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(54) **New anti-hCTLA-4 antibodies**

(57) The invention relates to new anti-hCTLA-4 antibodies that bind to a different epitope than prior art anti-CTLA4 antibodies. These antibodies show a similar affinity for the CTLA4 antigen and they are also able to block the binding of CTLA4 to CD80 and/or CD86.

Also part of the invention are methods to produce these antibodies and therapeutic and diagnostic uses of these antibodies.

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TITLE: New anti-hCTLA-4 antibodies

FIELD OF THE INVENTION

The present invention relates to treatments of conditions ameliorated by stimulation of an immune response, in particular by the stimulation of antigen-specific T-lymphocytes. More specifically, the present invention relates to anti-human CTLA-4 antibodies, as well as use of these antibodies in the treatment of diseases such as cancer and infectious disease.

BACKGROUND OF THE INVENTION

T lymphocytes play a central role in the adaptive immune response to antigen. Naive T cells require two signals for their full activation (Bretscher 1999, Proc Natl Acad Sci USA 96:185-90). The first signal is antigen-specific and is provided by interaction of the T-cell receptor (TCR) with MHC/peptide complex on an antigen-presenting cell (APC). The second signal is a co-stimulatory signal provided by the interactions between receptors on the T cell and their ligands on the APC. Engagement of both TCR/MHC and co-stimulatory interactions leads to T-cell activation via a number of intracellular pathways, including calcium calcineurin and RAS mitogen-activated protein kinase, and subsequent activation of transcription factors for a number of effector compounds, including cytokines such as IL-2.

Although multiple positive and negative costimulatory pathways are involved in T-cell regulation, the most critical are between CD28 on T cells and B7-1 (CD80) and B7-2 (CD86) on APCs. CD28 promotes T-cell differentiation and enhances antibody production by B cells and activation of T cells. CD80 and CD86, expressed on APCs such as dendritic cells and B cells, have overlapping but distinct functions. CD86 is constitutively expressed and is rapidly upregulated on APCs coincident with TCR/MHC engagement. CD80 expression is very low on the resting cell, but is typically induced after prolonged T-cell stimulation. These differences suggest that while CD86 may be important in initialization of T-cell activation, CD80 may play a greater role in perpetuating the immune response.

Subsequent to T-cell activation, a negative regulatory receptor Cytotoxic T Lymphocyte Antigen 4 (CTLA-4 or CTLA-4, also called CD152), is upregulated on T

cells (Alegre et al., 2001, Nat Rev Immunol 1:220-8). CTLA-4 is structurally homologous to CD28 but binds more tightly to both CD80 and CD86 ligands. CTLA-4 inhibits the immune response in two principal ways - it competes with CD28 for the CD80 and CD86 ligands and thus blocks co-stimulation, and it also signals in a negative way to inhibit T cell activation (Krummel and Allison, 1995, J Exp Med 182:459-465; Walunas et al., 1994, Immunity 1:405-413). It has further been shown that CD86 engages CD28 more than CTLA-4 at the immune synapse, while CD80 ligates more CTLA-4 than CD28 (Collins et al., 2002, Immunity 17:201-210; Jansson et al., 2005, J Immunol 175:1575-1585).

It has been reported that CTLA-4 blockade augments T cell responses in vitro and in vivo, exacerbates antitumor immunity, and enhances an induced autoimmune disease. It has also been reported that CTLA-4 has an alternative or additional impact on the initial character of the T cell immune response. This is consistent with the observation that some autoimmune patients have autoantibodies to CTLA-4. It is possible that CTLA-4 blocking autoantibodies play a pathogenic role in these patients. Furthermore, human antibodies against human CTLA-4 have been described as immunostimulation modulators in a number of disease conditions, such as treating or preventing viral and bacterial infection and for treating cancer. Ipilimumab is a human anti-human CTLA-4 antibody which blocks the binding of CTLA-4 to CD80 and CD86 expressed on APCs, blocking the negative downregulation of the immune responses elicited by the interaction of these molecules. Evidence of tumor regression with prolonged time to progression has been seen in patients with melanoma who received either ipilimumab (10D1) or another anti-CTLA-4 antibody, tremelimumab (CP-675,206) and durable responses have been observed with ipilimumab in patients with melanoma, ovarian cancer, prostate cancer and renal cell cancer. Interestingly, antitumor responses may be characterized by short-term progression followed by delayed regression, and an important, possibly unique, clinical characteristic of anti–CTLA-4 antibodies is that the duration of clinical responses and even stable disease is often quite prolonged. Preclinical and early clinical studies of patients with advanced melanoma show that ipilimumab promotes antitumor activity as monotherapy and in combination with treatments such as chemotherapy, antibodies, vaccines, or cytokines (Weber, J., The Oncologist, 12(7):864-872, 2007; Scott, A.M. et al., Nature Reviews (Cancer) 12:278-287, 2012; Hodi, F.S. et al., New Eng. J. Med. 363(8):711-723, 2010;

Schadendorf, D. et al., J. Clin. Oncol. 33(17):1889-1894, 2015; Larkin, J.V. et al., New Eng. J. Med. 2015; Ribas, A. et al., J. Clin. Oncol. 31(5):616-622, 2013).

A second proposed mechanism of CTLA-4 targeting by ipilimumab is depletion of CTLA-4+ regulatory T cells (Tregs), which has been shown to be a critical driver behind the efficacy of CTLA-4 targeting in mice (Peggs et al, J Exp Med, 2009, DOI: 10.1084/jem.20082492; Simpson et al, J Exp med, 2013, DOI: 10.1084/jem.20130579; Selby et al Cancer Immunol, 2013 DOI:10.1158/2326-6066.CIR-13-0013).

Although ipilimumab is already on the market for some cancer therapies and is being tested for other anti-cancer indications, and although tremelimumab is also advanced in the clinical test phase, there still is a need for alternative anti-CTLA-4 antibodies, especially where they have an activity that can be differentiated from the activities of the known anti-CTLA-4 antibodies.

SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to antibodies or antigen binding fragments thereof that binds to human CTLA-4, wherein the antibody or antigen binding fragment comprises one or more, and optionally each, of:

a heavy chain variable region CDR1 comprising the amino acid sequence of SEQ ID NO:1 or an amino acid sequence differing from SEQ ID NO: 1 by 1, 2, 3, or more conservative substitutions,

a heavy chain variable region CDR2 comprising the amino acid sequence of SEQ ID NO:2 or an amino acid sequence differing from SEQ ID NO: 2 by 1, 2, 3, or more conservative substitutions,

a heavy chain variable region CDR3 comprising the amino acid sequence of SEQ ID NO:3 or an amino acid sequence differing from SEQ ID NO: 3 by 1, 2, 3, or more conservative substitutions,

a light chain variable region CDR1 comprising the amino acid sequence of SEQ ID NO:4 or an amino acid sequence differing from SEQ ID NO: 4 by 1, 2, 3, or more conservative substitutions,

a light chain variable region CDR2 comprising the amino acid sequence of SEQ ID NO:5 or an amino acid sequence differing from SEQ ID NO: 5 by 1, 2, 3, or more conservative substitutions, and

a light chain variable region CDR3 comprising the amino acid sequence of SEQ ID NO:6 or an amino acid sequence differing from SEQ ID NO: 6 by 1, 2, 3, or more conservative substitutions.

Preferably, said antibody or antigen binding fragment comprises one or more and preferably each of:

a. a heavy chain variable region CDR1 comprising the amino acid sequence of SEQ ID NO: 1;
b. a heavy chain variable region CDR2 comprising the amino acid sequence of SEQ ID NO: 2;
c. a heavy chain variable region CDR3 comprising the amino acid sequence of SEQ ID NO: 3;
d. a light chain variable region CDR1 comprising the amino acid sequence of SEQ ID NO: 4;
e. a light chain variable region CDR2 comprising the amino acid sequence of SEQ ID NO: 5;
f. a light chain variable region CDR3 comprising the amino acid sequence of SEQ ID NO: 6

Preferably said antibody has a heavy chain according to SEQ ID NO: 7. Further preferably said antibody has a light chain according to SEQ ID NO: 8. More preferably, the heavy chain is chosen from any of SEQ ID NO: 10, 12, 14, 16, 18 or 20. More preferably, the light chain is chosen from any of SEQ ID NO: 22, 24, 26, 30.

These sequences are as follows:

Description	SEQ ID NO:	SEQUENCE
27A heavy chain CDR1 (amino acid sequence)	1	TYWMN
27A heavy chain CDR2 (amino acid sequence)	2	MIHPSDSETSLNQAFKD
27A heavy chain CDR3 (amino acid	3	MGRRNPYYFDY

sequence)		
27A light chain CDR1 (amino acid sequence)	4	RPSENLYTNLA
27A light chain CDR2 (amino acid sequence)	5	GATNLAD
27A light chain CDR3 (amino acid sequence)	6	QHLWGTPFT
Humanized 27 heavy chain variable region (consensus sequence)	7	EVQLX$_1$X$_2$X$_3$GX$_4$X$_5$X$_6$X$_7$X$_8$PGX$_9$SVKX$_{10}$SCKASGYSFTTYWM NWVX$_{11}$QX$_{12}$PGX$_{13}$GLEWX$_{14}$GMIHPSDSETSLNQAFKDX$_{15}$ X$_{16}$X$_{17}$X$_{18}$TX$_{19}$X$_{20}$X$_{21}$SX$_{22}$SX$_{23}$X$_{24}$YX$_{25}$X$_{26}$X$_{27}$SSLX$_{28}$X$_{29}$ED X$_{30}$AVYYCARX$_{31}$GRRNPYYFDYWGQGTX$_{32}$VTVSS wherein: X$_1$ = V, L X$_2$ = Q, E X$_3$ = S, A X$_4$ = A, P X$_5$ = V, E X$_6$ = L, V X$_7$ = A, V, K X$_8$ = R, K X$_9$ = A, T, S X$_{10}$ = I, V X$_{11}$ = K, R X$_{12}$ = R, A X$_{13}$ = K, Q X$_{14}$ = I, M X$_{15}$ = K, R X$_{16}$ = V, A X$_{17}$ = K, T X$_{18}$ = L, I, M X$_{19}$ = A, R X$_{20}$ = A, D X$_{21}$ = T, E, K X$_{22}$ = T, A X$_{23}$ = I, T X$_{24}$ = A, V X$_{25}$ = L, M X$_{26}$ = E, Q X$_{27}$ = F, L X$_{28}$ = R, T X$_{29}$ = S, N X$_{30}$ = S, T X$_{31}$ = M, I X$_{32}$ = L, T

Humanized 27 light chain variable region (consensus sequence)	8	DIQMTQX$_1$PSSLSASVGDX$_2$VTITCRPSENLYTNLAWYQQKP GKAPKLLX$_3$YGATNLADGVPSRFSGSGSGTX$_4$X$_5$X$_6$LX$_7$ISSL QX$_8$EDFATYYCQHLWGTPFTFGX$_9$GTKX$_{10}$EIK wherein: X$_1$ = S, A X$_2$ = R, T X$_3$ = L, I X$_4$ = D, E X$_5$ = Y, F X$_6$ = T, S X$_7$ = T, S X$_8$ = P, S, A X$_9$ = G, Q X$_{10}$ = L, V
hCTLA4.27VH1 (nucleotide sequence)	9	GAGGTGCAGCTGCTGCAGTCTGGCGCTGTGCTGGCCAGACCTGGC ACCAGCGTGAAGATCAGCTGCAAGGCCAGCGGCTACAGCTTCACC ACCTACTGGATGAACTGGGTCAAGCAGCGGCCAGGCCAGGGCCTG GAATGGATCGGAATGATCCACCCCAGCGACAGCGAGACAAGCCTG AACCAGGCCTTCAAGGACAAGGCCAAGCTGACCGCCGCCACCTCT GCCTCTATCGCCTACCTGGAATTTTCCAGCCTGACCAACGAGGAC AGCGCCGTGTACTACTGCGCCCGGATGGGCAGACGGAACCCCTAC TACTTCGACTACTGGGGCCAGGGCACCCTCGTGACAGTGTCTAGC
hCTLA4.27VH1 (amino acid sequence)	10	EVQLLQSGAVLARPGTSVKISCKASGYSFTTYWMNWVKQRPGQGL EWIGMIHPSDSETSLNQAFKDKAKLTAATSASIAYLEFSSLTNED SAVYYCARMGRRNPYYFDYWGQGTLVTVSS
hCTLA4.27VH2 (nucleotide sequence)	11	GAGGTGCAGCTGGTGCAGTCTGGCGCTGTGCTCGTGAAACCTGGC GCCTCCGTGAAGGTGTCCTGCAAGGCCAGCGGCTACAGCTTCACC ACCTACTGGATGAACTGGGTGCGCCAGAGGCCTGGCAAGGGCCTG GAATGGATCGGCATGATCCACCCCAGCGACAGCGAGACAAGCCTG AACCAGGCCTTCAAGGACAAAGTGACCATCACCGCCGACGAGAGC ACCAGCACCGCCTACATGCAGCTGAGCAGCCTGAGAAGCGAGGAC ACCGCCGTGTACTACTGCGCCCGGATGGGCAGACGGAACCCCTAC TACTTCGACTACTGGGGCCAGGGCACCACCGTGACAGTGTCTAGC
hCTLA4.27VH2 (amino acid sequence)	12	EVQLVQSGAVLVKPGASVKVSCKASGYSFTTYWMNWVRQRPGKGL EWIGMIHPSDSETSLNQAFKDKVTIITADESTSTAYMQLSSLRSED TAVYYCARMGRRNPYYFDYWGQGTTVTVSS
hCTLA4.27VH3 (nucleotide sequence)	13	GAGGTGCAGCTGGTGCAGTCTGGCGCCGTGGTGGCCAAGCCTGGC AGCAGCGTGAAGGTGTCCTGTAAAGCCAGCGGCTACAGCTTCACC ACCTACTGGATGAACTGGGTGCGCCAGGCCCCTGGACAGGGCCTG GAATGGATGGGCATGATCCACCCCAGCGACAGCGAGACAAGCCTG AACCAGGCCTTCAAGGACAGAGTGACCATCACCGCCGACAAGAGC ACCAGCACCGCCTACATGGAACTGAGCAGCCTGACCAGCGAGGAC ACCGCCGTGTACTACTGCGCCCGGATGGGCAGACGGAACCCCTAC TACTTCGACTACTGGGGCCAGGGCACCACCGTGACAGTGTCTAGC
hCTLA4.27VH3 (amino acid	14	EVQLVQSGAVVAKPGSSVKVSCKASGYSFTTYWMNWVRQAPGQGL EWMGMIHPSDSETSLNQAFKDRVTIITADKSTSTAYMELSSLTSED TAVYYCARMGRRNPYYFDYWGQGTTVTVSS

sequence)		
hCTLA4.27VH4 (nucleotide sequence)	15	GAGGTGCAGCTGGTGCAGTCTGGCGCCGAAGTGAAGAAACCAGGC GCCAGCGTGAAGGTGTCCTGCAAGGCCAGCGGCTACAGCTTCACC ACCTACTGGATGAACTGGGTGCGCCAGGCCCCTGGACAGGGCCTG GAATGGATGGGCATGATCCACCCCAGCGACAGCGAGACAAGCCTG AACCAGGCCTTCAAGGACAGAGTGACCATGACCCGGGACACCAGC ACCTCCACCGTGTACATGGAACTGAGCAGCCTGCGGAGCGAGGAC ACCGCCGTGTACTACTGCGCCCGGATGGGCAGACGGAACCCCTAC TACTTCGACTACTGGGGCCAGGGCACCCTCGTGACAGTGTCTAGC
hCTLA4.27VH4 (amino acid sequence)	16	EVQLVQSGAEVKKPGASVKVSCKASGYSFTTYWMNWVRQAPGQGL EWMGMIHPSDSETSLNQAFKDRVTMTRDTSTSTVYMELSSLRSED TAVYYCARMGRRNPYYFDYWGQGTLVTVSS
hCTLA4.27VH5 (nucleotide sequence)	17	GAGGTGCAGCTGCTGCAGGCTGGCGCTGTGCTGGCTAGACCTGGC ACCAGCGTGAAGATCAGCTGCAAGGCCAGCGGCTACAGCTTCACC ACCTACTGGATGAACTGGGTCAAGCAGAGGCCCGGCAAGGGCCTG GAATGGATCGGCATGATCCACCCCAGCGACAGCGAGACAAGCCTG AACCAGGCCTTCAAGGACAAGGCCAAGCTGACCGCCGCCACCTCT GCCTCTATCGCCTACCTGGAATTTTCCAGCCTGACCAACGAGGAC AGCGCCGTGTACTACTGCGCCCGGATCGGCAGACGGAACCCCTAC TACTTCGACTACTGGGGCCAGGGCACCCTCGTGACAGTGTCTAGC
hCTLA4.27VH5 (amino acid sequence)	18	EVQLLQAGAVLARPGTSVKISCKASGYSFTTYWMNWVKQRPGKGL EWIGMIHPSDSETSLNQAFKDKAKLTAATSASIAYLEFSSLTNED SAVYYCARIGRRNPYYFDYWGQGTLVTVSS
hCTLA4.27VH6 (nucleotide sequence)	19	GAGGTGCAGCTGCTGGAATCTGGCCCTGAACTCGTGCGGCCTGGC AGCAGCGTGAAGATCAGCTGTAAAGCCAGCGGCTACAGCTTCACC ACCTACTGGATGAACTGGGTCAAGCAGAGGCCCGGCAAGGGCCTG GAATGGATCGGCATGATCCACCCCAGCGACAGCGAGACAAGCCTG AACCAGGCCTTCAAGGACAAAGTGAAGCTGACCGCCGCCACCAGC GCCTCTATCGCCTACCTGGAATTTTCCAGCCTGCGGAACGAGGAC AGCGCCGTGTACTACTGCGCCCGGATGGGCAGACGGAACCCCTAC TACTTCGACTACTGGGGCCAGGGCACCCTCGTGACAGTGTCTAGC
hCTLA4.27VH6 (amino acid sequence)	20	EVQLLESGPELVRPGSSVKISCKASGYSFTTYWMNWVKQRPGKGL EWIGMIHPSDSETSLNQAFKDKVKLTAATSASIAYLEFSSLRNED SAVYYCARMGRRNPYYFDYWGQGTLVTVSS
hCTLA4.27VL1 (nucleotide sequence)	21	GACATCCAGATGACCCAGAGCCCCAGCAGCCTGTCTGCCAGCGTG GGCGACAGAGTGACCATCACCTGTCGGCCCAGCGAGAACCTGTAC ACCAACCTGGCCTGGTATCAGCAGAAGCCCGGCAAGGCCCCCAAA CTGCTGCTGTACGGCGCCACCAATCTGGCCGATGGCGTGCCCAGC AGATTTTCCGGCTCTGGCAGCGGCACCGACTACACCCTGACCATC TCTAGCCTGCAGCCCGAGGACTTCGCCACCTACTACTGTCAGCAC CTGTGGGGCACCCCCTTCACCTTTGGCCAGGGCACCAAGCTGGAA ATCAAG
hCTLA4.27VL1 (amino acid sequence)	22	DIQMTQSPSSLSASVGDRVTITCRPSENLYTNLAWYQQKPGKAPK LLLYGATNLADGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQH LWGTPFTFGQGTKLEIK
hCTLA4.27VL2 (nucleotide sequence)	23	GACATCCAGATGACCCAGAGCCCCAGCAGCCTGTCTGCCAGCGTG GGCGACAGAGTGACCATCACCTGTCGGCCCAGCGAGAACCTGTAC ACCAACCTGGCCTGGTATCAGCAGAAGCCCGGCAAGGCCCCCAAG CTGCTGATCTACGGCGCCACCAATCTGGCCGATGGCGTGCCCAGC

		AGATTTTCCGGCTCTGGCAGCGGCACCGAGTTCAGCCTGAGCATC TCTAGCCTGCAGCCCGAGGACTTCGCCACCTACTACTGTCAGCAC CTGTGGGGCACCCCCTTCACCTTTGGCGGCGGAACAAAGGTGGAA ATCAAG
hCTLA4.27VL2 (amino acid sequence)	24	DIQMTQSPSSLSASVGDRVTITCRPSENLYTNLAWYQQKPGKAPK LLIYGATNLADGVPSRFSGSGSGTEFSLSISSLQPEDFATYYCQH LWGTPFTFGGGTKVEIK
hCTLA4.27VL3 (nucleotide sequence)	25	GACATCCAGATGACCCAGAGCCCCAGCAGCCTGTCTGCCAGCGTG GGCGATACCGTGACCATCACCTGTCGGCCCAGCGAGAACCTGTAC ACCAACCTGGCCTGGTATCAGCAGAAGCCCGGCAAGGCCCCCAAA CTGCTGCTGTACGGCGCCACCAATCTGGCCGATGGCGTGCCCAGC AGATTTTCCGGCTCTGGCAGCGGCACCGACTACACCCTGACCATC TCTAGCCTGCAGAGCGAGGACTTCGCCACCTACTACTGTCAGCAC CTGTGGGGCACCCCCTTCACCTTTGGCCAGGGCACCAAGCTGGAA ATCAAG
hCTLA4.27VL3 (amino acid sequence)	26	DIQMTQSPSSLSASVGDTVTITCRPSENLYTNLAWYQQKPGKAPK LLLYGATNLADGVPSRFSGSGSGTDYTLTISSLQSEDFATYYCQH LWGTPFTFGQGTKLEIK
hCTLA4.27VL4 (nucleotide sequence)	27	GACATCCAGATGACCCAGAGCCCCAGCAGCCTGTCTGCCAGCGTG GGCGACAGAGTGACCATCACCTGTCGGCCCAGCGAGAACCTGTAC ACCAACCTGGCCTGGTATCAGCAGAAGCCCGGCAAGGCCCCTAAG CTGCTGCTGTACGGCGCCACCAATCTGGCCGATGGCGTGCCCAGC AGATTTTCCGGCTCTGGCAGCGGCACCGACTACACCCTGACCATC TCTAGCCTGCAGCCCGAGGACTTCGCCACCTACTACTGCCAGCAC CTGTGGGGCACCCCCTTCACATTTGGCGGAGGCACCAAGCTGGAA ATCAAG
hCTLA4.27VL4 (amino acid sequence)	28	DIQMTQSPSSLSASVGDRVTITCRPSENLYTNLAWYQQKPGKAPK LLLYGATNLADGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQH LWGTPFTFGGGTKLEIK
hCTLA4.27VL5 (nucleotide sequence)	29	GACATCCAGATGACCCAGGCCCCTAGCAGCCTGTCTGCCAGCGTG GGCGACAGAGTGACCATCACCTGTCGGCCCAGCGAGAACCTGTAC ACCAACCTGGCCTGGTATCAGCAGAAGCCCGGCAAGGCCCCCAAA CTGCTGCTGTACGGCGCCACCAATCTGGCCGATGGCGTGCCCAGC AGATTTTCCGGCTCTGGCAGCGGCACCGACTACACCCTGACAATC AGCTCCCTGCAGGCCGAGGACTTCGCCACCTACTACTGTCAGCAC CTGTGGGGCACCCCCTTCACCTTTGGCGGCGGAACAAAGCTGGAA ATCAAG
hCTLA4.27VL5 (amino acid sequence)	30	DIQMTQAPSSLSASVGDRVTITCRPSENLYTNLAWYQQKPGKAPK LLLYGATNLADGVPSRFSGSGSGTDYTLTISSLQAEDFATYYCQH LWGTPFTFGGGTKLEIK

The invention further relates to an antibody or antigen binding fragment thereof that binds to human CTLA-4 comprising a light chain immunoglobulin, a heavy

chain immunoglobulin or both a light chain and a heavy chain immunoglobulin selected from the group consisting of:

a. an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising the amino acid sequence of SEQ ID NO:7 and/or a variable light chain comprising the amino acid sequence of SEQ ID NO:8;

b. an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising the amino acid sequence of SEQ ID NO:10, 12, 14, 16, 18 or 20 and/or a variable light chain comprising the amino acid sequence of SEQ ID NO: 22, 24, 26 or 30;

c. an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising at least 90%, 95%, 96%, 97%, 98% or 99% identity any one of SEQ ID NO:10, 12, 14, 16, 18 or 20 and/or a variable light chain comprising at least 90%, 95%, 96%, 97%, 98% or 99% identity to any one of SEQ ID NO: 22, 24, 26 or 30; and

d. an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising at least 90%, 95%, 96%, 97%, 98% or 99% identity any one of SEQ ID NO: 10, 12, 14, 16, 18 or 20 and/or a variable light chain comprising at least 90%, 95%, 95%, 96%, 97%, 98% or 99% identity to any one of SEQ ID NO: 22, 24, 26 or 30, wherein any sequence variations occur in the framework regions of the antibody or antigen binding fragment;

e. an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising 1, 2, 3, 4, 5,6, 7, 8, 9 or 10 amino acid substitution with respect to any one of SEQ ID NO: 10, 12, 14, 16, 18 or 20 and/or a variable light chain 1, 2, 3, 4, 5,6, 7, 8, 9 or 10 amino acid substitution with respect to any one of SEQ ID NO: 22, 24, 26 or 30; and

f. an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising 1, 2, 3, 4, 5,6, 7, 8, 9 or 10 amino acid substitution with respect to any one of SEQ ID NO: 10, 12, 14, 16, 18 or 20 and/or a variable light chain 1, 2, 3, 4, 5,6, 7, 8, 9 or 10 amino acid substitution with respect to any one of SEQ ID NO: 22, 24, 26 or 30, wherein any said substitutions occur in the framework regions of the antibody or antigen binding fragment.

Preferably, said antibody or antigen binding fragment has at least one of the following characteristics:

a) binds to human CTLA-4 with a KD value of at least about 1 x 10^{-9} M as determined by surface plasmon resonance (e.g., BIACORE) or a similar technique (e.g. KinExa or OCTET);

b) blocks the binding of hCTLA-4 to hCD80 with an IC_{50} of about 100 nM or lower;

c) blocks the binding of hCTLA-4 to hCD86 with an IC_{50} of about 100 nM or lower;

d) binds to a different CTLA-4 epitope than ipilimumab or tremelimumab.

The invention further comprises an antibody or antigen binding fragment thereof that binds to an epitope of human CTLA-4 wherein said antibody or antigen binding fragment fragment does not bind to the mouse-human chimeric CTLA-4 molecule of SEQ ID NO: 44.

A further aspect of the invention is an antibody or antigen binding fragment thereof that binds to the same epitope of human CTLA-4 as an antibody comprising the variable heavy chain of SEQ ID NO:7 and the variable light chain of SEQ ID NO:8, wherein the antibody or fragment thereof does not bind to the mouse-human chimeric CTLA-4 molecule of SEQ ID NO: 44 and has one, two, three, or all four of the following characteristics:

a. binds to human CTLA-4 with a KD value of at least about 1 x 10^{-9} M as determined by surface plasmon resonance (e.g., BIACORE) or a similar technique (e.g. KinExa or OCTET);

b. blocks the binding of hCTLA-4 to hCD80 with an IC_{50} of about 100 nM or lower;

c. blocks the binding of hCTLA-4 to hCD86 with an IC_{50} of about 100 nM or lower;

d. binds to a different CTLA-4 epitope than ipilimumab or tremelimumab.

Preferably. the antibody or antigen binding fragment of the present invention is a humanized antibody comprising two heavy chains and two light chains.

In another aspect, the present invention relates to an isolated polypeptide comprising the amino acid sequence of any one of SEQ ID NOs: 1-8, 10, 12, 14, 16, 18, 20, 22, 24, 26 or 30.

In a further aspect, the invention is directed to an isolated nucleic acid encoding: any one of the antibodies or antigen binding fragments of th einvention, or

any one of the polypeptides as defined above. The invention also encompasses an expression vector comprising such an isolated nucleic acid.

The invention also comprises a host cell comprising the antibody, binding fragment, polypeptide, polynucleotide or expression vector of the invention. Said host cell preferably is a *Pichia* cell or a Chinese hamster ovary cell.

Another embodiment of the invention is formed by a composition comprising the antibody or antigen binding fragment of th einvention and a pharmaceutically acceptable carrier, diluent, excipient or stabilizer. Preferably, said composition further comprises an agent selected from the group consisting of:

a. an anti-PD1 antibody or an antigen binding fragment thereof;
b. an anti-LAG3 antibody or an antigen binding fragment thereof;
c. an anti-TIGIT antibody or an antigen binding fragment thereof;
d. an anti-VISTA antibody or an antigen binding fragment thereof;
e. an anti-BTLA antibody or an antigen binding fragment thereof;
f. an anti-TIM3 antibody or an antigen binding fragment thereof;
g. an anti-CD27 antibody or an antigen binding fragment thereof;
h. an anti-HVEM antibody or an antigen binding fragment thereof;
i. an anti-CD70 antibody or an antigen binding fragment thereof;
j. an anti-CD137 antibody or an antigen binding fragment thereof;
k. an anti-OX40 antibody or an antigen binding fragment thereof;
l. an anti-CD28 antibody or an antigen binding fragment thereof;
m. an anti-PDL1 antibody or an antigen binding fragment thereof;
n. an anti-PDL2 antibody or an antigen binding fragment thereof;
o. an anti-GITR antibody or an antigen binding fragment thereof;
p. an anti-ICOS antibody or an antigen binding fragment thereof ;
q. an anti-SIRPα antibody or an antigen binding fragment thereof;
r. an anti-ILT2 antibody or an antigen binding fragment thereof;
s. an anti-ILT3 antibody or an antigen binding fragment thereof;
t. an anti-ILT4 antibody or an antigen binding fragment thereof;
u. an anti-ILT5 antibody or an antigen binding fragment thereof;
v. an anti-4-1BB antibody or an antigen binding fragment thereof;
w. an anti-NK2GA antibody or an antigen binding fragment thereof;
x. an anti-NK2GC antibody or an antigen binding fragment thereof;

y. an anti-NK2GE antibody or an antigen binding fragment thereof;

z. an anti-TSLP antibody or an antigen binding fragment thereof, and;

aa. an anti-IL10 antibody or an antigen binding fragment thereof.

Also preferably, in said composition the anti-PD1 antibody or an antigen binding fragment thereof is selected from the group consisting of: pembrolizumab or an antigen binding fragment thereof and nivolumab or an antigen binding fragment thereof.

In another embodiment the composition of the invention further comprises a compound selected from the group of melphalan, vincristine, fludarabine, chlorambucil, bendamustine, etoposide, doxorubicin, cyclophosphamide, cisplatin, immune modulating agents such as corticosteroids, for example dexamethasone or prednisolone, thalidomide analogs, for example thalidomide, lenalidomide or pomalidomide, kinase inhibitors, for example ibrutinib, idealisib, antibody targeting CD20, for example rituximab, ofatumab or obinotuzumab, antibody targeting CD52, for example alemtuzumab, antibody targeting CD38, for example daratumumab, antibody targeting IL-6 or IL-6 receptor, for example sarilumab or tocilizumab, antibody targeting CS-1, for example elotuzumab, antibody targeting BCMA, for example GSK2857916, antibody targeting BAFF or BLyss, for example tabalumab, bisphosphonates, for example pamidronate or zolendronic acid, bortezomid, or combinations thereof.

The present invention also relates to a method of producing an antibody or antigen binding fragment comprising:

a. culturing a host cell comprising a polynucleotide encoding the heavy chain and/or the light chain of any one of the antibodies or antigen binding fragments of the invention under conditions favorable to expression of the polynucleotide; and

b. optionally, recovering the antibody or antigen binding fragment from the host cell and/or culture medium.

In certain embodiments the host cell comprises an expression vector comprising such a polynucleotide, wherein the expression vector comprises control sequences operably linked to the polynucleotide which drive expression of the antibody or antigen binding fragment. In preferred embodiments, the polynucleotide comprises a secretion signal sequence which mediates secretion of the antibody or antigen binding fragment by the host cell.

A further aspect of the present invention is a method of treating cancer in a subject, preferably a human subject, comprising administering to the subject an effective amount of the antibody or antigen binding fragment of the invention, or of an expression vector which mediates expression of the antibody or antigen binding fragment within the subject, optionally in association with a further therapeutic agent or therapeutic procedure.

Also part of the invention is a method of treating an infection or infectious disease in a human subject, comprising administering to the subject an effective amount of the antibody or antigen binding fragment according to th einvention, optionally in association with a further therapeutic agent or therapeutic procedure.

The invention further comprises a vaccine comprising the antibody or antigen binding fragment according to the invention and an antigen.

In another aspect, the invention comprises a method for detecting the presence of a CTLA-4 peptide or a fragment thereof in a sample comprising contacting the sample with an antibody or fragment of the invention and detecting the presence of a complex between the antibody or fragment and the peptide; wherein detection of the complex indicates the presence of the CTLA-4 peptide.

The invention also relates to a method of increasing the activity of an immune cell, comprising administering to a subject in need thereof an effective amount of an antibody or antigen binding fragment according to the invention, or of an expression vector which mediates expression of the antibody or antigen binding fragment within the subject. Preferably said method is used for:

a. the treatment of cancer;

b. the treatment of an infection or infectious disease; or

c. as a vaccine adjuvant.

In another aspect, the invention is directed to an antibody or antigen binding fragment according the invention, or an expression vector which mediates expression of the antibody or antigen binding fragment within the subject for use in the preparation of a medicament to:

a. increase immune cell activation;

b. treat cancer; or

c. treat an infection or infectious disease.

The invention comprises, in a following aspect, the use of the antibody or antigen binding fragment of the present invention for the manufacture of a medicament for the treatment of cancer for: increasing immune cell activation; treating cancer; or treating an infection or infectious disease.

The invention also comprises an antibody or antigen binding fragment thereof of the invention, wherein the fragment is a Fab, F(ab')2, Fv or a single chain Fv fragment (scFV).

In a following aspect, the antibody or antigen binding fragment thereof of th einvention comprises a heavy chain constant region selected from IgG1, IgG2, IgG3 and IgG4, preferably IgG1 or IgG4, and a light chain constant region chosen from the light chain constant regions kappa or lambda. In the embodiment wherein the antibody or antigen binding fragment thereof comprises a human IgG4 heavy chain constant region, said IgG4 sequence preferably has a Ser→Pro mutation at position 228, as depicted in SEQ ID NO: 50.

The invention is also directed to a method of stimulating an immune response in a subject, comprising administering to a subject in need thereof the antibody or antigen binding fragment thereof of the invention in an amount effective to stimulate the immune response. Preferably, in such a method the antibody molecule is administered in combination with an agonist of one or more costimulatory molecules for example one or more molecules selected from the group consisting of OX40, CD2, CD27, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD30, CD40, BAFFR, HVEM, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3 or CD83 ligand. Alternatively, the antibody molecule is administered in combination with one or more inhibitors of an immune checkpoint molecule, for example one or more inhibitors selected from the group consisting of PD-1, PD-L1, PD-L2, TIM-3, LAG-3, CEACAM-1, CEACAM-5, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 or TGFR

In a further embodiment, the invention comprise a method of treating cancer wherein the cancer is selected from the group consisting of a lung cancer, a melanoma, a renal cancer, a liver cancer, a myeloma, a prostate cancer, a breast cancer, a colorectal cancer, a gastric cancer, a pancreatic cancer, a thyroid cancer, a hematological cancer, a lymphoma, a myeloma, or a leukemia, or a metastatic lesion of the cancer.

Also in relation to a method of treating cancer the invention also is directed to a method wherein the antibody molecule is administered in combination with one or more second therapeutic agents or procedures, for example wherein the second therapeutic agent or procedure is selected from the group consisiting of chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cytotoxic agent, an immune-based therapy, a cytokine, surgical procedure, a radiation procedure, an activator of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, or a cellular immunotherapy.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Functionality of hCTLA4.27A antibody in the Jurkat-based reporter assay.

Figure 2: Differential hCD80 blocking profile of hCTLA4.27 antibodies.

Figure 3: Induction of IL2 production by hCTLA4.27 antibodies in the PBMC SEB assay.

Figure 4A: hCTLA4.27 and control antibodies: effector function in the ADCC assay.

Figure 4B: hCTLA4.27 and control antibodies: effector function in the CDC assay.

Figure 5: Unique binding profile of hCTLA4.27A chimeric hIgG4 to human/mouse CTLA-4 exhange mutants.

DETAILED DESCRIPTION

Throughout the detailed description and examples of the invention the following abbreviations will be used:

ADCC	Antibody-dependent cellular cytotoxicity
CDC	Complement-dependent cytotoxicity
CDR	Complementarity determining region in the immunoglobulin variable regions, defined using the Kabat numbering system
CHO	Chinese hamster ovary
EC_{50}	Concentration resulting in 50% of total binding
ELISA	Enzyme-linked immunosorbent assay
FR	Antibody framework region: the immunoglobulin variable regions excluding the CDR regions.
HRP	Horseradish peroxidase
IFN	interferon

IC_{50}	concentration resulting in 50% inhibition total signal
IgG	Immunoglobulin G
Kabat	An immunoglobulin alignment and numbering system pioneered by Elvin A. Kabat ((1991) Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md.)
mAb or Mab or MAb	Monoclonal antibody
SEB	Staphylococcus Enterotoxin B
TT	Tetanus toxoid
V region	The segment of IgG chains which is variable in sequence between different antibodies. It extends to Kabat residue 109 in the light chain and 113 in the heavy chain.
VH	Immunoglobulin heavy chain variable region
VL	Immunoglobulin light chain variable region
VK	Immunoglobulin kappa light chain variable region

"Treat" or "treating" means to administer a therapeutic agent, such as a composition containing any of the antibodies or antigen-binding fragments of the present invention, internally or externally to a subject or patient having one or more disease symptoms, or being suspected of having a disease, for which the agent has therapeutic activity. Typically, the agent is administered in an amount effective to alleviate one or more disease symptoms in the treated subject or population, whether by inducing the regression of or inhibiting the progression of such symptom(s) by any clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom may vary according to factors such as the disease state, age, and weight of the patient, and the ability of the drug to elicit a desired response in the subject. Whether a disease symptom has been alleviated can be assessed by any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of that symptom.

The present invention includes anti-CTLA-4 antibodies and methods of use thereof. As used herein, the term "antibody" refers to any form of antibody that exhibits the desired biological activity. Thus, it is used in the broadest sense and specifically covers, but is not limited to, monoclonal antibodies (including full length monoclonal

antibodies comprising two light chains and two heavy chains), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), humanized antibodies, fully human antibodies, chimeric antibodies and camelized single domain antibodies.

The present invention includes anti- CTLA-4 antigen-binding fragments and methods of use thereof. As used herein, unless otherwise indicated, "antibody fragment" or "antigen-binding fragment" refers to antigen-binding fragments of antibodies, *i.e.* antibody fragments that retain the ability to bind specifically to the antigen bound by the full-length antibody, *e.g.* fragments that retain one or more CDR regions. Examples of antigen-binding fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules, *e.g.*, sc-Fv; nanobodies and multispecific antibodies formed from antibody fragments.

The present invention includes anti- CTLA-4 Fab fragments and methods of use thereof. A "Fab fragment" is comprised of one light chain and the C_H1 and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. An "Fab fragment" can be the product of papain cleavage of an antibody.

The present invention includes anti- CTLA-4 antibodies and antigen-binding fragments thereof which comprise an Fc region and methods of use thereof. An "Fc" region contains two heavy chain fragments comprising the C_H2 and C_H3 domains of an antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the C_H3 domains.

The present invention includes anti- CTLA-4 Fab' fragments and methods of use thereof. A "Fab' fragment" contains one light chain and a portion or fragment of one heavy chain that contains the V_H domain and the C_H1 domain and also the region between the C_H1 and C_H2 domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form a $F(ab')_2$ molecule.

The present invention includes anti- CTLA-4 $F(ab')_2$ fragments and methods of use thereof. A "$F(ab')_2$ fragment" contains two light chains and two heavy chains containing a portion of the constant region between the C_{H1} and C_{H2} domains, such that an interchain disulfide bond is formed between the two heavy chains. A $F(ab')_2$ fragment thus is composed of two Fab' fragments that are held together by a disulfide

bond between the two heavy chains. An "$F(ab')_2$ fragment" can be the product of pepsin cleavage of an antibody.

The present invention includes anti- CTLA-4 Fv fragments and methods of use thereof. The "Fv region" comprises the variable regions from both the heavy and light chains, but lacks the constant regions.

The present invention includes anti- CTLA-4 scFv fragments and methods of use thereof. The term "single-chain Fv" or "scFv" antibody refers to antibody fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen-binding. For a review of scFv, see Pluckthun (1994) THE PHARMACOLOGY OF MONOCLONAL ANTIBODIES, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315. See also, International Patent Application Publication No. WO 88/01649 and U.S. Pat. Nos. 4,946, 778 and 5,260,203.

The present invention includes anti- CTLA-4 domain antibodies and methods of use thereof. A "domain antibody" is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. In some instances, two or more V_H regions are covalently joined with a peptide linker to create a bivalent domain antibody. The two V_H regions of a bivalent domain antibody may target the same or different antigens.

The present invention includes anti-CTLA-4 bivalent antibodies and methods of use thereof. A "bivalent antibody" comprises two antigen-binding sites. In some instances, the two binding sites have the same antigen specificities. However, bivalent antibodies may be bispecific (see below).

The present invention includes anti-CTLA-4 camelized single domain antibodies and methods of use thereof. In certain embodiments, antibodies herein also include camelized single domain antibodies. *See, e.g.,* Muyldermans *et al.* (2001) *Trends Biochem. Sci.* 26:230; Reichmann *et al.* (1999) *J. Immunol. Methods* 231:25; WO 94/04678; WO 94/25591; U.S. Pat. No. 6,005,079).

In one embodiment, the present invention provides single domain antibodies comprising two V_H domains with modifications such that single domain antibodies are formed.

The present invention includes anti-CTLA-4 diabodies and methods of use thereof. As used herein, the term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L or V_L-V_H). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, *e.g.*, EP 404,097; WO 93/11161; and Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 6444-6448. For a review of engineered antibody variants generally see Holliger and Hudson (2005) *Nat. Biotechnol.* 23:1126-1136.

Typically, an antibody or antigen-binding fragment of the invention which is modified in some way retains at least 10% of its binding activity (when compared to the parental antibody) when that activity is expressed on a molar basis. Preferably, an antibody or antigen-binding fragment of the invention retains at least 20%, 50%, 70%, 80%, 90%, 95% or 100% or more of the CTLA-4 binding affinity as the parental antibody. It is also intended that an antibody or antigen-binding fragment of the invention can include conservative or non-conservative amino acid substitutions (referred to as "conservative variants" or "function conserved variants" of the antibody) that do not substantially alter its biologic activity.

