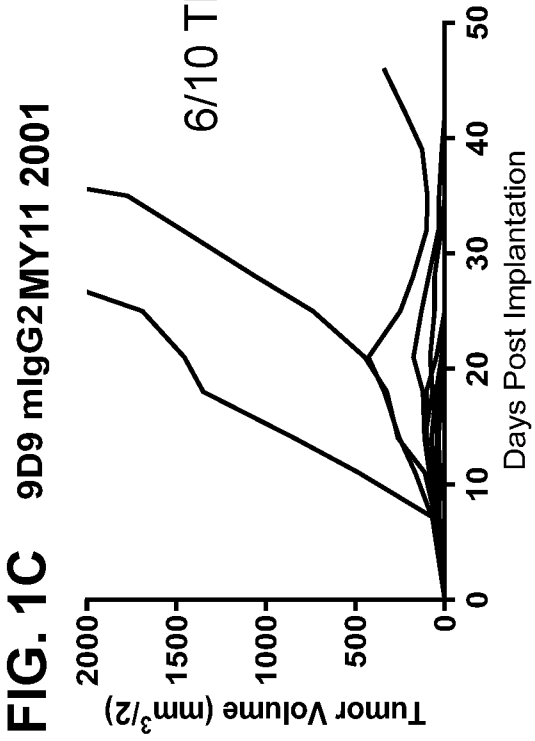


FIG. 1C



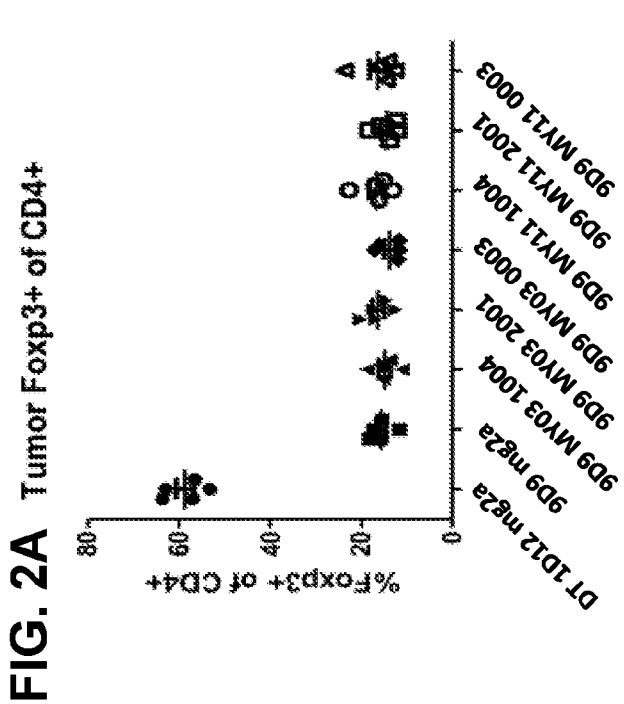


FIG. 2A

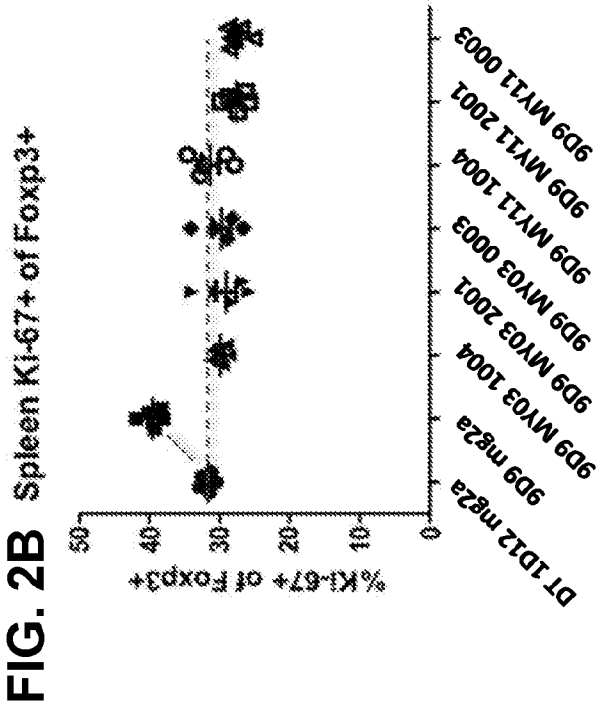


FIG. 2B

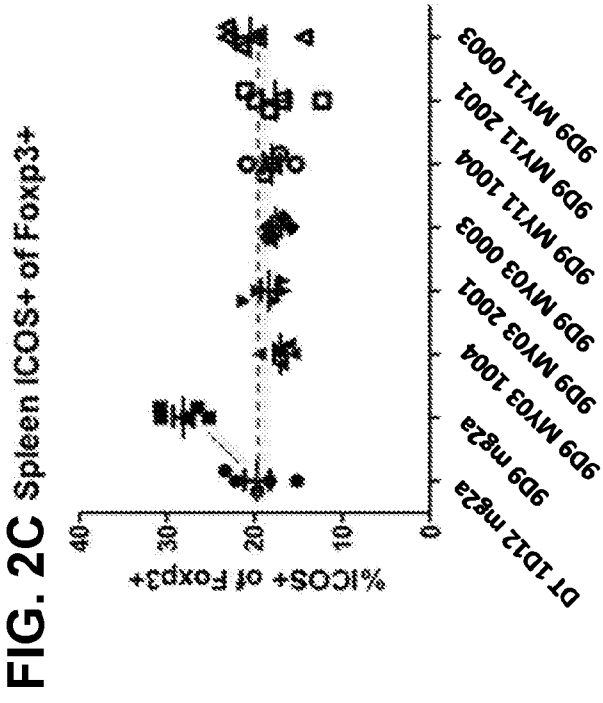


FIG. 2C

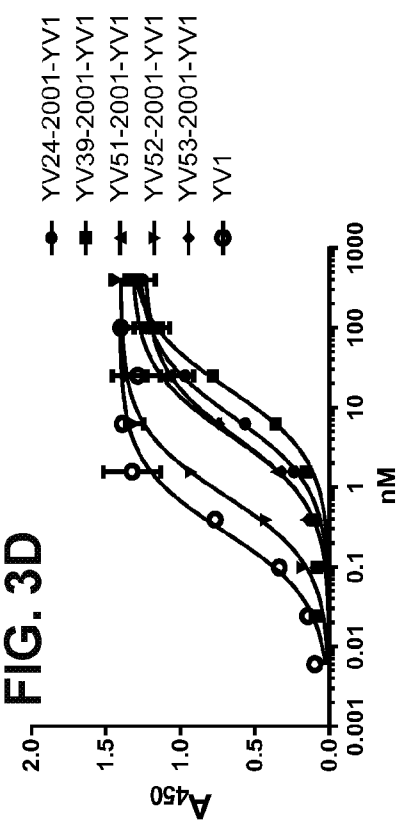


FIG. 3D

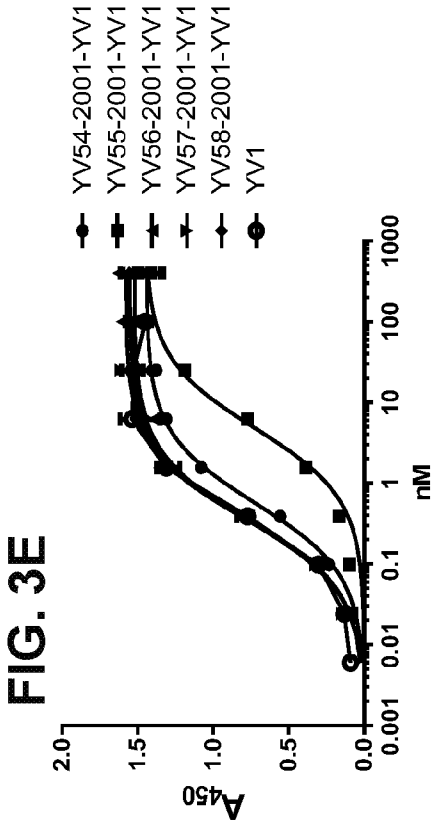


FIG. 3E

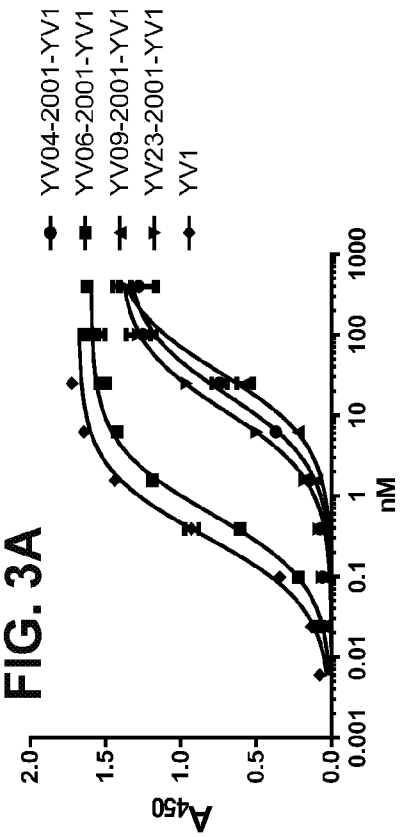


FIG. 3A

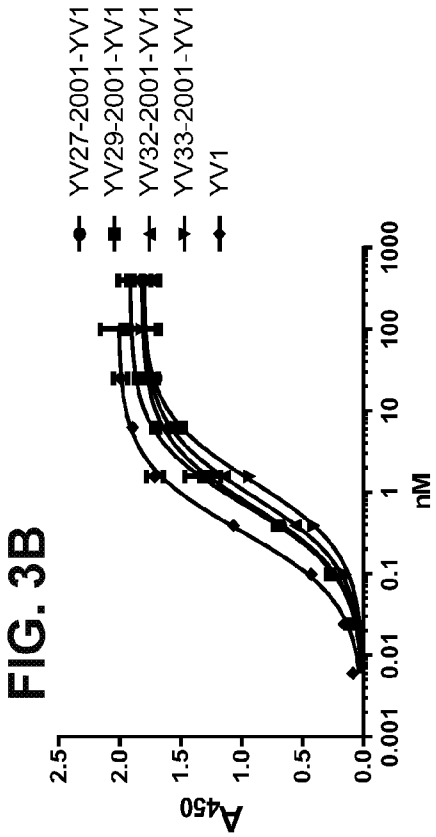


FIG. 3B

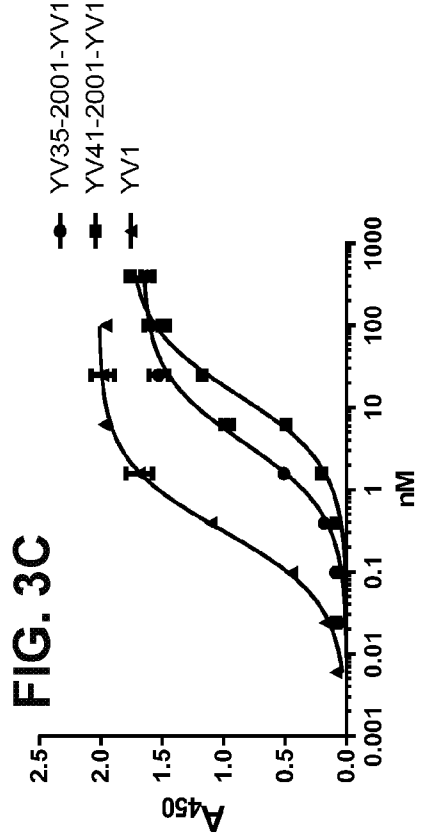


FIG. 3C

FIG. 4A

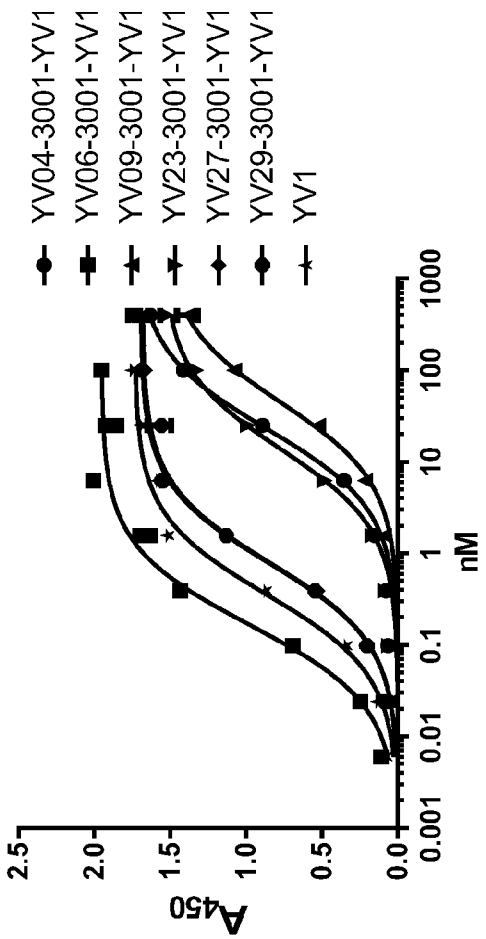


FIG. 4B

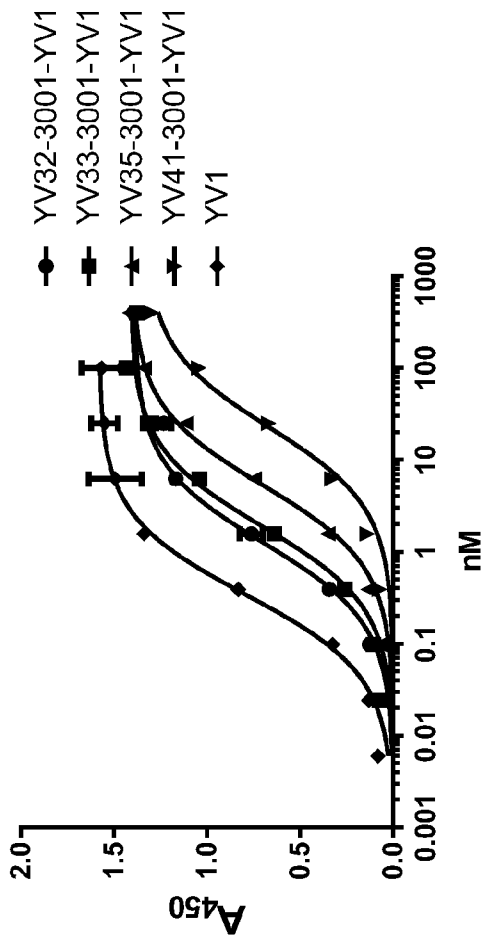


FIG. 4C

