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

(57) Abstract: Disclosed are isolated monoclonal antibodies, comprising a CD152-binding domain, wherein the antibodies bind specifically to human CD152. Methods of making and using the antibodies to treat diseases including cancers and autoimmune diseases are also provided.



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ANTIBODIES BINDING CTLA-4 AND USES THEREOF

FIELD OF THE INVENTION

[0001] The invention relates to monoclonal anti-CTLA-4 antibodies, nucleic acids encoding the antibodies, expression vectors and recombinant cells containing the nucleic acids, and pharmaceutical compositions comprising the antibodies. Methods of making the antibodies, and methods of using the antibodies to treat diseases including cancers and autoimmune diseases are also provided.

BACKGROUND OF THE INVENTION

[0002] Cancer immunotherapy, a recent breakthrough in cancer treatment, employs a patient's own immune system to attack tumor cells. Promoting a robust CD8 T cell dependent cytotoxic response in the tumor microenvironment is important for the generation of an effective antitumor immune response. However, tumor tends to evade the immune surveillance by taking advantage of the T cell suppression machinery. The exhaustion of tumor-infiltrating lymphocytes (TIL) results in the anergy of cytotoxic T cells and escape of tumor cells (Wherry and Kurachi, 2015, *Nat Rev Immunol.*, 2015, 15: 486-499; Dyck and Mills, 2017, *Eur. J. Immunol.*, 47(5): 765-779).

[0003] Inhibitors of immune checkpoint proteins have the potential to treat a variety of tumors, such as metastatic melanoma, lung cancer, breast cancer, and renal cell carcinoma. CTLA-4 (CD152) is such an inhibitory checkpoint molecule on the surface of T cells. It was originally identified by differential screening of a murine cytolytic T cell cDNA library (Brunet et al., 1987, *Nature*, 328:267-270). It is suggested that CTLA-4 can function as a negative regulator of T cell activation (Walunas et al., 1994, *Immunity*, 1:405-413). CTLA-4 is expressed constitutively on the surfaces of regulatory T cells, but the amount is relatively low. It is upregulated upon T cell activation. Upon activation, CTLA-4 interacts with CD80 (B7.1) and CD86 (B7.2) which are also the ligands for CD28, with a much higher binding affinity than CD28 (van der Merwe et al., 1997, *J Exp Med.* 185:393-403; Alegre et al., 2001, *Nat Rev Immunol*, 1: 220-228). CD28 signaling promotes T cell activation, while the interaction of CTLA-4 with its ligands B7.1 and B7.2 prevents further activation of T cells.

[0004] CTLA-4 antagonists are attractive since the blockade of CTLA-4 with the antagonists was shown as an efficient therapy against tumors (US patents #6,984,720). Inhibition of this surface receptor using an antagonist such as an anti CTLA-4 mAb augmented

effector CD4 and CD8 T-cell responses and reduced the suppressive function of Treg cells. The CTLA-4 antagonist based treatments progressed fast in recent years. Ipilimumab (VERYOR®), is a humanized antibody and blocks the effects of CTLA-4, which augments T-cell responses to tumor cells. Ipilimumab was the first medicament to show an improvement in overall survival of patients with metastatic melanoma in a randomized, controlled phase 3 trial. It has a manageable safety profile at a dosage of 3 mg/kg as a monotherapy in patients previously treated with other therapies and at a dosage of 10 mg/kg in combination with dacarbazine in treatment-naïve patients. In addition to malignant melanoma, Ipilimumab is also under development for prostate cancer and non-small cell lung cancer treatment.

[0005] However, despite the progresses mentioned above, CTLA-4 antagonists with improved affinity, specificity and developability are desired. Further, more effective therapeutics involving anti-CTLA-4 antibodies that effectively inhibit the CTLA-4 signaling activity while causing minimal adverse side effects in humans are also needed.