The present invention includes isolated anti- CTLA-4 antibodies and antigen-binding fragments thereof and methods of use thereof. "Isolated" antibodies or antigen-binding fragments thereof are at least partially free of other biological molecules from the cells or cell cultures in which they are produced. Such biological molecules include nucleic acids, proteins, lipids, carbohydrates, or other material such as cellular debris and growth medium. An isolated antibody or antigen-binding fragment may further be at least partially free of expression system components such as biological molecules from a host cell or of the growth medium thereof. Generally, the term "isolated" is not intended to refer to a complete absence of such biological molecules or to an absence of water, buffers, or salts or to components of a pharmaceutical formulation that includes the antibodies or fragments.

The present invention includes anti-CTLA-4 chimeric antibodies (*e.g.*, human constant domain/mouse variable domain) and methods of use thereof. As used herein, a "chimeric antibody" is an antibody having the variable domain from a first

antibody and the constant domain from a second antibody, where the first and second antibodies are from different species. (U.S. Pat. No. 4,816,567; and Morrison *et al.*, (1984) *Proc. Natl. Acad. Sci. USA* 81: 6851-6855). Typically, the variable domains are obtained from an antibody from an experimental animal (the "parental antibody"), such as a rodent, and the constant domain sequences are obtained from human antibodies, so that the resulting chimeric antibody will be less likely to elicit an adverse immune response in a human subject than the parental (*e.g.*, mouse) antibody.

The present invention includes anti-CTLA-4 humanized antibodies and antigen-binding fragments thereof (*e.g.*, rat or mouse antibodies that have been humanized) and methods of use thereof. The invention includes any humanized version of the hCTLA4.27A antibody as shown in the Examples. As used herein "27 antibody" and "hCTLA4.27" are used interchangeably to refer to an antibody comprising the VH region of SEQ ID NO:7 and the VL region of SEQ ID NO:8 more preferably, the VH region of any of SEQ ID NO: 10, 12, 14, 16, 18 or 20 and the VL region of any of SEQ ID NO: 22, 24, 26 or 30. As used herein, the term "humanized antibody" refers to forms of antibodies that contain sequences from both human and non-human (*e.g.*, mouse or rat) antibodies. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the framework (FR) regions are those of a human immunoglobulin sequence. The humanized antibody may optionally comprise at least a portion of a human immunoglobulin constant region (Fc).

In general, the basic antibody structural unit comprises a tetramer. Each tetramer includes 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 the heavy chain may define a constant region primarily responsible for effector function. Typically, human light chains are classified as kappa and lambda light chains. Furthermore, human heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D"

region of about 10 more amino acids. See generally, Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989).

The variable regions of each light/heavy chain pair form the antibody binding site. Thus, in general, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are, in general, the same.

Typically, the variable domains of both the heavy and light chains comprise three hypervariable regions, also called complementarity determining regions (CDRs), located within relatively conserved framework regions (FR). The CDRs are usually aligned by the framework regions, enabling binding to a specific epitope. In general, from N-terminal to C-terminal, both light and heavy chains variable domains comprise FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is, generally, in accordance with the definitions of Sequences of Proteins of Immunological Interest, Kabat, *et al.*; National Institutes of Health, Bethesda, Md. ; 5th ed.; NIH Publ. No. 91-3242 (1991); Kabat (1978) *Adv. Prot. Chem.* 32:1-75; Kabat, *et al.*, (1977) *J. Biol. Chem.* 252:6609-6616; Chothia, *et al.*, (1987) *J Mol. Biol.* 196:901-917 or Chothia, *et al.*, (1989) *Nature* 342:878-883.

As used herein, the term "hypervariable region" refers to the amino acid residues of an antibody or antigen-binding fragment thereof that are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* LCDR1, LCDR2 and LCDR3 in the light chain variable domain and HCDR1, HCDR2 and HCDR3 in the heavy chain variable domain). See Kabat *et al.* (1991) Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (defining the CDR regions of an antibody by sequence); see also Chothia and Lesk (1987) *J. Mol. Biol.* 196: 901-917 (defining the CDR regions of an antibody by structure). As used herein, the term "framework" or "FR" residues refers to those variable domain residues other than the hypervariable region residues defined herein as CDR residues.

"Isolated nucleic acid molecule" or “isolated polynucleotide” means a DNA or RNA of genomic, mRNA, cDNA, or synthetic origin or some combination thereof which is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, or is linked to a polynucleotide to which it is not linked in nature. For purposes of this disclosure, it should be understood that "a nucleic

acid molecule comprising" a particular nucleotide sequence does not encompass intact chromosomes. Isolated nucleic acid molecules "comprising" specified nucleic acid sequences may include, in addition to the specified sequences, coding sequences for up to ten or even up to twenty or more other proteins or portions or fragments thereof, or may include operably linked regulatory sequences that control expression of the coding region of the recited nucleic acid sequences, and/or may include vector sequences.

The phrase "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to use promoters, polyadenylation signals, and enhancers.

A nucleic acid or polynucleotide is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, but not always, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that not all progeny will have precisely identical DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

As used herein, "germline sequence" refers to a sequence of unrearranged immunoglobulin DNA sequences. Any suitable source of unrearranged

immunoglobulin sequences may be used. Human germline sequences may be obtained, for example, from JOINSOLVER germline databases on the website for the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the United States National Institutes of Health. Mouse germline sequences may be obtained, for example, as described in Giudicelli *et al.* (2005) *Nucleic Acids Res.* 33:D256-D261.

The antibodies of the present invention may comprise any combination of the heavy and light chains as defined in the present application and which are presented in SEQ ID NO: 7 and 8 and more preferably the heavy chains as presented in SEQ ID NO: 10, 12, 14, 16, 18 or 20 and the light chain as presented in SEQ ID NO: 22, 24, 26 and 30.

This means that the following combinations can be made:

An antibody with a heavy chain of SEQ ID NO: 10 and a light chain of SEQ ID NO: 22;
An antibody with a heavy chain of SEQ ID NO: 12 and a light chain of SEQ ID NO: 22;
An antibody with a heavy chain of SEQ ID NO: 14 and a light chain of SEQ ID NO: 22;
An antibody with a heavy chain of SEQ ID NO: 16 and a light chain of SEQ ID NO: 22;
An antibody with a heavy chain of SEQ ID NO: 18 and a light chain of SEQ ID NO: 22;
An antibody with a heavy chain of SEQ ID NO: 20 and a light chain of SEQ ID NO: 22;
An antibody with a heavy chain of SEQ ID NO: 10 and a light chain of SEQ ID NO: 24;
An antibody with a heavy chain of SEQ ID NO: 12 and a light chain of SEQ ID NO: 24;
An antibody with a heavy chain of SEQ ID NO: 14 and a light chain of SEQ ID NO: 24;
An antibody with a heavy chain of SEQ ID NO: 16 and a light chain of SEQ ID NO: 24;
An antibody with a heavy chain of SEQ ID NO: 18 and a light chain of SEQ ID NO: 24;
An antibody with a heavy chain of SEQ ID NO: 20 and a light chain of SEQ ID NO: 24;
An antibody with a heavy chain of SEQ ID NO: 10 and a light chain of SEQ ID NO: 26;
An antibody with a heavy chain of SEQ ID NO: 12 and a light chain of SEQ ID NO: 26;
An antibody with a heavy chain of SEQ ID NO: 14 and a light chain of SEQ ID NO: 26;
An antibody with a heavy chain of SEQ ID NO: 16 and a light chain of SEQ ID NO: 26;
An antibody with a heavy chain of SEQ ID NO: 18 and a light chain of SEQ ID NO: 26;
An antibody with a heavy chain of SEQ ID NO: 20 and a light chain of SEQ ID NO: 26;
An antibody with a heavy chain of SEQ ID NO: 10 and a light chain of SEQ ID NO: 30;
An antibody with a heavy chain of SEQ ID NO: 12 and a light chain of SEQ ID NO: 30;
An antibody with a heavy chain of SEQ ID NO: 14 and a light chain of SEQ ID NO: 30;
An antibody with a heavy chain of SEQ ID NO: 16 and a light chain of SEQ ID NO: 30;

An antibody with a heavy chain of SEQ ID NO: 18 and a light chain of SEQ ID NO: 30;
An antibody with a heavy chain of SEQ ID NO: 20 and a light chain of SEQ ID NO: 30.

Physical and Functional Properties of the Exemplary Anti-CTLA-4 Antibodies

The present invention provides anti-CTLA-4 antibodies and antigen-binding fragments thereof having specified structural and functional features, and methods of use of the antibodies or antigen-binding fragments thereof in the treatment or prevention of disease (e.g., cancer or infectious disease). These all origin from the mouse antibody that has been found as described in the Examples, which antibody has the heavy chain of SEQ ID NO: 32 and the light chain of SEQ ID NO: 34 (the nucleotide sequences encoding for these are SEQ ID NO: 31 and 33, respectively).

This antibody and the humanize antibodies derived therefrom are characterized because they bind to human CTLA-4 (hCTLA-4) with an EC50 of less than 20 nM, preferably less than 1 nM and they are able to block the binding of hCTLA-4 to hCD80 or hCD86 with an IC50 of less than 100 nM, preferably less than 10 nM for hCD80 blocking and preferably less than 10 nM, more preferably less than 2.5nM for hCD86 blocking. It should be remarked that the hCD80 blocking profile of the antibodies of the present invention lies in between the hCD80 blocking profiles of 10D1 (ipilimumab) and CP-675,206 (tremelimumab) (see Fig. 2). It differs from ipilimumab and tremelimumab because it binds to a different epitope on the CTLA-4 molecule. One of the differences is that the antibody or antigen binding fragment of the invention does not bind to the chimeric mouse-human CTLA4 molecule of which the sequence is provided in SEQ ID NO: 44 (see also Fig. 5), while 10D1 and CP-675,206 do bind.

There are several methods available for fine mapping antibody epitopes on target antigens, including: H/D-Ex Mass spec, X-ray crystallography, peptide array and site directed mutagenesis. For example, HDX (Hydrogen Deuterium Exchange) coupled with proteolysis and mass spectrometry can be used to determine the epitope of an antibody on a specific antigen Y. HDX-MS relies on the accurate measurement and comparison of the degree of deuterium incorporation by an antigen when incubated in D_2O on its own and in presence of its antibody at various time intervals. Deuterium is exchanged with hydrogen on the amide backbone of the proteins in exposed areas

whereas regions of the antigen bound to the antibody will be protected and will show less or no exchange after analysis by LC-MS/MS of proteolytic fragments.

The invention also comprises anti-CTLA-4 antibodies which bind to an epitope of human CTLA-4 but which do not bind to the mouse-human chimera CTLA-4 molecule of SEQ ID NO: 44.

In other embodiments, the invention provides antibodies or antigen-binding fragment thereof that bind human CTLA-4 (*e.g.*, humanized antibodies) and has V_L domains and V_H domains with at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with the amino acid sequences of SEQ ID NOs: 7-30, preferably with at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NOs: 10, 12, 14, 16, 18, 20, 22, 24, 26 or 30; wherein the variant exhibits the desired binding and properties, being the ability to bind to CTLA-4 and the ability to block CTLA-4 binding to CD80 and/or CD86.. In other embodiments, the invention provides antibodies or antigen-binding fragment thereof that bind human CTLA-4 (*e.g.*, humanized antibodies) and have V_L domains and V_H domains with at least 95% sequence identity with the amino acid sequences of SEQ ID NOs: 7-30, preferably with at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NOs: 10, 12, 14, 16, 18, 20, 22, 24, 26 or 30. In other embodiments, the invention provides antibodies or antigen-binding fragment thereof that bind human CTLA-4 (*e.g.*, humanized antibodies) and have V_L domains and V_H domains with at least 97% sequence identity with the amino acid sequences of SEQ ID NOs: 7-30, preferably with at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NOs: 10, 12, 14, 16, 18, 20, 22, 24, 26 or 30. In other embodiments, the invention provides antibodies or antigen-binding fragment thereof that bind human CTLA-4 (*e.g.*, humanized antibodies) and have V_L domains and V_H domains with at least 99% sequence identity with the amino acid sequences of SEQ ID NOs: 7-30, preferably with at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NOs: 10, 12, 14, 16, 18, 20, 22, 24, 26 or 30.

"Conservatively modified variants" or "conservative substitution" refers to substitutions of amino acids in a protein with other amino acids having similar characteristics (*e.g.* charge, side-chain size, hydrophobicity/hydrophilicity, backbone conformation and rigidity, etc.), such that the changes can frequently be made without altering the biological activity of the protein. Those of skill in this art recognize that, in

general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (*see, e.g.*, Watson *et al.* (1987) *Molecular Biology of the Gene*, The Benjamin/Cummings Pub. Co., p. 224 (4th Ed.)). In addition, substitutions of structurally or functionally similar amino acids are less likely to disrupt biological activity. Exemplary conservative substitutions are set forth in Table A.

TABLE A. Exemplary Conservative Amino Acid Substitutions

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys; His
Asn (N)	Gln; His
Asp (D)	Glu; Asn
Cys (C)	Ser; Ala
Gln (Q)	Asn
Glu (E)	Asp; Gln
Gly (G)	Ala
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; His
Met (M)	Leu; Ile; Tyr
Phe (F)	Tyr; Met; Leu
Pro (P)	Ala
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr; Phe
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Function-conservative variants of the antibodies of the invention are also contemplated by the present invention. "Function-conservative variants," as used herein, refers to antibodies or fragments in which one or more amino acid residues have

been changed without altering a desired property, such an antigen affinity and/or specificity. Such variants include, but are not limited to, replacement of an amino acid with one having similar properties, such as the conservative amino acid substitutions of Table 1. Also provided are isolated polypeptides comprising the V_L domains of the anti-CTLA-4 antibodies of the invention (*e.g.*, SEQ ID NOs: 22, 24, 26, 30), and isolated polypeptides comprising the V_H domains of the anti- CTLA-4 antibodies of the invention (*e.g.*, SEQ ID NOs: 10, 12, 14, 16, 18, 20) having up to 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid substitutions.

In another embodiment, provided is an antibody or antigen-binding fragment thereof that binds human CTLA-4 and has V_L domains and V_H domains with at least 99% 98%, 97%, 96%, 95%, 90%, 85%, 80% or 75% sequence identity to one or more of the V_L domains or V_H domains described herein, and exhibits specific binding to CTLA-4. In another embodiment the binding antibody or antigen-binding fragment thereof of the present invention comprises V_L and V_H domains (with and without signal sequence) having up to 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acid substitutions, and exhibits specific binding to CTLA-4.

Polynucleotides and Polypeptides

The present invention further comprises the polynucleotides encoding any of the polypeptides or immunoglobulin chains of anti-CTLA-4 antibodies and antigen-binding fragments thereof of the invention. For example, the present invention includes the polynucleotides encoding the amino acids described in any one of SEQ ID NOs: 1-30.

In one embodiment, an isolated polynucleotide, for example DNA, encoding the polypeptide chains of the isolated antibodies or antigen-binding fragments set forth herein is provided. In one embodiment, the isolated polynucleotide encodes an antibody or antigen-binding fragment thereof comprising at least one mature immunoglobulin light chain variable (V_L) domain according to the invention and/or at least one mature immunoglobulin heavy chain variable (V_H) domain according to the invention. In some embodiments the isolated polynucleotide encodes both a light chain and a heavy chain on a single polynucleotide molecule, and in other embodiments the

light and heavy chains are encoded on separate polynucleotide molecules. In another embodiment the polynucleotides further encodes a signal sequence.

In one embodiment, the invention comprises an isolated polynucleotide encoding an antibody heavy variable (V_H) domain or an antigen-binding fragment thereof comprising HCDR-1 (SEQ ID NO:1), HCDR-2 (SEQ ID NO:2) and HCDR-3 (SEQ ID NO:3).

In one embodiment, the invention comprises an isolated polynucleotide encoding an antibody light chain variable (V_L) domain or an antigen-binding fragment thereof comprising

LCDR-1 (SEQ ID NO:4), LCDR-2 (SEQ ID NO:5) and LCDR-3 (SEQ ID NO:6).

In one embodiment, the invention comprises an isolated polynucleotide encoding the immunoglobulin heavy chain variable (V_H) domain of SEQ ID NO: 7.

In one embodiment, the invention comprises an isolated polynucleotide encoding the immunoglobulin light chain variable (V_L) domain of SEQ ID NO: 8..

In one embodiment, the invention comprises an isolated polynucleotide according to any of SEQ ID NO: 9, 11, 13, 15, 17 or 19 encoding a humanized heavy chain

In one embodiment, the invention comprises an isolated polynucleotide according to any of SEQ ID NO: 21, 23, 25 or 29 encoding a humanized light chain.

In a further embodiment of the invention an isolated polynucleotide is comprised which comprises an isolated polynucleotide according to any of SEQ ID NO: 9, 11, 13, 15, 17 or 19 encoding a humanized heavy chain and an isolated polynucleotide according to any of SEQ ID NO: 21, 23, 25 or 29 encoding a humanized light chain. All possible combinations of these are included. Accordingly, the invention comprises a polynucleotide comprising SEQ ID NO 9 and SEQ ID NO 21, a polynucleotide comprising SEQ ID NO 11 and SEQ ID NO 21, a polynucleotide comprising SEQ ID NO 13 and SEQ ID NO 21, a polynucleotide comprising SEQ ID NO 15 and SEQ ID NO 21, a polynucleotide comprising SEQ ID NO 17 and SEQ ID NO 21, a polynucleotide comprising SEQ ID NO 19 and SEQ ID NO 21, a polynucleotide comprising SEQ ID NO 9 and SEQ ID NO 23. a polynucleotide comprising SEQ ID NO 11 and SEQ ID NO 23, a polynucleotide comprising SEQ ID NO 13 and SEQ ID NO 23, a polynucleotide comprising SEQ ID NO 15 and SEQ ID NO 23, a polynucleotide comprising SEQ ID NO 17 and SEQ ID NO 23, a

polynucleotide comprising SEQ ID NO 19 and SEQ ID NO 23, a polynucleotide comprising SEQ ID NO 9 and SEQ ID NO 25, a polynucleotide comprising SEQ ID NO 11 and SEQ ID NO 25, a polynucleotide comprising SEQ ID NO 13 and SEQ ID NO 25, a polynucleotide comprising SEQ ID NO 15 and SEQ ID NO 25, a polynucleotide comprising SEQ ID NO 17 and SEQ ID NO 25, a polynucleotide comprising SEQ ID NO 19 and SEQ ID NO 25 , a polynucleotide comprising SEQ ID NO 9 and SEQ ID NO 29, a polynucleotide comprising SEQ ID NO 11 and SEQ ID NO 29, a polynucleotide comprising SEQ ID NO 13 and SEQ ID NO 29, a polynucleotide comprising SEQ ID NO 15 and SEQ ID NO 29, a polynucleotide comprising SEQ ID NO 17 and SEQ ID NO 29, and/or a polynucleotide comprising SEQ ID NO 19 and SEQ ID NO 29.

This present invention also provides vectors, *e.g.*, expression vectors, such as plasmids, comprising the isolated polynucleotides of the invention, wherein the polynucleotide is operably linked to control sequences that are recognized by a host cell when the host cell is transfected with the vector. Also provided are host cells comprising a vector of the present invention and methods for producing the antibody or antigen-binding fragment thereof or polypeptide disclosed herein comprising culturing a host cell harboring an expression vector or a nucleic acid encoding the immunoglobulin chains of the antibody or antigen-binding fragment thereof in culture medium, and isolating the antigen or antigen-binding fragment thereof from the host cell or culture medium.

Also included in the present invention are polypeptides, *e.g.*, immunoglobulin polypeptides, comprising amino acid sequences that are at least about 75% identical, 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the amino acid sequences of the antibodies provided herein when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences (e.g. expect threshold: 10; word size: 3; max matches in a query range: 0; BLOSUM 62 matrix; gap costs: existence 11, extension 1; conditional compositional score matrix adjustment).

Sequence identity refers to the degree to which the amino acids of two polypeptides are the same at equivalent positions when the two sequences are optimally aligned.

The following references relate to BLAST algorithms often used for sequence analysis: BLAST ALGORITHMS: Altschul et al. (2005) *FEBS* J. 272(20): 5101-5109; Altschul, S.F., *et al.*, (1990) *J. Mol. Biol.* 215:403-410; Gish, W., *et al.*, (1993) *Nature Genet.* 3:266-272; Madden, T.L., *et al.*, (1996) *Meth. Enzymol.* 266:131-141; Altschul, S.F., *et al.*, (1997) *Nucleic Acids Res.* 25:3389-3402; Zhang, J., *et al.*, (1997) *Genome Res.* 7:649-656; Wootton, J.C., *et al.*, (1993) *Comput. Chem.* 17:149-163; Hancock, J.M. *et al.*, (1994) *Comput. Appl. Biosci.* 10:67-70; ALIGNMENT SCORING SYSTEMS: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, *Natl. Biomed. Res. Found.*, Washington, DC; Schwartz, R.M., *et al.*, "Matrices for detecting distant relationships." in *Atlas of Protein Sequence and Structure*, (1978) vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 353-358, *Natl. Biomed. Res. Found.*, Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219:555-565; States, D.J., *et al.*, (1991) *Methods* 3:66-70; Henikoff, S., *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36:290-300; ALIGNMENT STATISTICS: Karlin, S., *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268; Karlin, S., *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877; Dembo, A., *et al.*, (1994) *Ann. Prob.* 22:2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments." in *Theoretical and Computational Methods in Genome Research* (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

Binding Affinity

By way of example, and not limitation, the antibodies and antigen-binding fragments disclosed herein may bind human CTLA-4 (NCBI Accession No. NM_005214.4) comprising the following amino acid sequence: (SEQ ID NO: 36)

MACLGFQRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIASFVCEYASPGKATEVR
VTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYY
LGIGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFLLTAVSLSKMLKKRSPLTTGVYVKMPPTEPE
CEKQFQPYFIPIN

with a K_D value of at least about 1 x 10^{-9} M (i.e, a K_D value of 1 x 10^{-9} M or lower) as determined by surface plasmon resonance (e.g., BIACORE) or a similar technique (e.g. KinExa or OCTET).

Immune Cell Activation

In some embodiments, the antibodies or antigen binding fragments of the invention increase the activity of an immune cell. The increase of the activity of an immune cell can be detected using any method known in the art. In one embodiment, the increase in activity of an immune cell can be detected by measuring the proliferation of the immune cell. For example, an increase in activity of a T cell can be detected by measuring the proliferation of the T cell or signal transduction events such as tyrosine phosphorylation of immune receptors or downstream kinases that transmit signals to transcriptional regulators. In other embodiments, the increase in activity of an immune cell can be detected by measuring CTL or NK cell cytotoxic function on specific target cells or IFNγ cytokine responses, which are associated with stimulation of anti-tumor immunity. In yet other embodiments, the increase in activity of an immune cell can be detected by measuring T cell activation *ex vivo* in a sample derived from the subject. In one embodiment, the increase in T cell activity is determined by: (i) measuring SEB (Staphylococcus Enterotoxin B) induced production of one or more pro-inflammatory cytokines selected from the group consisting of: IL-2, TNFα, IL-17, IFNγ, IL-1β, GM-CSF, RANTES, IL-6, IL-8, IL-5 and IL-13 or upregulation of membrane activation markers from a group consisting of: CD25 and CD69 or induction of proliferation using detection of blast formation by flow cytometry or 3H-incorportationor (ii) measuring mixed lymphocyte reactions or direct anti-CD3 mAb stimulation of T cell receptor (TCR) signaling to induce production of a cytokine selected from the group consisting of: IL-2, TNFα, IL-17, IFNγ, IL-1β, GM-CSF, RANTES, IL-6, IL-8, IL-5 and IL-13 or upregulation of membrane activation markers from a group consisting of: CD25 and CD69 or induction of proliferation using detection of blast formation by flow cytometry or 3H-incorportation. In certain embodiments, the anti-CTLA-4 antibody or antigen binding fragment thereof of the present invention will stimulate CD3+ T cells, when these are presented to Raji cells expressing CD80 and CD86, to produce pro-inflammatory cytokines selected from the group consisting of: IL-2, TNFα, IL-17, IFNγ, IL-1β, GM-CSF, RANTES, IL-6, IL-8, IL-5 and IL-13 or upregulation of

membrane activation markers from a group consisting of: CD25 and CD69 or induction of proliferation using detection of blast formation by flow cytometry or 3H-incorportation. In certain embodiments, the anti- CTLA4 antibody or antigen binding fragment thereof of the present invention will stimulate production of IL-2 and/or IFNγ by activated T cells by at least 1.5 fold. As is clear from the experimental part, the T-cell activating characteristics of the antibodies of the present invention is about equal to the T-cell stimulating characteristics of the known prior art anti-hCTLA4 antibodies ipilimumab and tremelimumab.

Effector function of Anti-hCTLA-4 Antibodies

In some embodiments, the anti-CTLA-4 antibodies or antigen binding fragments of the invention can deplete CTLA-4+ regulatory T cells. The ability of the antibodies to exert such an effector function can be determined using any method known in the art. In one embodiment the ability of the antibodies to induce Antibody-Dependent Cell-mediated Cytoxicity is determined using natural killer cells as effector cells and a cell line that stably expresses human CTLA-4. As shown in the experimental section, the hCTLA-4 antibodies with a human IgG1 Fc portion are able to induce ADCC on CTLA-4+ cells.

In another embodiment the ability of the antibodies to induce Complement-Dependent Cytoxicity is determined using human complement and a cell line that stably expresses human CTLA-4. As shown in the experimental section, the hCTLA-4 antibodies with a human IgG1 Fc portion are able to induce CDC on CTLA-4+ cells.

In another embodiment the ability of the antibodies to induce cell-mediated lysis can be determined using Nonclassical CD14+CD16++ Monocytes that induce FcγRIIIA-Dependent Lysis of CTLA-4+ Tregs in the context of an hIgG1 CTLA-4 antibody such as ipilimumab (Romano et al; PNAS; 2015; 6140-6145; doi: 10.1073/pnas.1417320112)

Ability of Anti-hCTLA-4 Antibodies to Block Binding to hCD80 and hCD86

In some embodiments, the anti-CTLA-4 antibodies or antigen binding fragments of the invention are able to block binding of human CTLA-4 to human CD80 and/or human CD86. The ability to block binding of human CTLA-4 to human CD80

and/or human CD86 can be determined using any method known in the art. In one embodiment, the ability of the antibodies to block binding of human CTLA-4 to human CD80 and/or human CD86 is determined using an ELISA assay.

As is shown in the experimental section, the potency of blocking of CTLA-4 binding to human CD80 and/or human CD86 resembles the activities of the known anti-CTLA-4 antibodies ipilimumab and tremelimumab. It should be highlighted that the anti-CTLA-4 antibodies of the invention showed an intermediary efficacy in between the effects of ipilimumab and tremelimumab with respect to blocking of hCD80 (see Figure 2).

Methods of Making Antibodies and Antigen-binding Fragments Thereof

Thus, the present invention includes methods for making an anti-CTLA-4 antibody or antigen-binding fragment thereof of the present invention comprising culturing a hybridoma cell that expresses the antibody or fragment under condition favorable to such expression and, optionally, isolating the antibody or fragment from the hybridoma and/or the growth medium (e.g. cell culture medium).

Monoclonal antibodies derived from animals other than rats and mice offer unique advantages. Many protein targets relevant to signal transduction and disease are highly conserved between mice, rats and humans, and can therefore be recognized as self-antigens by a mouse or rat host, making them less immunogenic. This problem may be avoided when using rabbit as a host animal. See, e.g., Rossi et al., *Am. J. Clin. Pathol.,* 124, 295-302, 2005.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with an antigen of interest or a cell expressing such an antigen. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. B-cells were cultured, as described by *Steenbakkers et al., 1994, Mol. Biol. Rep. 19: 125-134.*

B-cell clones from reactive supernatants are then immortalized, e.g. by mini-electrofusion following published procedures *(Steenbakkers et al., 1992, J.*

Immunol. Meth. 152: 69-77; Steenbakkers et al., 1994, Mol. Biol. Rep. 19:125-34). Hybridomas are selected and cloned by limiting dilution.

The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding the antigen. Ascites fluid, which generally contains high levels of antibodies, can be generated by inoculating mice intraperitoneally with positive hybridoma clones.

Adjuvants that can be used in the methods of antibody generation include, but are not limited to, protein adjuvants; bacterial adjuvants, e.g., whole bacteria (BCG, *Corynebacterium parvum, Salmonella minnesota*) and bacterial components including cell wall skeleton, trehalose dimycolate, monophosphoryl lipid A, methanol extractable residue (MER) of tubercle bacillus, complete or incomplete Freund's adjuvant; viral adjuvants; chemical adjuvants, e.g., aluminum hydroxide, iodoacetate and cholesteryl hemisuccinateor; naked DNA adjuvants. Other adjuvants that can be used in the methods of the invention include, Cholera toxin, paropox proteins, MF-59 (Chiron Corporation; See also Bieg et al. (1999) "GAD65 And Insulin B Chain Peptide (9-23) Are Not Primary Autoantigens In The Type 1 Diabetes Syndrome Of The BB Rat," Autoimmunity, 31(1):15-24, which is incorporated herein by reference), MPL® (Corixa Corporation; See also Lodmell et al. (2000) "DNA Vaccination Of Mice Against Rabies Virus: Effects Of The Route Of Vaccination And The Adjuvant Monophosphoryl Lipid A (MPL)," Vaccine, 18: 1059-1066; Johnson et al. (1999) "3-O-Desacyl Monophosphoryl Lipid A Derivatives: Synthesis And Immunostimulant Activities," Journal of Medicinal Chemistry, 42: 4640-4649; Baldridge et al. (1999) "Monophosphoryl Lipid A (MPL) Formulations For The Next Generation Of Vaccines," Methods, 19: 103-107, all of which are incorporated herein by reference), RC-529 adjuvant (Corixa Corporation; the lead compound from Corixa's aminoalkyl glucosaminide 4-phosphate (AGP) chemical library, see also www.corixa.com), and DETOX™ adjuvant (Corixa Corporation; DETOX™ adjuvant includes MPL® adjuvant (monophosphoryl lipid A) and mycobacterial cell wall skeleton; See also Eton et al. (1998) "Active Immunotherapy With Ultraviolet B-Irradiated Autologous Whole Melanoma Cells Plus DETOX In Patients With Metastatic Melanoma," Clin. Cancer Res. 4(3):619-627; and Gupta et al. (1995) "Adjuvants For Human Vaccines—Current Status, Problems And Future Prospects," Vaccine, 13(14): 1263-1276, both of which are incorporated herein by reference).

Numerous publications discuss the use of phage display technology to produce and screen libraries of polypeptides for binding to a selected analyte. See, e.g, Cwirla et al., Proc. Natl. Acad. Sci. USA 87, 6378-82, 1990; Devlin et al., Science 249, 404-6, 1990, Scott and Smith, Science 249, 386-88, 1990; and Ladner et al., U.S. Pat. No. 5,571,698. A basic concept of phage display methods is the establishment of a physical association between DNA encoding a polypeptide to be screened and the polypeptide. This physical association is provided by the phage particle, which displays a polypeptide as part of a capsid enclosing the phage genome which encodes the polypeptide. The establishment of a physical association between polypeptides and their genetic material allows simultaneous mass screening of very large numbers of phage bearing different polypeptides. Phages displaying a polypeptide with affinity to a target bind to the target and these phages are enriched by affinity screening to the target. The identity of polypeptides displayed from these phages can be determined from their respective genomes. Using these methods a polypeptide identified as having a binding affinity for a desired target can then be synthesized in bulk by conventional means. See, e.g., U.S. Patent No. 6,057,098, which is hereby incorporated in its entirety, including all tables, figures, and claims.

The antibodies that are generated by these methods may then be selected by first screening for affinity and specificity with the purified polypeptide of interest and, if required, comparing the results to the affinity and specificity of the antibodies with polypeptides that are desired to be excluded from binding. The screening procedure can involve immobilization of the purified polypeptides in separate wells of microtiter plates. The solution containing a potential antibody or groups of antibodies is then placed into the respective microtiter wells and incubated for about 30 min to 2 h. The microtiter wells are then washed and a labeled secondary antibody (for example, an anti-mouse antibody conjugated to alkaline phosphatase if the raised antibodies are mouse antibodies) is added to the wells and incubated for about 30 min and then washed. Substrate is added to the wells and a color reaction will appear where antibody to the immobilized polypeptide(s) are present.

The antibodies so identified may then be further analyzed for affinity and specificity in the assay design selected. In the development of immunoassays for a target protein, the purified target protein acts as a standard with which to judge the sensitivity and specificity of the immunoassay using the antibodies that have been

selected. Because the binding affinity of various antibodies may differ; certain antibody pairs (e.g., in sandwich assays) may interfere with one another sterically, etc., assay performance of an antibody may be a more important measure than absolute affinity and specificity of an antibody.

Antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized using conventional methodologies with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg et al. (1995) "Human Antibodies From Transgenic Mice," Int. Rev. Immunol. 13:65-93, which is incorporated herein by reference in its entirety). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International Publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923, 5,625,126, 5,633,425, 5,569,825, 5,661,016, 5,545,806, 5,814,318, and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix/Amgen(Freemont, Calif.) and Medarex/BMS (Princeton, N.J.), Kymab

(Cambridge, UK) and Merus (Utrecht, Netherlands) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

The anti-CTLA-4 antibodies disclosed herein may also be produced recombinantly (*e.g.*, in an *E. coli*/T7 expression system, a mammalian cell expression system or a lower eukaryote expression system). In this embodiment, nucleic acids encoding the antibody immunoglobulin molecules of the invention (*e.g.*, V_H or V_L) may be inserted into a pET-based plasmid and expressed in the *E. coli*/T7 system. For example, the present invention includes methods for expressing an antibody or antigen-binding fragment thereof or immunoglobulin chain thereof in a host cell (*e.g.*, bacterial host cell such as *E.coli* such as BL21 or BL21DE3) comprising expressing T7 RNA polymerase in the cell which also includes a polynucleotide encoding an immunoglobulin chain that is operably linked to a T7 promoter. For example, in an embodiment of the invention, a bacterial host cell, such as a *E. coli*, includes a polynucleotide encoding the T7 RNA polymerase gene operably linked to a *lac* promoter and expression of the polymerase and the chain is induced by incubation of the host cell with IPTG (isopropyl-beta-D-thiogalactopyranoside).

Monoclonal antibody preparations can be produced using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS, pp. 563-681 (Elsevier, N.Y., 1981) (both of which are incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. One example of a method for recombinant production of antibodies is disclosed in U.S. Patent No. 4,816,567.

Thus, the present invention includes recombinant methods for making an anti-CTLA-4 antibody or antigen-binding fragment thereof of the present invention, or an immunoglobulin chain thereof, comprising introducing a polynucleotide encoding

one or more immunoglobulin chains of the antibody or fragment (*e.g.*, heavy and/or light immunoglobulin chain); culturing the host cell (*e.g.*, CHO or *Pichia* or *Pichia pastoris*) under condition favorable to such expression and, optionally, isolating the antibody or fragment or chain from the host cell and/or medium in which the host cell is grown.

Anti-CTLA-4 antibodies can also be synthesized by any of the methods set forth in U.S. Patent No. 6,331,415.

Eukaryotic and prokaryotic host cells, including mammalian cells as hosts for expression of the antibodies or fragments or immunoglobulin chains disclosed herein are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, *inter alia*, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), A549 cells, 3T3 cells, HEK-293 cells and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as Sf9 cells, amphibian cells, bacterial cells, plant cells and fungal cells. Fungal cells include yeast and filamentous fungus cells including, for example, *Pichia pastoris, Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia minuta* (*Ogataea minuta, Pichia lindneri*), *Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis, Pichia methanolica, Pichia* sp., *Saccharomyces cerevisiae, Saccharomyces* sp., *Hansenula polymorpha, Kluyveromyces sp., Kluyveromyces lactis, Candida albicans, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense, Fusarium* sp., *Fusarium gramineum, Fusarium venenatum*, *Physcomitrella patens* and *Neurospora crassa. Pichia sp.,* any *Saccharomyces sp., Hansenula polymorpha,* any *Kluyveromyces sp., Candida albicans,* any *Aspergillus sp., Trichoderma reesei, Chrysosporium lucknowense,* any *Fusarium sp., Yarrowia lipolytica,* and *Neurospora crassa.* When recombinant expression vectors encoding the heavy chain or antigen-binding portion or fragment thereof, the light chain and/or antigen-binding fragment thereof are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to

allow for expression of the antibody or fragment or chain in the host cells or secretion of the into the culture medium in which the host cells are grown.

A variety of host-expression vector systems may be utilized to express the antibodies of the invention. Such host-expression systems represent vehicles by which the coding sequences of the antibodies may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibodies of the invention in situ. These include, but are not limited to, microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing immunoglobulin coding sequences; yeast (e.g., *Saccharomyces, Pichia*) transformed with recombinant yeast expression vectors containing immunoglobulin coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the immunoglobulin coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing immunoglobulin coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (see U.S. Pat. No. 5,807,715), Per C.6 cells (rat retinal cells developed by Crucell) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al. (1983) "Easy Identification Of cDNA Clones," EMBO J. 2:1791-1794), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye et al. (1985) "Up-Promoter Mutations In The Lpp Gene Of Escherichia coli," Nucleic Acids Res. 13:3101-3110; Van Heeke et al. (1989) "Expression Of Human Asparagine

Synthetase In Escherichia coli," J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free gluta-thione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (e.g., the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts. (see e.g., see Logan et al. (1984) "Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection," Proc. Natl. Acad. Sci. (U.S.A.) 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al. (1987) "Expression And Secretion Vectors For Yeast," Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage)

of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express an antibody of the invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibodies of the invention. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibodies of the invention.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al. (1977) "Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells," Cell 11:223-232), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al. (1962) "Genetics Of Human Cess Line. IV. DNA-Mediated Heritable Transformation Of A Biochemical Trait," Proc. Natl. Acad. Sci. (U.S.A.) 48:2026-2034), and adenine phosphoribosyltransferase (Lowy et al. (1980) "Isolation Of Transforming DNA: Cloning The Hamster Aprt Gene," Cell 22:817-823) genes can be employed in tk–, hgprt– or aprt– cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate

(Wigler et al. (1980) "Transformation Of Mammalian Cells With An Amplfiable Dominant-Acting Gene," Proc. Natl. Acad. Sci. (U.S.A.) 77:3567-3570; O'Hare et al. (1981) "Transformation Of Mouse Fibroblasts To Methotrexate Resistance By A Recombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase," Proc. Natl. Acad. Sci. (U.S.A.) 78:1527-1531); gpt, which confers resistance to mycophenolic acid (Mulligan et al. (1981) "Selection For Animal Cells That Express The Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase," Proc. Natl. Acad. Sci. (U.S.A.) 78:2072-2076); neo, which confers resistance to the aminoglycoside G-418 (Tachibana et al. (1991) "Altered Reactivity Of Immunoglobutin Produced By Human-Human Hybridoma Cells Transfected By pSV2-Neo Gene," Cytotechnology 6(3):219-226; Tolstoshev (1993) "Gene Therapy, Concepts, Current Trials And Future Directions," Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan (1993) "The Basic Science Of Gene Therapy," Science 260:926-932; and Morgan et al. (1993) "Human gene therapy," Ann. Rev. Biochem. 62:191-217). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY; Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, CURRENT PROTOCOLS IN HUMAN GENETICS, John Wiley & Sons, NY.; Colbere-Garapin et al. (1981) "A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells," J. Mol. Biol. 150:1-14; and hygro, which confers resistance to hygromycin (Santerre et al. (1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418 Resistance As Dominant-Selection Markers In Mouse L Cells," Gene 30:147-156).

The expression levels of an antibody of the invention can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use Of Vectors Based On Gene Amplification For The Expression Of Cloned Genes In Mammaian Cells," in DNA CLONING, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing an antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the antibody, production of the antibody will also increase (Crouse et al. (1983) "Expression

And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Mol. Cell. Biol. 3:257-266).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot (1986) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Nature 322:562-565; Kohler (1980) "Immunoglobulin Chain Loss In Hybridoma Lines," Proc. Natl. Acad. Sci. (U.S.A.) 77:2197-2199). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Antibodies and antigen-binding fragments thereof and immunoglobulin chains can be recovered from the culture medium using standard protein purification methods. Further, expression of antibodies and antigen-binding fragments thereof and immunoglobulin chains of the invention (or other moieties therefrom) from production cell lines can be enhanced using a number of known techniques. For example, the glutamine synthetase gene expression system (the GS system) is a common approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with European Patent Nos. 0 216 846, 0 256 055, and 0 323 997 and European Patent Application No. 89303964.4. Thus, in an embodiment of the invention, the mammalian host cells (*e.g.*, CHO) lack a glutamine synthetase gene and are grown in the absence of glutamine in the medium wherein, however, the polynucleotide encoding the immunoglobulin chain comprises a glutamine synthetase gene which complements the lack of the gene in the host cell.

The present invention includes methods for purifying an anti-CTLA-4 antibody or antigen-binding fragment thereof of the present invention comprising introducing a sample comprising the antibody or fragment to a purification medium (*e.g.*, cation exchange medium, anion exchange medium, hydrophobic exchange medium, affinity purification medium (*e.g.*, protein-A, protein-G, protein-A/G, protein-L)) and either collecting purified antibody or fragment from the flow-through fraction of said sample that does not bind to the medium; or, discarding the flow-through

fraction and eluting bound antibody or fragment from the medium and collecting the eluate. In an embodiment of the invention, the medium is in a column to which the sample is applied. In an embodiment of the invention, the purification method is conducted following recombinant expression of the antibody or fragment in a host cell, *e.g.*, wherein the host cell is first lysed and, optionally, the lysate is purified of insoluble materials prior to purification on a medium.

In general, glycoproteins produced in a particular cell line or transgenic animal will have a glycosylation pattern that is characteristic for glycoproteins produced in the cell line or transgenic animal. Therefore, the particular glycosylation pattern of an antibody will depend on the particular cell line or transgenic animal used to produce the antibody. However, all antibodies encoded by the nucleic acid molecules provided herein, or comprising the amino acid sequences provided herein, comprise the instant invention, independent of the glycosylation pattern the antibodies may have. Similarly, in particular embodiments, antibodies with a glycosylation pattern comprising only non-fucosylated *N*-glycans may be advantageous, because these antibodies have been shown to typically exhibit more potent efficacy than their fucosylated counterparts both *in vitro* and *in vivo* (*See* for example, Shinkawa *et al.*, *J. Biol. Chem.* 278: 3466-3473 (2003); U.S. Patent Nos. 6,946,292 and 7,214,775). These antibodies with non-fucosylated *N*-glycans are not likely to be immunogenic because their carbohydrate structures are a normal component of the population that exists in human serum IgG.