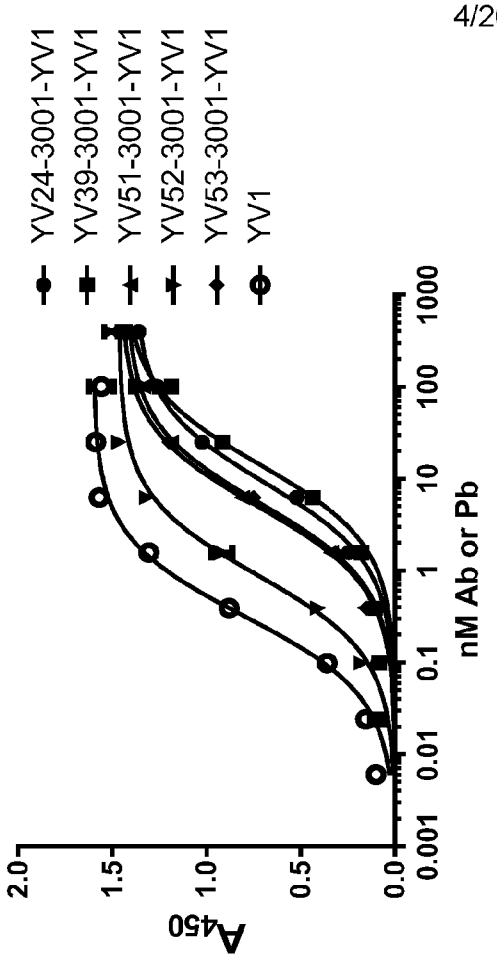


FIG. 4D

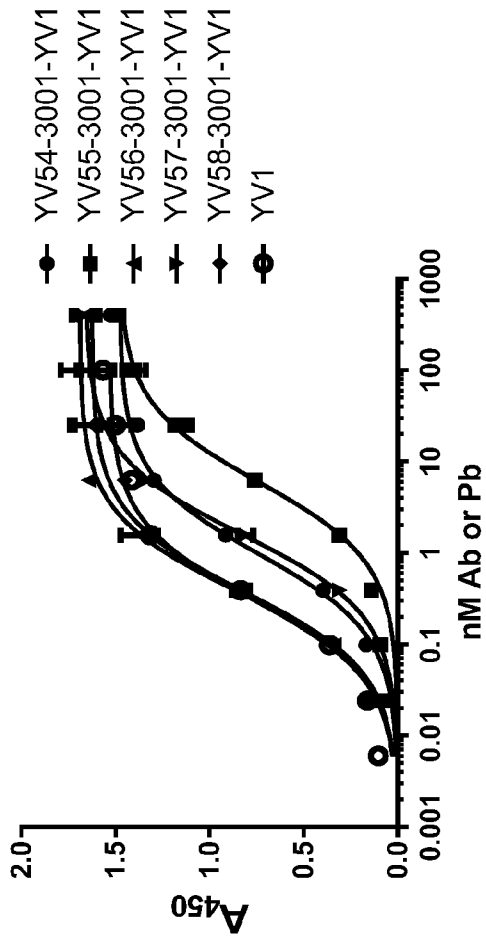


FIG. 5A

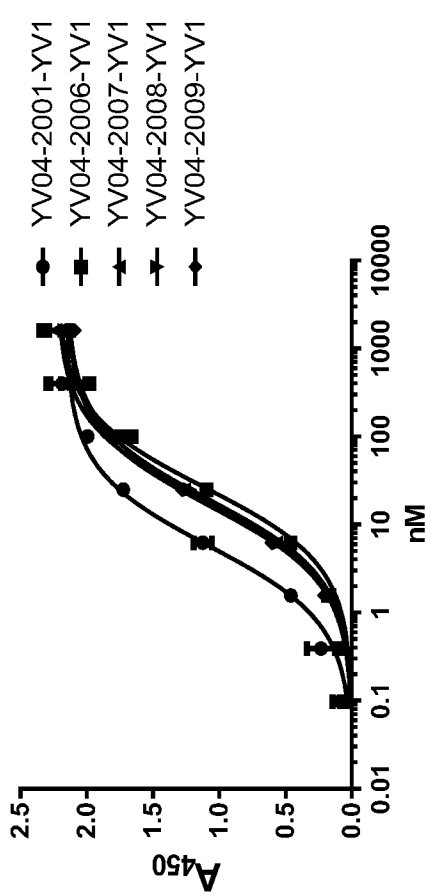


FIG. 5B

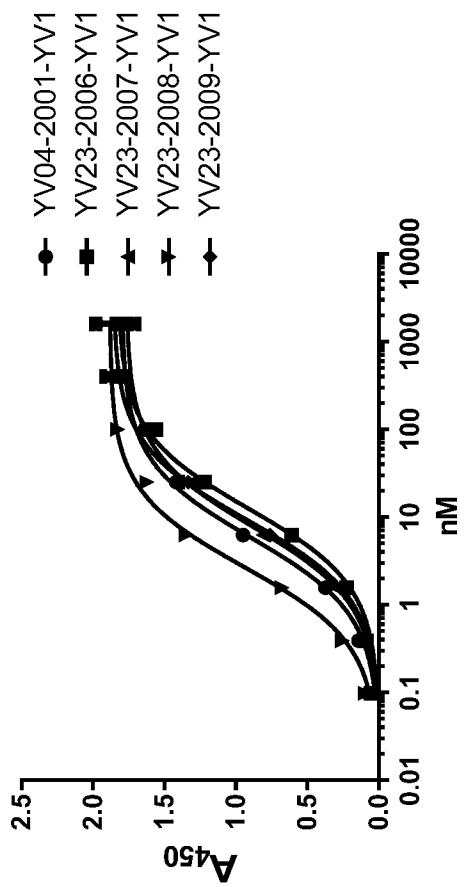


FIG. 5C

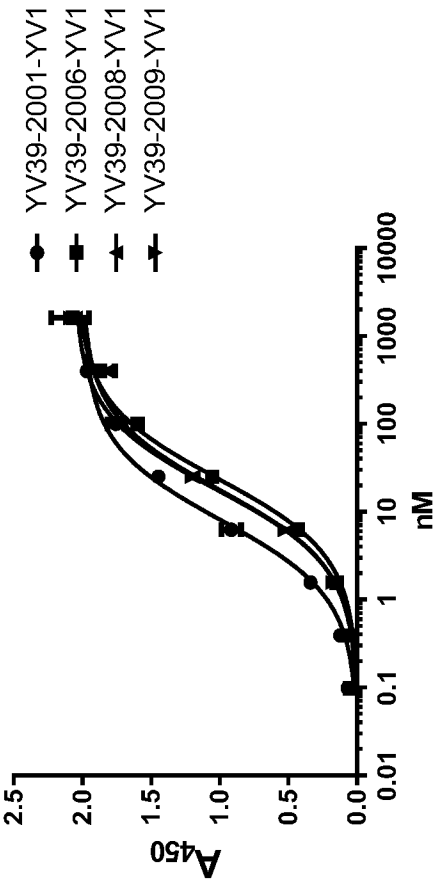


FIG. 5D

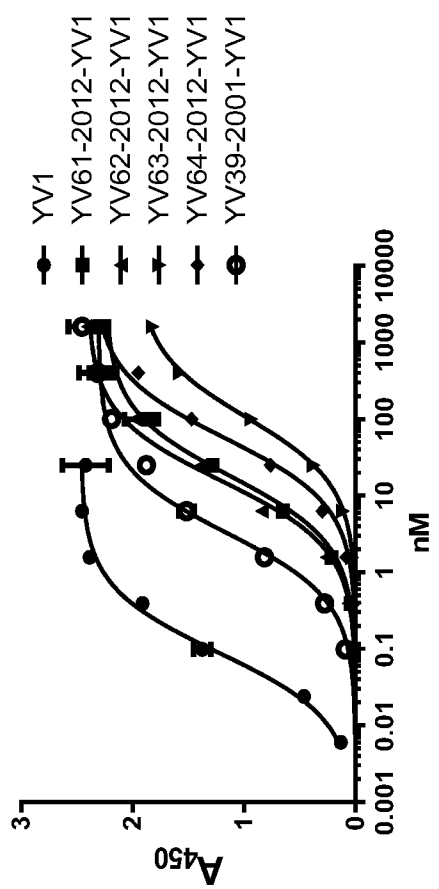


FIG. 5E

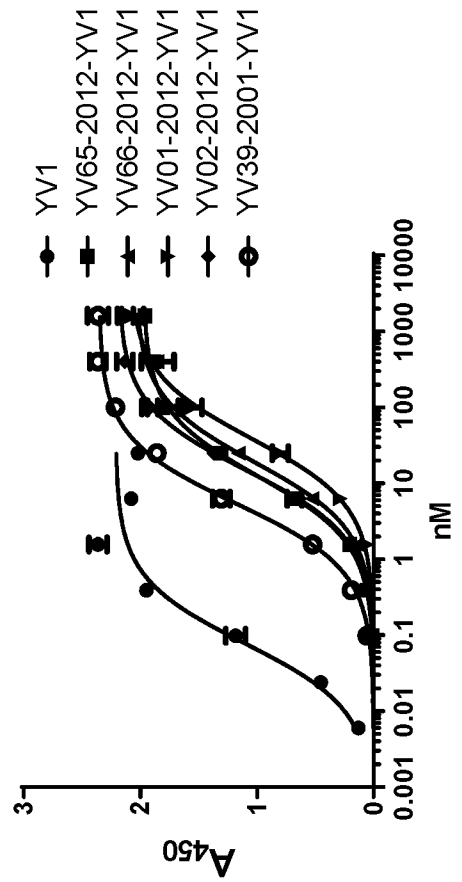


FIG. 5F

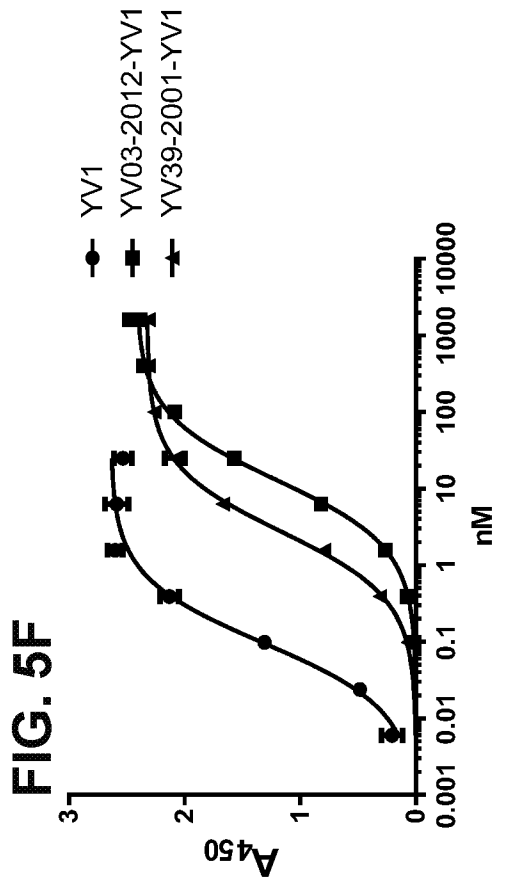


FIG. 6A

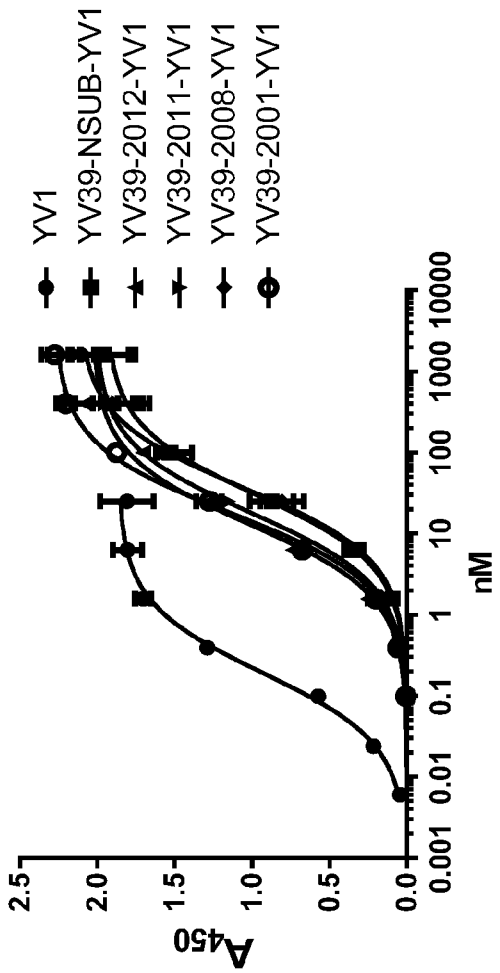

YV1
YV39-NSUB-YV1
YV39-2012-YV1
YV39-2011-YV1
YV39-2008-YV1
YV39-2001-YV1
A450
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
100
1000
10000
nM