SUMMARY OF THE INVENTION

[0006] Disclosed is an isolated monoclonal antibody, comprising a CD152-binding domain, wherein the CD152-binding domain comprises an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, wherein the CDR1, CDR2, and CDR3 comprise amino acid sequences having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to (1) SEQ ID NOs: 4, 5 and 6, respectively; (2) SEQ ID NOs: 10, 11 and 12, respectively; (3) SEQ ID NOs: 16, 17 and 18, respectively; (4) SEQ ID NOs: 22, 23 and 24, respectively; (5) SEQ ID NOs: 28, 29 and 30, respectively; (6) SEQ ID NOs: 34, 35 and 36, respectively; (7) SEQ ID NOs: 40, 41 and 42, respectively; (8) SEQ ID NOs: 46, 47 and 48, respectively; (9) SEQ ID NOs: 52, 53 and 54, respectively; (10) SEQ ID NOs: 58, 59 and 60, respectively; (11) SEQ ID NOs: 64, 65 and 66, respectively; (12) SEQ ID NOs: 70, 71 and 72, respectively; (13) SEQ ID NOs: 76, 77 and 78, respectively; (14) SEQ ID NOs: 82, 83 and 84, respectively; (15) SEQ ID NOs: 88, 89 and 90, respectively; (16) SEQ ID NOs: 94, 95 and 96, respectively; (17) SEQ ID NOs: 100, 101 and 102, respectively; (18) SEQ ID NOs: 106, 107 and 108, respectively; (19) SEQ ID NOs: 112, 113 and 114, respectively; (20) SEQ ID NOs: 118, 119 and 120, respectively; (21) SEQ ID NOs: 124, 125 and 126, respectively; (22) SEQ ID NOs: 130, 131 and 132, respectively; (23) SEQ ID NOs: 136, 137 and 138, respectively; (24) SEQ ID NOs: 142, 143 and 144, respectively; (25) SEQ ID NOs: 148, 149 and 150, respectively; (26) SEQ ID NOs: 154, 155 and 156, respectively; (27) SEQ ID NOs:

160, 161 and 162, respectively; (28) SEQ ID NOs: 166, 167 and 168, respectively; (29) SEQ ID NOs: 172, 173 and 174, respectively; (30) SEQ ID NOs: 178, 179 and 180, respectively; (31) SEQ ID NOs: 184, 185 and 186, respectively; or (32) SEQ ID NOs: 190, 191 and 192, respectively.

[0007] In some cases, the antibody is a heavy-chain-only antibody. In some cases, the antibody does not comprise an immunoglobulin light chain. In some cases, the antibody comprises one immunoglobulin heavy chain. In some cases, the antibody comprises two immunoglobulin heavy chains. In some cases, the antibody consists of two immunoglobulin heavy chains. In some cases, at least one of the two immunoglobulin heavy chains comprises an amino acid sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NOs: 2, 8, 14, 20, 26, 32, 38, 44, 50, 56, 62, 68, 74, 80, 86, 92, 98, 104, 110, 116, 122, 128, 134, 140, 146, 152, 158, 164, 170, 176, 182, or 188.

[0008] In some cases, the immunoglobulin heavy chain variable region comprises an amino acid sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NOs: 3, 9, 15, 21, 27, 33, 39, 45, 51, 57, 63, 69, 75, 81, 87, 93, 99, 105, 111, 117, 123, 129, 135, 141, 147, 153, 159, 165, 171, 177, 183, or 189.

[0009] In some cases, the antibody binds specifically to human CD152. In some cases, the antibody binds to human CD152 with high affinity. In some cases, the antibody binds to human CD152 with an affinity higher than an ipilimumab analogue. In some cases, the antibody binds to human CD152 with the affinity that is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold higher than an ipilimumab analogue.

[0010] In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-7} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-8} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-9} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-10} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-11} M or less. In some cases, the K_d is 6.0×10^{-11} M or less. In some cases, the antibody dissociates from human CD152 with the K_d that is lower than an ipilimumab analogue. In some cases, the antibody dissociates from human CD152 with the K_d that is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than an ipilimumab analogue. In some cases, the K_d is determined by surface plasmon resonance.

[0011] In some cases, the antibody binds specifically to monkey CD152. In some cases, the antibody does not bind specifically to monkey CD152. In some cases, the antibody blocks the binding of CD152 to CD80, CD86, or both. In some cases, the antibody promotes secretion of IL-2 by immune cells. In some cases, the antibody induces T-cell activation. In some cases, the antibody stimulates an anti-tumor immune response by immune cells. In some cases, the antibody is a human, humanized, or chimeric antibody.

[0012] In another aspect, disclosed herein is an isolated monoclonal heavy-chain-only antibody, comprising a CD152-binding domain, wherein the antibody binds specifically to human CD152. In some cases, the antibody does not comprise an immunoglobulin light chain. In some cases, the antibody comprises one immunoglobulin heavy chain. In some cases, the antibody comprises two immunoglobulin heavy chains. In some cases, the antibody consists of two immunoglobulin heavy chains.

[0013] In some cases, the antibody binds to human CD152 with high affinity. In some cases, the antibody binds to human CD152 with an affinity higher than an ipilimumab analogue. In some cases, the antibody binds to human CD152 with the affinity that is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold higher than an ipilimumab analogue.