The present invention includes bispecific and bifunctional antibodies and antigen-binding fragments having a binding specificity for CTLA-4 and another antigen such as, for example and antigen that plays a role in immune stimulation, such as, PD-1, PD-L1, TSLP, IL-10, 4-IBB, SIRP-alpha, ICOS, NKG2C, NKG2A, KR2DL and KIR3DL antigens, OX40, CD40, ITL-1 to ITL-8, GITR, CD137, CS1, CD27, APRIL, or LAG-3, or antigens that play a role in targeting to and recognition of cancer cells, such as EGFR (ERBB1), HER2 (ERBB2), ERBB3, CD19, CD20, CD30, CD33, CD52, CEA, alpha-fetoprotein, CC49, VEGF. VEGFR, HGFR (MET), CA-125, tenascin, integrin, FAB, IGF1R, EPHA3, TRAILR1, TRAILR2 or RANKL and methods of use thereof. In an embodiment of the invention, the anti-CTLA-4 chains comprise any one of the VH/VL sequences provided in SEQ ID NO: 7 -30 (or an antigen binding fragment of any of said sequences, such as provided in SEQ ID NO: 1 - 6). A bispecific or bifunctional antibody is an artificial hybrid antibody having two different heavy/light

chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai, *et al.*, (1990) *Clin. Exp. Immunol.* 79: 315-321, Kostelny, *et al.*, (1992) *J Immunol.* 148:1547- 1553. In addition, bispecific antibodies may be formed as "diabodies" (Holliger, *et al.*, (1993) *PNAS USA* 90:6444-6448) or as "Janusins" (Traunecker, *et al.*, (1991) *EMBO J.* 10:3655-3659 and Traunecker, *et al.*, (1992) *Int. J. Cancer Suppl.* 7:51-52). In addition, bispecific antibodies may be formed as "Duobodies" (Labrijn et al, PNAS 2013;110(13):5145-5150).

The present invention further includes anti-CTLA-4 antigen-binding fragments of the anti-CTLA-4 antibodies disclosed herein. The antibody fragments include $F(ab)_2$ fragments, which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of $F(ab)_2$ with dithiothreitol or mercaptoethylamine.

Immunoglobulins may be assigned to different classes depending on the amino acid sequences of the constant domain of their heavy chains. In some embodiments, different constant domains may be appended to humanized V_L and V_H regions derived from the CDRs provided herein. There are at least five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.* IgG1, IgG2, IgG3 and IgG4; IgA1 and IgA2. The invention comprises antibodies and antigen-binding fragments of any of these classes or subclasses of antibodies.

In one embodiment, the antibody or antigen-binding fragment comprises a heavy chain constant region, *e.g.* a human constant region, such as γ1, γ2, γ3, or γ4 human heavy chain constant region or a variant thereof. In another embodiment, the antibody or antigen-binding fragment comprises a light chain constant region, *e.g.* a human light chain constant region, such as lambda or kappa human light chain region or variant thereof. By way of example, and not limitation the human heavy chain constant region can be γ4 and the human light chain constant region can be kappa. In an alternative embodiment, the Fc region of the antibody is γ4 with a Ser228Pro mutation (*Angal S. et al., 1993, Mol Immunol. 30: 105-108* position 241 is based on the Kabat numbering system).

In one embodiment, the antibody or antigen-binding fragment comprises a heavy chain constant region of the IgG1 subtype. In one embodiment, the antibody or

antigen-binding fragment comprises a heavy chain constant region of the IgG2 subtype. In one embodiment, the antibody or antigen-binding fragment comprises a heavy chain constant region of the IgG4 subtype.

Antibody Engineering

Further included are embodiments in which the anti-CTLA-4 antibodies and antigen-binding fragments thereof are engineered antibodies to include modifications to framework residues within the variable domains of the parental hCTLA4.27A monoclonal antibody, *e.g.* to improve the properties of the antibody or fragment. Typically, such framework modifications are made to decrease the immunogenicity of the antibody or fragment. This is usually accomplished by replacing non-CDR residues in the variable domains (*i.e.* framework residues) in a parental (*e.g.* rodent) antibody or fragment with analogous residues from the immune repertoire of the species in which the antibody is to be used, *e.g.* human residues in the case of human therapeutics. Such an antibody or fragment is referred to as a "humanized" antibody or fragment. In some cases it is desirable to increase the affinity, or alter the specificity of an engineered (*e.g.* humanized) antibody. One approach is to "backmutate" one or more framework residues to the corresponding germline sequence. More specifically, an antibody or fragment that has undergone somatic mutation can contain framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody or fragment framework sequences to the germline sequences from which the antibody or fragment is derived. Another approach is to revert to the original parental (*e.g.*, rodent) residue at one or more positions of the engineered (*e.g.* humanized) antibody, *e.g.* to restore binding affinity that may have been lost in the process of replacing the framework residues. (See, *e.g.*, U.S. Patent No. 5,693,762, U.S. Patent No. 5,585,089 and U.S. Patent No. 5,530,101.).

In certain embodiments, the anti-CTLA-4 antibodies and antigen-binding fragments thereof are engineered (e.g. humanized) to include modifications to in the framework and/or CDRs to improve their properties. Such engineered changes can be based on molecular modelling. A molecular model for the variable region for the parental (non-human) antibody sequence can be constructed to understand the structural features of the antibody and used to identify potential regions on the antibody that can interact with the antigen. Conventional CDRs are based on alignment of

immunoglobulin sequences and identifying variable regions. Kabat et al., (1991) Sequences of Proteins of Immunological Interest, Kabat, *et al.*; National Institutes of Health, Bethesda, Md. ; 5th ed.; NIH Publ. No. 91-3242; Kabat (1978) *Adv. Prot. Chem.* 32:1-75; Kabat, *et al.*, (1977) *J. Biol. Chem.* 252:6609-6616. Chothia and coworkers carefully examined conformations of the loops in crystal structures of antibodies and proposed hypervariable loops. Chothia, *et al.*, (1987) *J Mol. Biol.* 196:901-917 or Chothia, *et al.*, (1989) *Nature* 342:878-883. There are variations between regions classified as "CDRs" and "hypervariable loops". Later studies (Raghunathan et al, (2012) *J. Mol Recog.* 25, 3, 103-113) analyzed several antibody –antigen crystal complexes and observed that the antigen binding regions in antibodies do not necessarily conform strictly to the "CDR" residues or "hypervariable" loops. The molecular model for the variable region of the non-human antibody can be used to guide the selection of regions that can potentially bind to the antigen. In practice the potential antigen binding regions based on model differ from the conventional "CDR"s or "hyper variable" loops. Commercial scientific software such as Discovery Studio (BIOVIA, Dassault Systemes) can be used for molecular modeling. Human frameworks can be selected based on best matches with the non-human sequence both in the frameworks and in the CDRs. For FR4 (framework 4) in VH, VJ regions for the human germlines are compared with the corresponding non-human region. In the case of FR4 (framework 4) in VL, J-kappa and J-Lambda regions of human germline sequences are compared with the corresponding non-human region. Once suitable human frameworks are identified, the CDRs are grafted into the selected human frameworks. In some cases certain residues in the VL-VH interface can be retained as in the non-human (parental) sequence. Molecular models can also be used for identifying residues that can potentially alter the CDR conformations and hence binding to antigen. In some cases, these residues are retained as in the non-human (parental) sequence. Molecular models can also be used to identify solvent exposed amino acids that can result in unwanted effects such as glycosylation, deamidation and oxidation. Developability filters can be introduced early on in the design stage to eliminate/minimize these potential problems.

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell epitopes to thereby reduce the potential immunogenicity of the antibody.

This approach is also referred to as "deimmunization" and is described in further detail in U.S. Patent No. 7,125,689.

In particular embodiments, it will be desirable to change certain amino acids containing exposed side-chains to another amino acid residue in order to provide for greater chemical stability of the final antibody, so as to avoid deamidation or isomerization. The deamidation of asparagine may occur on NG, DG, NG, NS, NA, NT, QG or QS sequences and result in the creation of an isoaspartic acid residue that introduces a kink into the polypeptide chain and decreases its stability (isoaspartic acid effect). Isomerization can occur at DG, DS, DA or DT sequences. In certain embodiments, the antibodies of the present disclosure do not contain deamidation or asparagine isomerism sites.

For example, an asparagine (Asn) residue may be changed to Gln or Ala to reduce the potential for formation of isoaspartate at any Asn-Gly sequences, particularly within a CDR. A similar problem may occur at a Asp-Gly sequence. Reissner and Aswad (2003) *Cell. Mol. Life Sci.* 60:1281. Isoaspartate formation may debilitate or completely abrogate binding of an antibody to its target antigen. *See*, Presta (2005) *J. Allergy Clin. Immunol.* 116:731 at 734. In one embodiment, the asparagine is changed to glutamine (Gln). It may also be desirable to alter an amino acid adjacent to an asparagine (Asn) or glutamine (Gln) residue to reduce the likelihood of deamidation, which occurs at greater rates when small amino acids occur adjacent to asparagine or glutamine. *See*, Bischoff & Kolbe (1994) *J. Chromatog.* 662:261. In addition, any methionine residues (typically solvent exposed Met) in CDRs may be changed to Lys, Leu, Ala, or Phe or other amino acids in order to reduce the possibility that the methionine sulfur would oxidize, which could reduce antigen-binding affinity and also contribute to molecular heterogeneity in the final antibody preparation. *Id.* Additionally, in order to prevent or minimize potential scissile Asn-Pro peptide bonds, it may be desirable to alter any Asn-Pro combinations found in a CDR to Gln-Pro, Ala-Pro, or Asn-Ala. Antibodies with such substitutions are subsequently screened to ensure that the substitutions do not decrease the affinity or specificity of the antibody for CTLA-4, or other desired biological activity to unacceptable levels.

TABLE 2. Exemplary stabilizing CDR variants

CDR Residue	Stabilizing Variant Sequence
Asn-Gly (N-G)	Gln-Gly, Ala-Gly, or Asn-Ala (Q-G), (A-G), or (N-A)
Asp-Gly (D-G)	Glu-Gly, Ala-Gly or Asp-Ala (E-G), (A-G), or (D-A)
Met (typically solvent exposed) (M)	Lys, Leu, Ala, or Phe (K), (L), (A), or (F)
Asn (N)	Gln or Ala (Q) or (A)
Asn-Pro (N-P)	Gln-Pro, Ala-Pro, or Asn-Ala (Q-P), (A-P), or (N-A)

Antibody Engineering of the Fc region

The antibodies (*e.g.*, humanized antibodies) and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) can also be engineered to include modifications within the Fc region, typically to alter one or more properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or effector function (*e.g.*, antigen-dependent cellular cytotoxicity). Furthermore, the antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) can be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more properties of the antibody or fragment. Each of these embodiments is described in further detail below. The numbering of residues in the Fc region is that of the EU index of Kabat.

The antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) also include antibodies and fragments with modified (or blocked) Fc regions to provide altered effector functions. See, *e.g.*, U.S. Pat. No. 5,624,821; WO2003/086310; WO2005/120571; WO2006/0057702. Such modifications can be used to enhance or suppress various reactions of the immune system, with possible beneficial effects in diagnosis and therapy. Alterations of the Fc region include amino acid changes (substitutions, deletions and insertions), glycosylation or deglycosylation, and adding multiple Fc regions. Changes to the Fc

can also alter the half-life of antibodies in therapeutic antibodies, enabling less frequent dosing and thus increased convenience and decreased use of material. *See* Presta (2005) *J. Allergy Clin. Immunol.* 116:731 at 734-35.

In one embodiment, the antibody or antigen-binding fragment of the invention (*e.g.*, antibody 27A and humanized versions thereof) is an IgG4 isotype antibody or fragment comprising a Serine to Proline mutation at a position corresponding to position 228 (S228P; EU index) in the hinge region of the heavy chain constant region. This mutation has been reported to abolish the heterogeneity of inter-heavy chain disulfide bridges in the hinge region (*Angal S. et al., 1993, Mol Immunol. 30: 105-108*; position 241 is based on the Kabat numbering system).

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

In another embodiment, the Fc hinge region of an antibody or antigen-binding fragment of the invention (*e.g.*, antibody 27A and humanized versions thereof) is mutated to decrease the biological half-life of the antibody or fragment. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody or fragment has impaired Staphylococcyl protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent No. 6,165,745.

In another embodiment, the antibody or antigen-binding fragment of the invention (*e.g.*, antibody 27A and humanized versions thereof) is modified to increase its biological half-life. Various approaches are possible. For example, one or more of the following mutations can be introduced: T252L, T254S, T256F, as described in U.S. Patent No. 6,277,375. Alternatively, to increase the biological half-life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022.

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector function(s) of

the antibody or antigen-binding fragment. For example, one or more amino acids selected from amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322 can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand and retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Patent Nos. 5,624,821 and 5,648,260.

In another example, one or more amino acids selected from amino acid residues 329, 331 and 322 can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent No. 6,194,551.

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

In yet another example, the Fc region is modified to decrease the ability of the antibody or antigen-binding fragment of the invention (*e.g.*, antibody 27A and humanized versions thereof) to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to decrease the affinity of the antibody or fragment for an Fcγ receptor by modifying one or more amino acids at the following positions: 238, 239, 243, 248, 249, 252, 254, 255, 256, 258, 264, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439. This approach is described further in PCT Publication WO 00/42072. Moreover, the binding sites on human IgG1 for FcγR1, FcγRII, FcγRIII and FcRn have been mapped and variants with improved binding have been described (*see* Shields *et al.* (2001) *J. Biol. Chem.* 276:6591-6604).

In one embodiment of the invention, the Fc region is modified to decrease the ability of the antibody of the invention (*e.g.*, antibody 27A and humanized versions thereof) to mediate effector function and/or to increase anti-inflammatory properties by modifying residues 243 and 264. In one embodiment, the Fc region of the antibody or fragment is modified by changing the residues at positions 243 and 264 to alanine. In

one embodiment, the Fc region is modified to decrease the ability of the antibody or fragment to mediate effector function and/or to increase anti-inflammatory properties by modifying residues 243, 264, 267 and 328.

Effector Function Enhancement

In some embodiments, the Fc region of an anti-CTLA-4 antibody is modified to increase the ability of the antibody or antigen-binding fragment to mediate effector function and/or to increase their binding to the Fcgamma receptors (FcγRs).

The term "Effector Function" as used herein is meant to refer to one or more of Antibody Dependent Cell-mediated Cytotoxic activity (ADCC), Complement-dependent cytotoxic activity (CDC) mediated responses, Fc-mediated phagocytosis or antibody dependent cellular phagocytosis (ADCP) and antibody recycling via the FcRn receptor.

The interaction between the constant region of an antigen binding protein and various Fc receptors (FcR) including FcgammaRI (CD64), FcgammaRII (CD32) and FcgammaRIII (CD16) is believed to mediate the effector functions, such as ADCC and CDC, of the antigen binding protein. The Fc receptor is also important for antibody cross-linking, which can be important for anti-tumor immunity.

Effector function can be measured in a number of ways including for example via binding of the FcgammaRIII to Natural Killer cells or via FcgammaRI to monocytes/macrophages to measure for ADCC effector function. For example an antigen binding protein of the present invention can be assessed for ADCC effector function in a Natural Killer cell assay. Examples of such assays can be found in *Shields et al, 2001 J. Biol. Chem., Vol. 276, p 6591-6604; Chappel et al, 1993 J. Biol. Chem., Vol 268, p 25124-25131; Lazar et al, 2006 PNAS, 103; 4005-4010.*

The ADCC or CDC properties of antibodies of the present invention, or their cross-linking properties, may be enhanced in a number of ways.

Human IgG1 constant regions containing specific mutations or altered glycosylation on residue Asn297 have been shown to enhance binding to Fc receptors. In some cases these mutations have also been shown to enhance ADCC and CDC (*Lazar et al. PNAS 2006, 103; 4005-4010; Shields et al. J Biol Chem 2001, 276; 6591-6604; Nechansky et al. Mol Immunol, 2007, 44; 1815-1817*).

In one embodiment of the present invention, such mutations are in one or more of positions selected from 239, 332 and 330 (IgG1), or the equivalent positions in other IgG isotypes. Examples of suitable mutations are S239D and I332E and A330L. In one embodiment, the antigen binding protein of the invention herein described is mutated at positions 239 and 332, for example S239D and I332E or in a further embodiment it is mutated at three or more positions selected from 239 and 332 and 330, for example S239D and I332E and A330L (EU index numbering).

In an alternative embodiment of the present invention, there is provided an antibody comprising a heavy chain constant region with an altered glycosylation profile such that the antigen binding protein has enhanced effector function. For example, wherein the antibody has enhanced ADCC or enhanced CDC or wherein it has both enhanced ADCC and CDC effector function. Examples of suitable methodologies to produce antigen binding proteins with an altered glycosylation profile are described in WO2003011878, WO2006014679 and EP1229125.

In a further aspect, the present invention provides "non-fucosylated" or "afucosylated" antibodies. Non-fucosylated antibodies harbour a tri-mannosyl core structure of complex-type N-glycans of Fc without fucose residue. These glycoengineered antibodies that lack core fucose residue from the Fc N-glycans may exhibit stronger ADCC than fucosylated equivalents due to enhancement of FcgammaRIIIa binding capacity.

The present invention also provides a method for the production of an antibody according to the invention comprising the steps of: a) culturing a recombinant host cell comprising an expression vector comprising the isolated nucleic acid as described herein, wherein the recombinant host cell does not comprise an alpha-1,6-fucosyltransferase; and b) recovering the antigen binding protein. The recombinant host cell may be not normally contain a gene encoding an alpha-1,6-fucosyltransferase (for example yeast host cells such as Pichia sp.) or may have been genetically modified to inactive an alpha-1,6-fucosyltransferase. Recombinant host cells which have been genetically modified to inactivate the FUT8 gene encoding an alpha-1,6-fucosyltransferase are available. See, e.g., the POTELLIGENT™ technology system available from BioWa, Inc. (Princeton, N.J.) in which CHOK1SV cells lacking a functional copy of the FUT8 gene produce monoclonal antibodies having enhanced antibody dependent cell mediated cytotoxicity (ADCC) activity that is increased relative

to an identical monoclonal antibody produced in a cell with a functional FUT8 gene. Aspects of the POTELLIGENT™ technology system are described in US7214775, US6946292, WO0061739 and WO0231240. Those of ordinary skill in the art will also recognize other appropriate systems.

It will be apparent to those skilled in the art that such modifications may not only be used alone but may be used in combination with each other in order to further enhance effector function.

Production of Antibodies with Modified Glycosylation

In still another embodiment, the antibodies or antigen-binding fragments of the invention (*e.g.*, antibody 27A and humanized versions thereof) comprise a particular glycosylation pattern. For example, an afucosylated or an aglycosylated antibody or fragment can be made (*i.e.*, the antibody lacks fucose or glycosylation, respectively). The glycosylation pattern of an antibody or fragment may be altered to, for example, increase the affinity or avidity of the antibody or fragment for a CTLA-4 antigen. Such modifications can be accomplished by, for example, altering one or more of the glycosylation sites within the antibody or fragment sequence. For example, one or more amino acid substitutions can be made that result removal of one or more of the variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity or avidity of the antibody or fragment for antigen. *See*, *e.g.*, U.S. Patent Nos. 5,714,350 and 6,350,861.

Antibodies and antigen-binding fragments disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may further include those produced in lower eukaryote host cells, in particular fungal host cells such as yeast and filamentous fungi have been genetically engineered to produce glycoproteins that have mammalian- or human-like glycosylation patterns (See for example, Choi *et al*, (2003) *Proc. Natl. Acad. Sci.* 100: 5022-5027; Hamilton *et al.*, (2003) *Science* 301: 1244-1246; Hamilton *et al.*, (2006) *Science* 313: 1441-1443; Nett et al., *Yeast* 28(3):237-52 (2011); Hamilton et al., *Curr Opin Biotechnol.* Oct;18(5):387-92 (2007)). A particular advantage of these genetically modified host cells over currently used mammalian cell lines is the ability to control the glycosylation profile of glycoproteins that are produced in the cells such that compositions of glycoproteins can be produced wherein a particular N-glycan structure predominates (see, *e.g.*, U.S. Patent No. 7,029,872 and U.S. Patent No. 7,449,308).

These genetically modified host cells have been used to produce antibodies that have predominantly particular *N*-glycan structures (See for example, Li *et al.*, (2006) *Nat. Biotechnol.* 24: 210-215).

In particular embodiments, the antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) further include those produced in lower eukaryotic host cells and which comprise fucosylated and non-fucosylated hybrid and complex *N*-glycans, including bisected and multiantennary species, including but not limited to *N*-glycans such as $GlcNAc_{(1-4)}Man_3GlcNAc_2$; $Gal_{(1-4)}GlcNAc_{(1-4)}Man_3GlcNAc_2$; $NANA_{(1-4)}Gal_{(1-4)}GlcNAc_{(1-4)}Man_3GlcNAc_2$.

In particular embodiments, the antibodies and antigen-binding fragments thereof provided herein (*e.g.*, antibody 27A and humanized versions thereof) may comprise antibodies or fragments having at least one hybrid *N*-glycan selected from the group consisting of $GlcNAcMan_5GlcNAc_2$; $GalGlcNAcMan_5GlcNAc_2$; and $NANAGalGlcNAcMan_5GlcNAc_2$. In particular aspects, the hybrid *N*-glycan is the predominant *N*-glycan species in the composition.

In particular embodiments, the antibodies and antigen-binding fragments thereof provided herein (*e.g.*, antibody 27A and humanized versions thereof) comprise antibodies and fragments having at least one complex *N*-glycan selected from the group consisting of $GlcNAcMan_3GlcNAc_2$; $GalGlcNAcMan_3GlcNAc_2$; $NANAGalGlcNAcMan_3GlcNAc_2$; $GlcNAc_2Man_3GlcNAc_2$; $GalGlcNAc_2Man_3GlcNAc_2$; $Gal_2GlcNAc_2Man_3GlcNAc_2$; $NANAGal_2GlcNAc_2Man_3GlcNAc_2$; and $NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$. In particular aspects, the complex *N*-glycan are the predominant *N*-glycan species in the composition. In further aspects, the complex *N*-glycan is a particular *N*-glycan species that comprises about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98%, 99%, or 100% of the complex *N*-glycans in the composition. In one embodiment, the antibody and antigen binding fragments thereof provided herein comprise complex N-glycans, wherein at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98%, 99%, or 100% of the complex *N*-glycans in comprise the structure $NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$, wherein such structure is afucosylated. Such structures can be produced, e.g., in engineered *Pichia pastoris* host cells.

In particular embodiments, the *N*-glycan is fucosylated. In general, the fucose is in an α1,3-linkage with the GlcNAc at the reducing end of the *N*-glycan, an

α1,6-linkage with the GlcNAc at the reducing end of the *N*-glycan, an α1,2-linkage with the Gal at the non-reducing end of the *N*-glycan, an α1,3-linkage with the GlcNac at the non-reducing end of the *N*-glycan, or an α1,4-linkage with a GlcNAc at the non-reducing end of the *N*-glycan.

Therefore, in particular aspects of the above the glycoprotein compositions, the glycoform is in an α1,3-linkage or α1,6-linkage fucose to produce a glycoform selected from the group consisting of $Man_5GlcNAc_2(Fuc)$, $GlcNAcMan_5GlcNAc_2(Fuc)$, $Man_3GlcNAc_2(Fuc)$, $GlcNAcMan_3GlcNAc_2(Fuc)$, $GlcNAc_2Man_3GlcNAc_2(Fuc)$, $GalGlcNAc_2Man_3GlcNAc_2(Fuc)$, $Gal_2GlcNAc_2Man_3GlcNAc_2(Fuc)$, $NANAGal_2GlcNAc_2Man_3GlcNAc_2(Fuc)$, and $NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2(Fuc)$; in an α1,3-linkage or α1,4-linkage fucose to produce a glycoform selected from the group consisting of $GlcNAc(Fuc)Man_5GlcNAc_2$, $GlcNAc(Fuc)Man_3GlcNAc_2$, $GlcNAc_2(Fuc_{1-2})Man_3GlcNAc_2$, $GalGlcNAc_2(Fuc_{1-2})Man_3GlcNAc_2$, Gal2GlcNAc2(Fuc1-2)Man3GlcNAc2, $NANAGal2GlcNAc2(Fuc_{1-2})Man_3GlcNAc_2$, and $NANA_2Gal_2GlcNAc_2(Fuc_{1-2})Man_3GlcNAc_2$; or in an α1,2-linkage fucose to produce a glycoform selected from the group consisting of $Gal(Fuc)GlcNAc_2Man_3GlcNAc_2$, $Gal_2(Fuc_{1-2})GlcNAc_2Man_3GlcNAc_2$, $NANAGal_2(Fuc_{1-2})GlcNAc_2Man_3GlcNAc_2$, and $NANA_2Gal_2(Fuc_{1-2})GlcNAc_2Man_3GlcNAc_2$.

In further aspects, the antibodies (*e.g.*, humanized antibodies) or antigen-binding fragments thereof comprise high mannose *N*-glycans, including but not limited to, $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, $Man_5GlcNAc_2$, $Man_4GlcNAc_2$, or *N*-glycans that consist of the $Man_3GlcNAc_2$ *N*-glycan structure.

In further aspects of the above, the complex *N*-glycans further include fucosylated and non-fucosylated bisected and multiantennary species.

As used herein, the terms "*N*-glycan" and "glycoform" are used interchangeably and refer to an *N*-linked oligosaccharide, for example, one that is attached by an asparagine-*N*-acetylglucosamine linkage to an asparagine residue of a polypeptide. *N*-linked glycoproteins contain an *N*-acetylglucosamine residue linked to the amide nitrogen of an asparagine residue in the protein. The predominant sugars found on glycoproteins are glucose, galactose, mannose, fucose, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) and sialic acid (*e.g.*, *N*-acetyl-neuraminic acid (NANA)). The processing of the sugar groups occurs co-translationally in the

lumen of the ER and continues post-translationally in the Golgi apparatus for *N*-linked glycoproteins.

N-glycans have a common pentasaccharide core of $Man_3GlcNAc_2$ ("Man" refers to mannose; "Glc" refers to glucose; and "NAc" refers to *N*-acetyl; GlcNAc refers to *N*-acetylglucosamine). Usually, *N*-glycan structures are presented with the non-reducing end to the left and the reducing end to the right. The reducing end of the *N*-glycan is the end that is attached to the Asn residue comprising the glycosylation site on the protein. *N*-glycans differ with respect to the number of branches (antennae) comprising peripheral sugars (*e.g.*, GlcNAc, galactose, fucose and sialic acid) that are added to the $Man_3GlcNAc_2$ ("Man3") core structure which is also referred to as the "trimannose core", the "pentasaccharide core" or the "paucimannose core". *N*-glycans are classified according to their branched constituents (*e.g.*, high mannose, complex or hybrid). A "high mannose" type *N*-glycan has five or more mannose residues. A "complex" type *N*-glycan typically has at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of a "trimannose" core. Complex *N*-glycans may also have galactose ("Gal") or *N*-acetylgalactosamine ("GalNAc") residues that are optionally modified with sialic acid or derivatives (*e.g.*, "NANA" or "NeuAc", where "Neu" refers to neuraminic acid and "Ac" refers to acetyl). Complex *N*-glycans may also have intrachain substitutions comprising "bisecting" GlcNAc and core fucose ("Fuc"). Complex *N*-glycans may also have multiple antennae on the "trimannose core," often referred to as "multiple antennary glycans." A "hybrid" N-glycan has at least one GlcNAc on the terminal of the 1,3 mannose arm of the trimannose core and zero or more mannoses on the 1,6 mannose arm of the trimannose core. The various N-glycans are also referred to as "glycoforms."

With respect to complex *N*-glycans, the terms "G-2", "G-1", "G0", "G1", "G2", "A1", and "A2" mean the following. "G-2" refers to an *N*-glycan structure that can be characterized as $Man_3GlcNAc_2$; the term "G-1" refers to an *N*-glycan structure that can be characterized as $GlcNAcMan_3GlcNAc_2$; the term "G0" refers to an *N*-glycan structure that can be characterized as $GlcNAc_2Man_3GlcNAc_2$; the term "G1" refers to an *N*-glycan structure that can be characterized as $GalGlcNAc_2Man_3GlcNAc_2$; the term "G2" refers to an *N*-glycan structure that can be characterized as $Gal_2GlcNAc_2Man_3GlcNAc_2$; the term "A1" refers to an *N*-glycan structure that can be

characterized as $NANAGal_2GlcNAc_2Man_3GlcNAc_2$; and, the term "A2" refers to an *N*-glycan structure that can be characterized as $NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$. Unless otherwise indicated, the terms G-2", "G-1", "G0", "G1", "G2", "A1", and "A2" refer to *N*-glycan species that lack fucose attached to the GlcNAc residue at the reducing end of the *N*-glycan. When the term includes an "F", the "F" indicates that the *N*-glycan species contains a fucose residue on the GlcNAc residue at the reducing end of the *N*-glycan. For example, G0F, G1F, G2F, A1F, and A2F all indicate that the *N*-glycan further includes a fucose residue attached to the GlcNAc residue at the reducing end of the *N*-glycan. Lower eukaryotes such as yeast and filamentous fungi do not normally produce *N*-glycans that produce fucose.

With respect to multiantennary *N*-glycans, the term "multiantennary *N*-glycan" refers to *N*-glycans that further comprise a GlcNAc residue on the mannose residue comprising the non-reducing end of the 1,6 arm or the 1,3 arm of the *N*-glycan or a GlcNAc residue on each of the mannose residues comprising the non-reducing end of the 1,6 arm and the 1,3 arm of the *N*-glycan. Thus, multiantennary *N*-glycans can be characterized by the formulas $GlcNAc_{(2-4)}Man_3GlcNAc_2$, $Gal_{(1-4)}GlcNAc_{(2-4)}Man_3GlcNAc_2$, or $NANA_{(1-4)}Gal_{(1-4)}GlcNAc_{(2-4)}Man_3GlcNAc_2$. The term "1-4" refers to 1, 2, 3, or 4 residues.

With respect to bisected *N*-glycans, the term "bisected *N*-glycan" refers to *N*-glycans in which a GlcNAc residue is linked to the mannose residue at the reducing end of the *N*-glycan. A bisected *N*-glycan can be characterized by the formula $GlcNAc_3Man_3GlcNAc_2$ wherein each mannose residue is linked at its non-reducing end to a GlcNAc residue. In contrast, when a multiantennary *N*-glycan is characterized as $GlcNAc_3Man_3GlcNAc_2$, the formula indicates that two GlcNAc residues are linked to the mannose residue at the non-reducing end of one of the two arms of the *N*-glycans and one GlcNAc residue is linked to the mannose residue at the non-reducing end of the other arm of the *N*-glycan.

Antibody Physical Properties

The antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may further contain one or more glycosylation sites in either the light or heavy chain immunoglobulin variable region.

Such glycosylation sites may result in increased immunogenicity of the antibody or fragment or an alteration of the pK of the antibody due to altered antigen-binding (Marshall *et al.* (1972) *Annu Rev Biochem* 41:673-702; Gala and Morrison (2004) *J Immunol* 172:5489-94; Wallick *et al* (1988) *J Exp Med* 168:1099-109; Spiro (2002) *Glycobiology* 12:43R-56R; Parekh *et al* (1985) *Nature* 316:452-7; Mimura *et al.* (2000) *Mol Immunol* 37:697-706). Glycosylation has been known to occur at motifs containing an N-X-S/T sequence.

Each antibody or antigen-binding fragment (*e.g.*, 27A or humanized versions thereof) will have a unique isoelectric point (pI), which generally falls in the pH range between 6 and 9.5. The pI for an IgG1 antibody typically falls within the pH range of 7-9.5 and the pI for an IgG4 antibody typically falls within the pH range of 6-8.

Each antibody or antigen-binding fragment (*e.g.*, 27A or humanized versions thereof) will have a characteristic melting temperature, with a higher melting temperature indicating greater overall stability *in vivo* (Krishnamurthy R and Manning MC (2002) *Curr Pharm Biotechnol* 3:361-71). In general, the T_{M1} (the temperature of initial unfolding) may be greater than 60°C, greater than 65°C, or greater than 70°C. The melting point of an antibody or fragment can be measured using differential scanning calorimetry (Chen *et al* (2003) *Pharm Res* 20:1952-60; Ghirlando *et al* (1999) *Immunol Lett* 68:47-52) or circular dichroism (Murray *et al.* (2002) *J. Chromatogr Sci* 40:343-9).

In a further embodiment, antibodies and antigen-binding fragments thereof (*e.g.*, antibody 27A and humanized versions thereof) are selected that do not degrade rapidly. Degradation of an antibody or fragment can be measured using capillary electrophoresis (CE) and MALDI-MS (Alexander AJ and Hughes DE (1995) *Anal Chem* 67:3626-32).

In a further embodiment, antibodies (*e.g.*, antibody 27A and humanized versions thereof) and antigen-binding fragments thereof are selected that have minimal aggregation effects, which can lead to the triggering of an unwanted immune response and/or altered or unfavorable pharmacokinetic properties. Generally, antibodies and fragments are acceptable with aggregation of 25% or less, 20% or less, 15% or less, 10% or less, or 5% or less. Aggregation can be measured by several techniques, including size-exclusion column (SEC), high performance liquid chromatography (HPLC), and light scattering.

Antibody Conjugates

The anti-CTLA-4 antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may also be conjugated to a chemical moiety. The chemical moiety may be, *inter alia*, a polymer, a radionuclide or a cytotoxic factor. In particular embodiments, the chemical moiety is a polymer which increases the half-life of the antibody or fragment in the body of a subject. Suitable polymers include, but are not limited to, hydrophilic polymers which include but are not limited to polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2kDa, 5 kDa, 10 kDa, 12kDa, 20 kDa, 30kDa or 40kDa), dextran and monomethoxypolyethylene glycol (mPEG). Lee, *et al.*, (1999) (*Bioconj. Chem.* 10:973-981) discloses PEG conjugated single-chain antibodies. Wen, *et al.*, (2001) (*Bioconj. Chem.* 12:545-553) disclose conjugating antibodies with PEG which is attached to a radiometal chelator (diethylenetriaminpentaacetic acid (DTPA)).

The antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may also be conjugated with labels such as ^{99}Tc, ^{90}Y, ^{111}In, ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{35}S, ^{51}Cr, ^{57}To, ^{226}Ra, ^{60}Co, ^{59}Fe, ^{57}Se, ^{152}Eu, ^{67}CU, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{234}Th, and ^{40}K, ^{157}Gd, ^{55}Mn, ^{52}Tr, and ^{56}Fe.

The antibodies and antigen-binding fragments disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may also be PEGylated, for example to increase its biological (*e.g.,* serum) half-life. To PEGylate an antibody or fragment, the antibody or fragment, typically is reacted with a reactive form of polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. In particular embodiments, the PEGylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. In certain embodiments, the antibody or fragment to be PEGylated is an aglycosylated antibody or fragment. Methods for PEGylating proteins are known in the art and can be applied to the antibodies of the invention. *See, e.g.*, EP 0 154 316 and EP 0 401 384.

The antibodies and antigen-binding fragments disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may also be conjugated with fluorescent or chemilluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthaladehyde, fluorescamine, ^{152}Eu, dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridimium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels and stable free radicals.

The antibodies and antigen-binding fragments thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) may also be conjugated to a cytotoxic factor such as diptheria toxin, *Pseudomonas aeruginosa* exotoxin A chain , ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins and compounds (*e.g.*, fatty acids), dianthin proteins, *Phytoiacca americana* proteins PAPI, PAPII, and PAP-S, *momordica charantia* inhibitor, curcin, crotin, *saponaria officinalis* inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

Any method known in the art for conjugating the antibodies and antigen-binding fragments thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) to the various moieties may be employed, including those methods described by Hunter, *et al.*, (1962) *Nature* 144:945; David, *et al.*, (1974) *Biochemistry* 13:1014; Pain, *et al.*, (1981) J. Immunol. Meth. 40:219; and Nygren, J., (1982) *Histochem. and Cytochem.* 30:407. Methods for conjugating antibodies and fragments are conventional and very well known in the art.

Antibodies or other polypeptides may be immobilized onto a variety of solid supports for use in assays. Solid phases that may be used to immobilize specific binding members include those developed and/or used as solid phases in solid phase binding assays. Examples of suitable solid phases include membrane filters, cellulose-based papers, beads (including polymeric, latex and paramagnetic particles), glass, silicon wafers, microparticles, nanoparticles, TentaGels, AgroGels, PEGA gels, SPOCC gels, and multiple-well plates. An assay strip could be prepared by coating the antibody or a plurality of antibodies in an array on solid support. This strip could then be dipped into the test sample and then processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot. Antibodies or other polypeptides

may be bound to specific zones of assay devices either by conjugating directly to an assay device surface, or by indirect binding. In an example of the later case, antibodies or other polypeptides may be immobilized on particles or other solid supports, and that solid support immobilized to the device surface.

Biological assays require methods for detection, and one of the most common methods for quantitation of results is to conjugate a detectable label to a protein or nucleic acid that has affinity for one of the components in the biological system being studied. Detectable labels may include molecules that are themselves detectable (*e.g.*, fluorescent moieties, electrochemical labels, metal chelates, *etc.*) as well as molecules that may be indirectly detected by production of a detectable reaction product (*e.g.*, enzymes such as horseradish peroxidase, alkaline phosphatase, *etc.*) or by a specific binding molecule which itself may be detectable (e.g., biotin, digoxigenin, maltose, oligohistidine, 2,4-dinitrobenzene, phenylarsenate, ssDNA, dsDNA, etc.).

Preparation of solid phases and detectable label conjugates often comprise the use of chemical cross-linkers. Cross-linking reagents contain at least two reactive groups, and are divided generally into homofunctional cross-linkers (containing identical reactive groups) and heterofunctional cross-linkers (containing non-identical reactive groups). Homobifunctional cross-linkers that couple through amines, sulfhydryls or react non- specifically are available from many commercial sources. Maleimides, alkyl and aryl halides, alpha-haloacyls and pyridyl disulfides are thiol reactive groups. Maleimides, alkyl and aryl halides, and alpha-haloacyls react with sulfhydryls to form thiol ether bonds, while pyridyl disulfides react with sulfhydryls to produce mixed disulfides. The pyridyl disulfide product is cleavable. Imidoesters are also very useful for protein-protein cross-links. A variety of heterobifunctional cross-linkers, each combining different attributes for successful conjugation, are commercially available.

Therapeutic Uses of Anti-CTLA-4 antibodies

Further provided are methods for treating subjects, including human subjects, in need of treatment with the isolated antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof). In one embodiment of the invention, such subject suffers from an infection or an infectious disease. In another embodiment of the invention, such subject suffers from cancer. In

one embodiment the cancer is , *e.g.*, osteosarcoma, rhabdomyosarcoma, neuroblastoma, kidney cancer, leukemia, renal transitional cell cancer, bladder cancer, Wilm's cancer, ovarian cancer, pancreatic cancer, breast cancer, prostate cancer, bone cancer, lung cancer (*e.g.*, non-small cell lung cancer), gastric cancer, colorectal cancer, cervical cancer, synovial sarcoma, head and neck cancer, squamous cell carcinoma, multiple myeloma, renal cell cancer, retinoblastoma, hepatoblastoma, hepatocellular carcinoma, melanoma, rhabdoid tumor of the kidney, Ewing's sarcoma, chondrosarcoma, brain cancer, glioblastoma, meningioma, pituitary adenoma, vestibular schwannoma, a primitive neuroectodermal tumor, medulloblastoma, astrocytoma, anaplastic astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, polycythemia vera, thrombocythemia, idiopathic myelfibrosis, soft tissue sarcoma, thyroid cancer, endometrial cancer, carcinoid cancer or liver cancer, breast cancer or gastric cancer. In an embodiment of the invention, the cancer is metastatic cancer, *e.g.*, of the varieties described above.

In an embodiment, the invention provides methods for treating subjects using an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof), wherein the subject suffers from a viral infection. In one embodiment, the viral infection is infection with a virus selected from the group consisting of human immunodeficiency virus (HIV), hepatitis virus (A, B, or C), herpes virus (*e.g.*, VZV, HSV-I, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, coronavirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus or arboviral encephalitis virus.

In an embodiment, the invention provides methods for treating subjects using an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention, wherein the subject suffers from a bacterial infection. In one embodiment, the bacterial infection is infection with a bacteria selected from the group consisting of *Chlamydia trachomatis*, rickettsial bacteria such as *Ehrlichia, Orientia* and *Ricekettsia*, mycobacteria, such as *Mycobacterium leprae*, or *Mycobacterium lepromatosis*, staphylococci, such as *Staphylococcus aureus*, streptococci, pneumonococci, meningococci and gonococci, *Klebsiella*, *Proteus, Serratia, Pseudomonas*, *Legionella*, *Corynebacterium diphtheriae*, *Salmonella*, bacilli, *Vibrio cholerae*, *Clostridium tetan*,

Clostridium botulinum, *Bacillus anthracis*, *Yersinia pestis*, *Haemophilus influenza*, *Actinomyces*, *Leptospira*, *Treponema*, *Shigella*, *Chlamydophila psittaci* and *Borriella*.

In an embodiment, the invention provides methods for treating subjects using an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention, wherein the subject suffers from a fungal infection. In one embodiment, the fungal infection is infection with a fungus selected from the group consisting of *Candida* (*albicans*, *krusei*, *glabrata*, *tropicalis*, *etc.*), *Cryptococcus neoformans*, *Aspergillus* (*fumigatus*, *niger*, *etc.*), Genus *Mucorales* (*mucor*, *absidia*, *rhizopus*), *Sporothrix schenkii*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis* and *Histoplasma capsulatum*.

In an embodiment, the invention provides methods for treating subjects using an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention, wherein the subject suffers from a parasitic infection. In one embodiment, the parasitic infection is infection with a parasite selected from the group consisting of *Entamoeba histolytica*, *Balantidium coli*, *Naegleria fowleri*, *Acanthamoeba*, *Giardia lambia*, *Cryptosporidium*, *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii* and *Nippostrongylus brasiliensis*.

A "subject" may be a mammal such as a human, dog, cat, horse, cow, mouse, rat, monkey (*Macaca fascicularis* (cynomolgus monkey)) or rabbit. In preferred embodiments of the invention, the subject is a human subject.