FIG. 6B

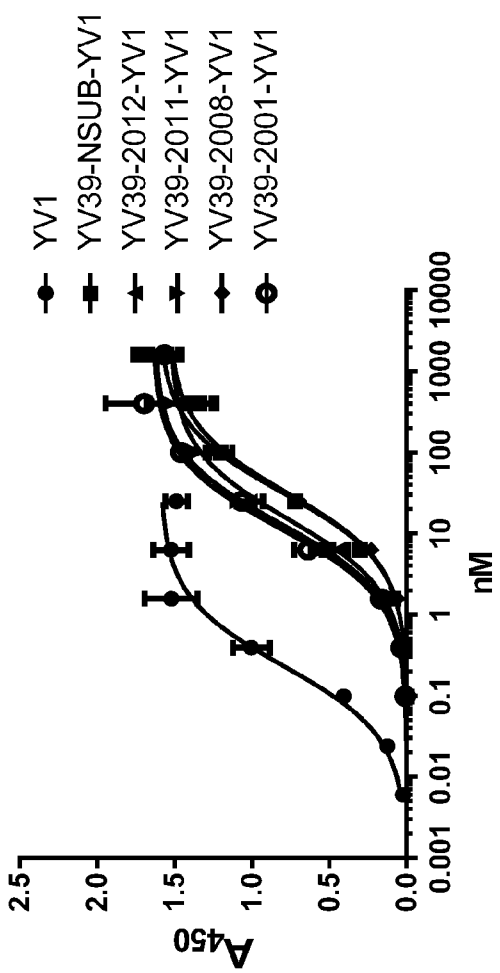

YV1
YV39-NSUB-YV1
YV39-2012-YV1
YV39-2011-YV1
YV39-2008-YV1
YV39-2001-YV1
A450
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
100
1000
10000
nM

FIG. 7A

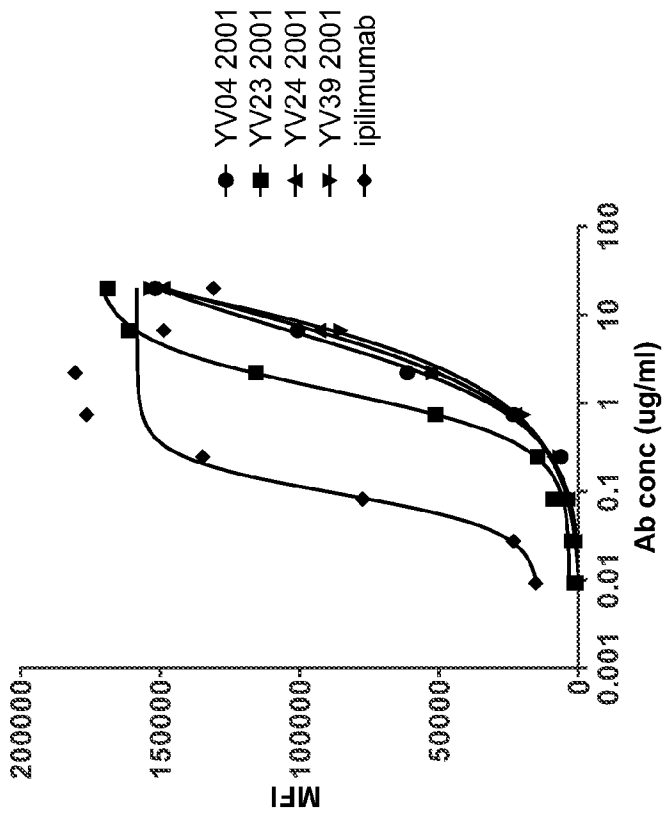

MFI
200000
150000
100000
50000
0
0.001
0.01
0.1
1
10
100
Ab conc (ug/ml)
YV04 2001
YV23 2001
YV24 2001
YV39 2001
ipilimumab

FIG. 7B

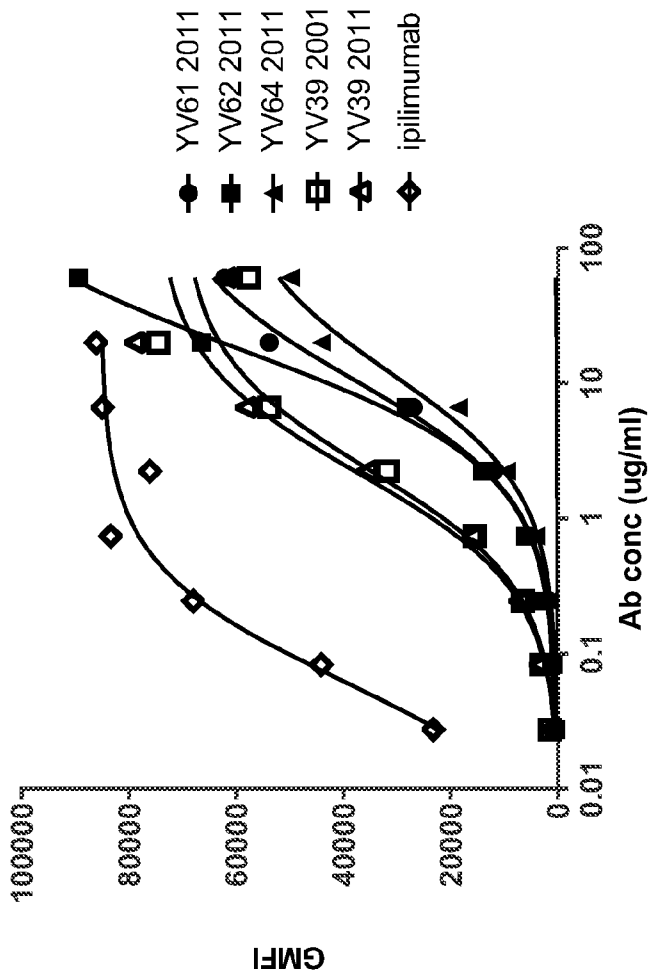

GMFI
100000
80000
60000
40000
20000
0
0.01
0.1
1
10
100
Ab conc (ug/ml)
YV61 2011
YV62 2011
YV64 2011
YV39 2001
YV39 2011
ipilimumab

FIG. 7C

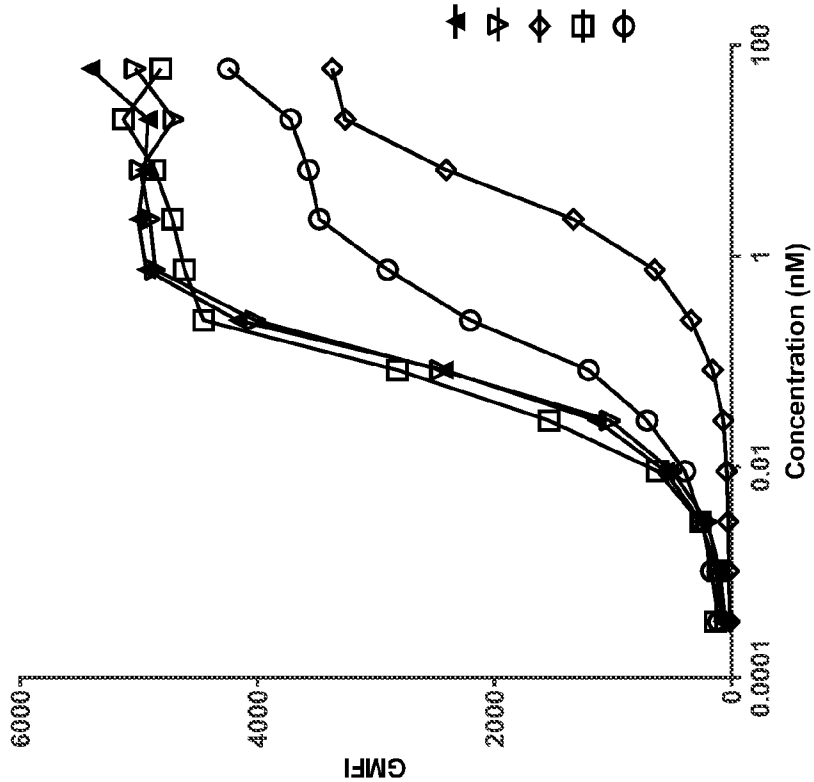
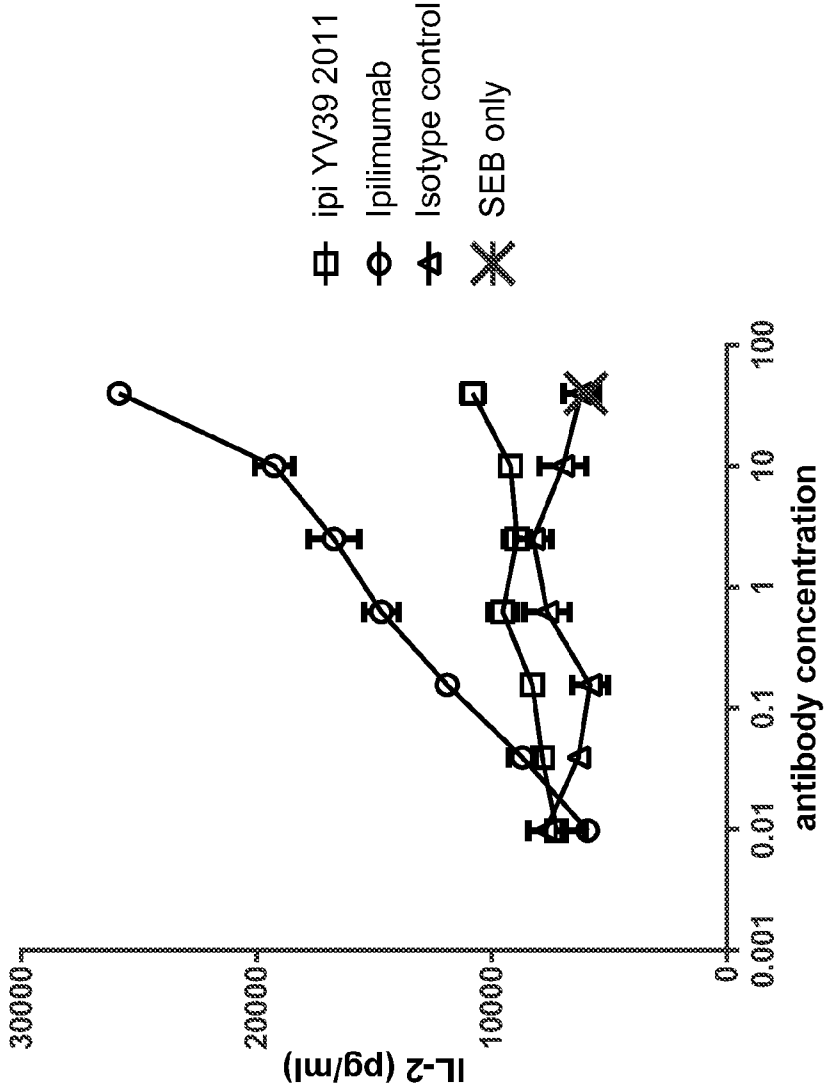


FIG. 7D

Sample	EC50 (nM)
Ipi YV39 2011	22
Ipilimumab	0.54
Ipi YV39 2011 MMP monoclipped	2.8
Ipi YV39 2011 MMP clipped	0.65
Ipi YV39 2011 µPA clipped	0.76

FIG. 8


ipi YV39 2011
Ipilimumab
Isotype control
SEB only
IL-2 (pg/ml)
30000
20000
10000
0
0.001
0.01
0.1
1
10
100
antibody concentration

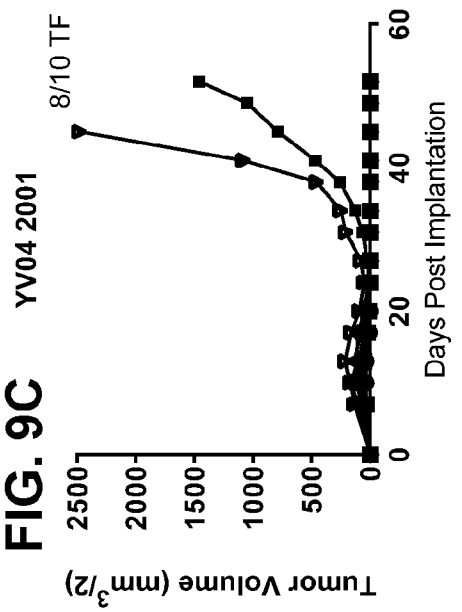


FIG. 9A
Control mg2a
0/10 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
2500
0
20
40
60