[0014] In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-7} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-8} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-9} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-10} M or less. In some cases, the antibody dissociates from human CD152 with a K_d of 1.0×10^{-11} M or less. In some cases, the K_d is 6.0×10^{-11} M or less. In some cases, the antibody dissociates from human CD152 with the K_d that is lower than an ipilimumab analogue. In some cases, the antibody dissociates from human CD152 with the K_d that is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than an ipilimumab analogue. In some cases, the K_d is determined by surface plasmon resonance.

[0015] In some cases, the antibody binds specifically to monkey CD152. In some cases, the antibody does not bind specifically to monkey CD152. In some cases, the CD152-binding domain comprises an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, wherein the CDR1, CDR2, and CDR3 comprise amino acid sequences having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to (1) SEQ ID NOs: 4, 5 and 6, respectively; (2) SEQ ID NOs: 10, 11 and 12, respectively; (3) SEQ ID NOs: 16, 17

and 18, respectively; (4) SEQ ID NOs: 22, 23 and 24, respectively; (5) SEQ ID NOs: 28, 29 and 30, respectively; (6) SEQ ID NOs: 34, 35 and 36, respectively; (7) SEQ ID NOs: 40, 41 and 42, respectively; (8) SEQ ID NOs: 46, 47 and 48, respectively; (9) SEQ ID NOs: 52, 53 and 54, respectively; (10) SEQ ID NOs: 58, 59 and 60, respectively; (11) SEQ ID NOs: 64, 65 and 66, respectively; (12) SEQ ID NOs: 70, 71 and 72, respectively; (13) SEQ ID NOs: 76, 77 and 78, respectively; (14) SEQ ID NOs: 82, 83 and 84, respectively; (15) SEQ ID NOs: 88, 89 and 90, respectively; (16) SEQ ID NOs: 94, 95 and 96, respectively; (17) SEQ ID NOs: 100, 101 and 102, respectively; (18) SEQ ID NOs: 106, 107 and 108, respectively; (19) SEQ ID NOs: 112, 113 and 114, respectively; (20) SEQ ID NOs: 118, 119 and 120, respectively; (21) SEQ ID NOs: 124, 125 and 126, respectively; (22) SEQ ID NOs: 130, 131 and 132, respectively; (23) SEQ ID NOs: 136, 137 and 138, respectively; (24) SEQ ID NOs: 142, 143 and 144, respectively; (25) SEQ ID NOs: 148, 149 and 150, respectively; (26) SEQ ID NOs: 154, 155 and 156, respectively; (27) SEQ ID NOs: 160, 161 and 162, respectively; (28) SEQ ID NOs: 166, 167 and 168, respectively; (29) SEQ ID NOs: 172, 173 and 174, respectively; (30) SEQ ID NOs: 178, 179 and 180, respectively; (31) SEQ ID NOs: 184, 185 and 186, respectively; or (32) SEQ ID NOs: 190, 191 and 192, respectively.

[0016] In some cases, the CD152-binding domain comprises at least one immunoglobulin heavy chain, wherein the immunoglobulin heavy chain comprises an amino acid sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NOs: 2, 8, 14, 20, 26, 32, 38, 44, 50, 56, 62, 68, 74, 80, 86, 92, 98, 104, 110, 116, 122, 128, 134, 140, 146, 152, 158, 164, 170, 176, 182, or 188. In some cases, the immunoglobulin heavy chain variable region comprises an amino acid sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NOs: 3, 9, 15, 21, 27, 33, 39, 45, 51, 57, 63, 69, 75, 81, 87, 93, 99, 105, 111, 117, 123, 129, 135, 141, 147, 153, 159, 165, 171, 177, 183, or 189.

[0017] In some cases, the antibody blocks the binding of CD152 to CD80, CD86, or both. In some cases, the antibody promotes secretion of IL-2 by immune cells. In some cases, the antibody induces T-cell activation. In some cases, the antibody stimulates an anti-tumor immune response by immune cells. In some cases, the antibody is a human, humanized, or chimeric antibody.

[0018] In another aspect, disclosed herein is a pharmaceutical composition, comprising any antibody disclosed herein, and a pharmaceutically acceptable excipient thereof. In some cases, the pharmaceutically acceptable excipient is selected from the group consisting of carriers,

surface active agents, thickening or emulsifying agents, solid binders, dispersion or suspension aids, solubilizers, colorants, flavoring agents, coatings, disintegrating agents, lubricants, sweeteners, preservatives, isotonic agents, and combinations thereof. In some cases, the pharmaceutical composition further comprises a second antibody, wherein the second antibody is an immunostimulatory antibody or costimulatory antibody. In some cases, the immunostimulatory antibody is selected from the group consisting of an anti-PD-1 antibody, an anti-PD-L1 antibody, an anti-LAG-3 antibody, an anti-TIM 3 antibody, an anti-STAT3 antibody, and an anti-ROR1 antibody. In some cases, the costimulatory antibody is an anti-CD137 antibody or an anti-GITR antibody.