In certain embodiments, the methods and compositions described herein are administered in combination with one or more of other antibody molecules, chemotherapy, other anti-cancer therapy (e.g., targeted anti-cancer therapies, gene therapy, viral therapy, RNA therapy bone marrow transplantation, nanotherapy, or oncolytic drugs), cytotoxic agents, immune-based therapies (e.g., cytokines or cell-based immune therapies), surgical procedures (e.g., lumpectomy or mastectomy) or radiation procedures, or a combination of any of the foregoing. The additional therapy may be in the form of adjuvant or neoadjuvant therapy. In some embodiments, the additional therapy is an enzymatic inhibitor (e.g., a small molecule enzymatic inhibitor) or a metastatic inhibitor. Exemplary cytotoxic agents that can be administered in combination with include antimicrotubule agents, topoisomerase inhibitors, anti-metabolites, mitotic inhibitors, alkylating agents, anthracyclines, vinca alkaloids,

intercalating agents, agents capable of interfering with a signal transduction pathway, agents that promote apoptosis, proteosome inhibitors, and radiation (e.g., local or whole body irradiation (e.g., gamma irradiation). In other embodiments, the additional therapy is surgery or radiation, or a combination thereof. In other embodiments, the additional therapy is a therapy targeting one or more of PI3K/AKT/mTOR pathway, an HSP90 inhibitor, or a tubulin inhibitor. Alternatively, or in combination with the aforesaid combinations, the methods and compositions described herein can be administered in combination with one or more of: an immunomodulator (e.g., an activator of a costimulatory molecule or an inhibitor of an inhibitory molecule, e.g., an immune checkpoint molecule); a vaccine, e.g., a therapeutic cancer vaccine; or other forms of cellular immunotherapy.

In particular embodiments, the antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A or humanized versions thereof) may be used alone, or in association with other, further therapeutic agents and/or therapeutic procedures, for treating or preventing any disease such as cancer, *e.g.*, as discussed herein, in a subject in need of such treatment or prevention. Compositions, *e.g.*, pharmaceutical compositions comprising a pharmaceutically acceptable carrier, comprising such antibodies and fragments in association with further therapeutic agents are also part of the present invention.

In particular embodiments, the antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may be used alone, or in association with tumor vaccines.

In particular embodiments, the antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may be used alone, or in association with chemotherapeutic agents.

In particular embodiments, the antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may be used alone, or in association with radiation therapy.

In particular embodiments, the antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may be used alone, or in association with targeted therapies. Examples of targeted therapies include: hormone therapies, signal transduction inhibitors (e.g., EGFR inhibitors, such as cetuximab (Erbitux) and erlotinib (Tarceva)); HER2 inhibitors (e.g., trastuzumab

(Herceptin) and pertuzumab (Perjeta)); BCR-ABL inhibitors (such as imatinib (Gleevec) and dasatinib (Sprycel)); ALK inhibitors (such as crizotinib (Xalkori) and ceritinib (Zykadia)); BRAF inhibitors (such as vemurafenib (Zelboraf) and dabrafenib (Tafinlar)), gene expression modulators, apoptosis inducers (e.g., bortezomib (Velcade) and carfilzomib (Kyprolis)), angiogenesis inhibitors (e.g., bevacizumab (Avastin) and ramucirumab (Cyramza) , monoclonal antibodies attached to toxins (e.g., brentuximab vedotin (Adcetris) and ado-trastuzumab emtansine (Kadcyla)).

In particular embodiments, the anti-CTLA-4 antibodies or antigen-binding fragments thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) may be used in combination with an anti-cancer therapeutic agent or immunomodulatory drug such as an immunomodulatory receptor inhibitor, *e.g.*, an antibody or antigen-binding fragment thereof that specifically binds to the receptor.

Thus, the present invention includes compositions comprising an anti-CTLA-4 antibody or antigen-binding fragment thereof of the present invention (*e.g.*, antibody 27A and humanized versions thereof) in association with one or more of PD-1/PD-L1 blocking antibodies: pembrolizumab, nivolumab, pidilizumab, REGN2810, MEDI-0680, PDR-001, SHR-1210, BGB-A317, PF-06801591, TSR-042, atezoluzimab, durvalumab, BMS-936559; as well as methods for treating or preventing cancer in a subject comprising administering an effective amount of the anti-CTLA-4 antibody or antigen-binding fragment thereof and one or more of pembrolizumab, nivolumab, pidilizumab, REGN2810 to the subject. Optionally, the subject is also administered a further therapeutic agent.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the present invention (*e.g.*, antibody 27A and humanized versions thereof) is in association with an isolated antibody encoding the heavy and light chain of pembrolizumab.

In an embodiment of the invention, an anti-CTLA-4 antibody) or antigen-binding fragment thereof of the present invention (*e.g.*, antibody 27A and humanized versions thereof) is in association with an isolated antibody encoding the heavy and light chain of nivolumab.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) is in association with one or more of: anti-PD1 antibody (*e.g.*, pembrolizumab,

nivolumab, pidilizumab (CT-011)), anti-PDL1 antibody, anti-TIGIT antibody, anti-CD27 antibody, anti-CS1 antibody (*e.g.*, elotuzumab), anti-KIR2DL1/2/3 antibody (*e.g.*, lirilumab), anti-CD137 antibody (*e.g.*, urelumab), anti-GITR antibody (*e.g.*, TRX518), anti-PD-L1 antibody (*e.g.*, BMS-936559, MSB0010718C or MPDL3280A), anti-PD-L2 antibody, anti-ILT1 antibody, anti-ILT2 antibody, anti-ILT3 antibody, anti-ILT4 antibody, anti-ILT5 antibody, anti-ILT6 antibody, anti-ILT7 antibody, anti-ILT8 antibody, anti-CD40 antibody, anti-OX40 antibody, anti-ICOS, anti-SIRPα, anti-KIR2DL1 antibody, anti-KIR2DL2/3 antibody, anti-KIR2DL4 antibody, anti-KIR2DL5A antibody, anti-KIR2DL5B antibody, anti-KIR3DL1 antibody, anti-KIR3DL2 antibody, anti-KIR3DL3 antibody, anti-NKG2A antibody, anti-NKG2C antibody, anti-NKG2E antibody, anti-4-1BB antibody (e.g., PF-05082566), anti-TSLP antibody, anti-IL-10 antibody, anti-APRIL (e.g. BION1301), anti-CD38 (daratumumab), anti-IL-10 or PEGylated IL-10, or any small organic molecule inhibitor of such targets.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-PD1 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-PDL1 antibody (*e.g.*, BMS-936559, MSB0010718C or MPDL3280A).

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-CD27 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-CS1 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR2DL1/2/3 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-CD137 (*e.g.*, urelumab) antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-GITR (*e.g.*, TRX518) antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-PD-L2 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL1 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL2 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL3 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL4 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL5 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL6 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL7 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ITL8 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-CD40 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-OX40 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR2DL1 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR2DL2/3 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR2DL4 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR2DL5A antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR2DL5B antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR3DL1 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR3DL2 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-KIR3DL3 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-NKG2A antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-NKG2C antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-ICOS antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (e.g., antibody 27A or a humanized version thereof) is in association with an anti-SIRPα antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-4-1BB antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-IL-10 antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an anti-TSLP antibody.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with IL-10 or PEGylated IL-10.

In an embodiment of the invention, an anti-CTKLA-4 antibody or antigen-binding fragment thereof of the invention is in association with a Tim-3 pathway antagonist, preferably as part of a pharmaceutical composition.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention is in association with a Vista pathway antagonist, preferably as part of a pharmaceutical composition.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention is in association with a BTLA pathway antagonist, preferably as part of a pharmaceutical composition.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention is in association with a LAG-3 pathway antagonist, preferably as part of a pharmaceutical composition.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention is in association with a TIGIT pathway antagonist, preferably as part of a pharmaceutical composition.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention is in association with a STING agonist, preferably as part of a pharmaceutical composition.. The cyclic-di-nucleotides (CDNs) cyclic-di-AMP (produced by Listeria monocytogenes and other bacteria) and its analogs cyclic-di-GMP and cyclic-GMP-AMP are recognized by the host cell as a pathogen associated molecular pattern (PAMP), which bind to the pathogen recognition receptor (PRR) known as Stimulator of INterferon Genes (STING). STING is an adaptor protein in the cytoplasm of host mammalian cells which activates the TANK binding kinase (TBK1)—IRF3 and the NF-kappaB signaling axis, resulting in the induction of IFN-β and other gene products that strongly activate innate immunity. It is now recognized that STING is a component of the host cytosolic surveillance pathway (Vance et al., 2009), that senses infection with intracellular pathogens and in response induces the production of IFN-β, leading to the development of an adaptive protective pathogen-specific immune response consisting of both antigen-specific CD4+ and CD8+ T cells as well as pathogen-specific antibodies. Examples of cyclic purine dinucleotides are described in some detail in, for example: U.S. Patents 7,709458 and 7,592,326; patent applications WO2007/054279, WO2014/093936, and WO2014/189805; and Yan et al., Bioorg. Med. Chem Lett. 18: 5631 (2008).

In some embodiments, the antibodies or antigen binding fragments of the invention increase the activity of an immune cell. The increase of the activity of an immune cell can be detected using any method known in the art. In one embodiment, the increase in activity of an immune cell can be detected by measuring the proliferation of the immune cell. For example, an increase in activity of a T cell can be detected by measuring the proliferation of the T cell or signal transduction events such as tyrosine phosphorylation of immune receptors or downstream kinases that transmit signals to transcriptional regulators. In other embodiments, the increase in activity of an immune cell can be detected by measuring CTL or NK cell cytotoxic function on specific target cells or IFNγ cytokine responses, which are associated with stimulation of anti-tumor immunity. In yet other embodiments, the increase in activity of an immune cell can be detected by measuring T cell activation *ex vivo* in a sample derived from the subject. In one embodiment, the increase in T cell activity is determined by: (i) measuring SEB (Staphylococcus Enterotoxin B) induced production of one or more pro-inflammatory cytokines selected from the group consisting of: IL-2, TNFα, IL-17, IFNγ, IL-1β, GM-

CSF, RANTES, IL-6, IL-8, IL-5 and IL-13; or (ii) measuring mixed lymphocyte reactions or direct anti-CD3 mAb stimulation of T cell receptor (TCR) signaling to induce production of a cytokine selected from the group consisting of: IL-2, TNFα, IL-17, IFNγ, IL-1β, GM-CSF, RANTES, IL-6, IL-8, IL-5 and IL-13. In certain embodiments, the anti-CTLA-4 antibody or antigen binding fragment thereof of the present invention will stimulate antigen-specific T-cell production of IL-2 and/or IFNγ and/or upregulation of CD25 and/or CD69 by at least 1.5 fold. In certain embodiments, the anti-CTLA-4 antibody or antigen binding fragment thereof of the present invention will stimulate CD3+ T cells, when these are presented to Raji cells expressing CD80 and CD86, to produce pro-inflammatory cytokines selected from the group consisting of: IL-2, TNFα, IL-17, IFNγ, IL-1β, GM-CSF, RANTES, IL-6, IL-8, IL-5 and IL-13.

Additional agents which are beneficial to raising a cytolytic T cell response may be used in combination with the anti-CTLA-4 antibody or antigen binding fragment thereof of the present invention. These include, without limitation, B7 costimulatory molecule, interleukin-2, interferon-γ, GM-CSF, PD-1 antagonists, OX-40/OX-40 ligand, CD40/CD40 ligand, sargramostim, levamisol, vaccinia virus, Bacille Calmette-Guerin (BCG), liposomes, alum, Freund's complete or incomplete adjuvant, detoxified endotoxins, mineral oils, surface active substances such as lipolecithin, pluronic polyols, polyanions, peptides, and oil or hydrocarbon emulsions.

Compositions for inducing a T cell immune response which preferentially stimulate a cytolytic T cell response versus an antibody response are preferred, although those that stimulate both types of response can be used as well. In cases where the agent is a polypeptide, the polypeptide itself or a polynucleotide encoding the polypeptide can be administered. The carrier can be a cell, such as an antigen presenting cell (APC) or a dendritic cell. Antigen presenting cells include such cell types as macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used. Examples of facultative antigen-presenting cells include astrocytes, follicular cells, endothelium and fibroblasts.

The composition can comprise a bacterial cell that is transformed to express the polypeptide or to deliver a polynucleoteide which is subsequently expressed in cells of the vaccinated individual. Adjuvants, such as aluminum hydroxide or aluminum

phosphate, can be added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response.

The composition can comprise a bacterial cell that is transformed to express the polypeptide or to deliver a polynucleotide which is subsequently expressed in cells of the vaccinated individual. A number of bacterial species have been developed for use as vaccines and can be used as a vaccine platform in present invention, including, but not limited to, *Shigella flexneri, Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella typhimurium, Salmonella typhi or mycobacterium species*. This list is not meant to be limiting. The present invention contemplates the use of attenuated, commensal, and/or killed but metabolically active bacterial strains as vaccine platforms. In preferred embodiments the bacterium is *Listeria monocytogenes*.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention with an inactivated tumor cell vaccine. By "inactivated tumor cell vaccine" is meant a tumor cell (either "autologous" or "allogeneic" to the patient) which has been treated to prevent division of the cells. For purposes of the present invention, such cells preserve their immunogenicity and their metabolic activity. Such tumor cells are genetically modified to express a transgene which is expressed within a patient as part of cancer therapy. Thus, a composition or vaccine of the invention comprises neoplastic (e.g., tumor) cells that are autologous or allogeneic to the patient undergoing treatment and is most preferably the same general type of tumor cell as is afflicting the patient. For example, a patient suffering from melanoma will typically be administered a genetically modified cell derived from a melanoma. Methods for inactivating tumor cells for use in the present invention, such as the use of irradiation, are well known in the art.

In some embodiments, the inactivated tumor cells of the present invention are modified to express and secrete one or more heat shock proteins. For example, gp96-Ig fusion proteins can be expressed and secreted to stimulate an immune response (Yamazaki et al., The Journal of Immunology, 1999, 163:5178-5182; Strbo et al., Immunol Res. 2013 Dec;57(1-3):311-25). In some embodiments the inactivated tumor cells are modified to express and secrete a gp96-Ig fusion protein.

The inactivated tumor cells of the present invention are administered to the patient together with one or more costimulatory molecules or agents. A preferred costimulatory agent comprises one or more cytokines which stimulate dendritic cell

induction, recruitment, and/or maturation. Methods for assessing such costimulatory agents are well known in the literature. Induction and maturation of DCs is typically assessed by increased expression of certain membrane molecules such as CD80 and CD86, and/or secretion of pro-inflammatory cytokines, such as IL-12 and type I interferons following stimulation.

In preferred embodiments, the inactivated tumor cells themselves are modified to express and secrete one or more cytokines which stimulate dendritic cell induction, recruitment, and/or maturation. The present invention is described in exemplary terms with regard to the use of GM-CSF. Thus, by way of example, the tumor cell may express a transgene encoding GM-CSF as described in U.S. Pat. Nos. 5,637,483, 5,904,920, 6,277,368 and 6,350,445, as well as in US Patent Publication No. 20100150946. A form of GM-CSF-expressing genetically modified cancer cells or a "cytokine-expressing cellular vaccine" for the treatment of pancreatic cancer is described in U.S. Pat. Nos. 6,033,674 and 5,985,290.

Other suitable cytokines which may be expressed by such inactivated tumor cells and/or bystander cells instead of, or together with, GM-CSF include, but are not limited to, one or more of CD40 ligand, FLT-3 ligand, IL-12, CCL3, CCL20, and CCL21. This list is not meant to be limiting.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention is administered in conjunction with one or more vaccines intended to stimulate an immune response to one or more predetermined antigens. Examples of target antigens that may find use in the invention are listed in the following table. The target antigen may also be a fragment or fusion polypeptide comprising an immunologically active portion of the antigens listed in the table. This list is not meant to be limiting.

Table B. List of antigens for use in combination with the anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention as described herein

Antigen	Reference
Tumor antigens	
Mesothelin	GenBank Acc. No. NM_005823; U40434; NM_013404; BC003512 (see also, e.g., Hassan, *et al.* (2004) Clin. Cancer Res. 10:3937-3942; Muminova, *et al.* (2004) BMC Cancer 4:19; Iacobuzio-Donahue, *et al.* (2003) Cancer Res. 63:8614-8622).
Wilms' tumor-1 associated protein (Wt-1), including isoform A; isoform B; isoform C; isoform D.	WT-1 isoform A (GenBank Acc. Nos. NM_000378; NP_000369). WT-1 isoform B (GenBank Acc. Nos. NM_024424; NP_077742). WT-1 isoform C (GenBank Acc. Nos. NM_024425; NP_077743). WT-1 isoform D (GenBank Acc. Nos. NM_024426; NP_077744).
Stratum corneum chymotryptic enzyme (SCCE), and variants thereof.	GenBank Acc. No. NM_005046; NM_139277; AF332583. See also, e.g., Bondurant, *et al.* (2005) Clin. Cancer Res. 11:3446-3454; Santin, *et al.* (2004) Gynecol. Oncol. 94:283-288; Shigemasa, *et al.* (2001) Int. J. Gynecol. Cancer 11:454-461; Sepehr, *et al.* (2001) Oncogene 20:7368-7374.
MHC class I chain-related protein A (MICA); MHC class I chain-related protein B (MICB).	See, e.g., Groh, *et al.* (2005) Proc. Natl. Acad. Sci. USA 102:6461-6466; GenBank Acc. Nos. NM_000247; BC_016929; AY750850; NM_005931.
Gastrin and peptides derived from gastrin; gastrin/CCK-2 receptor (also known as CCK-B).	Harris, *et al.* (2004) Cancer Res. 64:5624-5631; Gilliam, *et al.* (2004) Eur. J. Surg. Oncol. 30:536-543; Laheru and Jaffee (2005) Nature Reviews Cancer 5:459-467.
Glypican-3 (an antigen of, e.g., hepatocellular carcinoma and melanoma).	GenBank Acc. No. NM_004484. Nakatsura, *et al.* (2003) Biochem. Biophys. Res. Commun. 306:16-25; Capurro, *et al.* (2003) Gasteroenterol. 125:89-97; Nakatsura, *et al.* (2004) Clin. Cancer Res. 10:6612-6621).

Antigen	Reference
Coactosin-like protein.	Nakatsura, *et al.* (2002) Eur. J. Immunol. 32:826-836; Laheru and Jaffee (2005) Nature Reviews Cancer 5:459-467.
Prostate stem cell antigen (PSCA).	GenBank Acc. No. AF043498; AR026974; AR302232 (see also, e.g., Argani, *et al.* (2001) Cancer Res. 61:4320-4324; Christiansen, *et al.* (2003) Prostate 55:9-19; Fuessel, *et al.* (2003) 23:221-228).
Prostate acid phosphatase (PAP); prostate-specific antigen (PSA); PSM; PSMA.	Small, *et al.* (2000) J. Clin. Oncol. 18:3894-3903; Altwein and Luboldt (1999) Urol. Int. 63:62-71; Chan, *et al.* (1999) Prostate 41:99-109; Ito, *et al.* (2005) Cancer 103:242-250; Schmittgen, *et al.* (2003) Int. J. Cancer 107:323-329; Millon, *et al.* (1999) Eur. Urol. 36:278-285.
Six-transmembrane epithelial antigen of prostate (STEAP).	See, e.g., Machlenkin, *et al.* (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. NM_018234; NM_001008410; NM_182915; NM_024636; NM_012449; BC011802.
Prostate carcinoma tumor antigen-1 (PCTA-1).	See, e.g., Machlenkin, *et al.* (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. L78132.
Prostate tumor-inducing gene-1 (PTI-1).	See, e.g., Machlenkin, *et al.* (2005) Cancer Res. 65:6435-6442).
Prostate-specific gene with homology to G protein-coupled receptor.	See, e.g., Machlenkin, *et al.* (2005) Cancer Res. 65:6435-6442).
Prostase (an antrogen regulated serine protease).	See, e.g., Machlenkin, *et al.* (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. BC096178; BC096176; BC096175.
Proteinase 3.	GenBank Acc. No. X55668.
Cancer-testis antigens, e.g., NY-ESO-1; SCP-1; SSX-1; SSX-2; SSX-4; GAGE, CT7; CT8; CT10; MAGE-1; MAGE-2; MAGE-3; MAGE-4; MAGE-6; LAGE-1.	GenBank Acc. No. NM_001327 (NY-ESO-1) (see also, e.g., Li, *et al.* (2005) Clin. Cancer Res. 11:1809-1814; Chen, *et al.* (2004) Proc. Natl. Acad. Sci. U S A. 101(25):9363-9368; Kubuschok, *et al.* (2004) Int. J. Cancer. 109:568-575; Scanlan, *et al.* (2004) Cancer Immun. 4:1; Scanlan, *et al.* (2002) Cancer Res. 62:4041-4047; Scanlan, *et al.* (2000) Cancer Lett. 150:155-164; Dalerba, *et al.* (2001) Int. J.

Antigen	Reference
	Cancer 93:85-90; Ries, *et al.* (2005) Int. J. Oncol. 26:817-824.
MAGE-A1, MAGE-A2; MAGE-A3; MAGE-A4; MAGE-A6; MAGE-A9; MAGE-A10; MAGE-A12; GAGE-3/6; NT-SAR-35; BAGE; CA125.	Otte, *et al.* (2001) Cancer Res. 61:6682-6687; Lee, *et al.* (2003) Proc. Natl. Acad. Sci. USA 100:2651-2656; Sarcevic, *et al.* (2003) Oncology 64:443-449; Lin, *et al.* (2004) Clin. Cancer Res. 10:5708-5716.
GAGE-1; GAGE-2; GAGE-3; GAGE-4; GAGE-5; GAGE-6; GAGE-7; GAGE-8; GAGE-65; GAGE-11; GAGE-13; GAGE-7B.	De Backer, *et al.* (1999) Cancer Res. 59:3157-3165; Scarcella, *et al.* (1999) Clin. Cancer Res. 5:335-341.
HIP1R; LMNA; KIAA1416; Seb4D; KNSL6; TRIP4; MBD2; HCAC5; MAGEA3.	Scanlan, *et al.* (2002) Cancer Res. 62:4041-4047.
DAM family of genes, e.g., DAM-1; DAM-6.	Fleishhauer, *et al.* (1998) Cancer Res. 58:2969-2972.
RCAS1.	Enjoji, *et al.* (2004) Dig. Dis. Sci. 49:1654-1656.
RU2.	Van Den Eynde, *et al.* (1999) J. Exp. Med. 190:1793-1800.
CAMEL.	Slager, *et al.* (2004) J. Immunol. 172:5095-5102; Slager, *et al.* (2004) Cancer Gene Ther. 11:227-236.
Colon cancer associated antigens, e.g., NY-CO-8; NY-CO-9; NY-CO-13; NY-CO-16; NY-CO-20;	Scanlan, *et al.* (2002) Cancer Res. 62:4041-4047.

Antigen	Reference
NY-CO-38; NY-CO-45; NY-CO-9/HDAC5; NY-CO-41/MBD2; NY-CO-42/TRIP4; NY-CO-95/KIAA1416; KNSL6; seb4D.	
N-Acetylglucosaminyl-tranferase V (GnT-V).	Dosaka-Akita, *et al.* (2004) Clin. Cancer Res. 10:1773-1779.
Elongation factor 2 mutated (ELF2M).	Renkvist, *et al.* (2001) Cancer Immunol Immunother. 50:3-15.
HOM-MEL-40/SSX2	Neumann, *et al.* (2004) Int. J. Cancer 112:661-668; Scanlan, *et al.* (2000) Cancer Lett. 150:155-164.
BRDT.	Scanlan, *et al.* (2000) Cancer Lett. 150:155-164.
SAGE; HAGE.	Sasaki, *et al.* (2003) Eur. J. Surg. Oncol. 29:900-903.
RAGE.	See, e.g., Li, *et al.* (2004) Am. J. Pathol. 164:1389-1397; Shirasawa, *et al.* (2004) Genes to Cells 9:165-174.
MUM-1 (melanoma ubiquitous mutated); MUM-2; MUM-2 Arg-Gly mutation; MUM-3.	Gueguen, *et al.* (1998) J. Immunol. 160:6188-6194; Hirose, *et al.* (2005) Int. J. Hematol. 81:48-57; Baurain, *et al.* (2000) J. Immunol. 164:6057-6066; Chiari, *et al.* (1999) Cancer Res. 59:5785-5792.
LDLR/FUT fusion protein antigen of melanoma.	Wang, *et al.* (1999) J. Exp. Med. 189:1659-1667.
NY-REN series of renal cancer antigens.	Scanlan, *et al.* (2002) Cancer Res. 62:4041-4047; Scanlan, *et al.* (1999) Cancer Res. 83:456-464.
NY-BR series of breast cancer antigens, e.g.,	Scanlan, *et al.* (2002) Cancer Res. 62:4041-4047; Scanlan, *et al.* (2001) Cancer Immunity 1:4.

Antigen	Reference
NY-BR-62; NY-BR-75; NY-BR-85; NY-BR-62; NY-BR-85.	
BRCA-1; BRCA-2.	Stolier, *et al.* (2004) Breast J. 10:475-480; Nicoletto, *et al.* (2001) Cancer Treat Rev. 27:295-304.
DEK/CAN fusion protein.	Von Lindern, *et al.* (1992) Mol. Cell. Biol. 12:1687-1697.
Ras, e.g., wild type ras, ras with mutations at codon 12, 13, 59, or 61, e.g., mutations G12C; G12D; G12R; G12S; G12V; G13D; A59T; Q61H. K-RAS; H-RAS; N-RAS.	GenBank Acc. Nos. P01112; P01116; M54969; M54968; P01111; P01112; K00654. See also, e.g., GenBank Acc. Nos. M26261; M34904; K01519; K01520; BC006499; NM_006270; NM_002890; NM_004985; NM_033360; NM_176795; NM_005343.
BRAF (an isoform of RAF).	Tannapfel, *et al.* (2005) Am. J. Clin. Pathol. 123:256-260l; Tsao and Sober (2005) Dermatol. Clin. 23:323-333.
Melanoma antigens, including HST-2 melanoma cell antigens.	GenBank Acc. No. NM_206956; NM_206955; NM_206954; NM_206953; NM_006115; NM_005367; NM_004988; AY148486; U10340; U10339; M77481. See, e g., Suzuki, *et al.* (1999) J. Immunol. 163:2783-2791.
Survivin	GenBank Acc. No. AB028869; U75285 (see also, e.g., Tsuruma, *et al.* (2004) J. Translational Med. 2:19 (11 pages); Pisarev, *et al.* (2003) Clin. Cancer Res. 9:6523-6533; Siegel, *et al.* (2003) Br. J. Haematol. 122:911-914; Andersen, *et al.* (2002) Histol. Histopathol. 17:669-675).
MDM-2	NM_002392; NM_006878 (see also, e.g., Mayo, *et al.* (1997) Cancer Res. 57:5013-5016; Demidenko and Blagosklonny (2004) Cancer Res. 64:3653-3660).

Antigen	Reference
Methyl-CpG-binding proteins (MeCP2; MBD2).	Muller, *et al.* (2003) Br. J. Cancer 89:1934-1939; Fang, *et al.* (2004) World J. Gastreenterol. 10:3394-3398.
NA88-A.	Moreau-Aubry, *et al.* (2000) J. Exp. Med. 191:1617-1624.
Histone deacetylases (HDAC), e.g., HDAC5.	Waltregny, *et al.* (2004) Eur. J. Histochem. 48:273-290; Scanlan, *et al.* (2002) Cancer Res. 62:4041-4047.
Cyclophilin B (Cyp-B).	Tamura, *et al.* (2001) Jpn. J. Cancer Res. 92:762-767.
CA 15-3; CA 27.29.	Clinton, *et al.* (2003) Biomed. Sci. Instrum. 39:408-414.
Heat shock protein Hsp70.	Faure, *et al.* (2004) Int. J. Cancer 108:863-870.
GAGE/PAGE family, e.g., PAGE-1; PAGE-2; PAGE-3; PAGE-4; XAGE-1; XAGE-2; XAGE-3.	Brinkmann, *et al.* (1999) Cancer Res. 59:1445-1448.
MAGE-A, B, C, and D families. MAGE-B5; MAGE-B6; MAGE-C2; MAGE-C3; MAGE-3; MAGE-6.	Lucas, *et al.* (2000) Int. J. Cancer 87:55-60; Scanlan, *et al.* (2001) Cancer Immun. 1:4.
Kinesin 2; TATA element modulatory factor 1; tumor protein D53; NY	Scanlan, *et al.* (2001) Cancer Immun. 30:1-4.
Alpha-fetoprotein (AFP)	Grimm, *et al.* (2000) Gastroenterol. 119:1104-1112.
SART1; SART2; SART3; ART4.	Kumamuru, *et al.* (2004) Int. J. Cancer 108:686-695; Sasatomi, *et al.* (2002) Cancer 94:1636-1641; Matsumoto, *et al.* (1998) Jpn. J. Cancer Res. 89:1292-1295; Tanaka, *et al.* (2000) Jpn. J. Cancer Res. 91:1177-1184.
Preferentially expressed antigen of melanoma (PRAME).	Matsushita, *et al.* (2003) Leuk. Lymphoma 44:439-444; Oberthuer, *et al.* (2004) Clin. Cancer Res. 10:4307-4313.

Antigen	Reference
Carcinoembryonic antigen (CEA), CAP1-6D enhancer agonist peptide.	GenBank Acc. No. M29540; E03352; X98311; M17303 (see also, e.g., Zaremba (1997) Cancer Res. 57:4570-4577; Sarobe, *et al.* (2004) Curr. Cancer Drug Targets 4:443-454; Tsang, *et al.* (1997) Clin. Cancer Res. 3:2439-2449; Fong, *et al.* (2001) Proc. Natl. Acad. Sci. USA 98:8809-8814).
HER-2/neu.	Disis, *et al.* (2004) J. Clin. Immunol. 24:571-578; Disis and Cheever (1997) Adv. Cancer Res. 71:343-371.
Cdk4; cdk6; p16 (INK4); Rb protein.	Ghazizadeh, *et al.* (2005) Respiration 72:68-73; Ericson, *et al.* (2003) Mol. Cancer Res. 1:654-664.
TEL; AML1; TEL/AML1.	Stams, *et al.* (2005) Clin. Cancer Res. 11:2974-2980.
Telomerase (TERT).	Nair, *et al.* (2000) Nat. Med. 6:1011-1017.
707-AP.	Takahashi, *et al.* (1997) Clin. Cancer Res. 3:1363-1370.
Annexin, e.g., Annexin II.	Zimmerman, *et al.* (2004) Virchows Arch. 445:368-374.
BCR/ABL; BCR/ABL p210; BCR/ABL p190; CML-66; CML-28.	Cobaldda, *et al.* (2000) Blood 95:1007-1013; Hakansson, *et al.* (2004) Leukemia 18:538-547; Schwartz, *et al.* (2003) Semin. Hematol. 40:87-96; Lim, *et al.* (1999) Int. J. Mol. Med. 4:665-667.
BCL2; BLC6; CD10 protein.	Iqbal, *et al.* (2004) Am. J. Pathol. 165:159-166.
CDC27 (this is a melanoma antigen).	Wang, *et al.* (1999) Science 284:1351-1354.
Sperm protein 17 (SP17); 14-3-3-zeta; MEMD; KIAA0471; TC21.	Arora, *et al.* (2005) Mol. Carcinog. 42:97-108.
Tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2).	GenBank Acc. No. NM_001922. (see also, e.g., Bronte, *et al.* (2000) Cancer Res. 60:253-258).
Gp100/pmel-17.	GenBank Acc. Nos. AH003567; U31798; U31799;U31807; U31799 (see also, e.g., Bronte, *et al.* (2000) Cancer Res. 60:253-258).
TARP.	See, e.g., Clifton, *et al.* (2004) Proc. Natl. Acad. Sci. USA 101:10166-10171; Virok, *et al.* (2005) Infection Immunity 73:1939-1946.

Antigen	Reference
Tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2).	GenBank Acc. No. NM_001922. (see also, e.g., Bronte, *et al.* (2000) Cancer Res. 60:253-258).
Melanocortin 1 receptor (MC1R); MAGE-3; gp100; tyrosinase; dopachrome tautomerase (TRP-2); MART-1.	Salazar-Onfray, *et al.* (1997) Cancer Res. 57:4348-4355; Reynolds, *et al.* (1998) J. Immunol. 161:6970-6976; Chang, *et al.* (2002) Clin. Cancer Res. 8:1021-1032.
MUC-1; MUC-2.	See, e.g., Davies, *et al.* (1994) Cancer Lett. 82:179-184; Gambus, *et al.* (1995) Int. J. Cancer 60:146-148; McCool, *et al.* (1999) Biochem. J. 341:593-600.
Spas-1.	U.S. Published Pat. Appl. No. 20020150588 of Allison, *et al.*
CASP-8; FLICE; MACH.	Mandruzzato, *et al.* (1997) J. Exp. Med. 186:785-793.
CEACAM6; CAP-1.	Duxbury, *et al.* (2004) Biochem. Biophys. Res. Commun. 317:837-843; Morse, *et al.* (1999) Clin. Cancer Res. 5:1331-1338.
HMGB1 (a DNA binding protein and cytokine).	Brezniceanu, *et al.* (2003) FASEB J. 17:1295-1297.
ETV6/AML1.	Codrington, *et al.* (2000) Br. J. Haematol. 111:1071-1079.
Mutant and wild type forms of adenomatous polyposis coli (APC); beta-catenin; c-met; p53; E-cadherin; cyclooxygenase-2 (COX-2).	Clements, *et al.* (2003) Clin. Colorectal Cancer 3:113-120; Gulmann, *et al.* (2003) Appl. Immunohistochem. Mol. Morphol. 11:230-237; Jungck, *et al.* (2004) Int. J. Colorectal. Dis. 19:438-445; Wang, *et al.* (2004) J. Surg. Res. 120:242-248; Abutaily, *et al.* (2003) J. Pathol. 201:355-362; Liang, *et al.* (2004) Br. J. Surg. 91:355-361; Shirakawa, *et al.* (2004) Clin. Cancer Res. 10:4342-4348.
Renal cell carcinoma antigen bound by mAB G250.	Mulders, *et al.* (2003) Urol. Clin. North Am. 30:455-465; Steffens, *et al.* (1999) Anticancer Res. 19:1197-1200.
EphA2	See, e.g., U.S. Patent Publication No. 2005/0281783 A1; Genbank Accession No. NM_004431 (human); Genbank

Antigen	Reference
	Accession No. NM_010139 (Mouse); Genbank Accession No. AB038986 (Chicken, partial sequence); GenBank Accession Nos. NP_004422, AAH37166, and AAA53375 (human); GenBank Accession Nos. NP_034269 (mouse), AAH06954 (mouse), XP_345597 (rat), and BAB63910 (chicken).
EGFRvIII	See, e.g., WO/2012/068360
***Francisella tularensis* antigens**	
Francisella tularensis A and B.	Complete genome of subspecies Schu S4 (GenBank Acc. No. AJ749949); of subspecies Schu 4 (GenBank Acc. No. NC_006570). Outer membrane protein (43 kDa) Bevanger, *et al.* (1988) J. Clin. Microbiol. 27:922-926; Porsch-Ozcurumez, *et al.* (2004) Clin. Diagnostic. Lab. Immunol. 11:1008-1015). Antigenic components of *F. tularensis* include, e.g., 80 antigens, including 10 kDa and 60 kDa chaperonins (Havlasova, *et al.* (2002) Proteomics 2:857-86), nucleoside diphosphate kinase, isocitrate dehydrogenase, RNA-binding protein Hfq, the chaperone ClpB (Havlasova, *et al.* (2005) Proteomics 5:2090-2103). See also, e.g., Oyston and Quarry (2005) Antonie Van Leeuwenhoek 87:277-281; Isherwood, *et al.* (2005) Adv. Drug Deliv. Rev. 57:1403-1414; Biagini, *et al.* (2005) Anal. Bioanal. Chem. 382:1027-1034.
Malarial antigens	
Circumsporozoite protein (CSP); SSP2; HEP17; Exp-1 orthologs found in P. falciparum; and LSA-1.	See, e.g., Haddad, *et al.* (2004) Infection Immunity 72:1594-1602; Hoffman, *et al.* (1997) Vaccine 15:842-845; Oliveira-Ferreira and Daniel-Ribeiro (2001) Mem. Inst. Oswaldo Cruz, Rio de Janeiro 96:221-227. CSP (see, e.g., GenBank Acc. No. AB121024). SSP2 (see, e.g., GenBank Acc. No. AF249739). LSA-1 (see, e.g., GenBank Acc. No. Z30319).
Ring-infected erythrocyte survace protein (RESA); merozoite surface protein 2 (MSP2); Spf66;	See, e.g., Stirnadel, *et al.* (2000) Int. J. Epidemiol. 29:579-586; Krzych, *et al.* (1995) J. Immunol. 155:4072-4077. See also, Good, *et al.* (2004) Immunol. Rev. 201:254-267; Good, *et al.* (2004) Ann. Rev. Immunol. 23:69-99. MSP2 (see, e.g.,

Antigen	Reference
merozoite surface protein 1(MSP1); 195A; BVp42.	GenBank Acc. No. X96399; X96397). MSP1 (see, e.g., GenBank Acc. No. X03371). RESA (see, e.g., GenBank Acc. No. X05181; X05182).
Apical membrane antigen 1 (AMA1).	See, e.g. , Gupta, *et al.* (2005) Protein Expr. Purif. 41:186-198. AMA1 (see, e.g., GenBank Acc. No. A`13; AJ494905; AJ490565).
Viruses and viral antigens	
Hepatitis A	GenBank Acc. Nos., e.g., NC_001489; AY644670; X83302; K02990; M14707.
Hepatitis B	Complete genome (see, e.g., GenBank Acc. Nos. AB214516; NC_003977; AB205192; AB205191; AB205190; AJ748098; AB198079; AB198078; AB198076; AB074756).
Hepatitis C	Complete genome (see, e.g., GenBank Acc. Nos. NC_004102; AJ238800; AJ238799; AJ132997; AJ132996; AJ000009; D84263).
Hepatitis D	GenBank Acc. Nos, e.g. NC_001653; AB118847; AY261457.
Human papillomavirus, including all 200+ subtypes (classed in 16 groups), such as the high risk subtypes 16, 18, 30, 31, 33, 45.	See, e.g., Trimble, *et al.* (2003) Vaccine 21:4036-4042; Kim, *et al.* (2004) Gene Ther. 11:1011-1018; Simon, *et al.* (2003) Eur. J. Obstet. Gynecol. Reprod. Biol. 109:219-223; Jung, *et al.* (2004) J. Microbiol. 42:255-266; Damasus-Awatai and Freeman-Wang (2003) Curr. Opin. Obstet. Gynecol. 15:473-477; Jansen and Shaw (2004) Annu. Rev. Med. 55:319-331; Roden and Wu (2003) Expert Rev. Vaccines 2:495-516; de Villiers, *et al.* (2004) Virology 324:17-24; Hussain and Paterson (2005) Cancer Immunol. Immunother. 54:577-586; Molijn, *et al.* (2005) J. Clin. Virol. 32 (Suppl. 1) S43-S51. GenBank Acc. Nos. AY686584; AY686583; AY686582; NC_006169; NC_006168; NC_006164; NC_001355; NC_001349; NC_005351; NC_001596).
Human T-cell lymphotropic virus (HTLV) types I and II,	See, e.g., Capdepont, *et al.* (2005) AIDS Res. Hum. Retrovirus 21:28-42; Bhigjee, *et al.* (1999) AIDS Res. Hum. Restrovirus 15:1229-1233; Vandamme, *et al.* (1998) J. Virol.

Antigen	Reference
including the HTLV type I subtypes Cosmopolitan, Central African, and Austro-Melanesian, and the HTLV type II subtypes Iia, Iib, Iic, and Iid.	72:4327-4340; Vallejo, *et al.* (1996) J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 13:384-391. HTLV type I (see, e.g., GenBank Acc. Nos. AY563954; AY563953. HTLV type II (see, e.g., GenBank Acc. Nos. L03561; Y13051; AF139382).
Coronaviridae, including Coronaviruses, such as SARS-coronavirus (SARS-CoV), and Toroviruses.	See, e.g., Brian and Baric (2005) Curr. Top. Microbiol. Immunol. 287:1-30; Gonzalez, *et al.* (2003) Arch. Virol. 148:2207-2235; Smits, *et al.* (2003) J. Virol. 77:9567-9577; Jamieson, *et al.* (1998) J. Infect. Dis. 178:1263-1269 (GenBank Acc. Nos. AY348314; NC_004718; AY394850).
Rubella virus.	GenBank Acc. Nos. NC_001545; AF435866.
Mumps virus, including the genotypes A, C, D, G, H, and I.	See, e.g., Orvell, eta l. (2002) J. Gen. Virol. 83:2489-2496. See, e.g., GenBank Acc. Nos. AY681495; NC_002200; AY685921; AF201473.
Coxsackie virus A including the serotypes 1, 11, 13, 15, 17, 18, 19, 20, 21, 22, and 24 (also known as Human enterovirus C; HEV-C).	See, e.g., Brown, *et al.* (2003) J. Virol. 77:8973-8984. GenBank Acc. Nos. AY421768; AY790926: X67706.
Coxsackie virus B, including subtypes 1-6.	See, e.g., Ahn, *et al.* (2005) J. Med. Virol. 75:290-294; Patel, *et al.* (2004) J. Virol. Methods 120:167-172; Rezig, *et al.* (2004) J. Med. Virol. 72:268-274. GenBank Acc. No. X05690.
Human enteroviruses including, e.g., human enterovirus A (HEV-A, CAV2 to CAV8, CAV10, CAV12, CAV14, CAV16, and EV71) and	See, e.g., Oberste, *et al.* (2004) J. Virol. 78:855-867. Human enterovirus A (GenBank Acc. Nos. NC_001612); human enterovirus B (NC_001472); human enterovirus C (NC_001428); human enterovirus D (NC_001430). Simian enterovirus A (GenBank Acc. No. NC_003988).