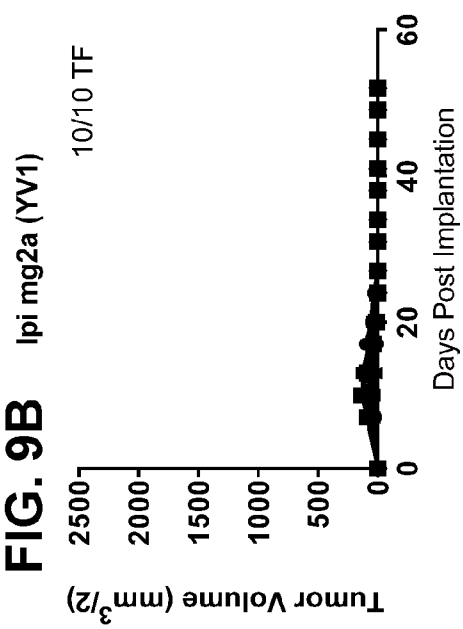


FIG. 9B
Ipi mg2a (YV1)
10/10 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
2500
0
20
40
60

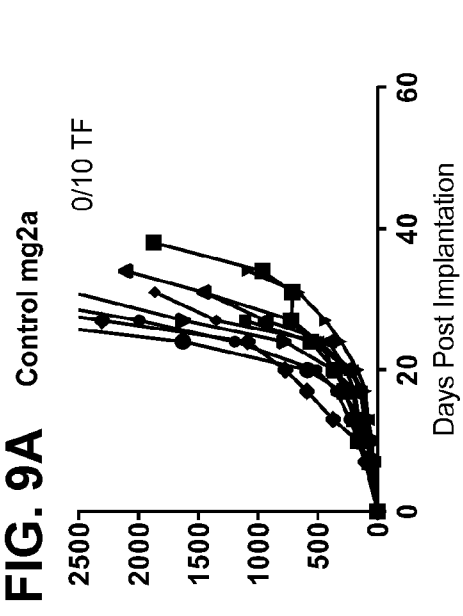


FIG. 9C
YV04 2001
8/10 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
2500
0
20
40
60

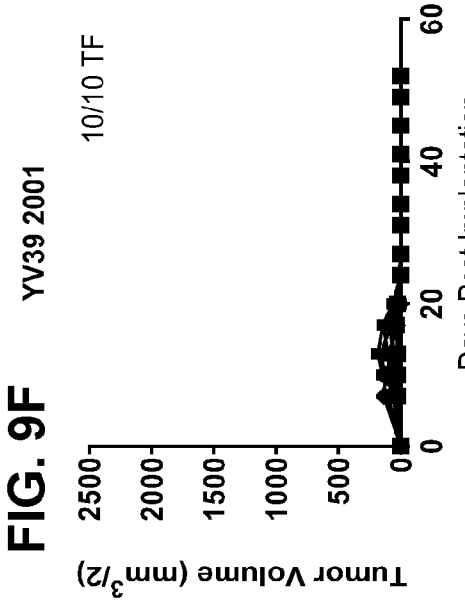


FIG. 9D
YV23 2001
9/10 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
2500
0
20
40
60

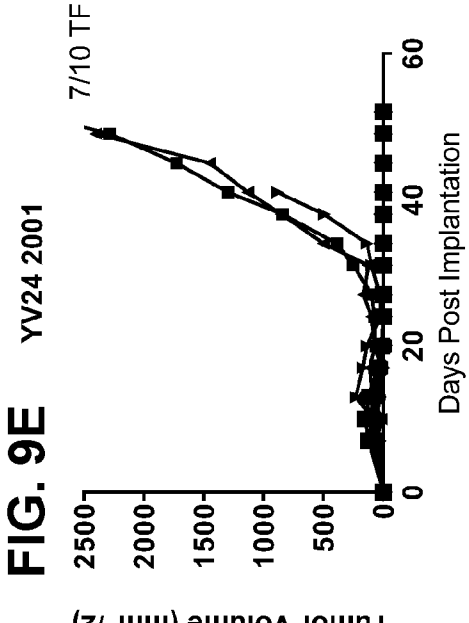


FIG. 9E
YV24 2001
7/10 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
2500
0
20
40
60

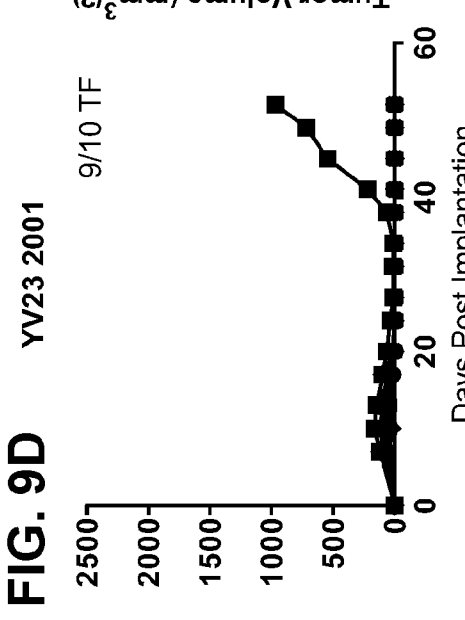


FIG. 9F
YV39 2001
10/10 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
2500
0
20
40
60

FIG. 10A

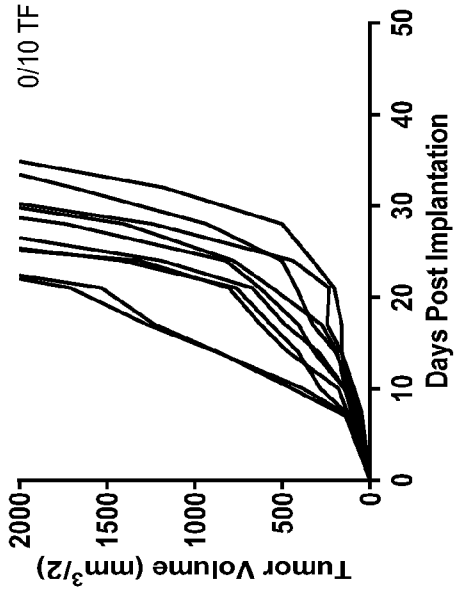


FIG. 10B

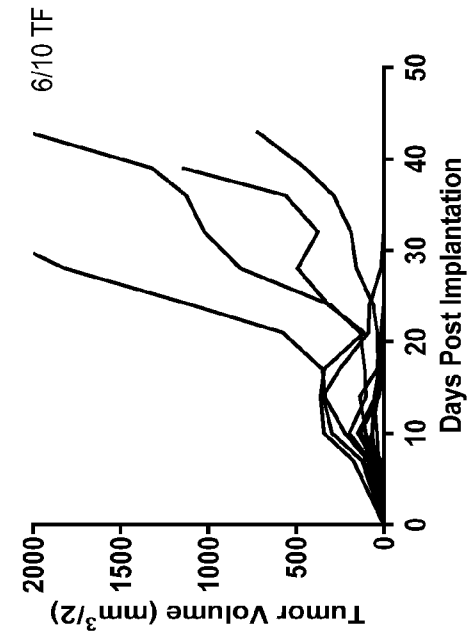


FIG. 10C

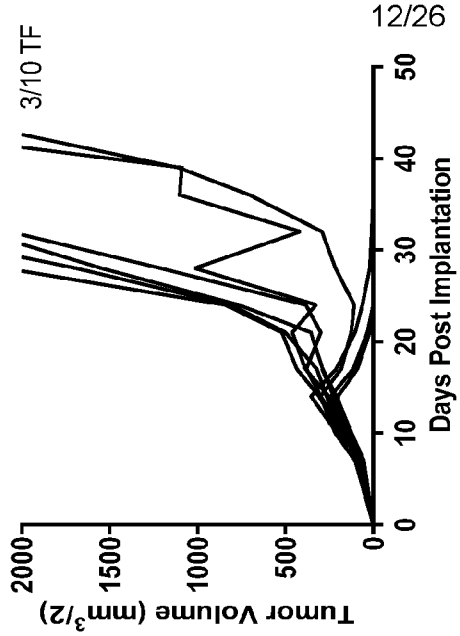


FIG. 10D

FIG. 10E

FIG. 10F

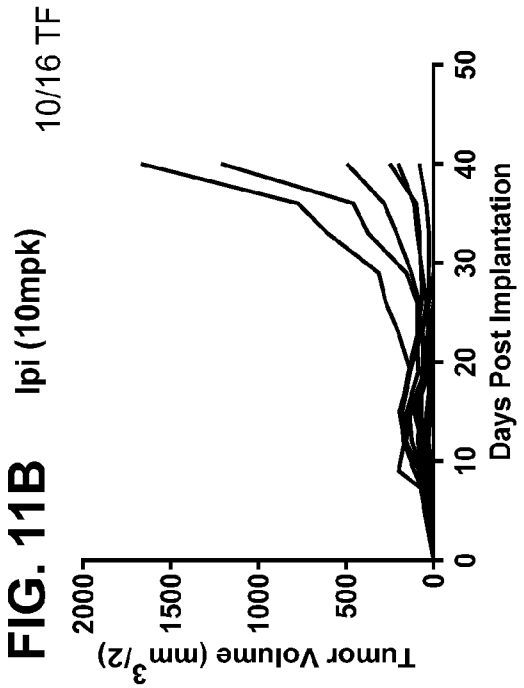


FIG. 11B
Ipi (10mpk)
10/16 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
0
10
20
30
40
50

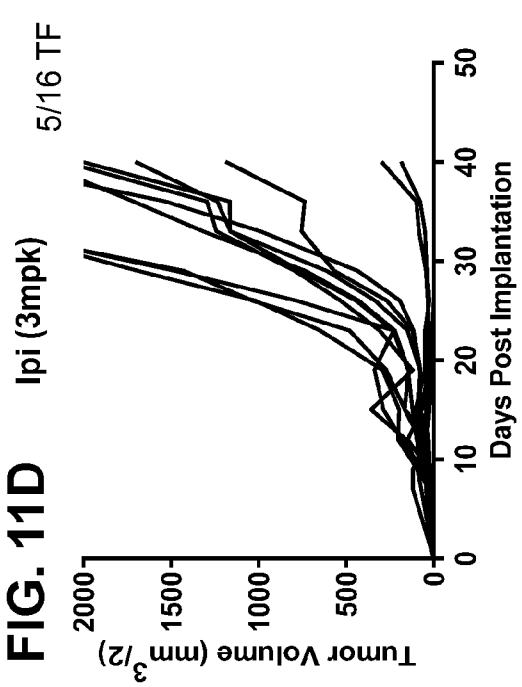


FIG. 11D
Ipi (3mpk)
5/16 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
0
10
20
30
40
50

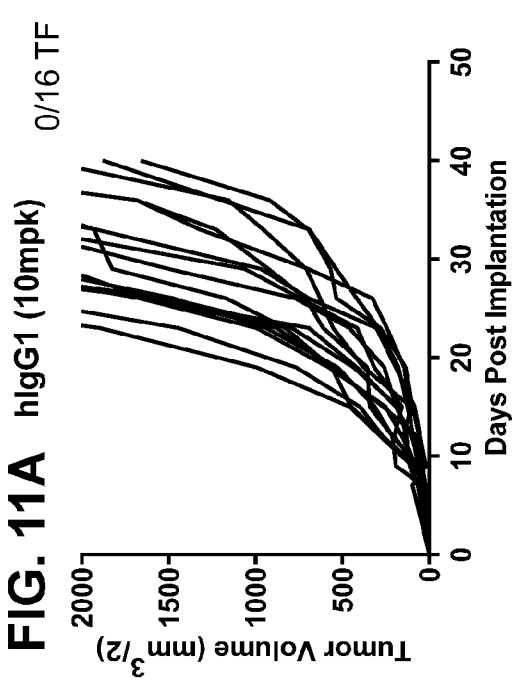


FIG. 11A
hIgG1 (10mpk)
0/16 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
0
10
20
30
40
50

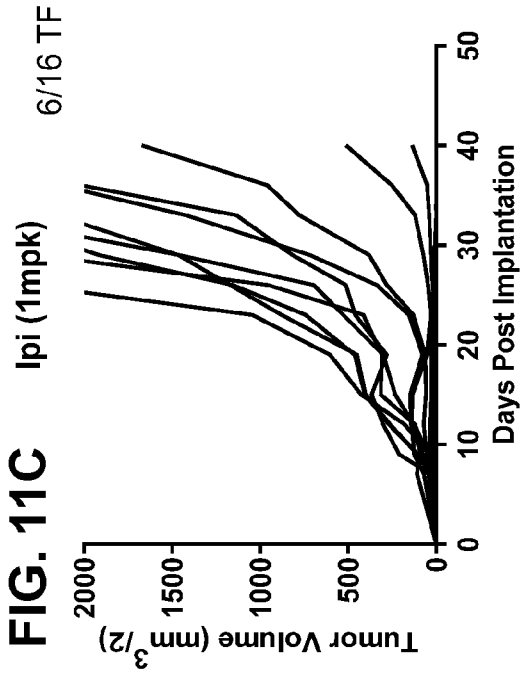


FIG. 11C
Ipi (1mpk)
6/16 TF
Tumor Volume (mm3/2)
Days Post Implantation
0
500
1000
1500
2000
0
10
20
30
40
50