[0019] In another aspect, disclosed herein is an isolated nucleic acid molecule encoding any antibody disclosed herein. In some cases, the nucleic acid molecule comprises a nucleotide sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 163, 169, 175, 181, or 187. In some cases, the nucleic acid molecule comprises the nucleotide sequence set forth in SEQ ID NO: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 163, 169, 175, 181, or 187.

[0020] In another aspect, disclosed herein is an expression vector comprising a nucleic acid segment encoding any antibody disclosed herein, wherein the nucleic acid segment is operatively linked to regulatory sequences suitable for expression of the nucleic acid segment in a host cell. In some cases, the nucleic acid segment comprises a nucleotide sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 163, 169, 175, 181, or 187. In some cases, the nucleic acid segment comprises the nucleotide sequence set forth in SEQ ID NO: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 163, 169, 175, 181, or 187.

[0021] In another aspect, disclosed herein is a host cell comprising any expression vector disclosed herein.

[0022] In another aspect, disclosed herein is a method for producing an CD152-binding monoclonal antibody, the method comprising: culturing a host cell comprising any expression vector under conditions whereby the nucleic acid segment is expressed, thereby producing the CD152-binding monoclonal antibody. In some cases, the host cell is a CHO, HEK293, or COS

host cell line. In some cases, the CHO host cell line is CHO-K1 cell line. In some cases, the method further comprises recovering the CD152-binding monoclonal antibody.

[0023] In another aspect, disclosed herein is a method of inducing an antibody-dependent cell-mediated cytotoxicity (ADCC) against a cell expressing a tumor associated antigen, the method comprising: contacting a T-cell with any antibody disclosed herein, wherein said contacting is under conditions whereby the ADCC against the cell expressing the tumor associated antigen is induced.

[0024] In another aspect, disclosed herein is a method for treating a disorder in a subject, the method comprising administering to the subject a therapeutically effective amount of any antibody disclosed herein or any pharmaceutical composition disclosed herein. In some cases, the disorder is a cancer. In some cases, the cancer is selected from the group consisting of leukemia, lymphoma, CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma, renal cell carcinoma, colon cancer, colorectal cancer, breast cancer, epithelial squamous cell cancer, melanoma, myeloma, stomach cancer, brain cancer, lung cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, prostate cancer, testicular cancer, thyroid cancer, and head and neck cancer. In some cases, the method further comprises a therapeutic agent. In some cases, the therapeutic agent is an anti-cancer drug. In some cases, the therapeutic agent is ipilimumab, or an biosimilar product thereof. In some cases, the disorder is an autoimmune disease.

[0025] In another aspect, disclosed herein is use of any pharmaceutical composition disclosed herein in preparation of a medicament for treating a disorder. In some cases, the disorder is a cancer. In some cases, the cancer is selected from the group consisting of leukemia, lymphoma, CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma, renal cell carcinoma, colon cancer, colorectal cancer, breast cancer, epithelial squamous cell cancer, melanoma, myeloma, stomach cancer, brain cancer, lung cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, prostate cancer, testicular cancer, thyroid cancer, and head and neck cancer. In some cases, the disorder is an autoimmune disease.

[0026] In another aspect, the invention pertains to an isolated monoclonal antibody, or an antigen-binding portion thereof, having a heavy chain variable region that comprises a CDR1 region, a CDR2 region and a CDR3 region, wherein the CDR1 region, the CDR2 region and the CDR3 region comprise amino acid sequences having at least 80%, 85%, 90%, 95%, 98%, 99% or 100% identity to (1) SEQ ID NOs: 4, 5 and 6, respectively; (2) SEQ ID NOs: 10, 11