Antigen	Reference
also including HEV-B (CAV9, CBV1 to CBV6, E1 to E7, E9, E11 to E21, E24 to E27, E29 to E33, and EV69 and E73), as well as HEV.	
Polioviruses including PV1, PV2, and PV3.	See, e.g., He, *et al.* (2003) J. Virol. 77:4827-4835; Hahsido, *et al.* (1999) Microbiol. Immunol. 43:73-77. GenBank Acc. No. AJ132961 (type 1); AY278550 (type 2); X04468 (type 3).
Viral encephalitides viruses, including equine encephalitis, Venezuelan equine encephalitis (VEE) (including subtypes IA, IB, IC, ID, IIIC, IIID), Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), St. Louis encephalitis, Murray Valley (Australian) encephalitis, Japanese encephalitis, and tick-born encephalitis.	See, e.g., Hoke (2005) Mil. Med. 170:92-105; Estrada-Franco, *et al.* (2004) Emerg. Infect. Dis. 10:2113-2121; Das, *et al.* (2004) Antiviral Res. 64:85-92; Aguilar, *et al.* (2004) Emerg. Infect. Dis. 10:880-888; Weaver, *et al.* (2004) Arch. Virol. Suppl. 18:43-64; Weaver, *et al.* (2004) Annu. Rev. Entomol. 49:141-174. Eastern equine encephalitis (GenBank Acc. No. NC_003899; AY722102); Western equine encephalitis (NC_003908).

Antigen	Reference
Human herpesviruses, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus-1 (HHV-1), HHV-2, HHV-3, HHV-4, HHV-5, HHV-6, HHV-7, HHV-8, herpes B virus, herpes simplex virus types 1 and 2 (HSV-1, HSV-2), and varicella zoster virus (VZV).	See, e.g., Studahl, *et al.* (2000) Scand. J. Infect. Dis. 32:237-248; Padilla, *et al.* (2003) J. Med. Virol. 70 (Suppl. 1) S103-S110; Jainkittivong and Langlais (1998) Oral Surg. Oral Med. 85:399-403. GenBank Nos. NC_001806 (herpesvirus 1); NC_001798 (herpesvirus 2); X04370 and NC_001348 (herpesvirus 3); NC_001345 (herpesvirus 4); NC_001347 (herpesvirus 5); X83413 and NC_000898 (herpesvirus 6); NC_001716 (herpesvirus 7). Human herpesviruses types 6 and 7 (HHV-6; HHV-7) are disclosed by, e.g., Padilla, *et al.* (2003) J. Med. Virol. 70 (Suppl. 1)S103-S110. Human herpesvirus 8 (HHV-8), including subtypes A-E, are disclosed in, e.g., Treurnicht, *et al.* (2002) J. Med. Virul. 66:235-240.
HIV-1 including group M (including subtypes A to J) and group O (including any distinguishable subtypes) (HIV-2, including subtypes A-E.	See, e.g., Smith, *et al.* (1998) J. Med. Virol. 56:264-268. See also, e.g., GenBank Acc. Nos. DQ054367; NC_001802; AY968312; DQ011180; DQ011179; DQ011178; DQ011177; AY588971; AY588970; AY781127; AY781126; AY970950; AY970949; AY970948; X61240; AJ006287; AJ508597; and AJ508596.
Epstein-Barr virus (EBV), including subtypes A and B.	See, e.g., Peh, *et al.* (2002) Pathology 34:446-450. Epstein-Barr virus strain B95-8 (GenBank Acc. No. V01555).
Reovirus, including serotypes and strains 1, 2, and 3, type 1 Lang, type 2 Jones, and type 3 Dearing.	See, e.g., Barthold, *et al.* (1993) Lab. Anim. Sci. 43:425-430; Roner, *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:12362-12366; Kedl, *et al.* (1995) J. Virol. 69:552-559. GenBank Acc. No. K02739 (sigma-3 gene surface protein).
Cytomegalovirus (CMV) subtypes include CMV subtypes I-VII.	See, e.g., Chern, *et al.* (1998) J. Infect. Dis. 178:1149-1153; Vilas Boas, *et al.* (2003) J. Med. Virol. 71:404-407; Trincado, *et al.* (2000) J. Med. Virol. 61:481-487. GenBank Acc. No.

Antigen	Reference
	X17403.
Rhinovirus, including all serotypes.	Human rhinovirus 2 (GenBank Acc. No. X02316); Human rhinovirus B (GenBank Acc. No. NC_001490); Human rhinovirus 89 (GenBank Acc. No. NC_001617); Human rhinovirus 39 (GenBank Acc. No. AY751783).
Adenovirus, including all serotypes.	AY803294; NC_004001; AC_000019; AC_000018; AC_000017; AC_000015; AC_000008; AC_000007; AC_000006; AC_000005; AY737798; AY737797;NC_003266; NC_002067; AY594256; AY594254; AY875648; AJ854486; AY163756; AY594255; AY594253; NC_001460; NC_001405; AY598970; AY458656; AY487947; NC_001454; AF534906; AY45969; AY128640; L19443; AY339865; AF532578.
Filoviruses, including Marburg virus and Ebola virus, and strains such as Ebola-Sudan (EBO-S), Ebola-Zaire (EBO-Z), and Ebola-Reston (EBO-R).	See, e.g., Geisbert and Jahrling (1995) Virus Res. 39:129-150; Hutchinson, *et al*. (2001) J. Med. Virol. 65:561-566. Marburg virus (see, e.g., GenBank Acc. No. NC_001608). Ebola virus (see, e.g., GenBank Acc. Nos. NC_006432; AY769362; NC_002549; AF272001; AF086833).
Arenaviruses, including lymphocytic choriomeningitis (LCM) virus, Lassa virus, Junin virus, and Machupo virus.	Junin virus, segment S (GenBank Acc. No. NC_005081); Junin virus, segment L (GenBank Acc. No. NC_005080).
Rabies virus.	See, e.g., GenBank Acc. Nos. NC_001542; AY956319; AY705373; AF499686; AB128149; AB085828; AB009663.
Arboviruses, including West Nile virus, Dengue viruses 1 to 4, Colorado tick fever virus, Sindbis virus, Togaviraidae, Flaviviridae, Bunyaviridae, Reoviridae,	Dengue virus type 1 (see, e.g., GenBank Acc. Nos. AB195673; AY762084). Dengue virus type 2 (see, e.g., GenBank Acc. Nos. NC_001474; AY702040; AY702039; AY702037). Dengue virus type 3 (see, e.g., GenBank Acc. Nos. AY923865; AT858043). Dengue virus type 4 (see, e.g., GenBank Acc. Nos. AY947539; AY947539; AF326573). Sindbis virus (see, e.g., GenBank Acc. Nos. NC_001547;

Antigen	Reference
Rhabdoviridae, Orthomyxoviridae, and the like.	AF429428; J02363; AF103728). West Nile virus (see, e.g., GenBank Acc. Nos. NC_001563; AY603654).
Poxvirus including orthopoxvirus (variola virus, monkeypox virus, vaccinia virus, cowpox virus), yatapoxvirus (tanapox virus, Yaba monkey tumor virus), parapoxvirus, and molluscipoxvirus.	Viriola virus (see, e.g., GenBank Acc. Nos. NC_001611; Y16780; X72086; X69198).
Yellow fever.	See, e.g., GenBank Acc. No. NC_002031; AY640589; X03700.
Hantaviruses, including serotypes Hantaan (HTN), Seoul (SEO), Dobrava (DOB), Sin Nombre (SN), Puumala (PUU), and Dobrava-like Saaremaa (SAAV).	See, e.g., Elgh, *et al.* (1997) J. Clin. Microbiol. 35:1122-1130; Sjolander, *et al.* (2002) Epidemiol. Infect. 128:99-103; Zeier, *et al.* (2005) Virus Genes 30:157-180. GenBank Acc. No. NC_005222 and NC_005219 (Hantavirus). See also, e.g., GenBank Acc. Nos. NC_005218; NC_005222; NC_005219.
Flaviviruses, including Dengue virus, Japanese encephalitis virus, West Nile virus, and yellow fever virus.	See, e.g., Mukhopadhyay, *et al.* (2005) Nature Rev. Microbiol. 3:13-22. GenBank Acc. Nos NC_001474 and AY702040 (Dengue). GenBank Acc. Nos. NC_001563 and AY603654.
Measles virus.	See, e.g., GenBank Acc. Nos. AB040874 and AY486084.
Human parainfluenzaviruses (HPV), including HPV types 1-56.	Human parainfluenza virus 2 (see, e.g., GenBank Acc. Nos. AB176531; NC003443). Human parainfluenza virus 3 (see, e.g., GenBank Acc. No. NC_001796).

Antigen	Reference
Influenza virus, including influenza virus types A, B, and C.	Influenza nucleocapsid (see, e.g., GenBank Acc. No. AY626145). Influenza hemagglutinin (see, e.g., GenBank Acc. Nos. AY627885; AY555153). Influenza neuraminidase (see, e.g., GenBank Acc. Nos. AY555151; AY577316). Influenza matrix protein 2 (see, e.g., GenBank Acc. Nos. AY626144(. Influenza basic protein 1 (see, e.g., GenBank Acc. No. AY627897). Influenza polymerase acid protein (see, e.g., GenBank Acc. No. AY627896). Influenza nucleoprotein (see, e.g., GenBank Acc. Nno. AY627895).
Influenza A viruses of various subtypes that originate from other species:, e.g., swine influenza viruses (SIV) (e.g. H1N1) and avian influenza virus (AIV) (e.g. H5N1; H7N7; H9N2)	Hemagglutinin of H1N1 (GenBank Acc. No. S67220). Influenza A virus matrix protein (GenBank Acc. No. AY700216). Influenza virus A H5H1 nucleoprotein (GenBank Acc. No. AY646426). H1N1 haemagglutinin (GenBank Acc. No. D00837). See also, GenBank Acc. Nos. BD006058; BD006055; BD006052. See also, e.g., Wentworth, *et al.* (1994) J. Virol. 68:2051-2058; Wells, *et al.* (1991) J.A.M.A. 265:478-481.
Respiratory syncytial virus (RSV), including subgroup A and subgroup B.	Respiratory syncytial virus (RSV) (see, e.g., GenBank Acc. Nos. AY353550; NC_001803; NC001781).
Rotaviruses, including human rotaviruses A to E, bovine rotavirus, rhesus monkey rotavirus, and human-RVV reassortments.	Human rotavirus C segment 8 (GenBank Acc. No. AJ549087); Human rotavirus G9 strain outer capsid protein (see, e.g., GenBank Acc. No. DQ056300); Human rotavirus B strain non-structural protein 4 (see, e.g., GenBank Acc. No. AY548957); human rotavirus A strain major inner capsid protein (see, e.g., GenBank Acc. No. AY601554).
Polyomavirus, including simian virus 40 (SV40), JC virus (JCV) and BK virus (BKV).	See, e.g., Engels, *et al.* (2004) J. Infect. Dis. 190:2065-2069; Vilchez and Butel (2004) Clin. Microbiol. Rev. 17:495-508; Shivapurkar, *et al.* (2004) Cancer Res. 64:3757-3760; Carbone, *et al.* (2003) Oncogene 2:5173-5180; Barbanti-Brodano, *et al.* (2004) Virology 318:1-9) (SV40 complete genome in, e.g., GenBank Acc. Nos. NC_001669; AF168994;

Antigen	Reference
	AY271817; AY271816; AY120890; AF345344; AF332562).
Coltiviruses, including Colorado tick fever virus, Eyach virus.	Attoui, *et al.* (1998) J. Gen. Virol. 79:2481-2489. Segments of Eyach virus (see, e.g., GenBank Acc. Nos. AF282475; AF282472; AF282473; AF282478; AF282476; NC_003707; NC_003702; NC_003703; NC_003704; NC_003705; NC_003696; NC_003697; NC_003698; NC_003699; NC_003701; NC_003706; NC_003700; AF282471; AF282477).
Calciviruses, including the genogroups Norwalk, Snow Mountain group (SMA), and Saaporo.	Snow Mountain virus (see, e.g., GenBank Acc. No. AY134748).
Parvoviridae, including dependovirus, parvovirus (including parvovirus B19), and erythrovirus.	See, e.g., Brown (2004) Dev. Biol. (Basel) 118:71-77; Alvarez-Lafuente, *et al.* (2005) Ann. Rheum. Dis. 64:780-782; Ziyaeyan, *et al.* (2005) Jpn. J. Infect. Dis. 58:95-97; Kaufman, *et al.* (2005) Virology 332:189-198.

Other organisms for which suitable antigens are known in the art include, but are not limited to, *Chlamydia trachomatis, Streptococcus pyogenes* (Group A Strep), *Streptococcus agalactia* (Group B Strep), *Streptococcus pneumonia, Staphylococcus aureus, Escherichia coli, Haemophilus influenzae, Neisseria meningitidis, Neisseria gonorrheae, Vibrio cholerae, Salmonella* species (including *typhi, typhimurium*), enterica *(including Helicobactor pylori Shigella flexneri* and other Group D *shigella* species), *Burkholderia mallei, Burkholderia pseudomallei, Klebsiella pneumonia, Clostridium* species (including *C. difficile*), *Vibrio parahaemolyticus* and *V. vulnificus*. This list is not meant to be limiting.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with one or more of an inhibitor (*e.g.*, a small organic

molecule or an antibody or antigen-binding fragment thereof) such as: an MTOR (mammalian target of rapamycin) inhibitor, a cytotoxic agent, a platinum agent, an EGFR inhibitor, a VEGF inhibitor, a microtubule stabilizer, a taxane, a CD20 inhibitor, a CD52 inhibitor, a CD30 inhibitor, a RANK (Receptor activator of nuclear factor kappa-B) inhibitor, a RANKL (Receptor activator of nuclear factor kappa-B ligand) inhibitor, an ERK inhibitor, a MAP Kinase inhibitor, an AKT inhibitor, a MEK inhibitor, a PI3K inhibitor, a HER1 inhibitor, a HER2 inhibitor, a HER3 inhibitor, a HER4 inhibitor, a Bcl2 inhibitor, a CD22 inhibitor, a CD79b inhibitor, an ErbB2 inhibitor, or a farnesyl protein transferase inhibitor.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with any one or more of: 13-cis-retinoic acid, 3-[5-(methylsulfonylpiperadinemethyl)-indolyl]-quinolone, 4-hydroxytamoxifen, 5-deooxyuridine, 5'-deoxy-5-fluorouridine, 5-fluorouracil, 6-mecaptopurine, 7-hydroxystaurosporine, A-443654, abirateroneacetate, abraxane, ABT-578, acolbifene, ADS-100380, ALT-110, altretamine, amifostine, aminoglutethimide, amrubicin, Amsacrine, anagrelide, anastrozole, angiostatin, AP-23573, ARQ-197, arzoxifene, AS-252424, AS-605240, asparaginase, AT-9263, atrasentan, axitinib, AZD1152, *Bacillus Calmette-Guerin* (BCG) vaccine, batabulin, BC-210, besodutox, bevacizumab, bicalutamide, Bio111, BIO140, bleomycin, BMS-214662, BMS-247550, BMS-275291, BMS-310705, bortezimib, buserelin, busulfan, calcitriol, camptothecin, canertinib, capecitabine, carboplatin, carmustine, CC8490, Cediranib, CG-1521, CG-781, chlamydocin, chlorambucil, chlorotoxin, cilengitide, cimitidine, cisplatin, cladribine, clodronate, COL-3, CP-724714, cyclophosphamide, cyproterone, cyproteroneacetate, cytarabine, cytosinearabinoside, dacarbazine, dacinostat, dactinomycin, dalotuzumab, danusertib, dasatanib, daunorubicin, decatanib, deguelin, denileukin, deoxycoformycin, depsipeptide, diarylpropionitrile, diethylstilbestrol, diftitox, docetaxel, dovitinib, doxorubicin, droloxifene, edotecarin, yttrium-90 labeled-edotreotide, edotreotide, EKB-569, EMD121974, endostatin, enzalutamide, enzastaurin, epirubicin, epithilone B, ERA-923, Erbitux, erlotinib, estradiol, estramustine, etoposide, everolimus, exemestane, ficlatuzumab, finasteride, flavopiridol, floxuridine, fludarabine, fludrocortisone, fluoxymesterone, flutamide, FOLFOX regimen, Fulvestrant, galeterone, gefitinib, gemcitabine, gimatecan, goserelin, goserelin acetate, gossypol, GSK461364,

GSK690693, HMR-3339, hydroxyprogesteronecaproate, hydroxyurea, IC87114, idarubicin, idoxyfene, ifosfamide, IM862, imatinib, IMC-1C11, INCB24360, INO1001, interferon, interleukin-12, ipilimumab, irinotecan, JNJ-16241199, ketoconazole, KRX-0402, lapatinib, lasofoxifene, letrozole, leucovorin, leuprolide, leuprolide acetate, levamisole, liposome entrapped paclitaxel, lomustine, lonafarnib, lucanthone, LY292223, LY292696, LY293646, LY293684, LY294002, LY317615, marimastat, mechlorethamine, medroxyprogesteroneacetate, megestrolacetate, melphalan, mercaptopurine, mesna, methotrexate, mithramycin, mitomycin, mitotane, mitoxantrone, tozasertib, MLN8054, neovastat, Neratinib , neuradiab, nilotinib, nilutimide, nolatrexed, NVP-BEZ235, oblimersen, octreotide, ofatumumab, oregovomab, orteronel, oxaliplatin, paclitaxel, palbociclib, pamidronate, panitumumab, pazopanib, PD0325901, PD184352, PEG-interferon, pemetrexed, pentostatin, perifosine, phenylalaninemustard, PI-103, pictilisib, PIK-75, pipendoxifene, PKI-166, plicamycin, porfimer, prednisone, procarbazine, progestins, PX-866, R-763, raloxifene, raltitrexed, razoxin, ridaforolimus, rituximab, romidepsin, RTA744, rubitecan, scriptaid, Sdx102, seliciclib, selumetinib, semaxanib, SF1126, sirolimus, SN36093, sorafenib, spironolactone, squalamine, SR13668, streptozocin, SU6668, suberoylanalide hydroxamic acid, sunitinib, synthetic estrogen, talampanel, talimogene laherparepvec, tamoxifen, temozolomide, temsirolimus, teniposide, tesmilifene, testosterone, tetrandrine, TGX-221, thalidomide, thioguanine, thiotepa, ticilimumab, tipifarnib, tivozanib, TKI-258, TLK286, topotecan, toremifene citrate, trabectedin, trastuzumab, tretinoin, trichostatin A, triciribinephosphate monohydrate, triptorelin pamoate, TSE-424, uracil mustard, valproic acid, valrubicin, vandetanib, vatalanib, VEGF trap, vinblastine, vincristine, vindesine, vinorelbine, vitaxin, vitespan, vorinostat, VX-745, wortmannin, Xr311, zanolimumab, ZK186619, ZK-304709, ZM336372, ZSTK474.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with one or more antiemetics including, but not limited to: casopitant (GlaxoSmithKline), Netupitant (MGI-Helsinn) and other NK-1 receptor antagonists, palonosetron (sold as Aloxi by MGI Pharma), aprepitant (sold as Emend by Merck and Co.; Rahway, NJ), diphenhydramine (sold as Benadryl® by Pfizer; New York, NY), hydroxyzine (sold as Atarax® by Pfizer; New York, NY), metoclopramide (sold as Reglan® by AH Robins Co,; Richmond, VA), lorazepam (sold as Ativan® by

Wyeth; Madison, NJ), alprazolam (sold as Xanax® by Pfizer; New York, NY), haloperidol (sold as Haldol® by Ortho-McNeil; Raritan, NJ), droperidol (Inapsine®), dronabinol (sold as Marinol® by Solvay Pharmaceuticals, Inc.; Marietta, GA), dexamethasone (sold as Decadron® by Merck and Co.; Rahway, NJ), methylprednisolone (sold as Medrol® by Pfizer; New York, NY), prochlorperazine (sold as Compazine® by Glaxosmithkline; Research Triangle Park, NC), granisetron (sold as Kytril® by Hoffmann-La Roche Inc.; Nutley, NJ), ondansetron (sold as Zofran® by Glaxosmithkline; Research Triangle Park, NC), dolasetron (sold as Anzemet® by Sanofi-Aventis; New York, NY), tropisetron (sold as Navoban® by Novartis; East Hanover, NJ).

Other side effects of cancer treatment include red and white blood cell deficiency. Accordingly, in an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is in association with an agent which treats or prevents such a deficiency, such as, *e.g.*, filgrastim, PEG-filgrastim, erythropoietin, epoetin alfa or darbepoetin alfa.

In an embodiment of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is administered in association with anti-cancer radiation therapy. For example, in an embodiment of the invention, the radiation therapy is external beam therapy (EBT): a method for delivering a beam of high-energy X-rays to the location of the tumor. The beam is generated outside the patient (*e.g.*, by a linear accelerator) and is targeted at the tumor site. These X-rays can destroy the cancer cells and careful treatment planning allows the surrounding normal tissues to be spared. No radioactive sources are placed inside the patient's body. In an embodiment of the invention, the radiation therapy is proton beam therapy: a type of conformal therapy that bombards the diseased tissue with protons instead of X-rays. In an embodiment of the invention, the radiation therapy is conformal external beam radiation therapy: a procedure that uses advanced technology to tailor the radiation therapy to an individual's body structures. In an embodiment of the invention, the radiation therapy is brachytherapy: the temporary placement of radioactive materials within the body, usually employed to give an extra dose—or boost—of radiation to an area.

In an embodiment of the invention, a surgical procedure that can be applied in association with an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) is surgical tumorectomy.

The term "in association with" indicates that the components administered in a method of the present invention (*e.g.*, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) along with pembrolizumab) can be formulated into a single composition for simultaneous delivery or formulated separately into two or more compositions (*e.g.*, a kit). Each component can be administered to a subject at a different time than when the other component is administered; for example, each administration may be given non-simultaneously (*e.g.*, separately or sequentially) at several intervals over a given period of time. Moreover, the separate components may be administered to a subject by the same or by a different route.

Experimental and Diagnostic Uses

The anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) may be used as affinity purification agent. In this process, the anti-CTLA-4 antibodies and antigen-binding fragments thereof are immobilized on a solid phase such a Sephadex, glass or agarose resin or filter paper, using methods well known in the art. The immobilized antibody or fragment is contacted with a sample containing the CTLA-4 protein (or a fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the CTLA-4 protein, which is bound to the immobilized antibody or fragment. Finally, the support is washed with a solvent which elutes the bound CTLA-4 (*e.g.*, protein A). Such immobilized antibodies and fragments form part of the present invention.

Further provided are antigens for generating secondary antibodies which are useful for example for performing Western blots and other immunoassays discussed herein.

Anti-CTLA-4 antibodies (*e.g.*, humanized antibodies) and antigen-binding fragments thereof may also be useful in diagnostic assays for CTLA-4 protein, *e.g.*, detecting its expression in specific cells, tissues, or serum, *e.g.*, tumor cells such as melanoma cells. Such diagnostic methods may be useful in various disease diagnoses.

The present invention includes ELISA assays (enzyme-linked immunosorbent assay) incorporating the use of an anti-CTLA-4 antibody or antigen-binding fragment thereof disclosed herein (*e.g.*, antibody 27A or a humanized version thereof).

For example, such a method comprises the following steps:

(a) coat a substrate (*e.g.*, surface of a microtiter plate well, *e.g.*, a plastic plate) with anti- CTLA-4 antibody or antigen-binding fragment thereof;

(b) apply a sample to be tested for the presence of CTLA-4 to the substrate;

(c) wash the plate, so that unbound material in the sample is removed;

(d) apply detectably labeled antibodies (*e.g.*, enzyme-linked antibodies) which are also specific to the CTLA-4 antigen;

(e) wash the substrate, so that the unbound, labeled antibodies are removed;

(f) if the labeled antibodies are enzyme linked, apply a chemical which is converted by the enzyme into a fluorescent signal; and

(g) detect the presence of the labeled antibody.

Detection of the label associated with the substrate indicates the presence of the CTLA-4 protein.

In a further embodiment, the labeled antibody or antigen-binding fragment thereof is labeled with peroxidase which react with ABTS (*e.g.*, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) or 3,3',5,5'-Tetramethylbenzidine to produce a color change which is detectable. Alternatively, the labeled antibody or fragment is labeled with a detectable radioisotope (*e.g.*, ^{3}H) which can be detected by scintillation counter in the presence of a scintillant.

An anti- CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) may be used in a Western blot or immune-protein blot procedure. Such a procedure forms part of the present invention and includes *e.g.*,:

(1) optionally transferring proteins from a sample to be tested for the presence of CTLA-4 (*e.g.*, from a PAGE or SDS-PAGE electrophoretic separation of the proteins in the sample) onto a membrane or other solid substrate using a method known in the art (*e.g.*, semi-dry blotting or tank blotting); contacting the membrane or other solid substrate to be tested for the presence of bound CTLA-4 or a fragment thereof with an anti- CTLA-4antibody or antigen-binding fragment thereof of the invention.

Such a membrane may take the form of a nitrocellulose or vinyl-based (*e.g.*, polyvinylidene fluoride (PVDF)) membrane to which the proteins to be tested for the presence of CTLA-4 in a non-denaturing PAGE (polyacrylamide gel electrophoresis) gel or SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel have been transferred (*e.g.*, following electrophoretic separation in the gel). Before contacting the membrane with the anti- CTLA-4 antibody or fragment, the membrane is optionally blocked, *e.g.*, with non-fat dry milk or the like so as to bind non-specific protein binding sites on the membrane.

(2) washing the membrane one or more times to remove unbound anti-CTLA-4 antibody or fragment and other unbound substances; and

(3) detecting the bound anti-CTLA-4 antibody or fragment.

Detection of the bound antibody or fragment indicates that the CTLA-4 protein is present on the membrane or substrate and in the sample. Detection of the bound antibody or fragment may be by binding the antibody or fragment with a secondary antibody (an anti-immunoglobulin antibody) which is detectably labeled and, then, detecting the presence of the secondary antibody.

The anti-CTLA-4 antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may also be used for immunohistochemistry. Such a method forms part of the present invention and comprises, *e.g.*,

(1) contacting a cell (*e.g.*, a tumor cell such as a melanoma cell) to be tested for the presence of CTLA-4 protein with an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention; and

(2) detecting the antibody or fragment on or in the cell.

If the antibody or fragment itself is detectably labeled, it can be detected directly. Alternatively, the antibody or fragment may be bound by a detectably labeled secondary antibody which is detected.

Certain anti-CTLA-4 antibodies and antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may also be used for *in vivo* tumor imaging. Such a method may include injection of a radiolabeled anti-CTLA-4 antibody or antigen-binding fragment thereof into the body of a patient to be tested for the presence of a tumor associated with CTLA-4 expression (*e.g.*, which expresses CTLA-4, for example, on tumor-infiltrating lymphocytes) followed by

nuclear imaging of the body of the patient to detect the presence of the labeled antibody or fragment *e.g.*, at loci comprising a high concentration of the antibody or fragment which are bound to the tumor. The detection of the loci indicates the presence of the CTLA-4^+ cells in the tumor.

Imaging techniques include SPECT imaging (single photon emission computed tomography) or PET imaging (positron emission tomography). Labels include *e.g.*, iodine-123 (^{123}I) and technetium-99m (^{99m}Tc), *e.g.*, in conjunction with SPECT imaging or ^{11}C, ^{13}N, ^{15}O or ^{18}F, *e.g.*, in conjunction with PET imaging or Indium-111 (See *e.g.*, Gordon *et al.*, (2005) International Rev. Neurobiol. 67:385-440).

Pharmaceutical Compositions and Administration

To prepare pharmaceutical or sterile compositions of the anti-CTLA-4 antibodies and antigen-binding fragments of the invention (*e.g.*, antibody 27A and humanized versions thereof), the antibody or antigen-binding fragment thereof is admixed with a pharmaceutically acceptable carrier or excipient. See, *e.g.*, *Remington's Pharmaceutical Sciences* and *U.S. Pharmacopeia: National Formulary*, Mack Publishing Company, Easton, PA (1984).

Formulations of therapeutic and diagnostic agents may be prepared by mixing with acceptable carriers, excipients, or stabilizers in the form of, *e.g.*, lyophilized powders, slurries, aqueous solutions or suspensions (see, *e.g.*, Hardman, *et al.* (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, NY; Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, NY; Avis, *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, NY).

Toxicity and therapeutic efficacy of the antibodies of the invention, administered alone or in combination with another therapeutic agent, can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and

therapeutic effects is the therapeutic index (LD_{50}/ ED_{50}). The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration.

In a further embodiment, a further therapeutic agent that is administered to a subject in association with an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or humanized versions thereof) in accordance with the Physicians' Desk Reference 2003 (Thomson Healthcare; 57th edition (November 1, 2002)).

The mode of administration can vary. Routes of administration include oral, rectal, transmucosal, intestinal, parenteral; intramuscular, subcutaneous, intradermal, intramedullary, intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intraocular, inhalation, insufflation, topical, cutaneous, transdermal, or intra-arterial.

In particular embodiments, the anti-CTLA-4 antibodies or antigen-binding fragments thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) can be administered by an invasive route such as by injection. In further embodiments of the invention, an anti-CTLA-4 antibody or antigen-binding fragment thereof, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intraarterially, intratumorally, or by inhalation, aerosol delivery. Administration by non-invasive routes (*e.g.*, orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

The present invention provides a vessel (*e.g.*, a plastic or glass vial, *e.g.*, with a cap or a chromatography column, hollow bore needle or a syringe cylinder) comprising any of the antibodies or antigen-binding fragments of the invention (*e.g.*, antibody 27A and humanized versions thereof) or a pharmaceutical composition thereof. The present invention also provides an injection device comprising any of the antibodies or antigen-binding fragments of the invention (*e.g.*, antibody 27A and humanized versions thereof) or a pharmaceutical composition thereof. An injection device is a device that introduces a substance into the body of a patient via a parenteral route, *e.g.*, intramuscular, subcutaneous or intravenous. For example, an injection

device may be a syringe (*e.g.*, pre-filled with the pharmaceutical composition, such as an auto-injector) which, for example, includes a cylinder or barrel for holding fluid to be injected (*e.g.*, antibody or fragment or a pharmaceutical composition thereof), a needle for piecing skin and/or blood vessels for injection of the fluid; and a plunger for pushing the fluid out of the cylinder and through the needle bore. In an embodiment of the invention, an injection device that comprises an antibody or antigen-binding fragment thereof of the present invention or a pharmaceutical composition thereof is an intravenous (IV) injection device. Such a device includes the antibody or fragment or a pharmaceutical composition thereof in a cannula or trocar/needle which may be attached to a tube which may be attached to a bag or reservoir for holding fluid (*e.g.*, saline; or lactated ringer solution comprising NaCl, sodium lactate, KCl, $CaCl_2$ and optionally including glucose) introduced into the body of the patient through the cannula or trocar/needle. The antibody or fragment or a pharmaceutical composition thereof may, in an embodiment of the invention, be introduced into the device once the trocar and cannula are inserted into the vein of a subject and the trocar is removed from the inserted cannula. The IV device may, for example, be inserted into a peripheral vein (*e.g.*, in the hand or arm); the superior vena cava or inferior vena cava, or within the right atrium of the heart (*e.g.*, a central IV); or into a subclavian, internal jugular, or a femoral vein and, for example, advanced toward the heart until it reaches the superior vena cava or right atrium (*e.g.*, a central venous line). In an embodiment of the invention, an injection device is an autoinjector; a jet injector or an external infusion pump. A jet injector uses a high-pressure narrow jet of liquid which penetrate the epidermis to introduce the antibody or fragment or a pharmaceutical composition thereof to a patient's body. External infusion pumps are medical devices that deliver the antibody or fragment or a pharmaceutical composition thereof into a patient's body in controlled amounts. External infusion pumps may be powered electrically or mechanically. Different pumps operate in different ways, for example, a syringe pump holds fluid in the reservoir of a syringe, and a moveable piston controls fluid delivery, an elastomeric pump holds fluid in a stretchable balloon reservoir, and pressure from the elastic walls of the balloon drives fluid delivery. In a peristaltic pump, a set of rollers pinches down on a length of flexible tubing, pushing fluid forward. In a multi-channel pump, fluids can be delivered from multiple reservoirs at multiple rates.

The pharmaceutical compositions disclosed herein may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 6,620,135; 6,096,002; 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556. Such needleless devices comprising the pharmaceutical composition are also part of the present invention. The pharmaceutical compositions disclosed herein may also be administered by infusion. Examples of well-known implants and modules for administering the pharmaceutical compositions include those disclosed in: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent. No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments. Many other such implants, delivery systems, and modules are well known to those skilled in the art and those comprising the pharmaceutical compositions of the present invention are within the scope of the present invention.

Alternately, one may administer the anti-CTLA-4 antibody or antigen-binding fragment of the invention (*e.g.*, antibody 27A and humanized versions thereof) in a local rather than systemic manner, for example, via injection of the antibody or fragment directly into a tumor. Furthermore, one may administer the antibody or fragment in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody, targeting, for example, a tumor *e.g.*, characterized by immunopathology. The liposomes will be targeted to and taken up selectively by the afflicted tissue. Such methods and liposomes are part of the present invention.

The administration regimen depends on several factors, including the serum or tissue turnover rate of the therapeutic antibody or antigen-binding fragment, the level of symptoms, the immunogenicity of the therapeutic antibody, and the accessibility of the target cells in the biological matrix. Preferably, the administration regimen delivers sufficient therapeutic antibody or fragment to effect improvement in the target disease state, while simultaneously minimizing undesired side effects. Accordingly, the amount of biologic delivered depends in part on the particular therapeutic antibody and the severity of the condition being treated. Guidance in selecting appropriate doses of therapeutic antibodies or fragments is available (see, *e.g.*, Wawrzynczak (1996)

Antibody Therapy, Bios Scientific Pub. Ltd, Oxfordshire, UK; Kresina (ed.) (1991) *Monoclonal Antibodies, Cytokines and Arthritis*, Marcel Dekker, New York, NY; Bach (ed.) (1993) *Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases*, Marcel Dekker, New York, NY; Baert, *et al.* (2003) *New Engl. J. Med.* 348:601-608; Milgrom *et al.* (1999) *New Engl. J. Med.* 341:1966-1973; Slamon *et al.* (2001) *New Engl. J. Med.* 344:783-792; Beniaminovitz *et al.* (2000) *New Engl. J. Med.* 342:613-619; Ghosh *et al.* (2003) *New Engl. J. Med.* 348:24-32; Lipsky *et al.* (2000) *New Engl. J. Med.* 343:1594-1602).

Determination of the appropriate dose is made by the clinician, *e.g.*, using parameters or factors known or suspected in the art to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, *e.g.*, the inflammation or level of inflammatory cytokines produced. In general, it is desirable that a biologic that will be used is derived from the same species as the animal targeted for treatment, thereby minimizing any immune response to the reagent. In the case of human subjects, for example, humanized and fully human antibodies are may be desirable.

Antibodies or antigen-binding fragments thereof disclosed herein (*e.g.*, antibody 27A and humanized versions thereof) may be provided by continuous infusion, or by doses administered, *e.g.*, daily, 1-7 times per week, weekly, bi-weekly, monthly, bimonthly, quarterly, semiannually, annually etc. Doses may be provided, *e.g.*, intravenously, subcutaneously, topically, orally, nasally, rectally, intramuscular, intracerebrally, intraspinally, or by inhalation. A total weekly dose is generally at least 0.05 μg/kg body weight, more generally at least 0.2 μg/kg, 0.5 μg/kg, 1 μg/kg, 10 μg/kg, 100 μg/kg, 0.25 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 5.0 mg/ml, 10 mg/kg, 25 mg/kg, 50 mg/kg or more (see, *e.g.*, Yang, *et al.* (2003) *New Engl. J. Med.* 349:427-434; Herold, *et al.* (2002) *New Engl. J. Med.* 346:1692-1698; Liu, *et al.* (1999) *J. Neurol. Neurosurg. Psych.* 67:451-456; Portielji, *et al.* (20003) *Cancer Immunol. Immunother.* 52:151-144). Doses may also be provided to achieve a pre-determined target concentration of anti-CTLA-4 antibody in the subject's serum, such as 0.1, 0.3, 1, 3, 10, 30, 100, 300 μg/ml or more. In other embodiments, An anti-CTLA-4 antibody of the present invention is administered, *e.g.*, subcutaneously or intravenously, on a weekly, biweekly, "every 4

weeks," monthly, bimonthly, or quarterly basis at 10, 20, 50, 80, 100, 200, 500, 1000 or 2500 mg/subject.

As used herein, the term "effective amount" refer to an amount of an anti-CTLA-4 or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) that, when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to cause a measurable improvement in one or more symptoms of disease, for example cancer or the progression of cancer. An effective dose further refers to that amount of the antibody or fragment sufficient to result in at least partial amelioration of symptoms, *e.g.,* tumor shrinkage or elimination, lack of tumor growth, increased survival time. When applied to an individual active ingredient administered alone, an effective dose refers to that ingredient alone. When applied to a combination, an effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. An effective amount of a therapeutic will result in an improvement of a diagnostic measure or parameter by at least 10%; usually by at least 20%; preferably at least about 30%; more preferably at least 40%, and most preferably by at least 50%. An effective amount can also result in an improvement in a subjective measure in cases where subjective measures are used to assess disease severity.

Kits

Further provided are kits comprising one or more components that include, but are not limited to, an anti-CTLA-4 antibody or antigen-binding fragment, as discussed herein (*e.g.*, antibody 27A or a humanized version thereof) in association with one or more additional components including, but not limited to a pharmaceutically acceptable carrier and/or a therapeutic agent, as discussed herein. The antibody or fragment and/or the therapeutic agent can be formulated as a pure composition or in combination with a pharmaceutically acceptable carrier, in a pharmaceutical composition.

In one embodiment, the kit includes an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A or a humanized version thereof) or a pharmaceutical composition thereof in one container (*e.g.*, in a sterile glass

or plastic vial) and a pharmaceutical composition thereof and/or a therapeutic agent in another container (*e.g.*, in a sterile glass or plastic vial).

In another embodiment, the kit comprises a combination of the invention, including an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, humanized 27A) along with a pharmaceutically acceptable carrier, optionally in combination with one or more therapeutic agents formulated together, optionally, in a pharmaceutical composition, in a single, common container.

If the kit includes a pharmaceutical composition for parenteral administration to a subject, the kit can include a device for performing such administration. For example, the kit can include one or more hypodermic needles or other injection devices as discussed above.

The kit can include a package insert including information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding a combination of the invention may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references, manufacturer/distributor information and patent information.

Detection Kits and Therapeutic Kits

As a matter of convenience, an anti-CTLA-4 antibody or antigen-binding fragment thereof of the invention (*e.g.*, antibody 27A and humanized versions thereof) can be provided in a kit, i.e., a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic or detection assay. Where the antibody or fragment is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.*, a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.*, a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized,

including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

Also provided are diagnostic or detection reagents and kits comprising one or more such reagents for use in a variety of detection assays, including for example, immunoassays such as ELISA (sandwich-type or competitive format). The kit's components may be pre-attached to a solid support, or may be applied to the surface of a solid support when the kit is used. In some embodiments of the invention, the signal generating means may come pre-associated with an antibody or fragment of the invention or may require combination with one or more components, *e.g.*, buffers, antibody-enzyme conjugates, enzyme substrates, or the like, prior to use. Kits may also include additional reagents, *e.g.*, blocking reagents for reducing nonspecific binding to the solid phase surface, washing reagents, enzyme substrates, and the like. The solid phase surface may be in the form of a tube, a bead, a microtiter plate, a microsphere, or other materials suitable for immobilizing proteins, peptides, or polypeptides. In particular aspects, an enzyme that catalyzes the formation of a chemilluminescent or chromogenic product or the reduction of a chemilluminescent or chromogenic substrate is a component of the signal generating means. Such enzymes are well known in the art. Kits may comprise any of the capture agents and detection reagents described herein. Optionally the kit may also comprise instructions for carrying out the methods of the invention.

Also provided is a kit comprising an anti-CTLA-4 antibody (*e.g.*, humanized antibody) or antigen-binding fragment thereof packaged in a container, such as a vial or bottle, and further comprising a label attached to or packaged with the container, the label describing the contents of the container and providing indications and/or instructions regarding use of the contents of the container to treat one or more disease states as described herein.

In one aspect, the kit is for treating cancer and comprises an anti-CTLA-4 antibody (*e.g.*, humanized antibody) or antigen-binding fragment thereof and a further therapeutic agent or a vaccine. The kit may optionally further include a syringe for parenteral, *e.g.*, intravenous, administration. In another aspect, the kit comprises an anti-CTLA-4 antibody (*e.g.*, humanized antibody) or antigen-binding fragment thereof and a label attached to or packaged with the container describing use of the antibody or fragment with the vaccine or further therapeutic agent. In yet another aspect, the kit

comprises the vaccine or further therapeutic agent and a label attached to or packaged with the container describing use of the vaccine or further therapeutic agent with the anti-CTLA-4 antibody or fragment. In certain embodiments, an anti-CTLA-4 antibody and vaccine or further therapeutic agent are in separate vials or are combined together in the same pharmaceutical composition.

As discussed above in the combination therapy section, concurrent administration of two therapeutic agents does not require that the agents be administered at the same time or by the same route, as long as there is an overlap in the time period during which the agents are exerting their therapeutic effect. Simultaneous or sequential administration is contemplated, as is administration on different days or weeks.

The therapeutic and detection kits disclosed herein may also be prepared that comprise at least one of the antibody, peptide, antigen-binding fragment, or polynucleotide disclosed herein and instructions for using the composition as a detection reagent or therapeutic agent. Containers for use in such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other suitable container, into which one or more of the detection and/or therapeutic composition(s) may be placed, and preferably suitably aliquoted. Where a second therapeutic agent is also provided, the kit may also contain a second distinct container into which this second detection and/or therapeutic composition may be placed. Alternatively, a plurality of compounds may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container. The kits disclosed herein will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vial(s) are retained. Where a radiolabel, chromogenic, fluorigenic, or other type of detectable label or detecting means is included within the kit, the labeling agent may be provided either in the same container as the detection or therapeutic composition itself, or may alternatively be placed in a second distinct container means into which this second composition may be placed and suitably aliquoted. Alternatively, the detection reagent and the label may be prepared in a single container means, and in most cases, the kit will also typically include a means for containing the vial(s) in close confinement for commercial sale and/or convenient packaging and delivery.

A device or apparatus for carrying out the detection or monitoring methods described herein is also provided. Such an apparatus may include a chamber or tube into which sample can be input, a fluid handling system optionally including valves or pumps to direct flow of the sample through the device, optionally filters to separate plasma or serum from blood, mixing chambers for the addition of capture agents or detection reagents, and optionally a detection device for detecting the amount of detectable label bound to the capture agent immunocomplex. The flow of sample may be passive (*e.g.*, by capillary, hydrostatic, or other forces that do not require further manipulation of the device once sample is applied) or active (*e.g.*, by application of force generated via mechanical pumps, electroosmotic pumps, centrifugal force, or increased air pressure), or by a combination of active and passive forces.