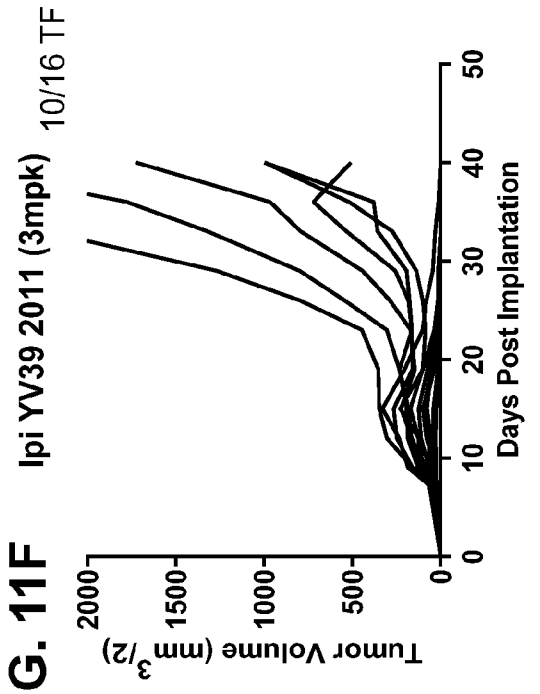


FIG. 11F

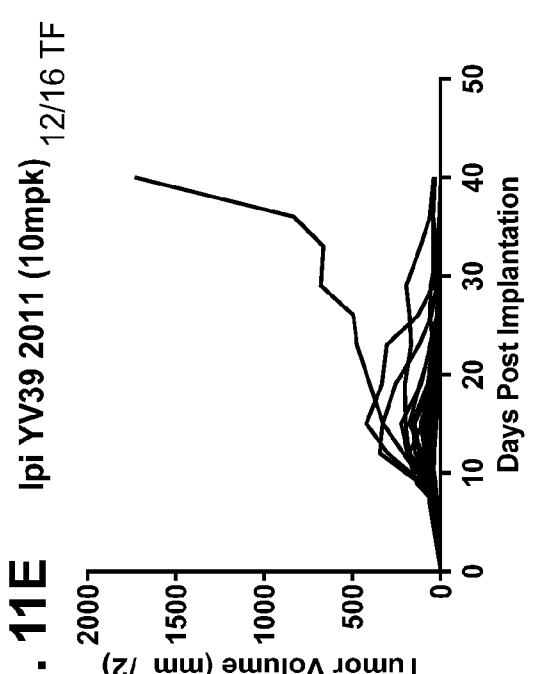


FIG. 11E

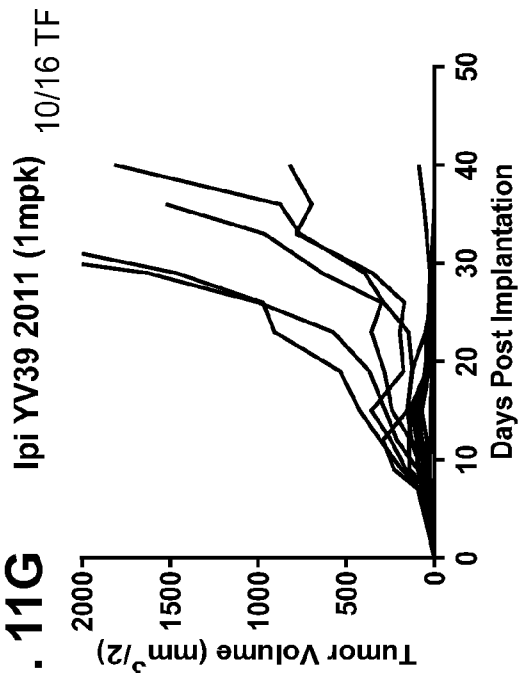


FIG. 11G

FIG. 12A

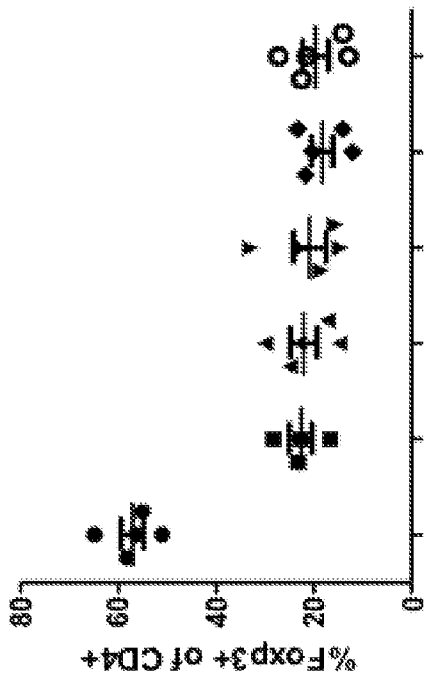


FIG. 12B

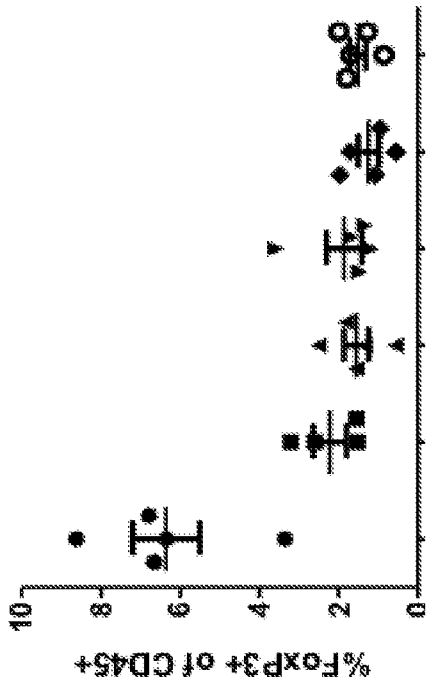
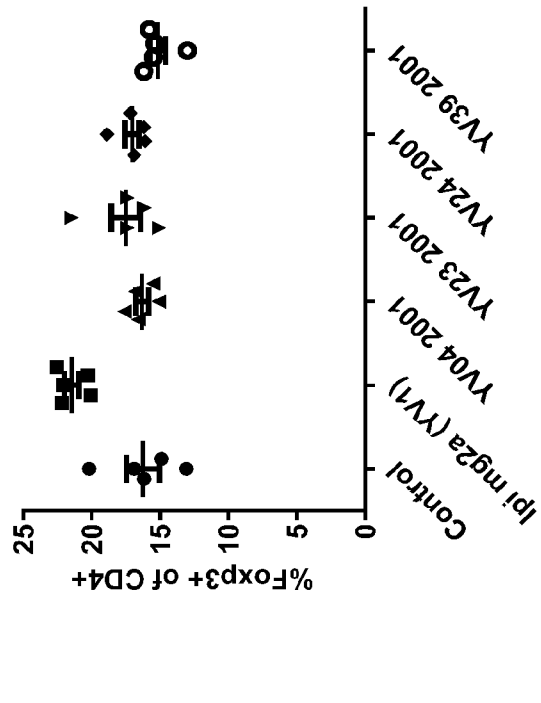


FIG. 12C



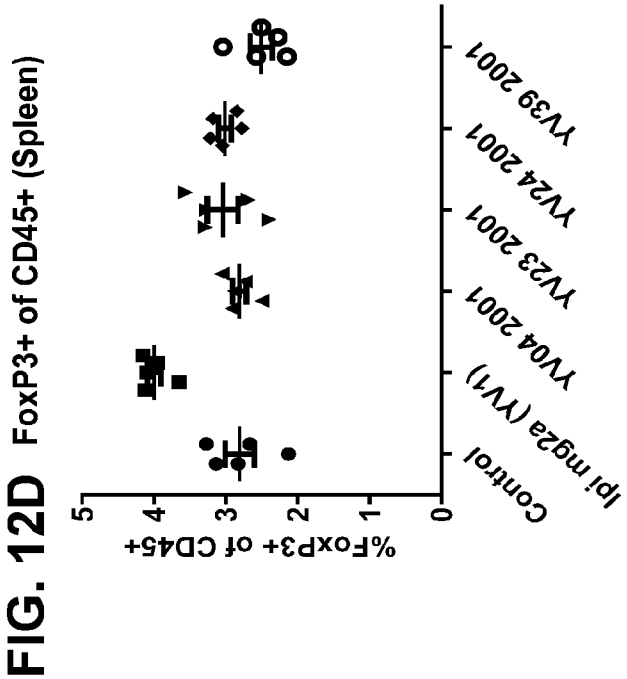


FIG. 12D
FoxP3+ of CD45+ (Spleen)
%FoxP3+ of CD45+
0
1
2
3
4
5
Control
Ipi mg2a (YV1)
YV04 2001
YV23 2001
YV24 2001
YV39 2001

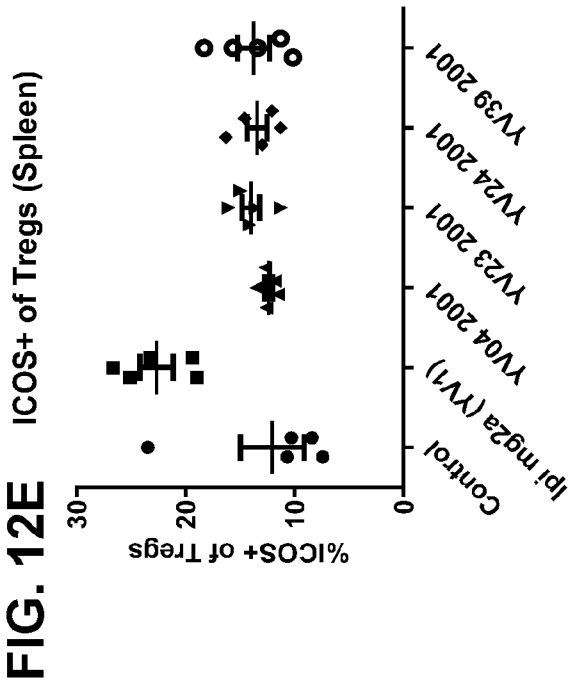


FIG. 12E
ICOS+ of Tregs (Spleen)
%ICOS+ of Tregs
0
10
20
30
Control
Ipi mg2a (YV1)
YV04 2001
YV23 2001
YV24 2001
YV39 2001

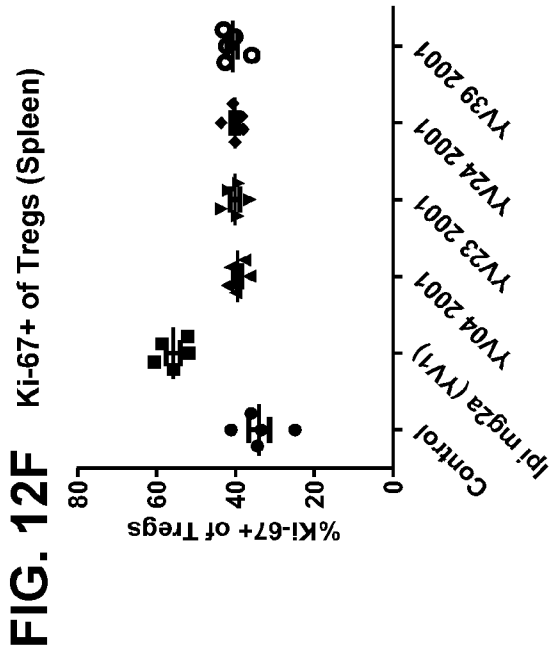


FIG. 12F
Ki-67+ of Tregs (Spleen)
%Ki-67+ of Tregs
0
20
40
60
80
Control
Ipi mg2a (YV1)
YV04 2001
YV23 2001
YV24 2001
YV39 2001

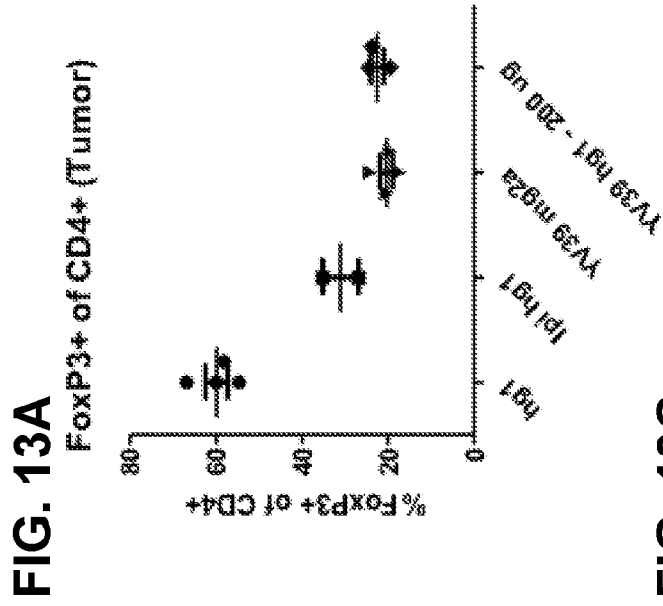


FIG. 13A
FoxP3+ of CD4+ (Tumor)
%FoxP3+ of CD4+
80
60
40
20
0
hIgG1
Ipi hIgG1
YV39 mIgG2a
YV39 hIgG1 - 200 ug

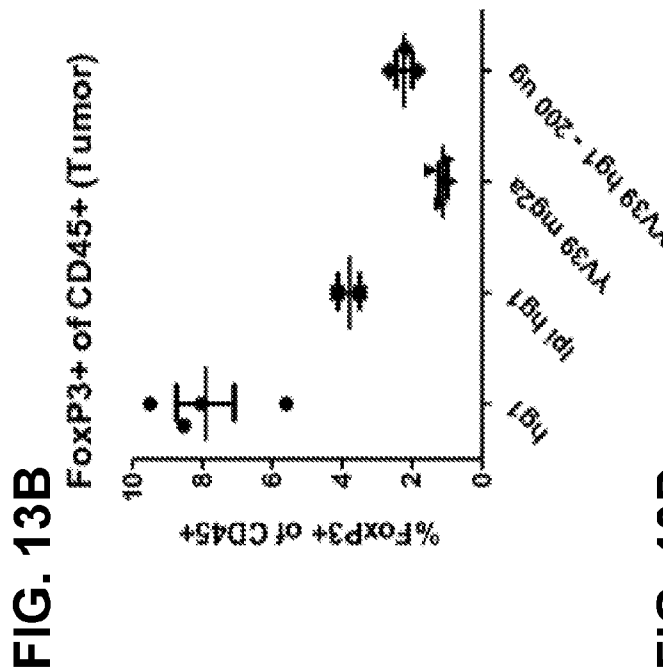


FIG. 13B
FoxP3+ of CD45+ (Tumor)
%FoxP3+ of CD45+
10
8
6
4
2
0
hIgG1
Ipi hIgG1
YV39 mIgG2a
YV39 hIgG1 - 200 ug