and 12, respectively; (3) SEQ ID NOs: 16, 17 and 18, respectively; (4) SEQ ID NOs: 22, 23 and 24, respectively; (5) SEQ ID NOs: 28, 29 and 30, respectively; (6) SEQ ID NOs: 34, 35 and 36, respectively; (7) SEQ ID NOs: 40, 41 and 42, respectively; (8) SEQ ID NOs: 46, 47 and 48, respectively; (9) SEQ ID NOs: 52, 53 and 54, respectively; (10) SEQ ID NOs: 58, 59 and 60, respectively; (11) SEQ ID NOs: 64, 65 and 66, respectively; (12) SEQ ID NOs: 70, 71 and 72, respectively; (13) SEQ ID NOs: 76, 77 and 78, respectively; (14) SEQ ID NOs: 82, 83 and 84, respectively; (15) SEQ ID NOs: 88, 89 and 90, respectively; (16) SEQ ID NOs: 94, 95 and 96, respectively; (17) SEQ ID NOs: 100, 101 and 102, respectively; (18) SEQ ID NOs: 106, 107 and 108, respectively; (19) SEQ ID NOs: 112, 113 and 114, respectively; (20) SEQ ID NOs: 118, 119 and 120, respectively; (21) SEQ ID NOs: 124, 125 and 126, respectively; (22) SEQ ID NOs: 130, 131 and 132, respectively; (23) SEQ ID NOs: 136, 137 and 138, respectively; (24) SEQ ID NOs: 142, 143 and 144, respectively; (25) SEQ ID NOs: 148, 149 and 150, respectively; (26) SEQ ID NOs: 154, 155 and 156, respectively; (27) SEQ ID NOs: 160, 161 and 162, respectively; (28) SEQ ID NOs: 166, 167 and 168, respectively; (29) SEQ ID NOs: 172, 173 and 174, respectively; (30) SEQ ID NOs: 178, 179 and 180, respectively; (31) SEQ ID NOs: 184, 185 and 186, respectively; or (32) SEQ ID NOs: 190, 191 and 192, respectively; wherein the antibody or antigen-binding fragment thereof binds CTLA-4.

[0027] In another aspect, an isolated monoclonal antibody, or an antigen-binding portion thereof, of the present invention comprises a heavy chain variable region comprising the amino acid sequence having at least 80%, 85%, 90%, 95%, 98%, 99% or 100% identity to SEQ ID NOs: 3, 9, 15, 21, 27, 33, 39, 45, 51, 57, 63, 69, 75, 81, 87, 93, 99, 105, 111, 117, 123, 129, 135, 141, 147, 153, 159, 165, 171, 177, 183 or 189.

[0028] In one embodiment, the antibody of the present invention comprises a heavy chain comprising the amino acid sequence having at least 80%, 85%, 90%, 95%, 98%, 99% or 100% identity to SEQ ID NOs: 2, 8, 14, 20, 26, 32, 38, 44, 50, 56, 62, 68, 74, 80, 86, 92, 98, 104, 110, 116, 122, 128, 134, 140, 146, 152, 158, 164, 170, 176, 182 or 188, which may be encoded by the nucleic acid sequence having at least 80%, 85%, 90%, 95%, 98%, 99% or 100% identity to SEQ ID No: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 157, 163, 169, 175, 181 or 187.

[0029] In some embodiments, the antibody of the present invention consists substantially of or consists of two heavy chains described above.

[0030] In another aspect of the invention, the antibody or an antigen-binding portion thereof is part of an immunoconjugate which comprises a therapeutic agent, e.g., a cytotoxin or

a radioactive isotope, linked to the antibody. In another aspect, the antibody is part of a bispecific molecule which comprises a second functional moiety (e.g., a second antibody) having a different binding specificity from said antibody, or the antigen binding portion thereof. In another aspect, the antibody or an antigen binding portions thereof of the present invention can be made into part of a chimeric antigen receptor (CAR) or an engineered T cell receptor. The antibody or an antigen binding portions thereof of the present invention can also be encoded by or used in conjunction with an oncolytic virus.

[0031] A pharmaceutical composition comprising an antibody, or an antigen-binding portion thereof, an immunoconjugate, a bispecific molecule, a chimeric antigen receptor, an engineered T cell receptor, or an oncolytic virus of the invention, optionally formulated in a pharmaceutically acceptable carrier, is also provided.

[0032] The pharmaceutical composition may further comprise other anticancer agents. The pharmaceutical composition may further comprise at least one additional immunostimulatory antibody selected from the group consisting of an anti-PD-1 antibody, an anti-PD-L1 antibody, an anti-LAG-3 antibody, an anti-TIM 3 antibody, an anti-STAT3 antibody, and an anti-ROR1 antibody, and/or an costimulatory antibody which can be an anti-CD137 antibody or an anti-GITR antibody.

[0033] A nucleic acid molecule encoding the antibody, or the antigen-binding portion (e.g., variable regions and/or CDRs) thereof, of the invention is also provided, as well as an expression vector comprising the nucleic acid and a host cell comprising the expression vector. A method for preparing an anti-CTLA-4 antibody using the host cell comprising the expression vector is also provided, and comprises steps of (i) expressing the antibody in the host cell and (ii) isolating the antibody from the host cell.