In further embodiments, also provided is a processor, a computer readable memory, and a routine stored on the computer readable memory and adapted to be executed on the processor to perform any of the methods described herein. Examples of suitable computing systems, environments, and/or configurations include personal computers, server computers, hand-held or laptop devices, multiprocessor systems, microprocessor-based systems, set top boxes, programmable consumer electronics, network PCs, minicomputers, mainframe computers, distributed computing environments that include any of the above systems or devices, or any other systems known in the art.

GENERAL METHODS

Standard methods in molecular biology are described Sambrook, Fritsch and Maniatis (1982 & 1989 2nd Edition, 2001 3rd Edition) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sambrook and Russell (2001) *Molecular Cloning, 3rd ed.,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, CA). Standard methods also appear in Ausbel, *et al.* (2001) *Current Protocols in Molecular Biology, Vols.1-4*, John Wiley and Sons, Inc. New York, NY, which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described (Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 1*, John Wiley and Sons, Inc., New York). Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described (see, *e.g.*, Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 2*, John Wiley and Sons, Inc., New York; Ausubel, *et al.* (2001) *Current Protocols in Molecular Biology, Vol. 3*, John Wiley and Sons, Inc., NY, NY, pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, MO; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391). Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described (Coligan, *et al.* (2001) *Current Protcols in Immunology, Vol. 1*, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Harlow and Lane, *supra*). Standard techniques for characterizing ligand/receptor interactions are available (see, *e.g.*, Coligan, *et al.* (2001) *Current Protocols in Immunology, Vol. 4*, John Wiley, Inc., New York).

Monoclonal, polyclonal, and humanized antibodies can be prepared (see, *e.g.*, Sheperd and Dean (eds.) (2000) *Monoclonal Antibodies*, Oxford Univ. Press, New York, NY; Kontermann and Dubel (eds.) (2001) *Antibody Engineering*, Springer-Verlag, New York; Harlow and Lane (1988) *Antibodies A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 139-243; Carpenter, *et al.* (2000) *J. Immunol.* 165:6205; He, *et al.* (1998) *J. Immunol.* 160:1029; Tang *et al.* (1999) J. Biol. Chem. 274:27371-27378; Baca *et al.* (1997) *J. Biol. Chem.* 272:10678-10684; Chothia *et al.* (1989) *Nature* 342:877-883; Foote and Winter (1992) *J. Mol. Biol.* 224:487-499; U.S. Pat. No. 6,329,511).

An alternative to humanization is to use human antibody libraries displayed on phage or human antibody libraries in transgenic mice (Vaughan *et al.* (1996) *Nature* Biotechnol. 14:309-314; Barbas (1995) *Nature Medicine* 1:837-839; Mendez *et al.* (1997) *Nature Genetics* 15:146-156; Hoogenboom and Chames (2000) *Immunol. Today* 21:371-377; Barbas *et al.* (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Kay *et al.* (1996) *Phage*

Display of Peptides and Proteins: A Laboratory Manual, Academic Press, San Diego, CA; de Bruin *et al.* (1999) *Nature Biotechnol.* 17:397-399).

Single chain antibodies and diabodies are described (see, *e.g.*, Malecki *et al.* (2002) *Proc. Natl. Acad. Sci. USA* 99:213-218; Conrath *et al.* (2001) *J. Biol. Chem.* 276:7346-7350; Desmyter *et al.* (2001) *J. Biol. Chem.* 276:26285-26290; Hudson and Kortt (1999) *J. Immunol. Methods* 231:177-189; and U.S. Pat. No. 4,946,778). Bifunctional antibodies are provided (see, *e.g.*, Mack, *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:7021-7025; Carter (2001) *J. Immunol. Methods* 248:7-15; Volkel, *et al.* (2001) *Protein Engineering* 14:815-823; Segal, *et al.* (2001) *J. Immunol. Methods* 248:1-6; Brennan, *et al.* (1985) *Science* 229:81-83; Raso, *et al.* (1997) *J. Biol. Chem.* 272:27623; Morrison (1985) *Science* 229:1202-1207; Traunecker, *et al.* (1991) *EMBO J.* 10:3655-3659; and U.S. Pat. Nos. 5,932,448, 5,532,210, and 6,129,914).

Bispecific antibodies are also provided (see, *e.g.*, Azzoni *et al.* (1998) *J. Immunol.* 161:3493; Kita *et al.* (1999) *J. Immunol.* 162:6901; Merchant *et al.* (2000) *J. Biol. Chem.* 74:9115; Pandey *et al.* (2000) *J. Biol. Chem.* 275:38633; Zheng *et al.* (2001) *J. Biol Chem.* 276:12999; Propst *et al.* (2000) *J. Immunol.* 165:2214; Long (1999) *Ann. Rev. Immunol.* 17:875).

Purification of antigen is not necessary for the generation of antibodies. Animals can be immunized with cells bearing the antigen of interest. Splenocytes can then be isolated from the immunized animals, and the splenocytes can fused with a myeloma cell line to produce a hybridoma (see, *e.g.*, Meyaard *et al.* (1997) *Immunity* 7:283-290; Wright *et al.* (2000) *Immunity* 13:233-242; Preston *et al.*, *supra*; Kaithamana *et al.* (1999) *J. Immunol.* 163:5157-5164).

Antibodies can be conjugated, *e.g.*, to small drug molecules, enzymes, liposomes, polyethylene glycol (PEG). Antibodies are useful for therapeutic, diagnostic, kit or other purposes, and include antibodies coupled, *e.g.*, to dyes, radioisotopes, enzymes, or metals, *e.g.*, colloidal gold (see, *e.g.*, Le Doussal *et al.* (1991) *J. Immunol.* 146:169-175; Gibellini *et al.* (1998) *J. Immunol.* 160:3891-3898; Hsing and Bishop (1999) *J. Immunol.* 162:2804-2811; Everts *et al.* (2002) *J. Immunol.* 168:883-889).

Methods for flow cytometry, including fluorescence activated cell sorting (FACS), are available (see, *e.g.*, Owens, *et al.* (1994) *Flow Cytometry Principles for Clinical Laboratory Practice*, John Wiley and Sons, Hoboken, NJ; Givan (2001) *Flow*

Cytometry, 2nd ed.; Wiley-Liss, Hoboken, NJ; Shapiro (2003) *Practical Flow Cytometry*, John Wiley and Sons, Hoboken, NJ). Fluorescent reagents suitable for modifying nucleic acids, including nucleic acid primers and probes, polypeptides, and antibodies, for use, *e.g.*, as diagnostic reagents, are available (Molecular Probes (2003) *Catalogue*, Molecular Probes, Inc., Eugene, OR; Sigma-Aldrich (2003) *Catalogue*, St. Louis, MO).

Standard methods of histology of the immune system are described (see, *e.g.*, Muller-Harmelink (ed.) (1986) *Human Thymus: Histopathology and Pathology*, Springer Verlag, New York, NY; Hiatt, *et al.* (2000) *Color Atlas of Histology*, Lippincott, Williams, and Wilkins, Phila, PA; Louis, *et al.* (2002) *Basic Histology: Text and Atlas*, McGraw-Hill, New York, NY).

Software packages and databases for determining, *e.g.*, antigenic fragments, leader sequences, protein folding, functional domains, glycosylation sites, and sequence alignments, are available (see, *e.g.*, GenBank, Vector NTI® Suite (Informax, Inc, Bethesda, MD); GCG Wisconsin Package (Accelrys, Inc., San Diego, CA); DeCypher® (TimeLogic Corp., Crystal Bay, Nevada); Menne, *et al.* (2000) *Bioinformatics* 16: 741-742; Menne, *et al.* (2000) *Bioinformatics Applications Note* 16:741-742; Wren, *et al.* (2002) *Comput. Methods Programs Biomed.* 68:177-181; von Heijne (1983) *Eur. J. Biochem.* 133:17-21; von Heijne (1986) *Nucleic Acids Res.* 14:4683-4690).

EXAMPLES

Example 1: Immunization and selection of anti-hCTLA-4 antibodies

To isolate antibodies against the human CTLA-4 protein mice were immunized with an expression construct encoding hCTLA-4. To generate this construct, cDNA encoding the full length open reading frame of hCTLA-4 (NCBI Reference Sequence: NM_005214.4, SEQ ID NO: 35) was subcloned into the pCI-neo vector (Promega, Madison, WI). Mice were immunized by gene gun immunization using a Helios Gene gun (BioRad, Hercules, CA) and DNA coated gold bullets (BioRad) following manufacturer's instructions. Briefly, 1 μm gold particles were coated with pCI-neo-CTLA-4 cDNA and commercial expression vectors for mouse Flt3L and mouse GM-CSF in a 2:1:1 ratio (both from Aldevron, Fargo, ND). A total of 1 μg of

plasmid DNA was used to coat 500 µg of gold particles. Specifically, 7-8 weeks old female BALB/C mice were immunized in the ears with a gene gun, receiving 4 administration cycles in both ears. Approximately, a 1:125-625 anti-hCTLA-4 titer was detected by flow Cytometry in mouse serum after three DNA immunizations. For this screening CHO-K1 cells were used, that were transiently transfected with the pCI-neo-CTLA-4 construct, using Lipofectamine 2000 (Invitrogen). Transfected cells were cultured overnight and a subsequently single cell suspension was obtained using cell dissocation solution (Sigma). $7,5*10^5$ cells were incubated with each sample of the diluted mouse sera for 30 minutes at 4°C. Then, cells were washed with Phosphate-buffered Saline (PBS)/2%Fetal Bovine serum (FBS) and stained with FITC-labelled goat-anti-mouse IgG (BD Pharmingen) for 30 minutes at 4°C. Again cells were washed with PBS/2% FBS followed by resuspension in PBS/2% FBS and antibody-bound cells were detected based on their FITC-labelling, assessed by flow cytometry (FACS Canto II; BD Biosciences). Mice that demonstrated reactivity against hCTLA-4 were immunized for a final, fourth time and sacrificed four days later. Erythrocyte-depleted spleen and lymph-node cell populations were prepared as described previously (*Steenbakkers et al., 1992, J. Immunol. Meth. 152: 69-77; Steenbakkers et al., 1994, Mol. Biol. Rep. 19: 125-134*) and frozen at -140°C.

For B-cell selection and CELISA purposes, CHO-K1.hCTLA-4 stable cell-lines were generated by transfecting CHO-K1 cells (American Type Culture Collection) with pCI-neo vector encoding a mutant hCTLA-4 cDNA (Y166G and Y183G, SEQ ID NO: 37). Stable clones were obtained by limiting dilution.

To select anti-hCTLA-4 antibody producing B-cells, a selection strategy was designed and developed that preferentially bound B-cells expressing antibodies that bind to hCTLA-4. Splenocyte and lymph nodes were harvested from the hCTLA-4 immunized mice and isolated cells werewere incubated with CHO-K1.hCTLA-4 cells that were irradiated at 3,000RAD. After 1 hour unbound cells were removed with multiple wash steps using culture medium. Subsequently CHO-K1.hCTLA-4 cells with bound lymphocytes were harvested with dissociation buffer. Bound B-cells were cultured, as described by *Steenbakkers et al., 1994, Mol. Biol. Rep. 19: 125-134.* Briefly, selected B-cells were mixed with 7.5% (v/v) T-cell supernatant and 50,000 irradiated (2,500 RAD) EL-4 B5 feeder cells in a final volume of 200 µl medium in a

96-well flat-bottom tissue culture plates. On day eight, supernatants were screened for hCTLA-4 reactivity by CELISA as described below.

CHO-K1.hCTLA-4 cells were seeded in culture medium (DMEM-F12 (Gibco) with 10% Fetal Bovine Serum (Hyclone) and Pen/Strep (Gibco)) in tissue culture plates and cultured at 37°C, 5% CO_2 and 95% humidity until they were confluent. Subsequently, culture medium was removed and cells were incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with supernatants from the B cell cultures. Next, cells were washed with PBS/0.05% Tween (PBST) and incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with goat-anti-mouse IgG-HRP (Southern Biotechnology). Subsequently, cells were washed 3 times with PBST and anti-hCTLA-4 immunoreactivity was visualized with TMB Stabilized Chromagen (Invitrogen). Reactions were stopped with 0.5 M H_2SO_4 and absorbances were read at 450 and 610 nm.

In addition, supernatants were evaluated for hCTLA-4/hCD80 interaction blockade using a Homogeneous Time Resolved Fluorescence (HTRF) assay format. Supernatants were co-incubated with biotinylated recombinant hCD80 in HTRF buffer (PBS/0.53M Kaliumfluoride/0.1% BSA). Recombinant human CTLA-4/Fc was added in combination with the detection reagents Streptavidin K (*Donor*) and anti-human Fc D2 (*Acceptor*). The complete mixtures were incubated at room temperature (RT) for 3 hours and subsequently fluorescence was measured at a wavelength of 615 and 665 nM using a Victor2 reader (Perkin Elmer). hCTLA-4 binding to hCD80 results in a fluorescent signal in this setup which was set a 100%. Supernatants containing blocking antibodies reduced fluorescence.

B-cell clones from the hCTLA-4 reactive supernatants, which were shown to block the hCTLA-4/hCD80 interaction were immortalized by mini-electrofusion following published procedures *(Steenbakkers et al., 1992, J. Immunol. Meth. 152: 69-77; Steenbakkers et al., 1994, Mol. Biol. Rep. 19:125-34)*. Briefly, B-cells were mixed with 10^6 Sp2/0-Ag14 myeloma cells in Electrofusion Isomolar Buffer (Eppendorf). Electrofusions were performed in a 50 µL fusion chamber by an alternating electric field of 30 s, 1 MHz, 15 Vrms followed by a square, high field pulse of 10 µs, 3 kV/cm and again by an alternating electric field of 30 s, 1 MHz, 15 Vrms. Content of the chamber was transferred to hybridoma selective medium and plated in a 96-well plate under limiting dilution conditions. On day 12 following the electrofusion, hybridoma

supernatants were screened for hCTLA-4 binding activity, as described above. Hybridomas that secreted antibodies in the supernatant that bound hCTLA-4 were subcloned by limited dilution to safeguard their integrity and stability. Stable hybridomas were cultured in serum-free media for 7-10 days; supernatants were harvested and antibodies were purified using MabSelect Sure Protein A resin according to the manufacturer's instructions (GE Healthcare). Antibody concentrations were quantified using spectrophotometry. Supernatants of the hybridoma cultures were used to isotype the hybridomas. In short, isotyping was done using a mouse monoclonal antibody isotyping kit (Biorad) based on a dipstick with immobilized goat-anti-mouse antibody bands to each of the common mouse isotypes and light chains. Recovered antibodies were all identified as mouse IgG1. Antibody sequences were elucidated by sequencing of variable regions of the mouse IgG1 hybridoma material, using the following method: the total RNA of the hybridoma cells was extracted, which allowed cDNA synthesis. Rapid Amplification of cDNA Ends (RACE) was performed that allowed cloning of positive fragments in a TOPO (ThermoFisher) vector. TOPO clones were sequenced and sequences were annotated using VBASE2 (http://www.vbase2.org).

In experiments binding and blocking was compared to 10D1 (US20020086014) or CP-675,206 (WO2007113648), which were expressed as human IgG1 kappa and IgG2 kappa, respectively. Plasmids encoding VH and VL constructs were transiently expressed by transfection into FreeStyle 293-F human embryonic kidney cells (HEK293T/17, ATCC-CRL-11268), using 293fectin transfection reagent (Invitrogen) following the manufacturer's instructions. Supernatants (30 ml) were harvested after 7 days and antibodies were purified using MabSelect Sure Protein A resin according to the manufacturer's instructions (GE Healthcare). Buffer was exchanged for 10 mM Histidine, 100 mM NaCl pH 5.5 buffer using Zeba desalting columns (Thermo Scientific). The concentration of purified antibodies was determined based on OD280 (Nanodrop ND-1000). Endotoxin level was determined by LAL-test according to the manufacturer's instructions (Lonza).

hCTLA-4 antibodies were characterized for binding to hCTLA-4, *Macaca fascicularis* (cynomolgus) CTLA-4 and blockade of ligand binding (hCD80/hCD86). Next, in vitro functionality was determined using a Jurkat-based reporter assay (Promega), following the manufacturer's procedures. In short, Raji cells expressing

hCD80/hCD86 were co-incubated with Jurkat T cells stably expressing membrane CTLA-4 and an IL2-RE-luciferase reporter. To this mixture a mouse anti-human CD3 antibody (BD Pharmingen) and goat anti-mouse IgG antibody (Thermo Fisher) were added followed by a dilution range of hCTLA-4 mouse antibodies (starting at 200 µg/ml and the dilutions thereof). After six hours of incubation at 37°C, 5% CO_2 and 95% humidity IL-2 promoter activity was detected by addition of Bio-Glo™ substrate (Promega) and using an Envision reader (Perkin Elmer). As shown in Figure 1, hCTLA4.27A more potently enhances IL-2 promoter as compared to 10D1.

Example 2: Humanized antibody design and CDR grafting

The mouse hCTLA4.27A antibody was humanized by CDR-grafting technology (*see e.g. U.S. Patent No. 5,225,539 and Williams, D.G. et al., 2010, Antibody Engineering, volume 1, Chapter 21*).

First, human germline sequences were identified using IgBLAST (Ye J. et al., 2013, Nucleic Acids Res. 41:W34-40). For the hCTLA4.27A VH human germline sequence, V-gene IGHV1-46*01 was identified (62.2% identity) and for the VL human germline sequence IGKV1-NL1*01 was identified (68.4% identity). These two germline sequences were used to directly graft the mouse CDRs, resulting in the following two cDNA constructs: SEQ ID NO: 15 (V_H) and SEQ ID NO: 27 (V_L). Next, a database was constructed containing all human sequences available in the IMGT database (*Lefranc, M.-P. et al., 1999, Nucleic Acid Res. 27:209-212*) identifying 82,958 individual sequences. These sequences were queried using TBLASTN (2.2.30+) to identify template sequences that demonstrated the highest identify to the framework of hCTLA4.27A V_H and V_L sequences. Three V_H and three V_L sequences were identified that demonstrated a similarity score of 70% or higher and that displayed similar CDR lengths, preferably identical to those in hCTLA4.27A V_H CDR1, CDR2, CDR3 and V_L CDR1, CDR2 and CDR3, respectively.

For the heavy chain, the frameworks encoded by GenBank (Benson, D.A. et al., 2013, Nucleic Acids Res. 41(D1):D36-42) accession # L39130, DI109259, and DD431634 were selected as templates for straight grafting of the hCTLA4.27A V_H CDRs, resulting in the following cDNA constructs: SEQ ID NO: 9, 11 and 13. respectively. For the light chain, the frameworks encoded by GenBank accession # AB063955, DI112350, and AB363305 were selected as templates for straight grafting

of the hCTLA4.27A V_L CDRs, resulting in the following cDNA constructs: SEQ ID NO: 21, 23 and 25. Framework and CDR definition were those as described by Kabat et al.

To study the effect of humanized framework residues on the structure of the Fv, a homology model of the mouse hCTLA4.27A Fv was made using the 'Antibody Modeling Cascade' (default parameters) within Discovery Studio 4.5. The homology model was built on basis of PDB ID 3V7A.

The CDRs were grafted in silico to study residues that are close to any of the CDRs and which might affect the loop conformation, referred as Vernier residues. Residues that might affect the loop conformation, and which are within < 5Å to the CDR surface were identified and substituted with the mouse amino acid at this position. The resulting templates were checked for the presence of post translational modification (PTM) motifs using Discovery Studio 4.5 and where possible (i.e. non-CDR, non-Vernier residues) changed to prevent a PTM. For the heavy chain, removal of the predicted sequence PTM motifs and structural considerations (i.e. rigidity of the backbone) in the hCTLA4.27A V_H resulted in the design of two additional constructs: SEQ ID NO: 17 and 19. For the light chain the PTM removal resulted in the following construct: SEQ ID NO: 29.

CDRs were grafted on each of the identified templates, expressed as a human IgG4 (SEQ ID 50), kappa (SEQ ID 52) antibody cloned in the pcDNA3.1(+) vector and transient transfection in HEK293 Free-style cells. An IgG4 version of humanized antibodies was produced, with the stabilizing Adair mutation (*Angal S. et al., 1993, Mol Immunol. 30: 105-108*), where Serine 228 is converted to Proline.

The hCTLA4.27IgG1 was also expressed as a human IgG1 (SEQ ID 48), kappa antibody (SEQ ID 52) cloned in the pcDNA3.1(+) vector and transiently transfected in HEK293 Free-style cells. The plasmids encoding the human IgG1 heavy chain and light chain constructs were mixed in a 1:1 ratio (1280 µg in total) and transiently expressed by transfection into FreeStyle 293-F human embryonic kidney cells (HEK293T/17, ATCC-CRL-11268), using 293fectin transfection reagent (Invitrogen) following the manufacturer's instructions. Supernatant (1250 ml) were harvested after 7 days and hCTLA4.27IgG1 was purified using MabSelect Sure Protein A resin according to the manufacturer's instructions (GE Healthcare). Buffer was exchanged for 10 mM Histidine, 100 mM NaCl pH 5.5 buffer using Zeba desalting

columns (Thermo Scientific). The concentration of purified hCTLA4.27IgG1 was determined based on OD280 (Nanodrop ND-1000).

In addition, the mouse hCTLA4.27A V_H and V_L (SEQ ID NO: 32 and 34) were expressed as a chimeric human IgG1 (hCTLA4.27A.C1) and IgG4 (hCTLA4.27A.C4), kappa antibody, cloned in the pcDNA3.1(+) vector and transient transfection in HEK293 Free-style cells.

Example 3: Synthesis, expression and purification of humanized constructs

The plasmids encoding the heavy chain and light chain constructs were mixed in a 1:1 ratio (30 µg in total) and transiently expressed by transfection into FreeStyle 293-F human embryonic kidney cells (HEK293T/17, ATCC-CRL-11268), using 293fectin transfection reagent (Invitrogen) following the manufacturer's instructions. Supernatants (30 ml) were harvested after 7 days and antibodies were purified using MabSelect Sure Protein A resin according to the manufacturer's instructions (GE Healthcare). Buffer was exchanged for 10 mM Histidine, 100 mM NaCl pH 5.5 buffer using Zeba desalting columns (Thermo Scientific). The concentration of purified antibodies was determined based on OD280 (Nanodrop ND-1000). Endotoxin level was determined by LAL-test according to the manufacturer's instructions (Lonza).

Example 4: Binding of humanized CTLA-4 antibodies

Binding of the humanized antibodies to hCTLA-4 was studied in CELISA format. CHO-K1.hCTLA-4 cells were seeded in culture medium (DMEM-F12 (Gibco) with 10% Fetal Bovine Serum (Hyclone) and Pen/Strep (Gibco)) in tissue culture plates and cultured at 37°C, 5% CO_2 and 95% humidity until they were confluent. Subsequently, culture medium was removed and cells were incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with purified hCTLA-4 antibodies (10 µg/ml and dilutions thereof). Next, cells were washed with PBS/0.05% Tween (PBST) and incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with goat-anti-human IgG-HRP (Southern Biotechnology) or goat-anti-mouse IgG-HRP (Southern Biotechnology). Subsequently, cells were washed 3 times with PBST and anti-hCTLA-4 immunoreactivity was visualized with TMB Stabilized Chromagen (Invitrogen).

Reactions were stopped with 0.5 M H_2SO_4 and absorbances were read at 450 and 610 nm. EC50 values, the concentration at which 50% of the total binding signal is observed, were calculated using Graphpad Prism 6. In Table 1 the EC50 values of the humanized hCTLA4.27 antibodies are depicted.

Table 1: Binding of humanized hCTLA4.27 antibodies, parental and chimeric antibodies to human CTLA-4 expressed on CHO-K1.hCTLA-4 cells. EC50 values represent the concentration at which 50% of the total binding signal is observed (average and SD were calculated from values of two independent experiment).

	hCTLA-4 Binding EC50 (nM)	
Antibody	**Average**	**SD**
hCTLA4.27H1L1	0,037	0,015
hCTLA4.27H1L2	0,040	0,013
hCTLA4.27H1L3	0,045	0,028
hCTLA4.27H1L5	0,046	0,037
hCTLA4.27H2L1	0,042	0,041
hCTLA4.27H2L2	0,044	0,003
hCTLA4.27H2L3	0,051	0,002
hCTLA4.27H2L5	0,045	0,010
hCTLA4.27H3L1	0,070	0,006
hCTLA4.27H3L2	0,065	0,008
hCTLA4.27H3L3	0,101	0,010
hCTLA4.27H3L5	0,082	0,022
hCTLA4.27H4L1	0,070	0,010
hCTLA4.27H4L2	0,047	0,008
hCTLA4.27H4L3	0,051	0,006
hCTLA4.27H4L5	0,061	0,002
hCTLA4.27H5L1	0,029	0,016
hCTLA4.27H5L2	0,042	0,032
hCTLA4.27H5L3	0,042	0,010
hCTLA4.27H5L5	0,035	0,011

hCTLA4.27H6L1	0,044	0,003
hCTLA4.27H6L2	0,027	0,004
hCTLA4.27H6L3	0,028	0,004
hCTLA4.27H6L5	0,036	0,005
hCTLA4.27A	0,048	0,005
hCTLA4.27A.C1	0,022	0,009
hCTLA4.27A.C4	0,033	0,005
Variants with the L4 light chain did not bind to human CTLA-4		

Binding of the hCTLA-4 antibodies to cynomolgus CTLA-4 was confirmed using CHO-K1 cells (American Type Culture Collection, Manassas, VA) that had been transiently transfected with cDNA encoding the full length open reading frame of cynomolgus CTLA-4 (SEQ ID NO: 39), subcloned into the pCI-neo vector (Promega). CHO-K1.cynoCTLA-4 cells were seeded in tissue culture plates and incubated at 37°C, 5% CO_2 and 95% humidity for until cell layers were confluent. Subsequently culture medium was removed and cells were incubated for 1 hour with purified hCTLA-4 antibodies (10 µg/ml and dilutions thereof) at 37°C, 5% CO_2 and 95% humidity. Next, cells were washed with PBST and incubated for 1 hour at 37°C with goat-anti-human IgG-HRP (Jackson Immuno Research). Subsequently, cells were washed 3 times with PBST and anti-CTLA-4 immunoreactivity was visualized with TMB Stabilized Chromagen (Invitrogen). Reactions were stopped with 0.5 M H2SO4 and absorbances were read at 450 and 610 nm.

Binding of hCTLA4.27 humanized antibodies to CTLA-4 expressed on human CD3+ T cells was confirmed by flow cytometry. Human CD3+ T cells were isolated from human buffy coat as follows. First, the Buffy coat was diluted to a total volume of 180 ml with PBS at room temperature. After mixing the cell suspension, aliquotes were loaded on a Ficoll-Paque Plus gradient in Sepmate tubes (Stemcell Technologies) and centrifuged at 1200 g for 10 min, at 20°C without a brake. Next, plasma was removed by aspiration and PBMCs were recovered from the plasma/Ficoll interface. PBMCs were washed three times in PBS. Subsequently, CD3+ T cell isolation was conducted with magnetic beads (CD3+ T-cell Biotin-Ab cocktail; Miltenyi Biotec). Next, T cells were stimulated with αCD3/αCD28 coated beads (Thermo Fisher Scientific) for 48 hours. First T-cell stimulation was confirmed by detection of blast

formation using flow cytometry. Binding of hCTLA4.27 humanized antibodies was assessed after fixation and permeabilization of T cells with Cytofix/Cytoperm (BD). Cells were washed twice in perm/wash buffer (BD), incubated with the hCTLA4.27 humanized antibodies, washed three times, and finally incubated with a FITC-labelled Goat-anti-human-hIgG detection antibody (Southern Biotech). After this labeling procedure, cells were washed two times, resuspended in FACS buffer and analysed by flow Cytometry on the FACS Canto II (BD). Data were processed and analysed with Flowjo Software.

Binding of hCTLA4.27 humanized antibodies to CTLA-4 expressed on *Macaca fascicularis* (cynomolgus) PBMCs was confirmed by flow cytometry. To this end cynomolgus blood was diluted 1:1 with PBS and added to 50ml tubes containing 13ml Lymphoprep 95%/PBS 5%. Cells were centrifuged for 30 minutesat 450 g and 20°C without brake. Next, plasma was removed by aspiration and PBMCs were recovered from the plasma/Ficoll interface. PBMCs were washed twice times in PBS. Cells were frozen in liquid nitrogen and retrieved from the freezer on the day of the experiment. Since endogenous expression of CTLA-4 on resting immune cells is low, the thawed PBMCs were stimulated with αCD3/αCD28/αCD2 coated beads (Milteny Biotec) for 48 hours. Subsequently, stimulation of the PBMCs was confirmed by detection of blast formation using flow cytometry. Next, the cells were analyzed by flow cytometry for intracellular binding of hCTLA4.27 antibodies.

Example 5: Blockade of hCD80 binding to hCTLA-4 by humanized hCTLA4.27 antibodies

hCD80 blockade was assessed in CELISA format for the full panel of humanized hCTLA4.27 antibodies. CHO-K1.hCTLA-4 cells were seeded in tissue culture plates and incubated at 37°C, 5% CO_2 and 95% humidity in culture medium. Once the cells were confluent culture medium was removed and cells were incubated for 1 hour with the humanized hCTL4.27 antibody variants (10 µg/ml and dilutions thereof) at 37°C, 5% CO_2 and 95% humidity. Next, cells were washed with PBS/0.05% Tween-20 (PBST) and incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with biotinylated recombinant hCD80/Fc-protein. Cells were then washed with PBST followed by addition of Streptavidin-HRP conjugate on the cells, which was incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity. Subsequently cells were washed three

times with PBST and binding of hCD80/Fc-protein was visualized with TMB Stabilized Chromagen (Invitrogen). Reactions were stopped with 0.5 M H_2SO_4 and absorbances were read at 450 and 610 nm. IC50 values for the blockade of hCD80 were calculated from this data and are represented in Table 2. IC50 values represent the concentration at which half of the inhibition is observed.

Table 2: Blockade of hCD80 binding by humanized hCTLA4.27 antibodies, parental and chimeric antibodies. IC50 values represent the concentration at which half of the inhibition is observed (average and SD were calculated from values of two independent experiment).

	hCD80 Blocking IC50 (nM)	
Antibody	**Average**	**SD**
hCTLA4.27H1L1	2,546	1,003
hCTLA4.27H1L2	2,716	1,193
hCTLA4.27H1L3	3,127	0,764
hCTLA4.27H1L5	3,444	1,048
hCTLA4.27H2L1	2,982	1,000
hCTLA4.27H2L2	2,586	0,872
hCTLA4.27H2L3	2,969	0,519
hCTLA4.27H2L5	2,722	0,197
hCTLA4.27H3L1	2,519	0,806
hCTLA4.27H3L2	2,061	0,399
hCTLA4.27H3L3	3,365	1,821
hCTLA4.27H3L5	3,666	1,468
hCTLA4.27H4L1	3,331	1,720
hCTLA4.27H4L2	2,519	0,706
hCTLA4.27H4L3	3,257	2,375
hCTLA4.27H4L5	3,077	2,272
hCTLA4.27H5L1	5,388	4,397
hCTLA4.27H5L2	10,334	n.a.
hCTLA4.27H5L3	2,574	0,513

hCTLA4.27H5L5	1,912	0,255
hCTLA4.27H6L1	2,056	0,482
hCTLA4.27H6L2	1,805	0,622
hCTLA4.27H6L3	1,821	1,089
hCTLA4.27H6L5	2,571	1,163
hCTLA4.27A	2,175	0,249
hCTLA4.27A.C1	1,535	0,471
hCTLA4.27A.C4	1,611	0,395
1) Variants with the L4 light chain did not bind CTLA-4		

Example 6: hCTLA4.27 affinity, binding to hCTLA-4 and its blocking abilities of the hCD80/hCD86 interaction compared to 10D1 (ipilimumab) and CP-675,206 (tremelimumab)

hCTLA4.27 binding kinetics and equilibrium binding constants were profiled using bio-light interferometry on the Octet RED96 and compared to several antibodies known in the art. First, anti-hCTLA-4 mAbs were coupled to amine-reactive second generation biosensors (Fortebio) using standard amine chemistry. hCTLA-4 binding to and dissociation from the biosensors was then observed at various hCTLA-4 concentrations. Amine-reactive biosensors were pre-wet by immersing them in wells containing 0.1M MES pH=5.5 for 10 minutes. The biosensors were then activated using a 0.1M NHS/0.4M EDC mixture for 5 minutes. Antibodies were coupled by immersing the biosensors in a solution of 2.5 or 12 ug/mL antibody in 0.1M MES for 7.5 minutes. The biosensor surface was quenched using a solution of 1M ethanolamine for 5 minutes. Biosensors were equilibrated in Octet kinetics buffer (ForteBio) for 5 minutes. Association of rhCTLA-4/Fc (R&D Systems) was observed by placing the biosensors in wells containing various rhCTLA-4/Fc concentrations (2.5-40 nM) and monitoring interferometry for 15 minutes. Dissociation was measured after transfer of the biosensors into kinetics buffer and monitoring of the interferometry signal for 45 minutes. The assay was run with a plate temperature of 30°C. The observed on and off rates (kon and kdis) were fit using a 1:1 binding global fit model comprising all concentrations tested, and the equilibrium binding constant KD was calculated. As shown in Table 3: hCTLA4.27 has similar binding affinity as control antibodies.

Table 3: Affinities of hCTLA4.27 formatted as human IgG1 and human IgG4 in relation to control antibodies 10D1 and CP-675,206

	KD (M)	KD error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error
hCTLA4.27A	2.0E-09	1.7E-09	5.0E+05	3.4E+05	4.5E-04	1.7E-04
hCTLA4.27IgG1	4.0E-09	3.7E-09	2.3E+05	1.8E+05	2.5E-04	1.3E-04
hCTLA4.27IgG4	1.2E-09	1.9E-10	5.7E+05	4.0E+05	6.4E-04	4.1E-04
10D1	1.2E-09	5.9E-10	4.1E+05	6.2E+04	4.4E-04	1.7E-04
CP-675,206	3.4E-09	2.6E-09	1.0E+05	4.2E+04	2.4E-04	1.3E-04

Next, binding of hCTLA4.27 antibodies to hCTLA-4 and its blocking abilities of the hCD80/hCD86 interaction were compared to 10D1 and CP-675,206 in CELISA format. In short, CELISA for hCTLA-4 binding was performed on CHO-K1.hCTLA-4 cells. Detection of bound antibody was done with goat-anti-mouse IgG HRP (Southern Biotech) for the mouse hCTLA4.27A and goat-anti-human IgG-HRP (Southern Biotech) for the hCTLA4.27A chimeric hIgG1 and hIgG4 and control antibodies respectively. For the assessment of hCD80 and hCD86 blockade CHO-K1.hCTLA-4 cells were seeded in tissue culture plates and incubated at 37°C, 5% CO_2 and 95% humidity in culture medium. Once the cells were confluent culture medium was removed and cells were incubated for 1 hour with the hCTL4.27 antibodies and control antibodies (10 µg/ml and dilutions thereof) at 37°C, 5% CO_2 and 95% humidity. Next, cells were washed with PBS/0.05% Tween-20 (PBST) and incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with biotinylated recombinant hCD80/Fc-protein or hCD86/Fc protein. Cells were then washed with PBST followed by addition of Streptavidin-HRP conjugate on the cells, which was incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity. Subsequently cells were washed three times with PBST and binding of hCD80/Fc-protein or hCD86/Fc-protein was visualized with TMB Stabilized Chromagen (Invitrogen). Reactions were stopped with 0.5 M H_2SO_4 and absorbances were read at 450 and 610 nm. IC50 values represent the concentration at which half of the inhibition is observed.

As depicted in figure 2 the hCD80 blocking profile of hCTLA4.27IgG1 and hCTLA4.27IgG4 antibodies lies between the hCD80 profiles of ipilimumab and tremelimumab. Thus although IC50 values are comparable, the efficacy plateau differs.

Table 4: Binding of hCTLA4.27A, hCTLA4.27A chimeric hIgG1 and hIgG4 and control antibodies to hCTLA-4 and their blocking abilities of the

hCD80/hCD86 interaction. EC50 values represent the concentration at which 50% of the total binding signal is observed. IC50 values represent the concentration at which half of the inhibition is observed (averages and SDs were calculated from values of two independent experiments).

	hCTLA-4 Binding EC50 (nM)		hCD80 Blocking IC50 (nM)		hCD86 Blocking IC50 (nM)	
	Average	**SD**	**Average**	**SD**	**Average**	**SD**
hCTLA4.27A	0,053	0,010	2,715	0,328	2,167	0,307
hCTLA4.27A.C1	0,034	0,001	1,454	0,565	1,175	0,040
hCTLA4.27A.C4	0,053	0,013	1,597	0,359	1,205	0,031
10D1	0,053	0,006	3,253	0,517	2,309	0,012
CP-675,206	0,037	0,006	1,754	0,234	1,690	0,194

Example 7: Functionality of humanized hCTLA4.27 antibodies in the Human PBMC SEB assay

To confirm the functionality of humanized hCTLA4.27 in primary immune cells, PBMCs were isolated from buffy coats of human donor blood. First, the buffy coat was diluted and mixed to a total volume of 180 ml with PBS at room temperature. Aliquots were loaded on a Ficoll-Paque Plus gradient in Sepmate tubes (Stemcell Technologies) and centrifuged at 1200 g for 10 min, at 20°C without a brake. Next, plasma was removed by aspiration and PBMCs were recovered from the plasma/Ficoll interface. PBMCs were washed three times in PBS before use in the assay. PBMCs were seeded at $2*10^5$ cells per well. Subsequently humanized hCTLA4.27 and control antibodies were diluted in RPMI 1640 medium (Gibco) supplemented with 10% Fetal Calf Serum and added in a concentration range starting at 100ug/ml with square root 10 dilution steps. Staphylococcus Enterotoxin B (Sigma) diluted in RPMI 1640 medium supplemented with 10% Fetal Calf Serum was added at a concentration of 10µg/ml. Plates were incubated for seventy-two hours at 37°C, 5% CO_2 and 95% humidity, followed by isolation of supernatants.

IL-2 secretion was detected in the supernatant as a measure of immune activation. Supernatants were cleared from any cell material by centrifugation and added to Nunc maxisorp ELISA plates that had been coated with anti-hIL-2 antibody

(BD Pharmingen) in PBS by incubation at 4°C for a minimal period of 16 hours. Prior to addition of the supernatant, wells were emptied and blocked with PBS/1%BSA for one hour at Room Temperature (RT). Supernatants were incubated in the anti-hIL-2 coated plates for one hour at RT after which plates were washed three times with PBST (PBS with 0.05% Tween 20). Subsequently, 0.5 µg/ml of anti-hIL2-biotin (BD Pharmingen) was added in PBST/0.5%BSA and incubated for one hour at RT. After three washes with PBST, 1:5000 diluted streptavidin-HRP (BD Pharmingen) was added in PBST/0.5%BSA. After six washes with PBST, IL-2 was detected by addition of TMB stabilized chromogen (Invitrogen). Reactions were stopped with 0.5 M H_2SO_4 and absorbances were read at 450 and 610 nm. In this assay, recombinant human IL-2 (Sigma) was used as a reference to quantify IL-2 protein levels in the supernatants. Fig. 3 shows that hCTLA4.27 antibodies enhance immune activation.

Example 8: hCTLA4.27 in effector function assays

The ability of the chimeric hCTLA4.27A antibodies: hCTLA4.27A.C1 and hCTLA4.27A.C4 to induce Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC) was studied. For the ADCC assay, human NK cells were used as effector cells. NK cells were isolated from human blood. Buffy coats were enriched for NK cells with the Rosette SEP NK enrichment cocktail (Stemcell Technologies). Subsequent the buffy coat was mixed 1:1 with PBS/2% FBS and layered on Ficol Paque plus (GE Healthcare). After centrifugation the interphase was collected and cells were washed with PBS/2% FBS. Characterization of the isolated NK cells by flow cytometry confirmed CD16 and CD56 expression.

CHO-K1.hCTLA-4 were used as target cells and seeded in a flat bottom cell culture plate together with NK cells in an effector:target ratio of 10:1. hCTLA4.27 antibodies or control antibodies (10D1 or isotype-matched control antibodies (hIgG1, hIgG4)) were added (100 µg/ml and dilutions thereof). Plates were incubated overnight at 37°C, 5% CO_2 and 95% humidity. After overnight incubation, cells were washed with PBST (PBS and 0.01% Tween-20) and incubated in RPMI (Gibco) supplemented with 10% Fetal Bovine Serum (Hyclone) and 1% Pen/Strep (Gibco) incubated at 37°C, 5% CO_2 and 95% humidity for 30 minutes. Subsequently Celltiter 96 Aqueous One solution (Promega) was added followed by 3 hours incubation at 37°C, 5% CO_2 and 95% humidity. Cell viability was assessed by analyzing the OD492-690 using an iEMS

reader (Labsystems). As shown in Figure 4A, hCTLA4.27A.C1 induced NK-mediated cell lysis in two different donors, while formatted as an hIgG4 it was not able to induce cytotoxicity.

To assess Complement-dependent Cytotoxicity a concentration range of hCTLA4.27 antibodies was added to confluent monolayers of CHO-K1.hCTLA-4 target cells. After a 15 minutes incubation period 50% human complement serum (Sigma) was added to the cells. After 3,5 hours incubation cells were washed with PBST (PBS and 0.01% Tween-20), incubated with supplemented RPMI (Gibco) and incubated at 37°C, 5% CO_2 and 95% humidity for 30 minutes. Subsequently Celltiter 96 Aqueous One solution (Promega) was added followed by 3 hours incubated at 37°C, 5% CO_2 and 95% humidity. Cell viability was assessed by analyzing the OD492-690 in an iEMS reader (Labsystems). As shown in Figure 4B, hCTLA4.27A.C1does induce Complement-mediated cell lysis in the CDC assay, while formatted as an hIgG4 it does not induce complement-mediated cell cytotoxicity.