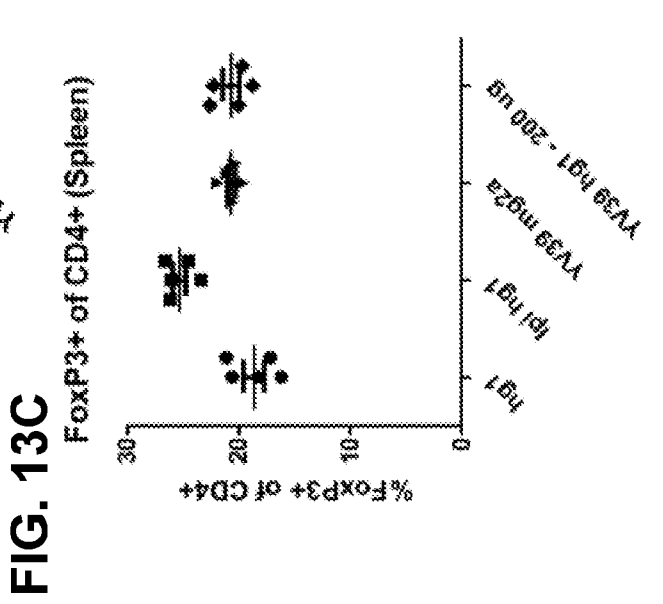


FIG. 13C
FoxP3+ of CD4+ (Spleen)
%FoxP3+ of CD4+
30
20
10
0
hIgG1
Ipi hIgG1
YV39 mIgG2a
YV39 hIgG1 - 200 ug

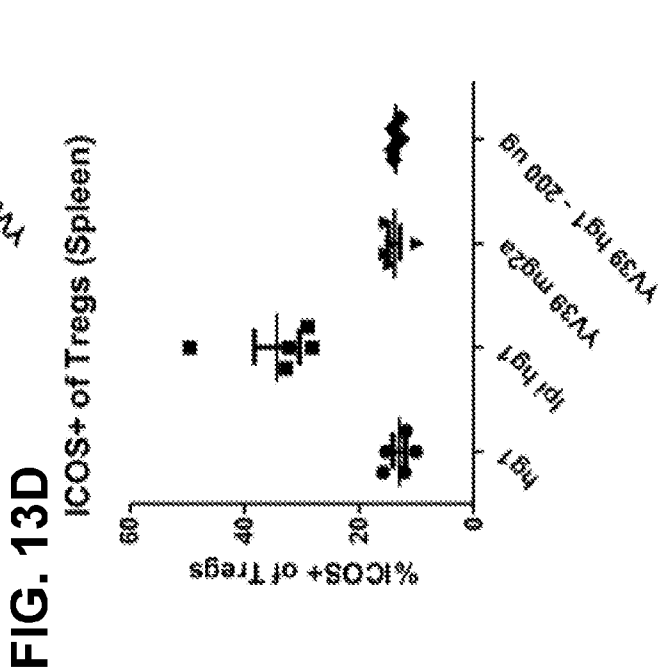


FIG. 13D
ICOS+ of Tregs (Spleen)
%ICOS+ of Tregs
60
40
20
0
hIgG1
Ipi hIgG1
YV39 mIgG2a
YV39 hIgG1 - 200 ug

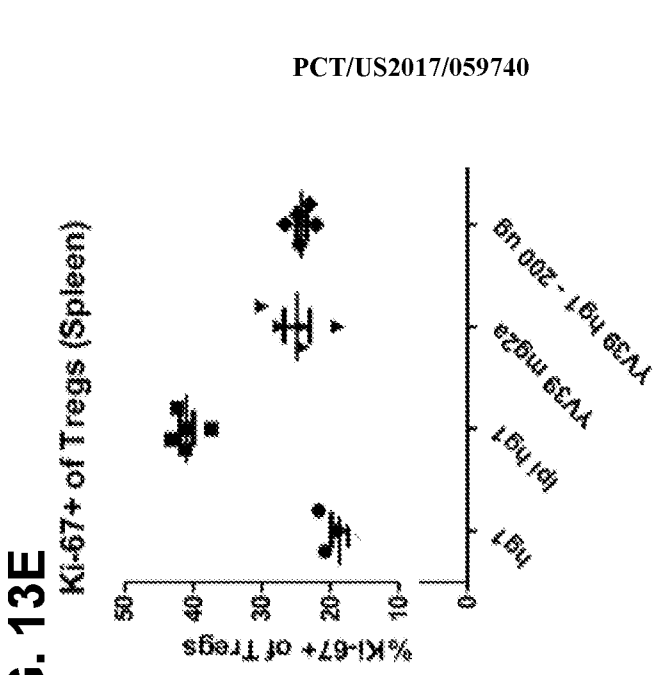


FIG. 13E
Ki-67+ of Tregs (Spleen)
%Ki-67+ of Tregs
50
40
30
20
10
0
hIgG1
Ipi hIgG1
YV39 mIgG2a
YV39 hIgG1 - 200 ug

FIG. 14A

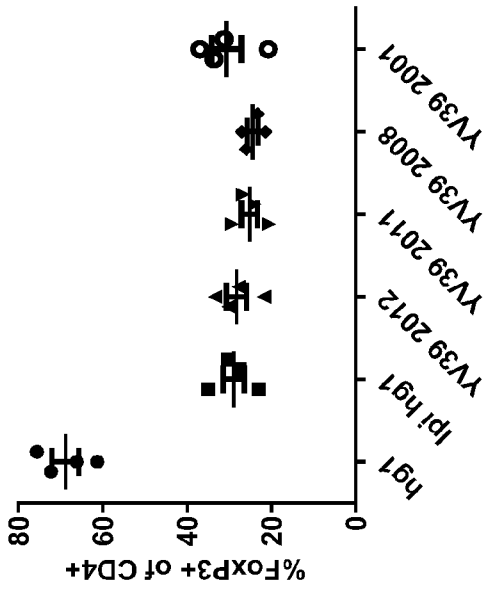


FIG. 14B

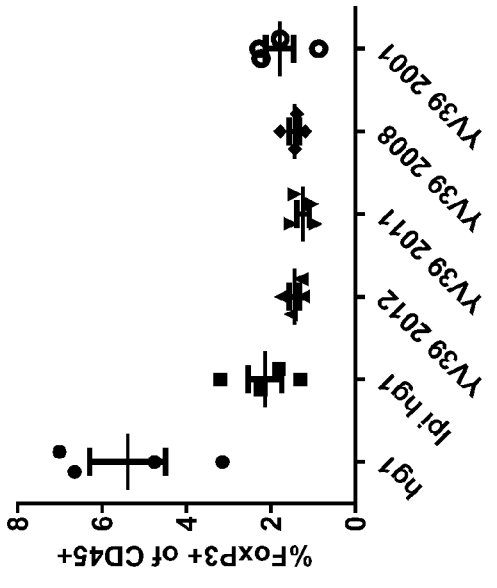
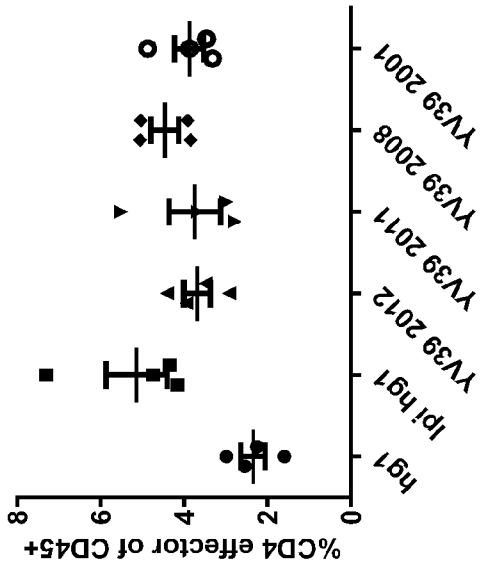


FIG. 14C



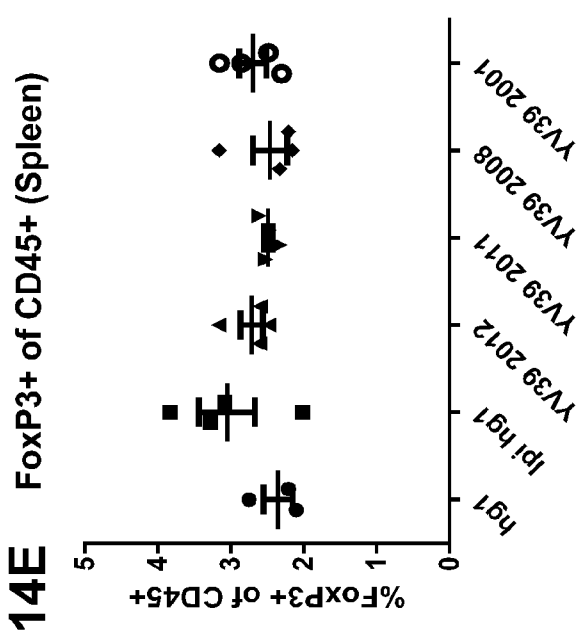


FIG. 14E
FoxP3+ of CD45+ (Spleen)
%FoxP3+ of CD45+
0
1
2
3
4
5
hγ1
Ipi hγ1
YV39 2012
YV39 2011
YV39 2008
YV39 2001

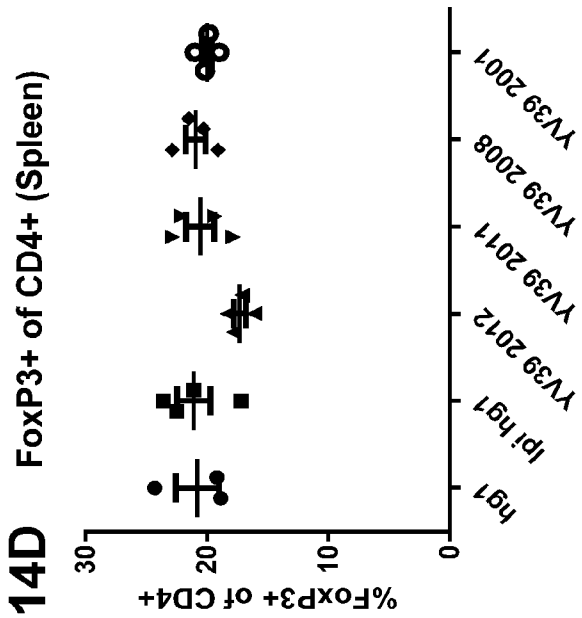


FIG. 14D
FoxP3+ of CD4+ (Spleen)
%FoxP3+ of CD4+
0
10
20
30
hγ1
Ipi hγ1
YV39 2012
YV39 2011
YV39 2008
YV39 2001

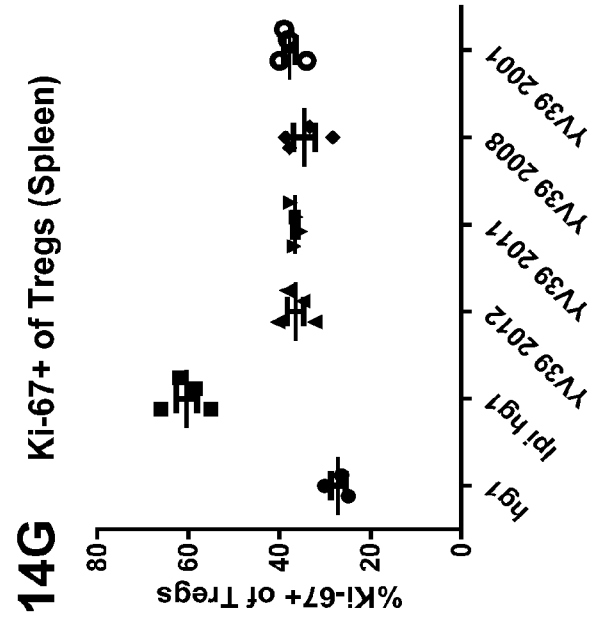


FIG. 14G
Ki-67+ of Tregs (Spleen)
%Ki-67+ of Tregs
0
20
40
60
80
hγ1
Ipi hγ1
YV39 2012
YV39 2011
YV39 2008
YV39 2001