[0034] In yet another embodiment, the invention provides a method for inhibiting growth of tumor cells in a subject, comprising administering to the subject an therapeutically effective amount of an antibody, or an antigen-binding portion thereof, of the invention. The tumor may be a solid or non-solid tumor, selected from the group consisting of leukemia, lymphoma, CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma, renal cell carcinoma, colon cancer, colorectal cancer, breast cancer, epithelial squamous cell cancer, melanoma, myeloma, stomach cancer, brain cancer, lung cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, prostate cancer, testicular cancer, thyroid cancer, and head and neck cancer. Preferably, the tumor is melanoma, prostate cancer or non-small cell lung cancer. In yet another embodiment, the invention provides a method for

treating autoimmune disease in a subject, comprising administering to the subject an therapeutically effective amount of an antibody, or an antigen-binding portion thereof, of the invention. In still another embodiment, the invention provides a method for treating viral infection in a subject, comprising administering to the subject an therapeutically effective amount of an antibody, or an antigen-binding portion thereof, of the invention. In another embodiment, the method comprises administering a pharmaceutical composition, a bispecific, or an immunoconjugate of the invention.

[0035] In another aspect, the invention provides an anti-CTLA-4 antibody and a composition of the invention for use in the foregoing methods, or for the manufacture of a medicament for use in the foregoing methods (e.g., for treatment).

[0036] Other features and advantages of the instant disclosure will be apparent from the following detailed description and examples, which should not be construed as limiting. The contents of all references, Genbank entries, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] The foregoing summary, as well as the following detailed description of the invention, will be better understood when construed in conjunction with the drawings. It should be understood that the invention is not limited to the embodiments as shown in the drawings.

[0038] In the drawings:

[0039] Fig. 1 shows the binding activity of the anti-CTLA-4 antibodies of the present invention to human CTLA-4 expressed on 293 cells.

[0040] Fig. 2 shows the binding activity of the anti-CTLA-4 antibodies of the present invention to cynomolgus CTLA-4 expressed on 293 cells.

[0041] Fig. 3 shows the capacity of the anti-CTLA-4 antibodies of the present invention on blocking interaction between CTLA-4 and B7.1.

[0042] Fig. 4A and 4B show the effect of the anti-CTLA-4 antibody CL3, CL5, CL11 and CL22 (**A**), and CL24, CL25 and CL30 (**B**) on IL-2 secretion in a SEB dependent T lymphocyte stimulation assay using PBMC's from donor 1.

[0043] Fig. 5A and 5B show the binding capacity of the anti-CTLA-4 antibodies antibody CL5, CL11, CL22, CL24 and CL25 (**A**) and CL5 (**B**) to human CTLA-4 or mouse CTLA-4.

[0044] Fig. 6A and 6B show the effect of anti-CTLA-4 antibody CL5, CL11 and their mutants (**A**), and CL22, CL25 and their mutants (**B**) on IL-2 secretion in a SEB dependent T lymphocyte stimulation assay using PBMC's from donor 2.

[0045] Fig. 7 A and 7B show the binding activity of anti-CTLA-4 antibody mutants to human CTLA-4.

[0046] Fig. 8A, 8B, and 8C show the activity of anti-CTLA-4 antibody mutants on blocking interaction between CTAL-4 and biotin labeled B7.1 (**A** and **B**) and B7.2 (**C**).

[0047] Fig. 9 shows the effect of anti-CTLA-4 antibody mutants on IL-2 secretion in a SEB dependent T lymphocyte stimulation assay using PBMC's from donor 3.

[0048] Fig. 10 shows the binding activity of anti-CTLA-4 antibody CL5 and its mutants to human or cynomolgus CTLA-4 as measured by BiaCore.

[0049] Fig. 11A and 11B show the *in vitro* ADCC activity of anti-CTLA-4 antibody CL5 and its mutant on CHO K1-CTLA-4 cells.

[0050] Fig. 12 shows the serum concentration-time profile of anti-CTLA-4 antibody CL5 in male C57BL/6 mice.

[0051] Fig. 13 shows the tumor to serum ratio of anti-CTLA4 HCAb concentration.

[0052] Fig. 14A, 14B, and 14C show the anti-tumor activity of anti-CTLA-4 antibodies in MC38 tumor bearing mice. (**A**) Tumor growth curves in different groups. (**B**) Time-to-End point Kaplan-Meier survival curve. (**C**) Mice body weights kept relatively constant along with the human anti-CTLA-4 antibody treatment. Data were expressed as Mean + SEM, N = 9.

[0053] Fig. 15A and 15B show the inhibition of tumor growth by human anti-CTLA-4 antibodies in MC38 bearing mice. (**A**) Tumor growth curve. (**B**) Mice body weights kept relatively constant along with the human anti-CTLA-4 antibody treatment. Data were expressed as Mean + SEM, N = 6.