Example 9: Cross competition of hCTLA4.27 and control antibodies

To characterize the difference in binding site of hCTLA4.27 compared to 10D1 (ipilimumab) and CP-675,206 (tremelimumab) competition between the antibodies was profiled using bio-light interferometry as described previously. Amine-reactive biosensors were pre-wet by immersing them in wells containing 0.1M MES pH=5.5 for 10 minutes. The biosensors were then activated using a 0.1M NHS/0.4M EDC mixture for 5 minutes. hCTLA4.27 formatted as human IgG1 or human IgG4 was coupled by immersing the biosensors in a solution of 12 µg/ml mAb in 0.1M MES for 7.5 minutes. The biosensor surface was quenched using a solution of 1M ethanolamine for 5 minutes. Biosensors were equilibrated in Octet kinetics buffer (ForteBio) for 5 minutes. Association of rhCTLA-4/Fc was observed by placing the biosensors in wells containing a fixed concentration rhCTLA-4/Fc (12 µg/ml) and monitoring interferometry for 15 minutes. Next, for an additional 2 minutes the same anti-hCTLA-4 mAb as coupled to the biosensor was allowed to bind, to ensure binding of all available rhCTLA-4/Fc binding sites. Competition or non-competition was determined by placing the biosensors for 5 minutes in wells containing a fixed concentration (6 µg/ml) of another or the same anti-hCTLA-4 mAb or a reference well containing kinetics buffer

only. In this direct competition assay, binding of hCTLA4.27 to rhCTLA-4 does not block binding of control antibodies to rhCTLA-4/Fc as shown in Table 5.

Table 5: Cross competition of hCTLA4.27 and control antibodies. Binding of the second antibody in nm shift, zero or negative value means no binding is observed.

	Binding of second antibody (in nm shift)			
Coupled antibody	**hCTLA4.27IgG1**	**hCTLA4.27IgG4**	**10D1**	**CP-675,206**
hCTLA4.27IgG1	-0.0144	-0.0164	0.0534	0.0877
hCTLA4.27IgG4	-0.0096	-0.0132	0.0395	0.0714

Example 10: Binding to human/mouse CTLA-4 exchange mutants

The difference in binding regions between hCTLA4.27 in comparison to 10D1 and CP-675,206 was confirmed using two hCTLA-4 mutants. hCTLA-4 mutants were designed which were half human and half mouse. Based on the fold of CTLA-4, an Ig-like V-type (immunoglobulin-like) domain, the protein can be divided into two subdomains: one containing beta-strand 1, 2, 5, and 6, including connecting loops and one containing beta-strand 3, 4, 7, and 8, including connecting loops. The human-mouse variant (SEQ ID NO: 42, Hum-Mou-CTLA-4) contains human residues on strand 1, 2, 5 and 6 and mouse residues on strand 3, 4, 7, and 8. The mouse-human variant (SEQ ID NO 44, Mou-Hum-CTLA-4) contains mouse residues on strand 1, 2, 5 and 6 and human residues.

The cDNAs encoding these constructs (SEQ ID NO: 41 and 43, respectively) were synthesized and subcloned into the pCI-Neo vector (GeneArt). Binding of hCTLA4.27A chimeric hIgG4 (hCTLA4.27A.C4), 10D1 and CP-675,206 to the exchange mutants was tested using CELISA. To this end CHO-K1 cells were transiently transfected, using Lipofectamine 2000 (Invitrogen), with the pCI-Neo vectors expressing human CTLA-4 (hCTLA-4), mouse CTLA-4 (mCTLA-4), Hum-Mou-CTLA-4, and Mou-Hum-CTLA-4 respectively. The transfected cells were cultured at 37°C, 5% CO_2 and 95% humidity in medium (DMEM-F12 (Gibco) with 5% New Born Calf serum (Biowest) and Pen/Strep (Gibco)) until confluent. Subsequently, cells were trypsinized and seeded in tissue culture plates and cultured at 37°C, 5% CO_2 and 95% humidity in culture medium until confluent. Then, culture medium was removed and cells were incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with

hCTLA-4 antibodies. Next, cells were washed with PBS/0.05% Tween (PBST) and incubated for 1 hour at 37°C, 5% CO_2 and 95% humidity with 1:2,000 goat-anti-human IgG-HRP (Jackson Immunoresearch). After that, cells were washed 3 times with PBST and anti-CTLA-4 immunoreactivity was visualized with TMB Stabilized Chromagen (Invitrogen). Reactions were stopped with 0.5 M H_2SO_4 and absorbances were read at 450 and 610 nm. hCTLA4.27A.C4 showed binding to the human-mouse exchange mutant, while 10D1 and CP-675,206 could bind to the mouse-human exchange mutant (figure 5).

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Leu Glu Phe Ser Ser Leu Thr Asn Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

gcc cgg atc ggc aga cgg aac ccc tac tac ttc gac tac tgg ggc cag        336
Ala Arg Ile Gly Arg Arg Asn Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110

ggc acc ctc gtg aca gtg tct agc                                       360
Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

<210> 18
<211> 120
<212> PRT
<213> Artificial Sequence
<220>
<223> Synthetic Construct
<400> 18

```
Glu Val Gln Leu Leu Gln Ala Gly Ala Val Leu Ala Arg Pro Gly Thr
1               5                   10                  15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Thr Tyr
            20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Ser Leu Asn Gln Ala Phe
    50                  55                  60

Lys Asp Lys Ala Lys Leu Thr Ala Ala Thr Ser Ala Ser Ile Ala Tyr
65                  70                  75                  80

Leu Glu Phe Ser Ser Leu Thr Asn Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Ile Gly Arg Arg Asn Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

<210> 19
<211> 360
<212> DNA
<213> Artificial Sequence
<220>
<223> hCTLA4.27VH6
<220>
<221> CDS
<222> (1)..(360)
<400> 19

```
gag gtg cag ctg ctg gaa tct ggc cct gaa ctc gtg cgg cct ggc agc         48
Glu Val Gln Leu Leu Glu Ser Gly Pro Glu Leu Val Arg Pro Gly Ser
1               5                   10                  15

agc gtg aag atc agc tgt aaa gcc agc ggc tac agc ttc acc acc tac         96
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Thr Tyr
            20                  25                  30

tgg atg aac tgg gtc aag cag agg ccc ggc aag ggc ctg gaa tgg atc        144
Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45
```

```
ggc atg atc cac ccc agc gac agc gag aca agc ctg aac cag gcc ttc        192
Gly Met Ile His Pro Ser Asp Ser Glu Thr Ser Leu Asn Gln Ala Phe
    50                  55                  60

aag gac aaa gtg aag ctg acc gcc gcc acc agc gcc tct atc gcc tac        240
Lys Asp Lys Val Lys Leu Thr Ala Ala Thr Ser Ala Ser Ile Ala Tyr
65                  70                  75                  80

ctg gaa ttt tcc agc ctg cgg aac gag gac agc gcc gtg tac tac tgc        288
Leu Glu Phe Ser Ser Leu Arg Asn Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

gcc cgg atg ggc aga cgg aac ccc tac tac ttc gac tac tgg ggc cag        336
Ala Arg Met Gly Arg Arg Asn Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110

ggc acc ctc gtg aca gtg tct agc                                       360
Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

<210> 20
<211> 120
<212> PRT
<213> Artificial Sequence
<220>
<223> Synthetic Construct
<400> 20

```
Glu Val Gln Leu Leu Glu Ser Gly Pro Glu Leu Val Arg Pro Gly Ser
1               5                   10                  15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Thr Tyr
            20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Ser Leu Asn Gln Ala Phe
    50                  55                  60

Lys Asp Lys Val Lys Leu Thr Ala Ala Thr Ser Ala Ser Ile Ala Tyr
65                  70                  75                  80

Leu Glu Phe Ser Ser Leu Arg Asn Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Met Gly Arg Arg Asn Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

<210> 21
<211> 321
<212> DNA
<213> Artificial Sequence
<220>
<223> hCTLA4.27VL1
<220>
<221> CDS
<222> (1)..(321)
<400> 21

```
gac atc cag atg acc cag agc ccc agc agc ctg tct gcc agc gtg ggc         48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15
```

```
gac aga gtg acc atc acc tgt cgg ccc agc gag aac ctg tac acc aac          96
Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

ctg gcc tgg tat cag cag aag ccc ggc aag gcc ccc aaa ctg ctg ctg         144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

tac ggc gcc acc aat ctg gcc gat ggc gtg ccc agc aga ttt tcc ggc         192
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

tct ggc agc ggc acc gac tac acc ctg acc atc tct agc ctg cag ccc         240
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

gag gac ttc gcc acc tac tac tgt cag cac ctg tgg ggc acc ccc ttc         288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

acc ttt ggc cag ggc acc aag ctg gaa atc aag                             321
Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105

<210>  22
<211>  107
<212>  PRT
<213>  Artificial Sequence
<220>
<223>  Synthetic Construct
<400>  22

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105

<210>  23
<211>  321
<212>  DNA
<213>  Artificial Sequence
<220>
<223>  hCTLA4.27VL2
<220>
<221>  CDS
<222>  (1)..(321)
<400>  23

gac atc cag atg acc cag agc ccc agc agc ctg tct gcc agc gtg ggc          48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15
```

```
gac aga gtg acc atc acc tgt cgg ccc agc gag aac ctg tac acc aac          96
Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

ctg gcc tgg tat cag cag aag ccc ggc aag gcc ccc aag ctg ctg atc         144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

tac ggc gcc acc aat ctg gcc gat ggc gtg ccc agc aga ttt tcc ggc         192
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

tct ggc agc ggc acc gag ttc agc ctg agc atc tct agc ctg cag ccc         240
Ser Gly Ser Gly Thr Glu Phe Ser Leu Ser Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

gag gac ttc gcc acc tac tac tgt cag cac ctg tgg ggc acc ccc ttc         288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

acc ttt ggc ggc gga aca aag gtg gaa atc aag                             321
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
            100                 105

<210>  24
<211>  107
<212>  PRT
<213>  Artificial Sequence
<220>
<223>  Synthetic Construct
<400>  24

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Glu Phe Ser Leu Ser Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
            100                 105

<210>  25
<211>  321
<212>  DNA
<213>  Artificial Sequence
<220>
<223>  hCTLA4.27VL3
<220>
<221>  CDS
<222>  (1)..(321)
<400>  25
gac atc cag atg acc cag agc ccc agc agc ctg tct gcc agc gtg ggc          48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15
```

```
gat acc gtg acc atc acc tgt cgg ccc agc gag aac ctg tac acc aac          96
Asp Thr Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

ctg gcc tgg tat cag cag aag ccc ggc aag gcc ccc aaa ctg ctg ctg         144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

tac ggc gcc acc aat ctg gcc gat ggc gtg ccc agc aga ttt tcc ggc         192
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

tct ggc agc ggc acc gac tac acc ctg acc atc tct agc ctg cag agc         240
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Ser
65                  70                  75                  80

gag gac ttc gcc acc tac tac tgt cag cac ctg tgg ggc acc ccc ttc         288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

acc ttt ggc cag ggc acc aag ctg gaa atc aag                             321
Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

<210> 26
<211> 107
<212> PRT
<213> Artificial Sequence
<220>
<223> Synthetic Construct
<400> 26

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Thr Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Ser
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

<210> 27
<211> 321
<212> DNA
<213> Artificial Sequence
<220>
<223> hCTLA4.27VL4
<220>
<221> CDS
<222> (1)..(321)
<400> 27

```
gac atc cag atg acc cag agc ccc agc agc ctg tct gcc agc gtg ggc          48
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15
```

```
gac aga gtg acc atc acc tgt cgg ccc agc gag aac ctg tac acc aac          96
Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

ctg gcc tgg tat cag cag aag ccc ggc aag gcc cct aag ctg ctg ctg         144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

tac ggc gcc acc aat ctg gcc gat ggc gtg ccc agc aga ttt tcc ggc         192
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

tct ggc agc ggc acc gac tac acc ctg acc atc tct agc ctg cag ccc         240
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

gag gac ttc gcc acc tac tac tgc cag cac ctg tgg ggc acc ccc ttc         288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

aca ttt ggc gga ggc acc aag ctg gaa atc aag                             321
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105

<210>  28
<211>  107
<212>  PRT
<213>  Artificial Sequence
<220>
<223>  Synthetic Construct
<400>  28

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105

<210>  29
<211>  321
<212>  DNA
<213>  Artificial Sequence
<220>
<223>  hCTLA4.27VL5
<220>
<221>  CDS
<222>  (1)..(321)
<400>  29
gac atc cag atg acc cag gcc cct agc agc ctg tct gcc agc gtg ggc          48
Asp Ile Gln Met Thr Gln Ala Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15
```

```
gac aga gtg acc atc acc tgt cgg ccc agc gag aac ctg tac acc aac         96
Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

ctg gcc tgg tat cag cag aag ccc ggc aag gcc ccc aaa ctg ctg ctg        144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

tac ggc gcc acc aat ctg gcc gat ggc gtg ccc agc aga ttt tcc ggc        192
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

tct ggc agc ggc acc gac tac acc ctg aca atc agc tcc ctg cag gcc        240
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Ala
65                  70                  75                  80

gag gac ttc gcc acc tac tac tgt cag cac ctg tgg ggc acc ccc ttc        288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

acc ttt ggc ggc gga aca aag ctg gaa atc aag                            321
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

<210> 30
<211> 107
<212> PRT
<213> Artificial Sequence
<220>
<223> Synthetic Construct
<400> 30

```
Asp Ile Gln Met Thr Gln Ala Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Leu
        35                  40                  45

Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Ala
65                  70                  75                  80
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

<210> 31
<211> 360
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(360)
<400> 31

```
cag gtc caa ctg cag cag cct ggg gct gta ctg gtg agg cct gga gtt          48
Gln Val Gln Leu Gln Gln Pro Gly Ala Val Leu Val Arg Pro Gly Val
1               5                   10                  15

tca gtg aag ctg tcc tgc aag gct tct ggc tac tcc ttc acc acc tac         96
```

```
Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Thr Tyr
            20                  25                  30

tgg atg aac tgg gtg aag cag agg cct gga caa ggc ctt gag tgg att        144
Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

ggc atg att cat cct tcc gat agt gaa act agt tta aat cag gcg ttc        192
Gly Met Ile His Pro Ser Asp Ser Glu Thr Ser Leu Asn Gln Ala Phe
    50                  55                  60

aag gac aag gcc aca ttg act ata gac aaa tcc tcc agc aca gcc tac        240
Lys Asp Lys Ala Thr Leu Thr Ile Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

atg caa ctc agc agc ccg aca tct gaa gac tct gcg gtc tat ttc tgt        288
Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

gca aga atg gga cgt cgt aat ccc tat tac ttt gac tac tgg ggc caa        336
Ala Arg Met Gly Arg Arg Asn Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110

ggc acc act ctc aca gtc tcc tca                                      360
Gly Thr Thr Leu Thr Val Ser Ser
        115                 120

<210>  32
<211>  120
<212>  PRT
<213>  Mus musculus
<400>  32

Gln Val Gln Leu Gln Gln Pro Gly Ala Val Leu Val Arg Pro Gly Val
1               5                   10                  15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Thr Tyr
            20                  25                  30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Ser Leu Asn Gln Ala Phe
    50                  55                  60

Lys Asp Lys Ala Thr Leu Thr Ile Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Gln Leu Ser Ser Pro Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

Ala Arg Met Gly Arg Arg Asn Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Thr Leu Thr Val Ser Ser
        115                 120

<210>  33
<211>  321
<212>  DNA
<213>  Mus musculus

<220>
<221>  CDS
<222>  (1)..(321)
<400>  33
```

```
gac atc caa atg act cag tct cca gtc tcc cta tct gta tct gtg gga         48
Asp Ile Gln Met Thr Gln Ser Pro Val Ser Leu Ser Val Ser Val Gly
1               5                   10                  15

gaa act gtc acc atc aca tgt cga cca agt gag aat ctt tat act aat         96
Glu Thr Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

tta gca tgg tat caa cag aaa cag gga aaa tct cct cag ctc ctg gtc        144
Leu Ala Trp Tyr Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val
        35                  40                  45

tat ggt gca aca aac cta gca gat ggt gtg cca tca agg ttc agt ggc        192
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

agt gga tca ggc aca cag tac tcc ctc agg atc aac agc ctg cag tct        240
Ser Gly Ser Gly Thr Gln Tyr Ser Leu Arg Ile Asn Ser Leu Gln Ser
65                  70                  75                  80

gaa gat ttc ggg act tat tac tgt caa cat ttg tgg ggt act cct ttc        288
Glu Asp Phe Gly Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

acg ttc ggc tcg ggg aca aag ttg gaa cta aaa                            321
Thr Phe Gly Ser Gly Thr Lys Leu Glu Leu Lys
            100                 105

<210>  34
<211>  107
<212>  PRT
<213>  Mus musculus
<400>  34

Asp Ile Gln Met Thr Gln Ser Pro Val Ser Leu Ser Val Ser Val Gly
1               5                   10                  15

Glu Thr Val Thr Ile Thr Cys Arg Pro Ser Glu Asn Leu Tyr Thr Asn
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val
        35                  40                  45

Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Gln Tyr Ser Leu Arg Ile Asn Ser Leu Gln Ser
65                  70                  75                  80

Glu Asp Phe Gly Thr Tyr Tyr Cys Gln His Leu Trp Gly Thr Pro Phe
                85                  90                  95

Thr Phe Gly Ser Gly Thr Lys Leu Glu Leu Lys
            100                 105

<210>  35
<211>  669
<212>  DNA
<213>  Homo sapiens

<220>
<221>  CDS
<222>  (1)..(669)
<400>  35

atg gct tgc ctt gga ttt cag cgg cac aag gct cag ctg aac ctg gct         48
Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
```

```
1               5                   10                  15

acc agg acc tgg ccc tgc act ctc ctg ttt ttt ctt ctc ttc atc cct          96
Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
            20                  25                  30

gtc ttc tgc aaa gca atg cac gtg gcc cag cct gct gtg gta ctg gcc         144
Val Phe Cys Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
        35                  40                  45

agc agc cga ggc atc gcc agc ttt gtg tgt gag tat gca tct cca ggc         192
Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly
    50                  55                  60

aaa gcc act gag gtc cgg gtg aca gtg ctt cgg cag gct gac agc cag         240
Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

gtg act gaa gtc tgt gcg gca acc tac atg atg ggg aat gag ttg acc         288
Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr
                85                  90                  95

ttc cta gat gat tcc atc tgc acg ggc acc tcc agt gga aat caa gtg         336
Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110

aac ctc act atc caa gga ctg agg gcc atg gac acg gga ctc tac atc         384
Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile
        115                 120                 125

tgc aag gtg gag ctc atg tac cca ccg cca tac tac ctg ggc ata ggc         432
Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly
    130                 135                 140

aac gga acc cag att tat gta att gat cca gaa ccg tgc cca gat tct         480
Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

gac ttc ctc ctc tgg atc ctt gca gca gtt agt tcg ggg ttg ttt ttt         528
Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

tat agc ttt ctc ctc aca gct gtt tct ttg agc aaa atg cta aag aaa         576
Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

aga agc cct ctt aca aca ggg gtc tat gtg aaa atg ccc cca aca gag         624
Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys Met Pro Pro Thr Glu
        195                 200                 205

cca gaa tgt gaa aag caa ttt cag cct tat ttt att ccc atc aat             669
Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe Ile Pro Ile Asn
    210                 215                 220

<210>  36
<211>  223
<212>  PRT
<213>  Homo sapiens
<400>  36

Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1               5                   10                  15

Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
            20                  25                  30

Val Phe Cys Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
```

```
        35                  40                  45

Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly
    50                  55                  60
Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr
                85                  90                  95

Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110

Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile
        115                 120                 125

Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly
    130                 135                 140

Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys Met Pro Pro Thr Glu
        195                 200                 205

Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe Ile Pro Ile Asn
    210                 215                 220

<210>  37
<211>  669
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  mutant CTLA4
<220>
<221>  CDS
<222>  (1)..(669)
<400>  37

atg gct tgc ctt gga ttt cag cgg cac aag gct cag ctg aac ctg gct          48
Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1               5                   10                  15

acc agg acc tgg ccc tgc act ctc ctg ttt ttt ctt ctc ttc atc cct          96
Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
            20                  25                  30

gtc ttc tgc aaa gca atg cac gtg gcc cag cct gct gtg gta ctg gcc         144
Val Phe Cys Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
        35                  40                  45

agc agc cga ggc atc gcc agc ttt gtg tgt gag tat gca tct cca ggc         192
Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly
    50                  55                  60

aaa gcc act gag gtc cgg gtg aca gtg ctt cgg cag gct gac agc cag         240
Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

gtg act gaa gtc tgt gcg gca acc tac atg atg ggg aat gag ttg acc         288
```

```
Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr
                85                  90                  95

ttc cta gat gat tcc atc tgc acg ggc acc tcc agt gga aat caa gtg        336
Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110

aac ctc act atc caa gga ctg agg gcc atg gac acg gga ctc tac atc        384
Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile
        115                 120                 125

tgc aag gtg gag ctc atg tac cca ccg cca tac tac ctg ggc ata ggc        432
Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly
    130                 135                 140

aac gga acc cag att tat gta att gat cca gaa ccg tgc cca gat tct        480
Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

gac ttc ctc ctc tgg atc ctt gca gca gtt agt tcg ggg ttg ttt ttt        528
Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

tat agc ttt ctc ctc aca gct gtt tct ttg agc aaa atg cta aag aaa        576
Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

aga agc cct ctt aca aca ggg gtc ggt gtg aaa atg ccc cca aca gag        624
Arg Ser Pro Leu Thr Thr Gly Val Gly Val Lys Met Pro Pro Thr Glu
        195                 200                 205

cca gaa tgt gaa aag caa ttt cag cct ggt ttt att ccc atc aat            669
Pro Glu Cys Glu Lys Gln Phe Gln Pro Gly Phe Ile Pro Ile Asn
    210                 215                 220

<210>  38
<211>  223
<212>  PRT
<213>  Artificial Sequence
<220>
<223>  Synthetic Construct
<400>  38

Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1               5                   10                  15

Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
            20                  25                  30

Val Phe Cys Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
        35                  40                  45

Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly
    50                  55                  60

Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr
                85                  90                  95

Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110
Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile
        115                 120                 125

Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly
```

```
    130                 135                 140

Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

Arg Ser Pro Leu Thr Thr Gly Val Gly Val Lys Met Pro Pro Thr Glu
        195                 200                 205

Pro Glu Cys Glu Lys Gln Phe Gln Pro Gly Phe Ile Pro Ile Asn
    210                 215                 220
```

<210> 39
<211> 669
<212> DNA
<213> Macaca fascicularis

<220>
<221> CDS
<222> (1)..(669)
<400> 39

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atg gct tgc ctt gga ttt cag cgg cac aag gct cgg ctc aac ctg gct          48
Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Arg Leu Asn Leu Ala
1               5                   10                  15

acc agg acc cgg ccc tac act ctc ctg ttt tct ctt ctc ttc atc cct          96
Thr Arg Thr Arg Pro Tyr Thr Leu Leu Phe Ser Leu Leu Phe Ile Pro
            20                  25                  30

gtc ttc tcc aaa gca atg cac gtg gcc cag cct gct gtg gtg ctg gcc         144
Val Phe Ser Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
        35                  40                  45

aac agc cga ggg atc gcc agc ttt gtg tgt gag tat gca tct cca ggc         192
Asn Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly
    50                  55                  60

aaa gcc act gag gtc cgg gtg aca gtg ctt cgg cag gcc gac agc cag         240
Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

gtg act gaa gtc tgt gcg gca acg tac atg atg ggg aat gag ttg acc         288
Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr
                85                  90                  95

ttc cta gat gat tcc atc tgc acg ggc acc tcc agt gga aat caa gtg         336
Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110

aac ctc act atc caa gga ctg agg gct atg gac aca gga ctc tac atc         384
Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile
        115                 120                 125

tgc aag gtg gag ctc atg tac cca cca cca tac tac atg ggc ata ggc         432
Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Met Gly Ile Gly
    130                 135                 140

aat gga acc cag att tat gta att gat cca gaa ccg tgc cca gat tct         480
Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160
```

```
gac ttc ctc ctc tgg atc ctt gca gca gtt agt tcg ggg ttg ttt ttt        528
Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

tat agc ttt ctc ctc aca gct gtt tct ttg agc aaa atg cta aag aaa        576
Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

aga agc cct ctc aca aca ggg gtc tat gtg aaa atg ccc cca aca gag        624
Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys Met Pro Pro Thr Glu
        195                 200                 205

cca gaa tgt gaa aag caa ttt cag cct tat ttt att ccc atc aat            669
Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe Ile Pro Ile Asn
    210                 215                 220
```

<210> 40
<211> 223
<212> PRT
<213> Macaca fascicularis
<400> 40

```
Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Arg Leu Asn Leu Ala
1               5                   10                  15

Thr Arg Thr Arg Pro Tyr Thr Leu Leu Phe Ser Leu Leu Phe Ile Pro
            20                  25                  30

Val Phe Ser Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
        35                  40                  45

Asn Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly
    50                  55                  60

Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr
                85                  90                  95

Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110

Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile
        115                 120                 125

Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Met Gly Ile Gly
    130                 135                 140

Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys Met Pro Pro Thr Glu
        195                 200                 205

Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe Ile Pro Ile Asn
    210                 215                 220
```

<210> 41
<211> 669
212> DNA
<213> Artificial Sequence

<220>
<223> Human-mouse CTLA4
<220>
<221> CDS
<222> (1)..(669)
<400> 41

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atg gcc tgc ctg ggc ttc cag aga cac aag gcc cag ctg aac ctg gcc          48
Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1               5                  10                  15

acc agg acc tgg cct tgt acc ctg ctg ttc ttc ctg ctg ttt atc ccc          96
Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
            20                  25                  30

gtg ttc tgc aag gcc atg cac gtg gcc cag cct gct gtg gtg ctg gcc         144
Val Phe Cys Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
        35                  40                  45

tct tcc aga gga atc gcc tcc ttc gtg tgc gag tac gcc tcc ccc cac         192
Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro His
    50                  55                  60

aac acc gat gaa gtg cgc gtg acc gtg ctg cgg cag acc aac gac cag         240
Asn Thr Asp Glu Val Arg Val Thr Val Leu Arg Gln Thr Asn Asp Gln
65                  70                  75                  80

atg acc gaa gtg tgc gcc acc acc ttc acc gag aag aac gag ctg acc         288
Met Thr Glu Val Cys Ala Thr Thr Phe Thr Glu Lys Asn Glu Leu Thr
                85                  90                  95

ttc ctg gac gac tct atc tgc acc ggc acc tcc agc ggc aac caa gtg         336
Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
            100                 105                 110

aac ctg aca atc cag ggc ctg cgg gcc atg gac acc ggc ctg tac ctg         384
Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Leu
        115                 120                 125

tgc aag gtg gaa ctg atg tac ccc cct ccc tac ttc gtg ggc atg ggc         432
Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Phe Val Gly Met Gly
    130                 135                 140

aac ggc acc cag atc tac gtg atc gac ccc gag cct tgc ccc gac tcc         480
Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

gac ttt ctg ctg tgg atc ctg gct gcc gtg tcc tcc ggc ctg ttc ttc         528
Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

tac tct ttc ctg ctg acc gcc gtg tcc ctg tcc aag atg ctg aag aag         576
Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

cgg tcc ccc ctg acc acc ggc gtg gga gtg aaa atg cct ccc acc gag         624
Arg Ser Pro Leu Thr Thr Gly Val Gly Val Lys Met Pro Pro Thr Glu
        195                 200                 205

ccc gag tgc gag aag cag ttc cag ccc ggc ttc atc ccc atc aac             669
Pro Glu Cys Glu Lys Gln Phe Gln Pro Gly Phe Ile Pro Ile Asn
    210                 215                 220
```

<210> 42
<211> 223
<212> PRT
<213> Artificial Sequence
<220>

<223> Synthetic Construct
<400> 42

Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1 5 10 15

Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
20 25 30

Val Phe Cys Lys Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala
35 40 45

Ser Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro His
50 55 60

Asn Thr Asp Glu Val Arg Val Thr Val Leu Arg Gln Thr Asn Asp Gln
65 70 75 80

Met Thr Glu Val Cys Ala Thr Thr Phe Thr Glu Lys Asn Glu Leu Thr
85 90 95

Phe Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val
100 105 110

Asn Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Leu
115 120 125

Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Phe Val Gly Met Gly
130 135 140

Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145 150 155 160

Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
165 170 175

Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
180 185 190

Arg Ser Pro Leu Thr Thr Gly Val Gly Val Lys Met Pro Pro Thr Glu
195 200 205

Pro Glu Cys Glu Lys Gln Phe Gln Pro Gly Phe Ile Pro Ile Asn
210 215 220

<210> 43
<211> 669
<212> DNA
<213> Artificial Sequence
<220>
<223> Mouse-Human CTLA4

<220>
<221> CDS
<222> (1)..(669)
<400> 43

atg gcc tgc ctg ggc ttc cag aga cac aag gcc cag ctg aac ctg gcc 48
Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1 5 10 15

acc agg acc tgg cct tgt acc ctg ctg ttc ttc ctg ctg ttt atc ccc 96
Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
20 25 30

gtg ttc tgc gag gcc atc caa gtg acc cag ccc tct gtg gtg ctg gcc 144
Val Phe Cys Glu Ala Ile Gln Val Thr Gln Pro Ser Val Val Leu Ala

```
        35                  40                  45

tct tct cat ggc gtg gcc agc ttc cct tgc gag tac tcc cca tct ggc        192
Ser Ser His Gly Val Ala Ser Phe Pro Cys Glu Tyr Ser Pro Ser Gly
    50                  55                  60

aag gcc acc gaa gtg cgc gtg acc gtg ctg aga cag gcc gac tcc caa        240
Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80

gtg aca gaa gtg tgc gcc gcc acc tac atg atg ggc aac acc gtg ggc        288
Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Thr Val Gly
                85                  90                  95

ttt ctg gac tac ccc ttc tgc tcc ggc acc ttc aac gag tcc aga gtg        336
Phe Leu Asp Tyr Pro Phe Cys Ser Gly Thr Phe Asn Glu Ser Arg Val
            100                 105                 110

aac ctg aca atc cag ggc ctg cgg gcc gtg gat acc ggc ctg tat atc        384
Asn Leu Thr Ile Gln Gly Leu Arg Ala Val Asp Thr Gly Leu Tyr Ile
        115                 120                 125

tgc aag gtg gaa ctg atg tac ccc cct ccc tac tac ctg ggc atc ggc        432
Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly
    130                 135                 140

aac ggc acc cag atc tac gtg atc gac ccc gag cct tgc ccc gac tcc        480
Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

gac ttt ctg ctg tgg atc ctg gcc gcc gtg tcc tcc ggc ctg ttc ttc        528
Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

tac tct ttc ctg ctg acc gct gtg tcc ctg tcc aag atg ctg aag aag        576
Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

cgg tcc ccc ctg acc acc ggc gtg gga gtg aaa atg cct ccc acc gag        624
Arg Ser Pro Leu Thr Thr Gly Val Gly Val Lys Met Pro Pro Thr Glu
        195                 200                 205

ccc gag tgc gag aag cag ttc cag ccc ggc ttc atc ccc atc aac            669
Pro Glu Cys Glu Lys Gln Phe Gln Pro Gly Phe Ile Pro Ile Asn
    210                 215                 220

<210>  44
<211>  223
<212>  PRT
<213>  Artificial Sequence
<220>
<223>  Synthetic Construct
<400>  44

Met Ala Cys Leu Gly Phe Gln Arg His Lys Ala Gln Leu Asn Leu Ala
1               5                   10                  15

Thr Arg Thr Trp Pro Cys Thr Leu Leu Phe Phe Leu Leu Phe Ile Pro
            20                  25                  30

Val Phe Cys Glu Ala Ile Gln Val Thr Gln Pro Ser Val Val Leu Ala
        35                  40                  45
Ser Ser His Gly Val Ala Ser Phe Pro Cys Glu Tyr Ser Pro Ser Gly
    50                  55                  60

Lys Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln
65                  70                  75                  80
```

```
Val Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Thr Val Gly
                85                  90                  95

Phe Leu Asp Tyr Pro Phe Cys Ser Gly Thr Phe Asn Glu Ser Arg Val
            100                 105                 110

Asn Leu Thr Ile Gln Gly Leu Arg Ala Val Asp Thr Gly Leu Tyr Ile
        115                 120                 125

Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly
    130                 135                 140

Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

Asp Phe Leu Leu Trp Ile Leu Ala Ala Val Ser Ser Gly Leu Phe Phe
                165                 170                 175

Tyr Ser Phe Leu Leu Thr Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

Arg Ser Pro Leu Thr Thr Gly Val Gly Val Lys Met Pro Pro Thr Glu
        195                 200                 205

Pro Glu Cys Glu Lys Gln Phe Gln Pro Gly Phe Ile Pro Ile Asn
    210                 215                 220
```

<210> 45
<211> 669
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(669)
<400> 45

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atg gct tgt ctt gga ctc cgg agg tac aaa gct caa ctg cag ctg cct          48
Met Ala Cys Leu Gly Leu Arg Arg Tyr Lys Ala Gln Leu Gln Leu Pro
1               5                   10                  15

tct agg act tgg cct ttt gta gcc ctg ctc act ctt ctt ttc atc cca          96
Ser Arg Thr Trp Pro Phe Val Ala Leu Leu Thr Leu Leu Phe Ile Pro
            20                  25                  30

gtc ttc tct gaa gcc ata cag gtg acc caa cct tca gtg gtg ttg gct         144
Val Phe Ser Glu Ala Ile Gln Val Thr Gln Pro Ser Val Val Leu Ala
        35                  40                  45

agc agc cat ggt gtc gcc agc ttt cca tgt gaa tat tca cca tca cac         192
Ser Ser His Gly Val Ala Ser Phe Pro Cys Glu Tyr Ser Pro Ser His
    50                  55                  60

aac act gat gag gtc cgg gtg act gtg ctg cgg cag aca aat gac caa         240
Asn Thr Asp Glu Val Arg Val Thr Val Leu Arg Gln Thr Asn Asp Gln
65                  70                  75                  80

atg act gag gtc tgt gcc acg aca ttc aca gag aag aat aca gtg ggc         288
Met Thr Glu Val Cys Ala Thr Thr Phe Thr Glu Lys Asn Thr Val Gly
                85                  90                  95

ttc cta gat tac ccc ttc tgc agt ggt acc ttt aat gaa agc aga gtg         336
Phe Leu Asp Tyr Pro Phe Cys Ser Gly Thr Phe Asn Glu Ser Arg Val
            100                 105                 110

aac ctc acc atc caa gga ctg aga gct gtt gac acg gga ctg tac ctc         384
Asn Leu Thr Ile Gln Gly Leu Arg Ala Val Asp Thr Gly Leu Tyr Leu
```

```
        115                 120                 125

tgc aag gtg gaa ctc atg tac cca ccg cca tac ttt gtg ggc atg ggc        432
Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Phe Val Gly Met Gly
    130                 135                 140

aac ggg acg cag att tat gtc att gat cca gaa cca tgc ccg gat tct        480
Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

gac ttc ctc ctt tgg atc ctt gtc gca gtt agc ttg ggg ttg ttt ttt        528
Asp Phe Leu Leu Trp Ile Leu Val Ala Val Ser Leu Gly Leu Phe Phe
                165                 170                 175

tac agt ttc ctg gtc tct gct gtt tct ttg agc aag atg cta aag aaa        576
Tyr Ser Phe Leu Val Ser Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190

aga agt cct ctt aca aca ggg gtc tat gtg aaa atg ccc cca aca gag        624
Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys Met Pro Pro Thr Glu
        195                 200                 205

cca gaa tgt gaa aag caa ttt cag cct tat ttt att ccc atc aac            669
Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe Ile Pro Ile Asn
    210                 215                 220

<210>  46
<211>  223
<212>  PRT
<213>  Mus musculus
<400>  46

Met Ala Cys Leu Gly Leu Arg Arg Tyr Lys Ala Gln Leu Gln Leu Pro
1               5                   10                  15

Ser Arg Thr Trp Pro Phe Val Ala Leu Leu Thr Leu Leu Phe Ile Pro
            20                  25                  30

Val Phe Ser Glu Ala Ile Gln Val Thr Gln Pro Ser Val Val Leu Ala
        35                  40                  45

Ser Ser His Gly Val Ala Ser Phe Pro Cys Glu Tyr Ser Pro Ser His
    50                  55                  60

Asn Thr Asp Glu Val Arg Val Thr Val Leu Arg Gln Thr Asn Asp Gln
65                  70                  75                  80

Met Thr Glu Val Cys Ala Thr Thr Phe Thr Glu Lys Asn Thr Val Gly
                85                  90                  95

Phe Leu Asp Tyr Pro Phe Cys Ser Gly Thr Phe Asn Glu Ser Arg Val
            100                 105                 110

Asn Leu Thr Ile Gln Gly Leu Arg Ala Val Asp Thr Gly Leu Tyr Leu
        115                 120                 125

Cys Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Phe Val Gly Met Gly
    130                 135                 140

Asn Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser
145                 150                 155                 160

Asp Phe Leu Leu Trp Ile Leu Val Ala Val Ser Leu Gly Leu Phe Phe
                165                 170                 175

Tyr Ser Phe Leu Val Ser Ala Val Ser Leu Ser Lys Met Leu Lys Lys
            180                 185                 190
```

```
Arg Ser Pro Leu Thr Thr Gly Val Tyr Val Lys Met Pro Pro Thr Glu
        195                 200                 205

Pro Glu Cys Glu Lys Gln Phe Gln Pro Tyr Phe Ile Pro Ile Asn
    210                 215                 220

<210>  47
<211>  990
<212>  DNA
<213>  Artificial Sequence
<220>
<223>  human IgG1 constant domains
<220>
<221>  CDS
<222>  (1)..(990)
<400>  47

gcc agc aca aag ggc cca tcg gtc ttc ccc ctg gca ccc tcc tcc aag          48
Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
1               5                   10                  15

agc acc tct ggg ggc aca gcg gcc ctg ggc tgc ctg gtc aag gac tac          96
Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
            20                  25                  30

ttc ccc gaa ccg gtg acg gtg tcg tgg aac tca ggc gcc ctg acc agc         144
Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

ggc gtg cac acc ttc ccg gct gtc cta cag tcc tca gga ctc tac tcc         192
Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

ctc agc agc gtg gtg acc gtg ccc tcc agc agc ttg ggc acc cag acc         240
Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
65                  70                  75                  80

tac atc tgc aac gtg aat cac aag ccc agc aac acc aag gtg gac aag         288
Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
                85                  90                  95

aaa gtt gag ccc aaa tct tgt gac aaa act cac aca tgc cca ccg tgc         336
Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
            100                 105                 110
cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca         384
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
        115                 120                 125

aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc         432
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
    130                 135                 140

gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg         480
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
145                 150                 155                 160

tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag         528
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
                165                 170                 175

gag cag tac aac agc acg tac cgg gtg gtc agc gtc ctc acc gtc ctg         576
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
            180                 185                 190

cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac         624
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
        195                 200                 205
```

```
aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg        672
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
    210                 215                 220

cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag        720
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
225                 230                 235                 240

ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat        768
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                245                 250                 255

ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac        816
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
            260                 265                 270

aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc        864
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
        275                 280                 285

ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac        912
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
    290                 295                 300

gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg        960
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
305                 310                 315                 320

cag aag agc ctc tcc ctg tct ccg ggt aaa                                990
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                325                 330
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<210> 48
<211> 330
<212> PRT
<213> Artificial Sequence
<220>
<223> Synthetic Construct
<400> 48

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Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
1               5                   10                  15

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
            20                  25                  30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
65                  70                  75                  80

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
                85                  90                  95

Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
            100                 105                 110
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
        115                 120                 125
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
    130                 135                 140

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
145                 150                 155                 160
```

```
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
                165                 170                 175

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
            180                 185                 190

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
        195                 200                 205

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
    210                 215                 220

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
225                 230                 235                 240

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
                245                 250                 255

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
            260                 265                 270

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
        275                 280                 285

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
    290                 295                 300

Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
305                 310                 315                 320

Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
                325                 330
```

<210> 49
<211> 981
<212> DNA
<213> Artificial Sequence
<220>
<223> human IgG4 constant domains (including S228P)

<220>
<221> CDS
<222> (1)..(981)
<400> 49

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gcc agc aca aag ggc ccc agc gtg ttc cct ctg gcc cct tgt agc aga         48
Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg
1               5                   10                  15

agc acc agc gag tct aca gcc gcc ctg ggc tgc ctc gtg aag gac tac         96
Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
            20                  25                  30

ttt ccc gag ccc gtg acc gtg tcc tgg aac tct ggc gct ctg aca agc        144
Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

ggc gtg cac acc ttt cca gcc gtg ctg cag agc agc ggc ctg tac tct        192
Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

ctg agc agc gtc gtg act gtg ccc agc agc tct ctg ggc acc aag acc        240
Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Lys Thr
65                  70                  75                  80

tac acc tgt aac gtg gac cac aag ccc agc aac acc aag gtg gac aag        288
Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
```

```
                85                  90                  95

cgg gtg gaa tct aag tac ggc cct ccc tgc cct cct tgc cca gcc cct        336
Arg Val Glu Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro
            100                 105                 110

gaa ttt ctg ggc gga ccc tcc gtg ttc ctg ttc ccc cca aag ccc aag        384
Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        115                 120                 125

gac acc ctg atg atc agc cgg acc ccc gaa gtg acc tgc gtg gtg gtg        432
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    130                 135                 140

gat gtg tcc cag gaa gat cct gag gtg cag ttc aat tgg tac gtg gac        480
Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp
145                 150                 155                 160

ggc gtg gaa gtg cac aac gcc aag acc aag cct aga gag gaa cag ttc        528
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe
                165                 170                 175

aac agc acc tac cgg gtg gtg tcc gtg ctg aca gtg ctg cac cag gac        576
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            180                 185                 190

tgg ctg aac ggc aaa gag tac aag tgc aag gtg tcc aac aag gga ctg        624
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu
        195                 200                 205

ccc agc tcc atc gag aaa acc atc agc aag gcc aag ggc cag ccc cgc        672
Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    210                 215                 220

gaa ccc cag gtg tac aca ctg cct cca agc cag gaa gag atg acc aag        720
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys
225                 230                 235                 240

aac cag gtg tcc ctg acc tgt ctc gtg aaa ggc ttc tac ccc tcc gat        768
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                245                 250                 255

atc gcc gtg gaa tgg gag agc aac ggc cag ccc gag aac aac tac aag        816
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            260                 265                 270

acc acc ccc cct gtg ctg gac agc gac ggc tca ttc ttc ctg tac agc        864
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        275                 280                 285

aga ctg acc gtg gac aag agc cgg tgg cag gaa ggc aac gtg ttc agc        912
Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser
    290                 295                 300

tgc agc gtg atg cac gag gcc ctg cac aac cac tac acc cag aag tcc        960
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
305                 310                 315                 320

ctg tct ctg agc ctg ggc aaa                                           981
Leu Ser Leu Ser Leu Gly Lys
                325
```