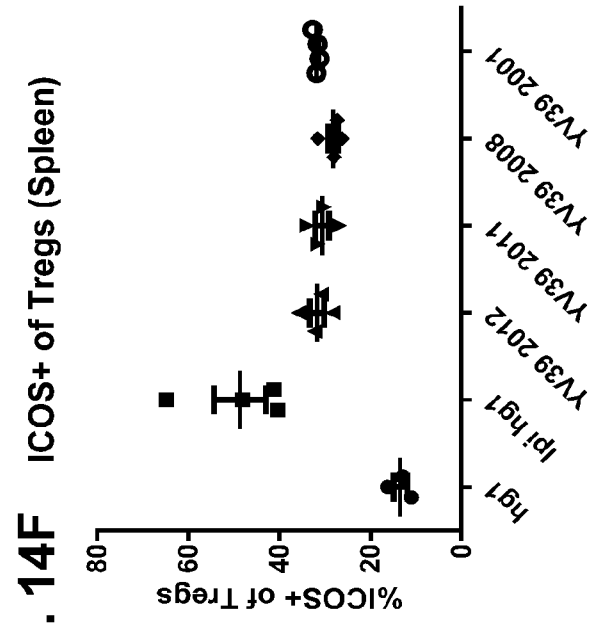


FIG. 14F
ICOS+ of Tregs (Spleen)
%ICOS+ of Tregs
0
20
40
60
80
hγ1
Ipi hγ1
YV39 2012
YV39 2011
YV39 2008
YV39 2001

FIG. 15

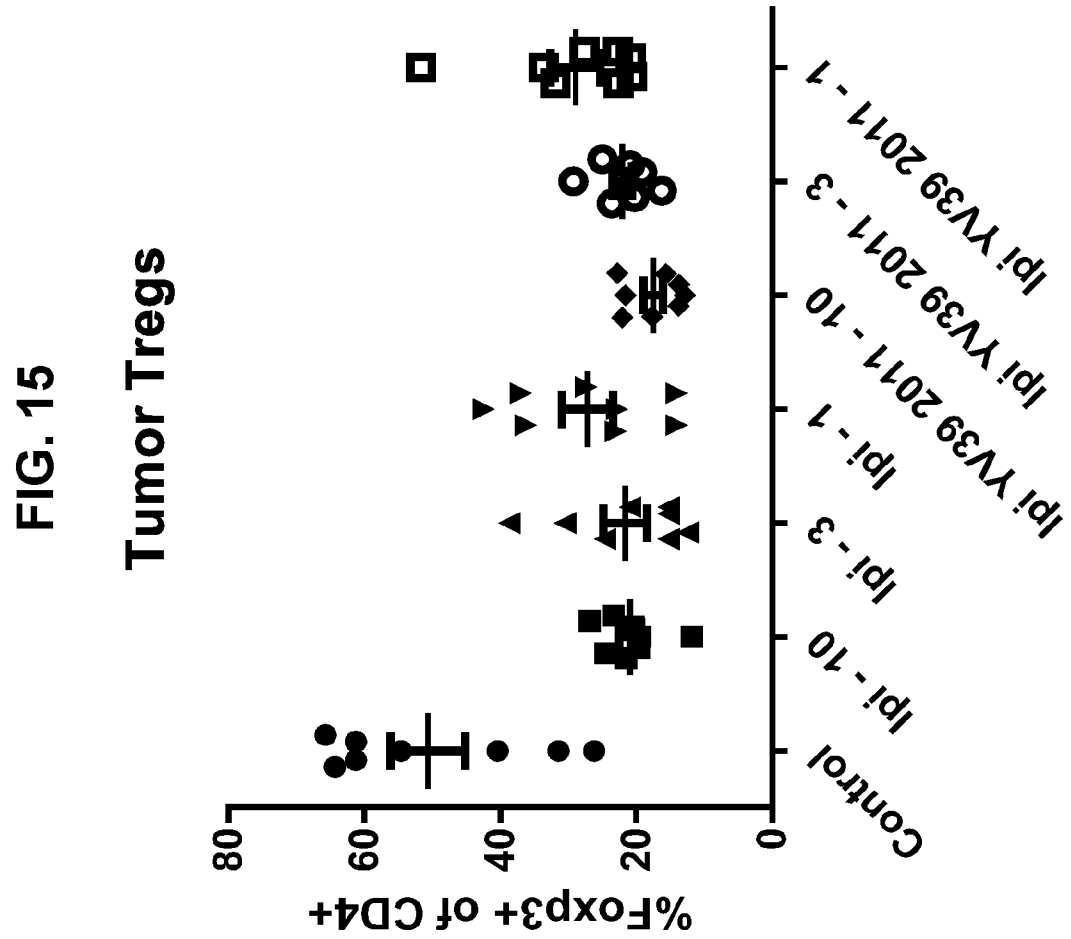

Tumor Tregs
%Foxp3+ of CD4+
0
20
40
60
80
Control
Ipi - 10
Ipi - 3
Ipi - 1
Ipi YV39 2011 - 10
Ipi YV39 2011 - 3
Ipi YV39 2011 - 1

FIG. 16B

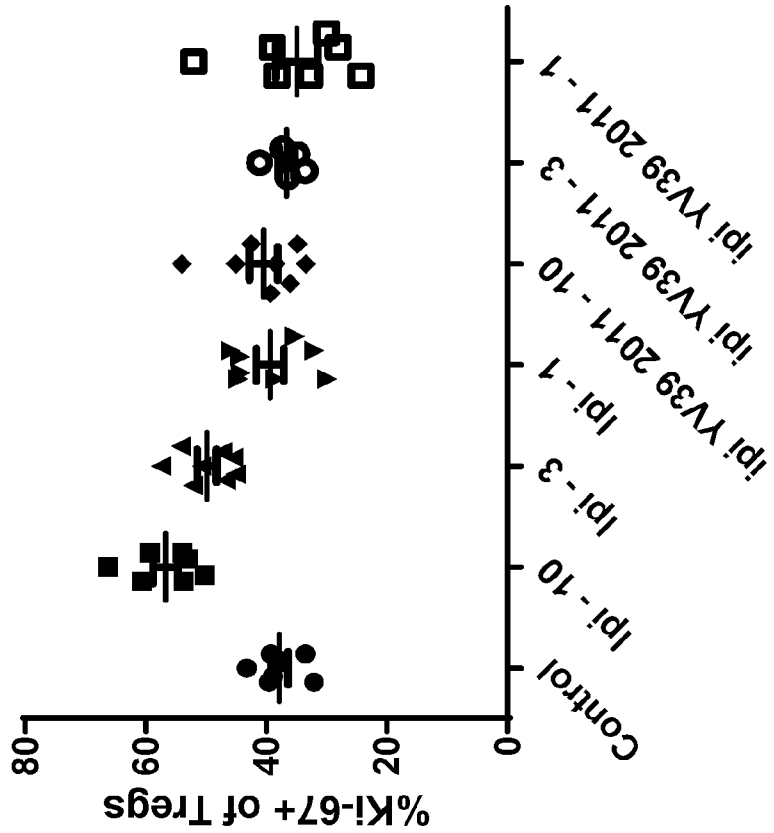


FIG. 16A

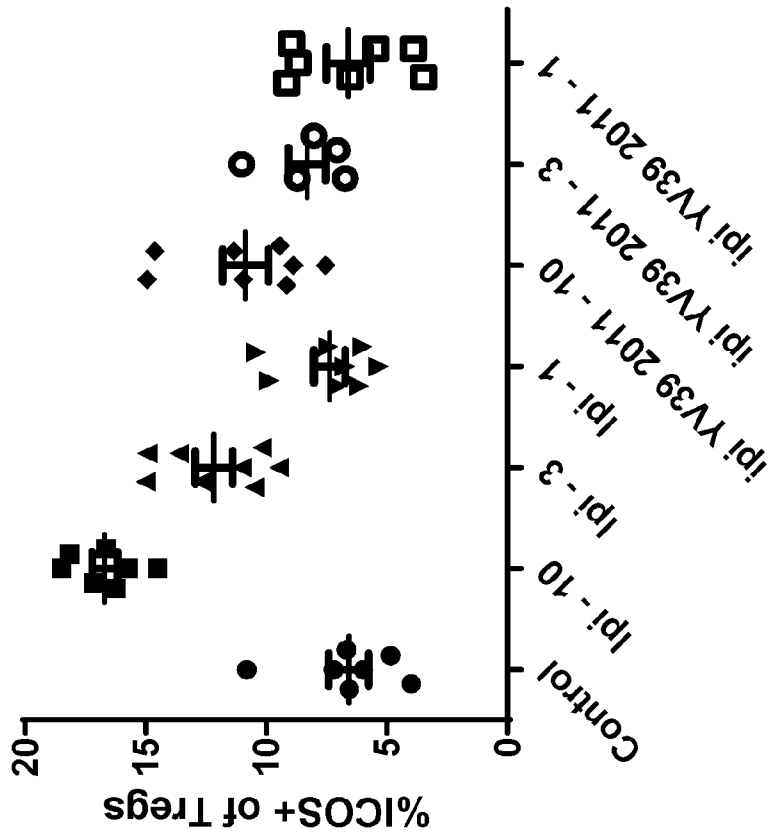


FIG. 17A

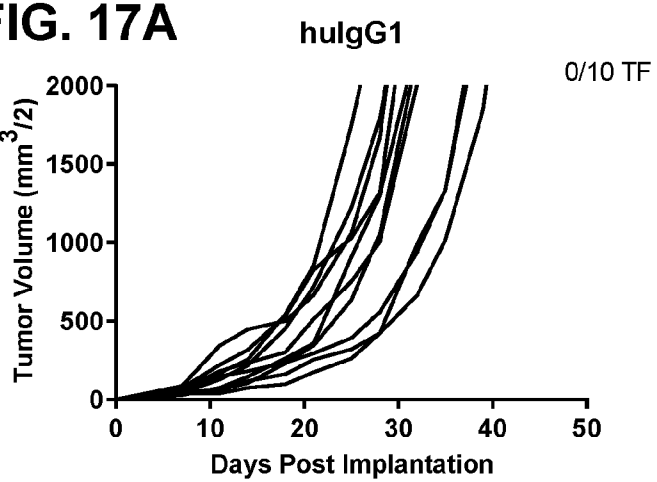
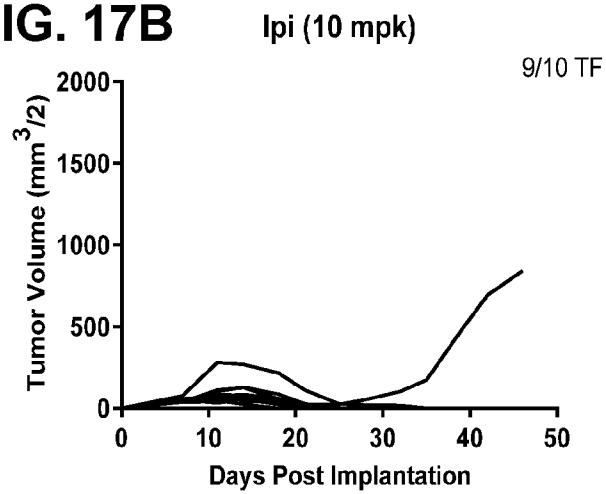
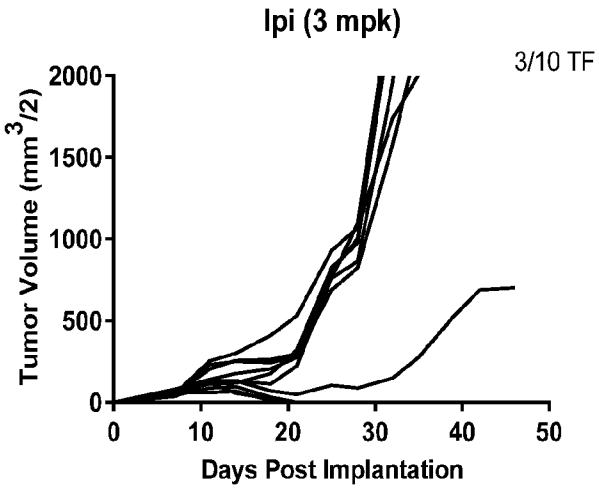


FIG. 17B





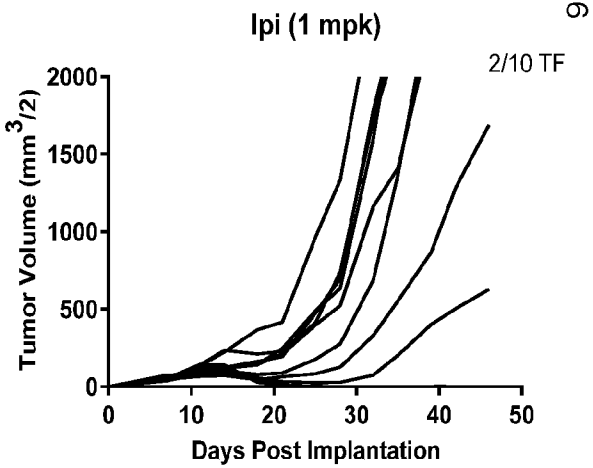
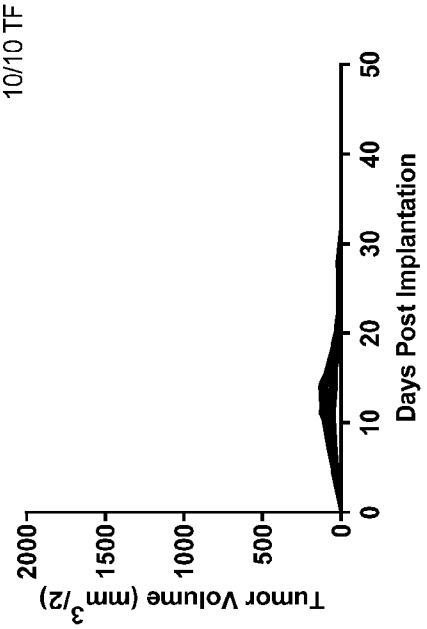
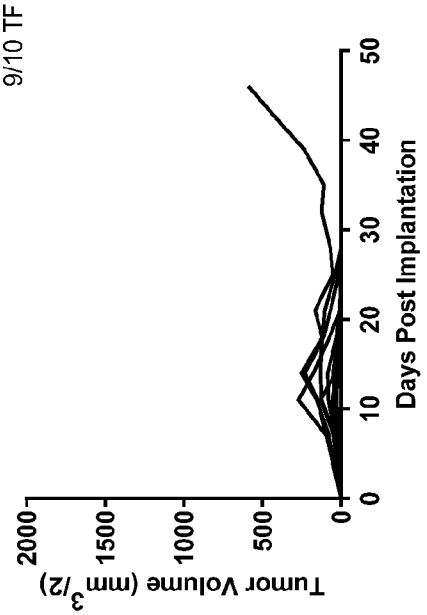