DETAILED DESCRIPTION OF THE INVENTION

General overview

[0054] The disclosure provides antibodies that specifically bind to CD152. These binding molecules may bind specifically to CD152 and to another target. Administration of a therapeutically effective amount of a CD152-binding antibody to a patient in need thereof is useful for treatment of certain disorders, including certain cancers. The binding of the antibody to a T-cell expressing CD152 induces an antibody-dependent cell-mediated cytotoxicity against

a cell expressing a tumor associated antigen. The CD152-binding therapeutics of the disclosure offer various advantages in treating patients, for example, effective binding to CD152, efficient induction of the antibody-dependent cell-mediated cytotoxicity and/or a lower risk of adverse events (e.g., toxicity). In certain aspects, the CD152-binding antibodies bind to CD152 more effectively in certain formats (e.g., heavy-chain-only antibody compared to typical full-length antibody), leading to higher potency and improved utility in treating disorders associated with CD152.

Definitions

[0055] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited herein, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated documents or portions of documents define a term that contradicts that term's definition in the application, the definition that appears in this application controls. However, mention of any reference, article, publication, patent, patent publication, and patent application cited herein is not, and should not be taken as an acknowledgment, or any form of suggestion, that they constitute valid prior art or form part of the common general knowledge in any country in the world.

[0056] In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components unless otherwise indicated. The use of the alternative (e.g., "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include" and "comprise" are used synonymously. In addition, it should be understood that the polypeptides comprising the various combinations of the components (e.g., domains or regions) and substituents described herein, are disclosed by the present application to the same extent as if each polypeptide was set forth individually. Thus, selection of particular components of individual polypeptides is within the scope of the present disclosure.

[0057] The term "about" and its grammatical equivalents in relation to a reference numerical value and its grammatical equivalents as used herein can include a range of values plus or minus 10% from that value, such as a range of values plus or minus 10%, 9%, 8%, 7%,

6%, 5%, 4%, 3%, 2%, or 1% from that value. For example, the amount “about 10” includes amounts from 9 to 11.

[0058] As used herein, a “polypeptide” or “polypeptide chain” is a single, linear and contiguous arrangement of covalently linked amino acids. It does not include two polypeptide chains that link together in a non-linear fashion, such as via an interchain disulfide bond (*e.g.*, a half immunoglobulin molecule in which a light chain links with a heavy chain via a disulfide bond). Polypeptides can have or form one or more intrachain disulfide bonds. With regard to polypeptides as described herein, reference to amino acid residues corresponding to those specified by SEQ ID NO includes post-translational modifications of such residues.

[0059] A “protein” is a macromolecule comprising one or more polypeptide chains. A protein can also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents can be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

[0060] The term “antibody”, “immunoglobulin” or “Ig” may be used interchangeably herein and means an immunoglobulin molecule that recognizes and specifically binds to a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or combinations of the foregoing through at least one antigen recognition site within the variable region of the immunoglobulin molecule. As used herein, the term “antibody” encompasses intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (such as Fab, Fab', F(ab')₂, and F_μ fragments), single chain F_μ (scFv) mutants, multispecific antibodies such as bispecific antibodies (including dual binding antibodies), chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen determination portion of an antibody, and any other modified immunoglobulin molecule comprising an antigen recognition site so long as the antibodies exhibit the desired biological activity. The term “antibody” can also refer to a Y-shaped glycoprotein with a molecular weight of approximately 150 kDa that is made up of four polypeptide chains: two light (L) chains and two heavy (H) chains. There are five types of mammalian Ig heavy chain isotypes denoted by the Greek letters alpha (α), delta (δ), epsilon (ε), gamma (γ), and mu (μ). The type of heavy chain defines the class of antibody, *i.e.*, IgA, IgD, IgE, IgG, and IgM, respectively. The γ and α classes are further divided into subclasses on the basis of differences in the constant domain sequence and

function, *e.g.*, IgG1, IgG2A, IgG2B, IgG3, IgG4, IgA1 and IgA2. In mammals there are two types of immunoglobulin light chains, λ and κ .

[0061] The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (*e.g.*, isomerizations, amidations) that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler and Milstein, *Nature*, 256:495-97 (1975); Hongo et al., *Hybridoma*, 14 (3): 253-260 (1995), 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* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567), phage-display technologies (see, *e.g.*, Clackson et al., *Nature*, 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.*, WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

[0062] As used herein, the term “heavy-chain-only antibody” (HCAb) refers to an antibody which consists only of two heavy chains and lacks the two light chains usually found in full-length antibodies.