<210> 50
<211> 327
<212> PRT
<213> Artificial Sequence
<220>

<223> Synthetic Construct
<400> 50

```
Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg
1               5                  10                  15

Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
            20                  25                  30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        35                  40                  45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
    50                  55                  60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Lys Thr
65                  70                  75                  80

Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys
                85                  90                  95

Arg Val Glu Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro
            100                 105                 110

Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        115                 120                 125

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    130                 135                 140

Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp
145                 150                 155                 160

Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe
                165                 170                 175

Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            180                 185                 190

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu
        195                 200                 205

Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    210                 215                 220

Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys
225                 230                 235                 240

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                245                 250                 255

Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            260                 265                 270

Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        275                 280                 285

Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser
    290                 295                 300
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
305                 310                 315                 320

Leu Ser Leu Ser Leu Gly Lys
                325
```

<210> 51
<211> 321

<212> DNA
<213> Artificial Sequence
<220>
<223> human kappa constant domain

<220>
<221> CDS
<222> (1)..(321)
<400> 51

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cgg aca gtg gcc gct ccc agc gtg ttc atc ttc cca cct agc gac gag          48
Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
1               5                   10                  15

cag ctg aag tcc ggc aca gcc tct gtc gtg tgc ctg ctg aac aac ttc          96
Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
            20                  25                  30

tac ccc cgc gag gcc aag gtg cag tgg aag gtg gac aat gcc ctg cag         144
Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
        35                  40                  45

agc ggc aac agc cag gaa agc gtg acc gag cag gac agc aag gac tcc         192
Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
    50                  55                  60

acc tac agc ctg agc agc acc ctg aca ctg agc aag gcc gac tac gag         240
Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
65                  70                  75                  80

aag cac aag gtg tac gcc tgc gaa gtg acc cac cag ggc ctg tct agc         288
Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
                85                  90                  95

ccc gtg acc aag agc ttc aac cgg ggc gag tgc                             321
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
            100                 105
```

<210> 52
<211> 107
<212> PRT
<213> Artificial Sequence
<220>
<223> Synthetic Construct
<400> 52

```
Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
1               5                   10                  15

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
            20                  25                  30

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
        35                  40                  45

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
    50                  55                  60

Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
65                  70                  75                  80

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
                85                  90                  95

Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
            100                 105
```

Conclusies

1. Een antilichaam of antigeen bindend fragment daarvan dat bindt aan humaan CTLA-4, waarin het antilichaam of antigeen bindend fragment een of meer van de polypeptide sequenties gedefinieerd in a-f omvat en optioneel elk van de polypeptide sequenties gedefinieerd in a-c en/of elk van de polypeptide sequenties gedefinieerd in d-f:
    a. een zware keten variabel gebied CDR1 omvattende de aminozuur sequentie van SEQ ID NO: 1, of een aminozuur sequentie die verschilt van SEQ ID NO: 1 in 1, 2, 3 of meer conservatieve substituties;
    b. een zware keten variabel gebied CDR2 omvattende de aminozuur sequentie van SEQ ID NO: 2, of een aminozuur sequentie die verschilt van SEQ ID NO: 2 in 1, 2, 3 of meer conservatieve substituties;
    c. een zware keten variabel gebied CDR3 omvattende de aminozuur sequentie van SEQ ID NO: 3, of een aminozuur sequentie die verschilt van SEQ ID NO: 3 in 1, 2, 3 of meer conservatieve substituties;
    d. een lichte keten variabel gebied CDR1 omvattende de aminozuur sequentie van SEQ ID NO: 4, of een aminozuur sequentie die verschilt van SEQ ID NO: 4 in 1, 2, 3 of meer conservatieve substituties;
    e. een lichte keten variabel gebied CDR2 omvattende de aminozuur sequentie van SEQ ID NO: 5, of een aminozuur sequentie die verschilt van SEQ ID NO: 5 in 1, 2, 3 of meer conservatieve substituties;
    f. een lichte keten variabel gebied CDR3 omvattende de aminozuur sequentie van SEQ ID NO: 6, of een aminozuur sequentie die verschilt van SEQ ID NO: 6 in 1, 2, 3 of meer conservatieve substituties.

2. Het antilichaam of antigeen bindend fragment volgens conclusie 1, waarin het antilichaam of antigeen bindend fragment een van de polypeptide sequenties gedefinieerd in a-f omvat en optioneel elk van de polypeptide sequenties gedefinieerd in a-c en/of elk van de polypeptide sequenties gedefinieerd in d-f:
    a. een zware keten variabel gebied CDR1 omvattende de aminozuur sequentie van SEQ ID NO: 1;
    b. een zware keten variabel gebied CDR2 omvattende de aminozuur sequentie van SEQ ID NO: 2;
    c. een zware keten variabel gebied CDR3 omvattende de aminozuur sequentie van SEQ ID NO: 3;
    d. een lichte keten variabel gebied CDR1 omvattende de aminozuur sequentie van SEQ ID NO: 4;
    e. een lichte keten variabel gebied CDR2 omvattende de aminozuur sequentie van SEQ ID NO: 5;
    f. een lichte keten variabel gebied CDR3 omvattende de aminozuur sequentie van SEQ ID NO: 6.

3. Een antilichaam of antigeen bindend fragment daarvan dat bindt aan humaan CTLA-4 omvattende een lichte keten immuunglobuline, een zware keten immuunglobuline of zowel een lichte keten als een zware keten immuunglobuline gekozen uit de groep bestaande uit:
    a. een antilichaam of antigeen bindend fragment daarvan omvattende een variabele zware keten omvattende de aminozuur sequentie van SEQ ID NO: 7 en/of een variabele lichte keten omvattende de aminozuur sequentie van SEQ ID NO: 8;
    b. een antilichaam of antigeen bindend fragment daarvan omvattende een variabele zware keten omvattende de aminozuur sequentie van SEQ ID NO: 10, 12, 14, 16, 18 of 20 en/of een variabele lichte keten omvattende de aminozuur sequentie van SEQ ID NO: 22, 24, 26 of 30;

c. een antilichaam of antigeen bindend fragment daarvan omvattende een variabele zware keten omvattende ten minste 90%, 95%, 96%, 97%, 98% of 99% identiteit met een van SEQ ID NO: 10, 12, 14, 16, 18 of 20 en/of een variabele lichte keten omvattende ten minste 90%, 95%, 96%, 97%, 98% of 99% identiteit met een van SEQ ID NO: 22, 24, 26 of 30;

d. een antilichaam of antigeen bindend fragment daarvan omvattende een variabele zware keten omvattende ten minste 90%, 95%, 96%, 97%, 98% of 99% identiteit met een van SEQ ID NO: 10, 12, 14, 16, 18 of 20 en/of een variabele lichte keten omvattende ten minste 90%, 95%, 96%, 97%, 98% of 99% identiteit met een van SEQ ID NO: 22, 24, 26 of 30, waarin elke variatie in de sequentie voorkomt in de raamwerk gebieden van het antilichaam of antigeen bindende fragment;

e. een antilichaam of antigeen bindend fragment daarvan omvattende een variabele zware keten omvattende 1, 2, 3, 4, 5, 6, 7, 8, 9 of 10 aminozuur substituties ten opzichte van een van SEQ ID NO: 10, 12, 14, 16, 18 of 20 en/of een variabele lichte keten omvattende 1, 2, 3, 4, 5, 6, 7, 8, 9 of 10 aminozuur substituties ten opzichte van SEQ ID NO: 22, 24, 26 of 30;

f. een antilichaam of antigeen bindend fragment daarvan omvattende een variabele zware keten omvattende 1, 2, 3, 4, 5, 6, 7, 8, 9 of 10 aminozuur substituties ten opzichte van een van SEQ ID NO: 10, 12, 14, 16, 18 of 20 en/of een variabele lichte keten omvattende 1, 2, 3, 4, 5, 6, 7, 8, 9 of 10 aminozuur substituties ten opzichte van SEQ ID NO: 22, 24, 26 of 30, waarin elke variatie in de sequentie voorkomt in de raamwerk gebieden van het antilichaam of antigeen bindende fragment.

4. Het antilichaam of antigeen bindende fragment volgens conclusie 3, waarin antilichaam of antigeen bindende fragment daarvan een, twee, drie of alle vier van de volgende karakteristieken heeft:

a. bindt aan humaan CTLA-4 met een KD waarde van ten minste ongeveer 1 x 10^{-9} M zoals bepaald via oppervlakte plasmon resonantie (bv. BIACORE) of een soortgelijke techniek (bv. KinExa of OCTET);
b. blokkeert de binding van hCTLA-4 aan hCD80 met een IC_{50} van ongeveer 100 nM of lager;
c. blokkeert de binding van hCTLA-4 aan hCD86 met een IC_{50} van ongeveer 100 nM of lager;
d. bindt aan een ander CTLA-4 epitoop dan ipilimumab of tremelimumab.

5. Een antilichaam of antigeen bindende fragment daarvan dat bindt aan een epitoop van humaan CTLA-4, waarin genoemd antilichaam of antigeen bindende fragment niet bindt aan het muis-mens chimeer CTLA-4 molecuul van SEQ ID NO: 44.

6. Een antilichaam of antigeen bindende fragment daarvan dat bindt aan hetzelfde epitoop van humaan CTLA-4 als een antilichaam dat de variabele zware keten van SEQ ID NO: 7 en de variabele lichte keten van SEQ ID NO: 8 omvat, waarin het antilichaam of antigeen bindende fragment daarvan niet bindt aan het muis-mens chimeer CTLA-4 molecuul van SEQ ID NO: 44 en ten minste een van de volgende karakteristieken heeft:
a. bindt aan humaan CTLA-4 met een KD waarde van ten minste ongeveer 1 x 10^{-9} M zoals bepaald via oppervlakte plasmon resonantie (bv. BIACORE) of een soortgelijke techniek (bv. KinExa of OCTET);
b. blokkeert de binding van hCTLA-4 aan hCD80 met een IC_{50} van ongeveer 100 nM of lager;
c. blokkeert de binding van hCTLA-4 aan hCD86 met een IC_{50} van ongeveer 100 nM of lager;
d. bindt aan een ander CTLA-4 epitoop dan ipilimumab of tremelimumab.

7. Het antilichaam of antigeen bindende fragment van een van de bovenstaande conclusies, welk een gehumaniseerd antilichaam is welke

twee zware ketens en twee lichte ketens omvat.

8. Een geïsoleerd polypeptide omvattende de aminozuur sequentie van een van de SEQ ID NOs: 1-8, 10, 12, 14, 16, 18, 20, 22, 24, 26 of 30.

9. Een geïsoleerd nucleïnezuur dat codeert voor een eenvan de antilichamen of antigeen bindende fragmenten volgens conclusie 1-7, of een van de polypeptiden volgens conclusie 8.

10. Een expressievector die het geïsoleerde nucleïnezuur volgens conclusie 9 omvat.

11. Een gastheercel omvattende het antilichaam, bindende fragment, polypeptide, polynucleotide of expressievector volgens een der conclusies 1-10 omvat.

12. De gastheercel volgens conclusie 11, welke een *Pichia* cel of een Chinese hamster eierstokcel is.

13. Een samenstelling omvattende het antilichaam of antigeen bindende fragment volgens een der conclusies 1-7 en een farmaceutisch aanvaardbare drager, verdunningsmiddel, excipiëns of stabilisator.

14. De samenstelling volgens conclusie 13, verder omvattende een middel gekozen uit de groep bestaande uit:
    a. een anti-PD1 antilichaam of antigeen bindend fragment daarvan;
    b. een anti-LAG3 antilichaam of antigeen bindend fragment daarvan;
    c. een anti-TIGIT antilichaam of antigeen bindend fragment daarvan;
    d. een anti-VISTA antilichaam of antigeen bindend fragment daarvan;
    e. een anti-BTLA antilichaam of antigeen bindend fragment daarvan;
    f. een anti-TIM3 antilichaam of antigeen bindend fragment daarvan;
    g. een anti-CD27 antilichaam of antigeen bindend fragment daarvan;

h. een anti-HVEM antilichaam of antigeen bindend fragment daarvan;
i. een anti-CD70 antilichaam of antigeen bindend fragment daarvan;
j. een anti-CD137 antilichaam of antigeen bindend fragment daarvan;
k. een anti-OX40 antilichaam of antigeen bindend fragment daarvan;
l. een anti-CD28 antilichaam of antigeen bindend fragment daarvan;
m. een anti-PDL1 antilichaam of antigeen bindend fragment daarvan;
n. een anti-PDL2 antilichaam of antigeen bindend fragment daarvan;
o. een anti-GITR antilichaam of antigeen bindend fragment daarvan;
p. een anti-ICOS antilichaam of antigeen bindend fragment daarvan;
q. een anti-SIRPα antilichaam of antigeen bindend fragment daarvan;
r. een anti-ILT2 antilichaam of antigeen bindend fragment daarvan;
s. een anti-ILT3 antilichaam of antigeen bindend fragment daarvan;
t. een anti-ILT4 antilichaam of antigeen bindend fragment daarvan;
u. een anti-ILT5 antilichaam of antigeen bindend fragment daarvan;
v. een anti-4-1BB antilichaam of antigeen bindend fragment daarvan;
w. een anti-NK2GA antilichaam of antigeen bindend fragment daarvan;
x. een anti-NK2GC antilichaam of antigeen bindend fragment daarvan;
y. een anti-NK2GE antilichaam of antigeen bindend fragment daarvan;
z. een anti-TSLP antilichaam of antigeen bindend fragment daarvan;
aa. een anti-IL10 antilichaam of antigeen bindend fragment daarvan.

15. De samenstelling volgens conclusie 14, waarin het anti-PD1 antilichaam of een antigeen bindend fragment daarvan is gekozen uit de groep bestaande uit pembrolizumab of een antigeen binden fragment daarvan en nivolumab of een antigeen binden fragment daarvan.

16. De samenstelling volgens conclusie 13, verder omvattende een verbinding gekozen uit de groep van melfalan, vincristine, fludarabine, chloorambucil, bendamustine, etoposide, doxorubicine, cyclofosfamide, cisplatin, immuun

modulerende middelen zoals corticosteroïden, bij voorbeeld dexamethason of prednisolon, thalidomide analoga, bij voorbeeld thalidomide, lenalidomide of pomalidomide, kinase remmers, bij voorbeeld ibrutinib, idealisib, antilichaam gericht op CD20, bij voorbeeld rituximab, ofatumab of obinotuzumab, antilichaam gericht op CD52, bij voorbeeld alemtuzumab, antilichaam gericht op CD38, bij voorbeeld daratumumab, antilichaam gericht op IL-6 of IL-6 receptor, bij voorbeeld sarilumab of tocilizumab, antilichaam gericht op CS-1, bij voorbeeld elotuzumab, antilichaam gericht op BCMA, bij voorbeeld GSK2857916, antilichaam gericht op BAFF of BLyss, bij voorbeeld tabalumab, bisfosfonaten, bij voorbeeld pamidronaat or zolendronzuur, bortezomide, of combinaties daarvan.

17. Een werkwijze voor het produceren van een antilichaam of antigeen bindend fragment omvattende:
    a. het kweken van een gastheercel omvattende een polynucleotide coderend voor de zware keten en/of de lichte keten van een van de antilichamen of antigeen bindende fragmenten volgens conclusie 1-7 onder omstandigheden die gunstig zijn voor de expressie van het polynucleotide; en
    b. optioneel, het winnen van het antilichaam of antigeen bindend fragment uit de gastheercel en/of kweekmedium.

18. Een werkwijze voor het behandelen van kanker in een subject, bij voorkeur een menselijk subject, omvattende toediening aan het subject van een effectieve hoeveelheid van het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7, of van een expressievector welke expressie van het antilichaam of antigeen bindend fragment in het subject medieert, optioneel in samenhang met een verder therapeutisch middel of therapeutische procedure.

19. Een werkwijze voor het behandelen van een infectie of infectieziekte in een subject, bij voorkeur een menselijk subject, omvattende toediening aan het subject van een effectieve hoeveelheid van het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7, of van een expressievector welke expressie van het antilichaam of antigeen bindend fragment in het subject medieert, optioneel in samenhang met een verder therapeutisch middel of therapeutische procedure.

20. Een vaccin dat het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7 en een antigeen omvat.

21. Een werkwijze voor het detecteren van de aanwezigheid van een CTLA-4 peptide of een fragment daarvan in een monster omvattende het in contact brengen van het monster met een antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7 en het detecteren van de aanwezigheid van een complex tussen het antilichaam of fragment en het peptide, waarin detectie van het complex de aanwezigheid van het CTLA-4 peptide aangeeft.

22. Werkwijze voor het verhogen van de activiteit van een immuuncel, omvattende het toedienen aan een subject dat daaraan behoefte heeft van een effectieve hoeveelheid van een antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7, of van een expressievector welke expressie van het antilichaam of antigeen bindend fragment in het subject medieert.

23. De werkwijze volgens conclusie 22, waarin genoemde werkwijze wordt gebruikt voor:
    a. de behandeling van kanker;
    b. de behandeling van een infectie of infectieziekte; of
    c. als een vaccin adjuvans.

24. Een antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7, of van een expressievector welke expressie van het antilichaam of antigeen bindend fragment in het subject medieert, voor toepassing in de bereiding van een medicament om:
    a. immuuncel activatie te verhogen;
    b. kanker te behandelen; of
    c. een infectie of infectieziekte te behandelen.

25. Toepassing van het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7 voor de bereiding van een medicament voor de behandeling van kanker voor het verhogen van immuuncel activatie, behandelen van kanker of het behandelen van een infectie of infectieziekte.

26. Het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7, waarin het fragment een Fab, F(ab')2, Fv of enkele keten Fv fragment (scFv) is.

27. Het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7, welke een zware keten constant gebied omvat gekozen uit IgG1, IgG2, IgG3 en IgG4, bij voorkeur IgG1 of IgG4, en een lichte keten constant gebied gekozen uit de lichte keten constante gebieden kappa of lambda.

28. Het antilichaam of antigeen bindend fragment volgens conclusie 27, welke een humaan IgG4 zware keten constant gebied omvat met een Ser→Pro mutatie op positie 228 van SEQ ID NO: 50.

29. Werkwijze voor het stimuleren van een immuunrespons in een subject, omvattende het toedienen aan genoemd subject dat daaraan behoefte heeft van het antilichaam of antigeen bindend fragment volgens een der conclusies 1 -7 in een hoeveelheid die effectief is om de immuunrespons te stimuleren.

30. Werkwijze voor het behandelen van kanker volgens conclusie 18, waarin de kanker gekozen is uit de groep bestaande uit een longkanker, een melanoom, een nierkanker, een leverkanker, een myeloom, een prostaatkanker, een borstkanker, een colorectale kanker, een maagkanker, een alvleesklier kanker, een schildklierkanker, een hematologische kanker, een lymfoom, een myeloom, of een leukemie, of een metastatische laesie van de kanker.

31. Werkwijze voor het behandelen van kanker volgens conclusie 18, waarin het antilichaam molecuul wordt toegediend in combinatie met een of meer therapeutische middelen of procedures, waarin het tweede therapeutische middel of procedure is gekozen uit de groep bestaande uit chemotherapie, een gerichte anti-kanker therapie, een oncolytisch geneesmiddel, een cytotoxisch middel, een therapie op immuunbasis, een cytokine, chirurgische procedure, een bestralingsprocedure, een activator van een costimulatoir molecuul, een remmer van een remmend molecuul, een vaccin of een cellulaire immuuntherapie.

32. Werkwijze volgens conclusie 31, waarin het antilichaam molecuul wordt toegediend in combinatie met een agonist van een of meer costimulatoire moleculen gekozen uit de groep bestaande uit OX40, CD2, CD27, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD30, CD40, BAFFR, HVEM, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3 of CD83 ligand.

33. Werkwijze volgens conclusie 31, waarin het antilichaam molecuul wordt toegediend in combinatie met een of meer remmers van een immuun checkpoint molecuul gekozen uit de groep bestaande uit PD-1, PD-L1, PD-L2, TIM-3, LAG-3, CEACAM-1, CEACAM-5, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 of TGFR.

Figure 1

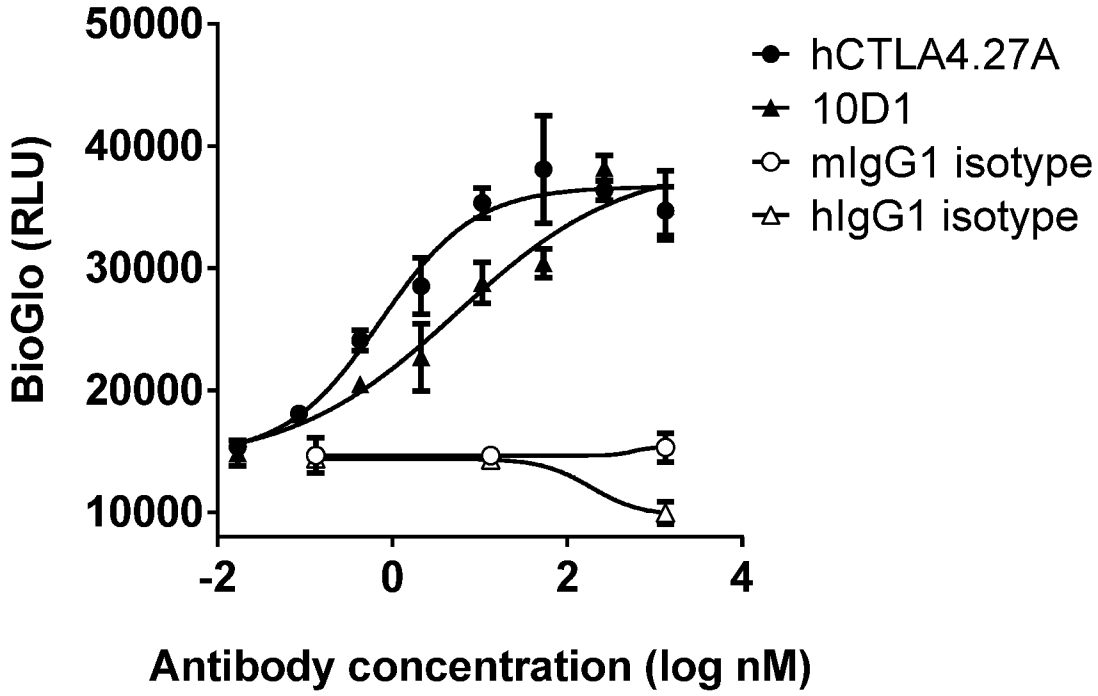


Figure 2

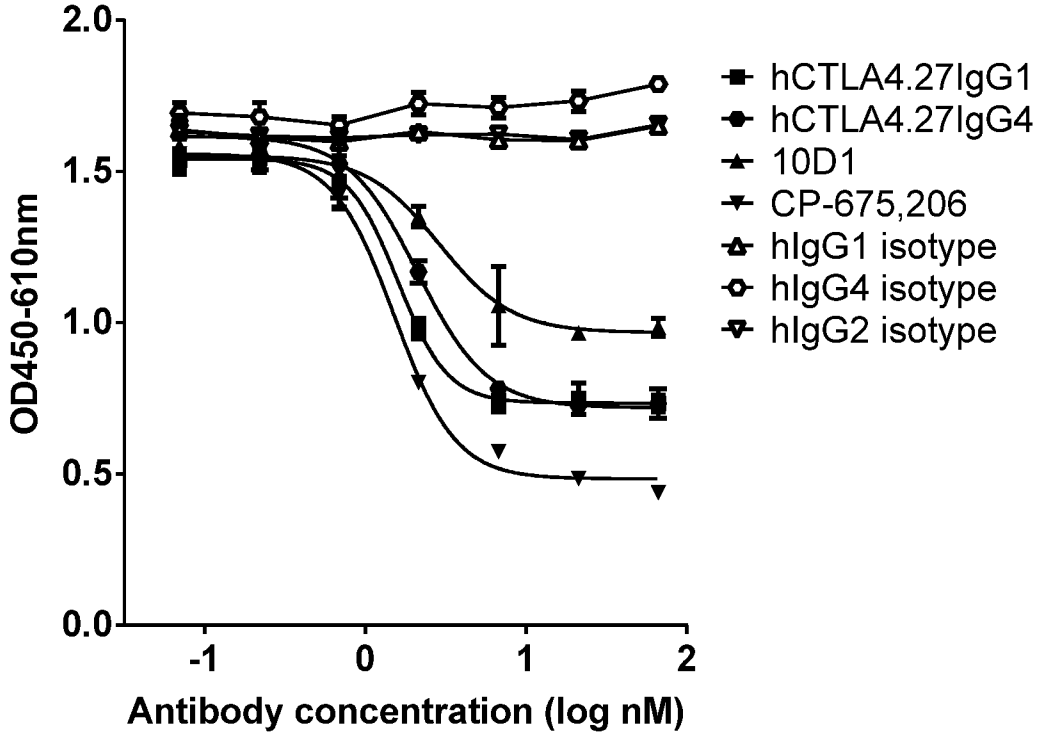


Figure 3

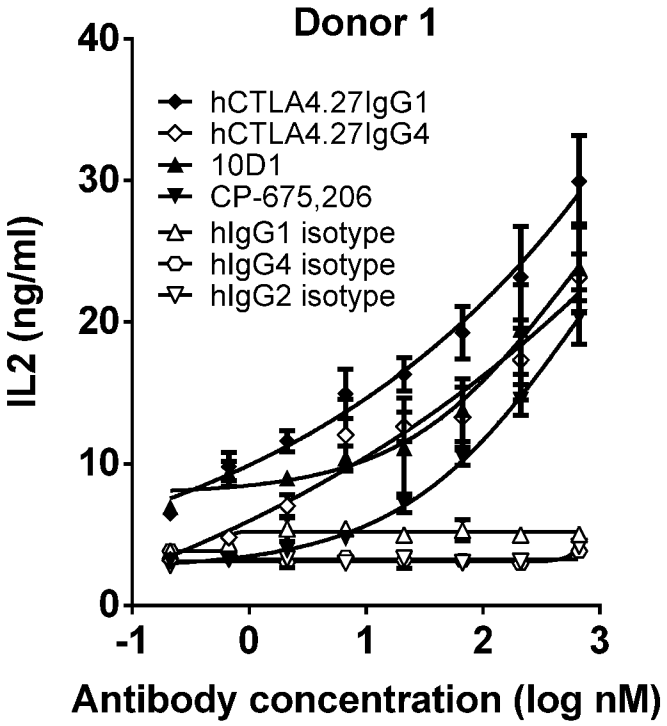


Figure 4A

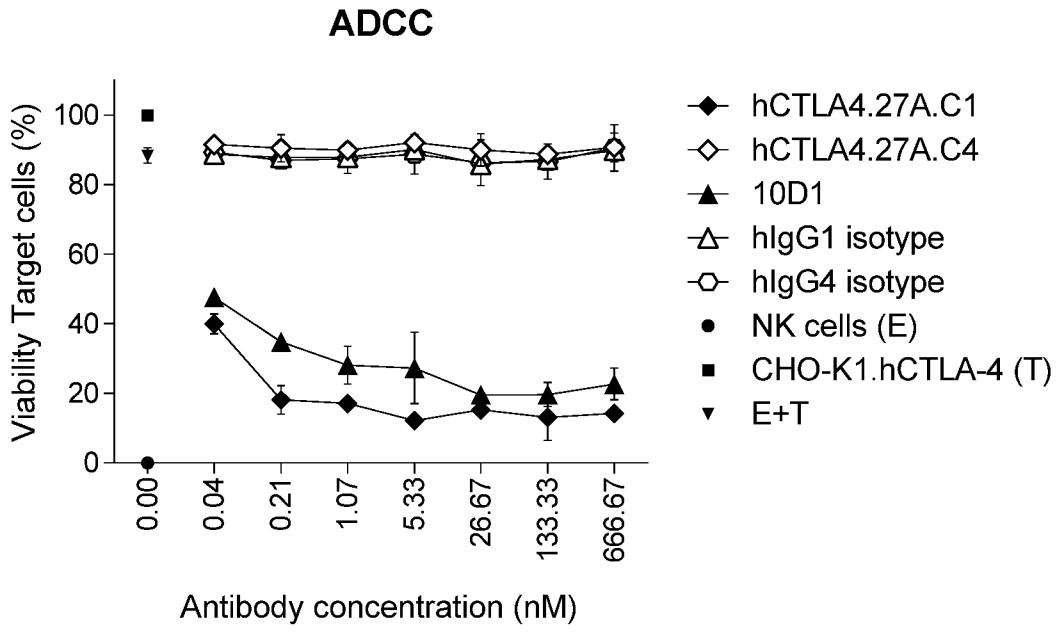


Figure 4B

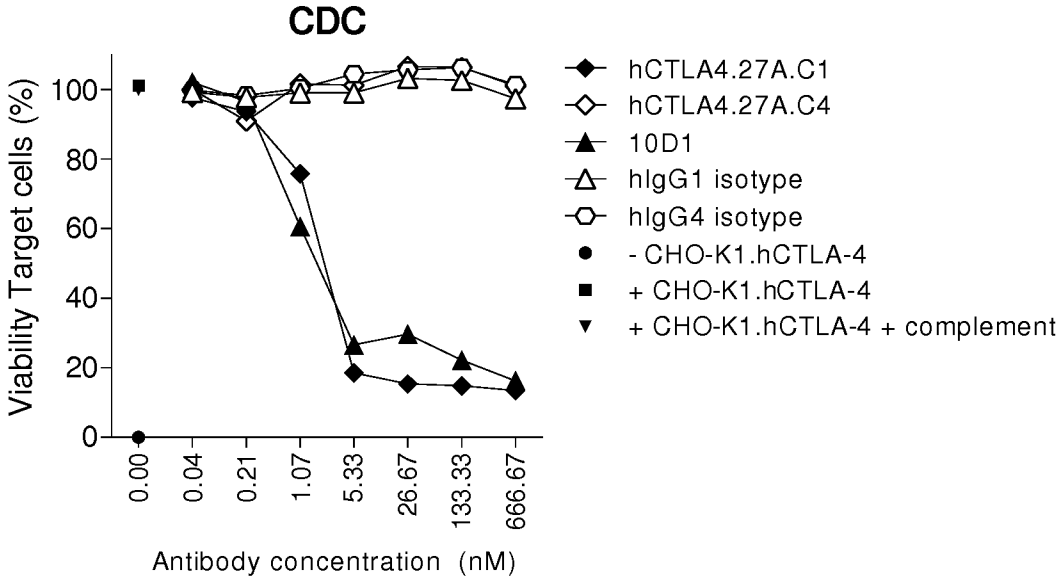
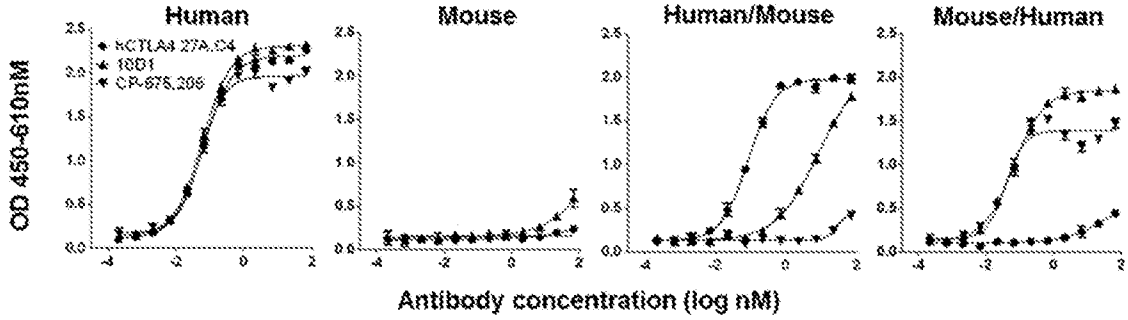


Figure 5



TITLE: New anti-hCTLA-4 antibodies

ABSTRACT

The invention relates to new anti-hCTLA-4 antibodies that bind to a different epitope than prior art anti-CTLA4 antibodies. These antibodies show a similar affinity for the CTLA4 antigen and they are also able to block the binding of CTLA4 to CD80 and/or CD86.

Also part of the invention are methods to produce these antibodies and therapeutic and diagnostic uses of these antibodies.

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
	P112407NL00
Nederlands aanvraag nr. **2017270**	Indieningsdatum **02-08-2016**
	Ingeroepen voorrangsdatum
Aanvrager (Naam) **Aduro Biotech Holdings, Europe B.V.**	
Datum van het verzoek voor een onderzoek van internationaal type **15-10-2016**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN 67582**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

C07K16/28

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**C07K**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III.	☐	GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES	(opmerkingen op aanvullingsblad)
IV.	☐	GEBREK AAN EENHEID VAN UITVINDING	(opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2017270

A. CLASSIFICATIE VAN HET ONDERWERP
INV. C07K16/28
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimum documentatie (classificatie gevolgd door classificatiesymbolen)
C07K

Onderzochte andere documentatie dan de minimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie *	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	WO 2006/066568 A2 (TEGENERO AG [DE]; HANKE THOMAS [DE]; HORLING FRANK [DE]; TRISCHLER MAR) 29 juni 2006 (2006-06-29) * figuren 1-12; voorbeelden 1,2; tabellen 1,2 *	1-33
X	WO 2009/100140 A1 (MEDAREX INC [US]; KORMAN ALAN [US]; HALK EDWARD L [US]; WANG CHANGYU [) 13 augustus 2009 (2009-08-13) * voorbeelden 1-4 *	1-33
X	WO 01/14424 A2 (MEDAREX INC [US]; KORMAN ALAN J [US]; HALK EDWARD L [US]; LONBERG NILS) 1 maart 2001 (2001-03-01) * voorbeelden 1-10 *	1-33

-/--

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

* Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
6 februari 2017	

Naam en adres van de instantie	De bevoegde ambtenaar
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cilensek, Zoran

1

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2017270

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie *	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
A	Li-Te Chin ET AL: "Immune Intervention with Monoclonal Antibodies Targeting CD152 (CTLA-4) for Autoimmune and Malignant Diseases", Chang Gung Med J., 1 februari 2008 (2008-02-01), XP055342512, Gevonden op het Internet: URL:http://memo.cgu.edu.tw/cgmj/3101/31010 1.pdf [gevonden op 2017-02-06] * figuur 3 * -----	1-33

Formulier PCT/ISA/201 (vervolg tweede blad) (Januari 2004)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek: NL 2017270

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
WO 2006066568 A2	29-06-2006	DE 102004063494 A1	13-07-2006
		EP 2111415 A2	28-10-2009
		US 2009123477 A1	14-05-2009
		WO 2006066568 A2	29-06-2006
WO 2009100140 A1	13-08-2009	EP 2240204 A1	20-10-2010
		US 2011081354 A1	07-04-2011
		WO 2009100140 A1	13-08-2009
WO 0114424 A2	01-03-2001	AT 354655 T	15-03-2007
		AU 784012 B2	12-01-2006
		AU 2006201520 A1	11-05-2006
		AU 2009202447 A1	09-07-2009
		CA 2381770 A1	01-03-2001
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		CN 1371416 A	25-09-2002
		CN 102766210 A	07-11-2012
		CY 1107628 T1	18-04-2013
		DE 60033530 T2	31-10-2007
		DE 122012000001 I1	15-03-2012
		DK 1212422 T3	02-07-2007
		EP 1212422 A2	12-06-2002
		EP 1792991 A1	06-06-2007
		EP 2829609 A1	28-01-2015
		ES 2282133 T3	16-10-2007
		HK 1048831 A1	07-06-2013
		IL 148079 A	13-04-2008
		JP 4093757 B2	04-06-2008
		JP 5599158 B2	01-10-2014
		JP 2004512005 A	22-04-2004
		JP 2006151993 A	15-06-2006
		JP 2009213478 A	24-09-2009
		JP 2012100674 A	31-05-2012
		KR 20060100950 A	21-09-2006
		KR 20090036598 A	14-04-2009
		LU 91928 I2	09-03-2012
		MX PA02001911 A	21-07-2003
		NZ 517202 A	28-05-2004
		PT 1212422 E	30-04-2007
		US 6984720 B1	10-01-2006
		WO 0114424 A2	01-03-2001
		ZA 200201190 B	28-05-2003

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN67582	02.08.2016		NL2017270

International Patent Classification (IPC)
INV. C07K16/28

Applicant
Aduro Biotech Holdings, Europe B.V.

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

Examiner
Cilensek, Zoran

Application number

WRITTEN OPINION

NL2017270

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.
2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ on paper

   ☒ in electronic form

   c. time of filing/furnishing:

   ☒ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	3-33
	No:	Claims	1, 2
Inventive step	Yes:	Claims	
	No:	Claims	1-33
Industrial applicability	Yes:	Claims	1-33
	No:	Claims	

2. Citations and explanations

see separate sheet

WRITTEN OPINION

Box No. VIII Certain observations on the application

see separate sheet

Reference is made to the following documents:

D1 WO 2006/066568 A2 (TEGENERO AG [DE]; HANKE THOMAS [DE]; HORLING FRANK [DE]; TRISCHLER MAR) 29 juni 2006 (2006-06-29)

D2 WO 2009/100140 A1 (MEDAREX INC [US]; KORMAN ALAN [US]; HALK EDWARD L [US]; WANG CHANGYU []) 13 augustus 2009 (2009-08-13)

D3 WO 01/14424 A2 (MEDAREX INC [US]; KORMAN ALAN J [US]; HALK EDWARD L [US]; LONBERG NILS) 1 maart 2001 (2001-03-01)

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The patentability can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound in medical treatment, but may allow claims to a product, in particular substances or compositions for in a first or further medical treatment. Patentability, in particular novelty and inventive step, of claims 17-25 and 29-33 has been assessed on the basis of a purpose-limited product claim taking into account the alleged effects of the compound/composition.

There are 4 independent product claims:

Claim 1 is directed to an antibody binding human CTLA-4, defined by reference to any of the CDRs, as set out in SEQ ID NOs:1-6, respectively, each having up to 3 conservative amino-acid changes.

Claim 3 is directed to an antibody binding human CTLA-4, defined by reference to the heavy and light chain variable region consensus sequences SEQ ID NO:7 and 8, or SEQ ID NOs: 10, 12, 14, 16, 18 and 20 and/or SEQ ID NOs: 22, 24, 26 and 30, or sequences exhibiting a certain degree of amino acid identity thereto.

Claim 5 is directed to an antibody binding an epitope of human CTLA-4 and failing to bind the murine-human chimera comprising exchange mutations as set forth in SEQ ID NO:44.

Claim 6 is directed to an antibody binding human CTLA-4, conforming with the consensus sequences as set forth in SEQ ID NOs:7 and 8, whilst failing to bind SEQ ID NO:44, and exhibiting at least one of the functional properties recited in items (a) to (d) therein.

NOVELTY

D1 discloses an anti CTLA-4 antibody termed 4.3F6B5, which light chain CDR2 is 100% identical to SEQ ID NO:5. The antibody does not bind the C"D-Loop, while being capable of antagonizing the activities of rat and human CTLA-4 *in vitro* and *in vivo* (Examples 1-2; Tables 1-2; Figures 1-12).

Thus at least claims 1 and 2 cannot be considered new over D1.

In view of the prior art cited, claims 3-33 appear to be novel.

INVENTIVE STEP

Document D3 can be considered to represent the most relevant state of the art.

D3 discloses several human antibodies against human CTLA-4, falling into six epitope binds. The antibodies termed 10D1.3 (10D1), 4B6.12 and 11E8 were characterized in greater detail. 10D1 (later also known as MDX-010, Yervoy and ipilimumab) blocks CTLA-4/B7 interactions, cf. Examples 1-10.

The current claims differ from D3 in the definition of at least one of its CDR sequences, or one of the variable regions (independent claims 1 and) or in functional features (independent claim 5), or in combination thereof (independent claim 6).

It is not possible to discern any particular technical effect resulting from the above difference even for an antibody comprising all 6 CDRs as set out in SEQ ID NOs:1-6, or both variable regions as set out in SEQ ID NOs: 10, 12, 14, 16, 18 and 20 and SEQ ID NOs:22, 24, 26 and 30, let alone for an extremely high number of possible sequences encompassed by the claims.

The current antibody (hCTLA4.27A) is inferior in affinity to 10D1 (Table 3), as well in the ability to block CTLA-4/CD86(B7-2) interaction (Figure 2). Both antibodies mediate ADCC and CDC to a similar extent (Figures 4A and 4B). The fact that the antibodies apparently bind different epitopes does not translate in any surprising or unexpected technical effects.

The problem to be solved by the present invention may therefore be regarded as providing an alternative antibody against CTLA4.

The solution cannot be considered as involving an inventive step, since the provision of further antibodies with similar properties, even if they bind different epitopes, would not have required inventive skill. The dependent claims either cover standard technical options, or treatments which are not substantiated by technical evidence.

Similar reasons as to lack of inventive step would apply if D1 (as discussed supra) or D2 were considered as the closest prior art in lieu of D3.

D2 discloses human antibodies against human CTLA-4, termed 3A4, 1H5 and 6C10, compared to the previously characterized 10D1 antibody. In contrast to 10D1, these antibodies are capable of increasing the response of T cells to antigenic stimulation in vivo yet the antibodies do not substantially inhibit the binding of a soluble human CTLA-4 protein to cells expressing B7-1, Examples 1-4.

In view of the above, claims 1-33 are considered to lack an inventive step.

Re Item VIII

Certain observations on the national application

CLARITY

The present application comprises 33 claims relating to numerous possible antibodies The number of claims shall be reasonable in consideration of the nature of the invention claimed, which is not the case here.

Although claims 1, 3, 5 and 6 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought and/or in respect of the terminology used for the features of that subject-matter. The aforementioned claims therefore lack conciseness.

Furthermore, the application relates to sequence combinations which may or may not generate an antibody with defined technical properties. In view of the importance of the role played by each single amino acid in the binding of an antibody to its target, especially at the level of its CDRs, a claim to an antibody which is structurally characterised by less than the full sequence of all the CDRs, and their specific order, cannot be seen to sufficiently disclose an antibody with any particular properties. Thus, it is apparent that a search of all possible combinations of claimed sequences would not be useful, since only an antibody defined by at least 6 CDRs (HCDR1-3, LCDR1-3) has any defined technical properties of binding.

Moreover, it is not sufficient to characterize an antibody by one or any less than all six of the CDR sequences, since an antibody is structurally made of two light and two heavy chains, both necessary to confer antigen binding specificity. Unless the contrary is shown, it is considered that a CDR is neither equivalent to an antibody, nor sufficient to define the specificity of an antibody, and therefore an antibody defined by any less than six CDRs lacks the technical features essential to the performance of the invention.

Claims 5 and 6 define an antibody in terms of the absence of ability to bind to SEQ ID NO:44. Since SEQ ID NO:44, does not appear to be disclosed in the state of the art, the novelty of the claim is questionable, since each and every antibody of the prior art has to be tested for this property. This unusual definition also places an undue burden on the person skilled in the art to determine the scope of the claims.