FIG. 17C





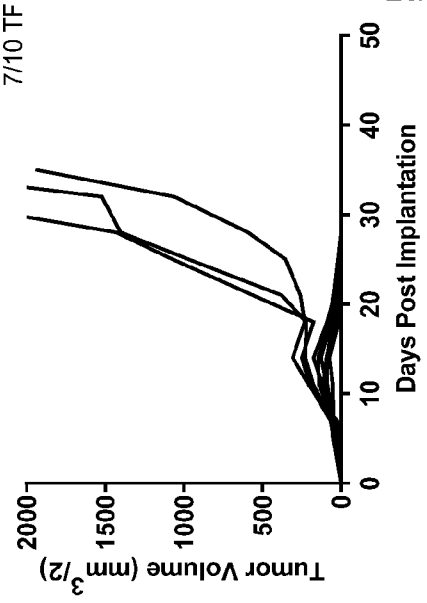
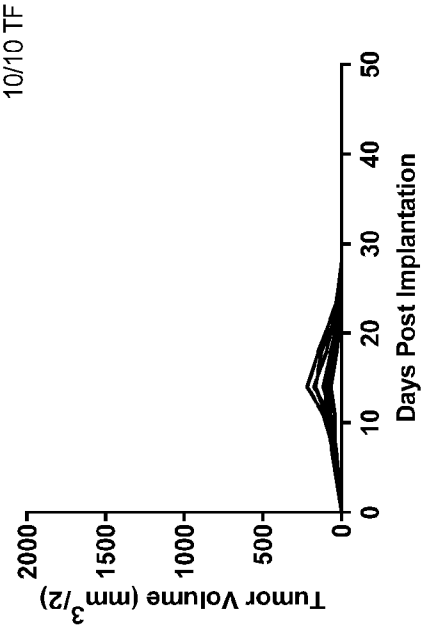
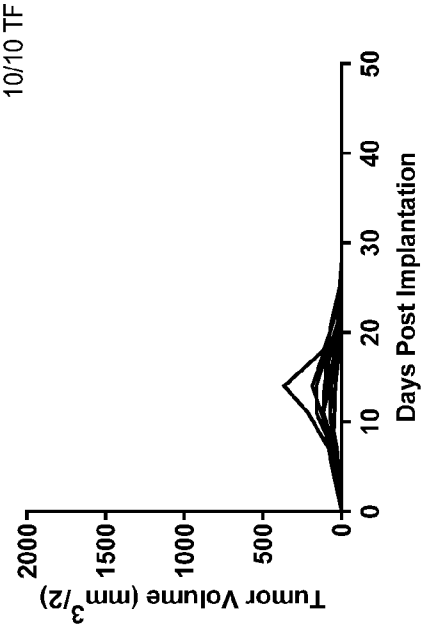
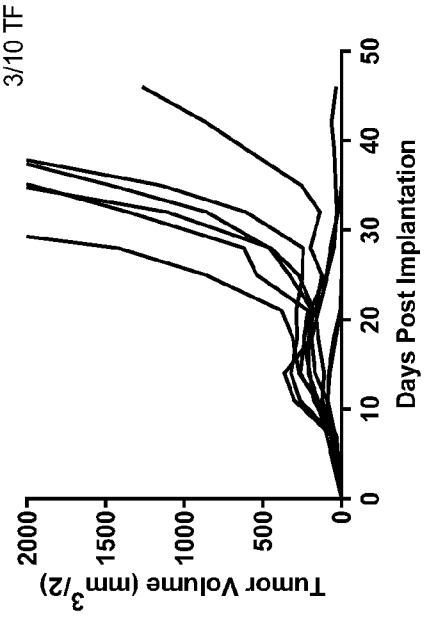


FIG. 17D







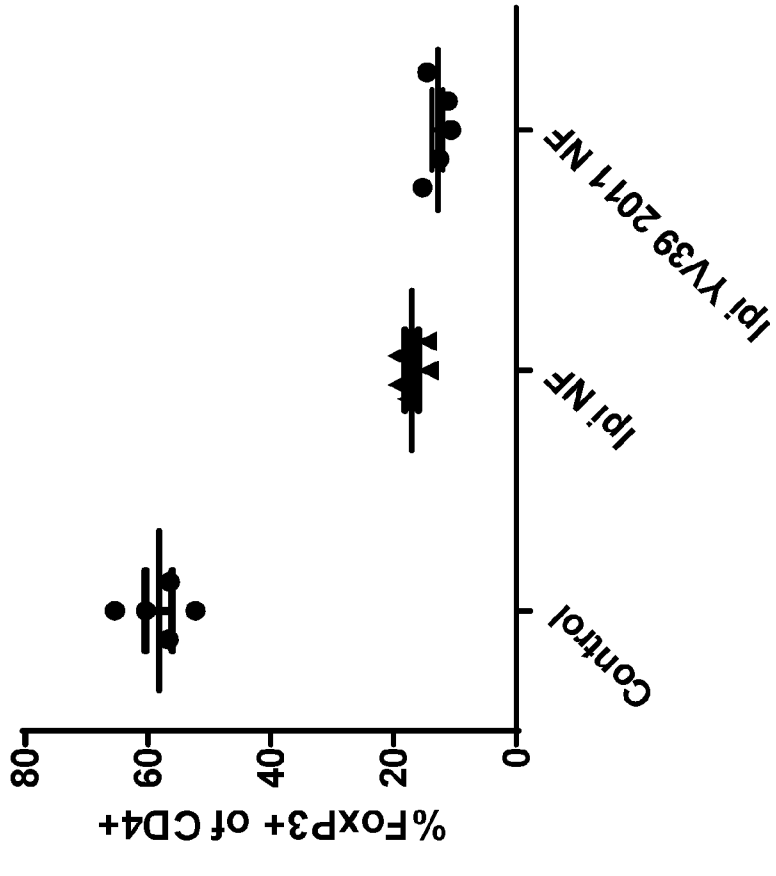
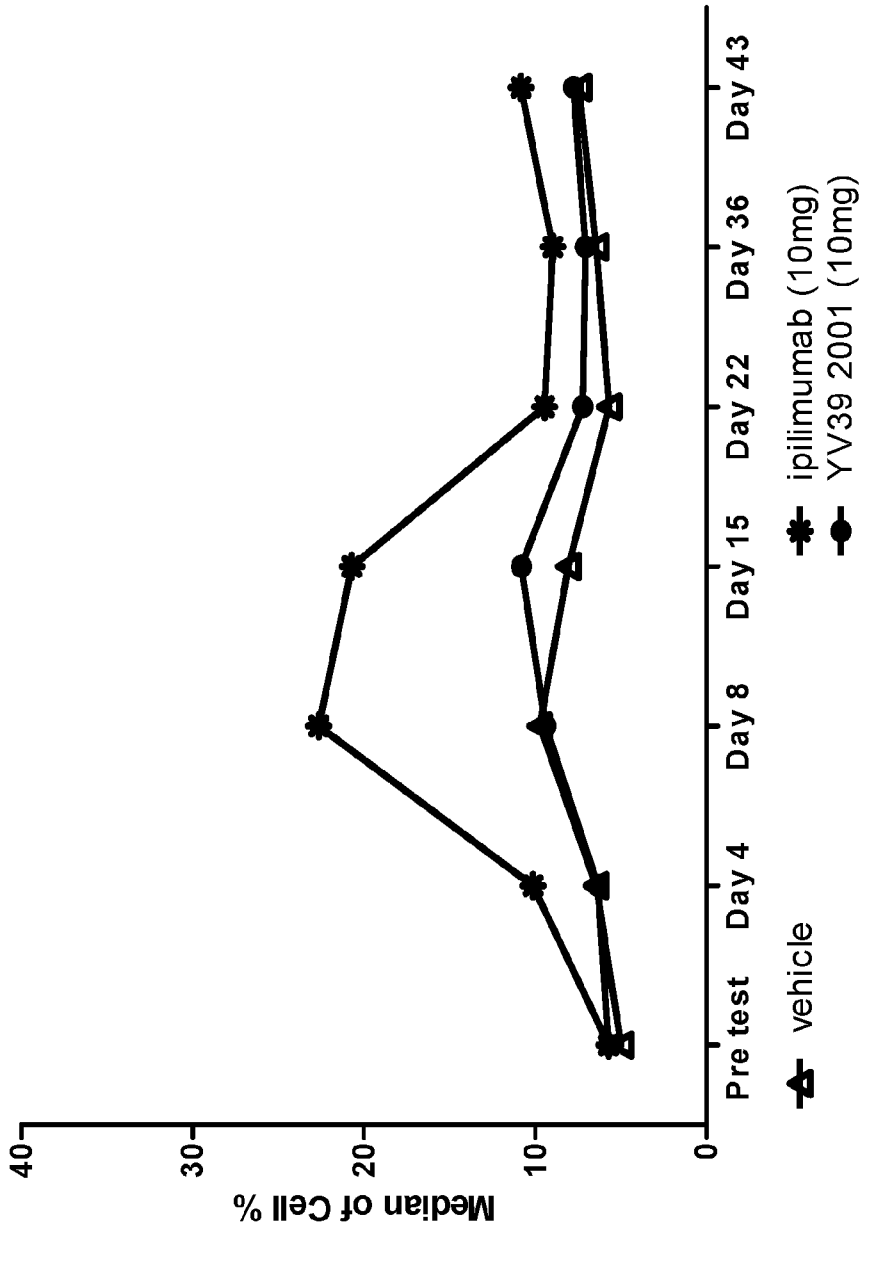


FIG. 18

FIG. 19

	FcR	IgG1 Ipi (nM)	IgG1 Ipi NF (nM)	NF
Human FcR	hCD64	0.2	0.18	
	hCD32a-H131	920	900	
	hCD32a-R131	1100	730	
	hCD32b	>5000	4200	
	hCD16a-V158	310	9.5	
	hCD16a-F158	4600	190	
	hCD16B-NA1	>5000	1800	
	hCD16B-NA2	4200	110	
Cyno FcR	cyCD64	11	5.6	
	cyCD32a	2700	2300	
	cyCD32b	1900	2000	
	cyCD16	**370**	**7.5**	
Mouse FcR	mCD64	62	69	
	mCD32	1300	1100	
	mCD16	3100	2700	
	mFcγRIV	29	6.3	

FIG. 20


Ki67 on CD4+ T cells
Median of Cell %
40
30
20
10
0
Pre test
Day 4
Day 8
Day 15
Day 22
Day 36
Day 43
vehicle
ipilimumab (10mg)
YV39 2001 (10mg)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/059740

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

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   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

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2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/059740

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 C07K19/00 A61K39/395 A61P35/00
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/081173 A2 (CYTOMX THERAPEUTICS LLC [US]; STAGLIANO NANCY E [US]; WEST JAMES W [US) 15 July 2010 (2010-07-15) cited in the application paragraphs [0131], [0196], [0197], [0210], [0242], [0257], [0258] paragraphs [0297] - [0299], [0376], [0391]	1-6,9-27
X	WO 2016/149201 A2 (CYTOMX THERAPEUTICS INC [US]) 22 September 2016 (2016-09-22) cited in the application paragraph [0233] table 23 paragraphs [0198], [0202], [0236], [0259], [0371]	1-6,9-23

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

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2018	23/03/2018

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/059740

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 3 059 319 A1 (PRESTIGE BIOPHARMA PTE LTD [SG]) 24 August 2016 (2016-08-24) sequence 36 claim 12 paragraph [0056]	21,22
A	WO 2016/115275 A1 (HOPE CITY [US]; THOMAS JEFFERSON UNIVERSITY AND HEALTH SYSTEM INNOVATI) 21 July 2016 (2016-07-21) examples 10-14 page 2, lines 1-7	1-27
A	JOSEPH M. RYAN ET AL: "Enhancing the safety of antibody-based immunomodulatory cancer therapy without compromising therapeutic benefit: Can we have our cake and eat it too?", EXPERT OPINION ON BIOLOGICAL THERAPY, vol. 16, no. 5, 25 February 2016 (2016-02-25), pages 655-674, XP055448265, ASHLEY, LONDON; GB ISSN: 1471-2598, DOI: 10.1517/14712598.2016.1152256	1-27
T	DATABASE EMBASE [Online] ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL; 1 January 2018 (2018-01-01), PRICE K D ET AL: "Nonclinical safety evaluation of two distinct second generation variants of anti-CTLA4 monoclonal antibody, ipilimumab, in monkeys", XP002779024, Database accession no. EMB-620384233	
T	DATABASE EMBASE [Online] ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL; 1 July 2017 (2017-07-01), KORMAN A J ET AL: "Next-generation anti-CTLA-4 antibodies", XP002779025, Database accession no. EMB-619155523	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/059740

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 2016115275 A1	21-07-2016