[0063] The term an “isolated antibody” when used to describe the various antibodies disclosed herein, means an antibody that has been identified and separated and/or recovered from a cell or cell culture from which it was expressed. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and can include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For a review of methods for assessment of antibody purity, see, e.g., Flatman et al., *J. Chromatogr. B* 848:79-87 (2007). In preferred embodiments, the antibody will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes antibodies in situ within recombinant cells, because at least one component of the polypeptide natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

[0064] The term an “isolated” nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

[0065] The terms “light chain variable region” (also referred to as “light chain variable domain” or “VL” or V_L) and “heavy chain variable region” (also referred to as “heavy chain variable domain” or “VH” or V_H) refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of discrete, well-defined sub-regions known as “complementarity determining regions” (CDRs) and “framework regions” (FRs), generally comprising in order FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 from amino-terminus to carboxyl-terminus. In one embodiment, the FRs are humanized. The term “CL” refers to an “immunoglobulin light chain constant region” or a “light chain constant region,” i.e., a constant region from an antibody light chain. The term

“CH” refers to an “immunoglobulin heavy chain constant region” or a “heavy chain constant region,” which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM). A “Fab” (fragment antigen binding) is the part of an antibody that binds to antigens and includes the variable region and CH1 domain of the heavy chain linked to the light chain via an inter-chain disulfide bond.

[0066] As used herein, the term “binding domain” or “binding region” refers to the domain, region, portion, or site of a protein, polypeptide, oligopeptide, or peptide or antibody or binding domain derived from an antibody that possesses the ability to specifically recognize and bind to a target molecule, such as an antigen, ligand, receptor, substrate, or inhibitor (*e.g.*, CD152). Exemplary binding domains include single-chain antibody variable regions (*e.g.*, domain antibodies, sFv, scFv, scFab), receptor ectodomains, and ligands (*e.g.*, cytokines, chemokines). In certain embodiments, the binding domain comprises or consists of an antigen binding site (*e.g.*, comprising a variable heavy chain sequence and variable light chain sequence or three light chain complementary determining regions (CDRs) and three heavy chain CDRs from an antibody placed into alternative framework regions (FRs) (*e.g.*, human FRs optionally comprising one or more amino acid substitutions). A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, including Western blot, ELISA, phage display library screening, and BIACORE® interaction analysis. As used herein, a “CD152-binding domain” can have an immunoglobulin heavy chain variable regions comprising three heavy chain CDRs: CDR1, CDR2, and CDR3.

[0067] An antibody or binding domain “specifically binds” a target if it binds the target with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of $1/M$) equal to or greater than $10^5 M^{-1}$, while not significantly binding other components present in a test sample. Antibodies or binding domains can be classified as “high affinity” antibodies or binding domains and “low affinity” antibodies or binding domains. “High affinity” antibodies or binding domains refer to those antibodies or binding domains with a K_a of at least $10^7 M^{-1}$, at least $10^8 M^{-1}$, at least $10^9 M^{-1}$, at least $10^{10} M^{-1}$, at least $10^{11} M^{-1}$, at least $10^{12} M^{-1}$, or at least $10^{13} M^{-1}$. “Low affinity” antibodies or binding domains refer to those antibodies or binding domains with a K_a of up to $10^7 M^{-1}$, up to $10^6 M^{-1}$, up to $10^5 M^{-1}$. Alternatively, affinity can be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, $10^{-5} M$ to $10^{-13} M$). In the case of an

antibody binding to an antigen, $K_a = 1/K_d$. Affinities of antibodies or binding domains according to the present disclosure can be readily determined using conventional techniques, such as surface plasmon resonance (*see, e.g.*, Scatchard et al. (1949) Ann. N.Y. Acad. Sci. 51:660; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

[0068] As used herein, “CD152” refers to cluster of differentiation 152, which is also known as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). The terms, “CD152,” “CTLA-4,” and “CTLA4” are used interchangeably herein. Similarly, “anti-CD152,” “anti-CTLA-4,” “anti-CTLA4” are also used interchangeably herein.

[0069] As used herein, “CD80” refers to cluster of differentiation 80, which is a protein found on dendritic cells, activated B cells and monocytes that provides a costimulatory signal necessary for T cell activation and survival. The terms, “CD80,” “B7-1,” and “B7.1” are used interchangeably herein.

[0070] As used herein, “CD86” refers to cluster of differentiation 86, which is a protein expressed on antigen-presenting cells that provides costimulatory signals necessary for T cell activation and survival. The terms, “CD86,” “B7-2,” and “B7.2” are used interchangeably herein.

[0071] As used herein, a “conservative substitution” is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well-known in the art (*see, e.g.*, WO 97/09433, page 10, published March 13, 1997; Lehninger, Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge, MA (1990), p. 8). In certain embodiments, a conservative substitution includes a leucine to serine substitution.

[0072] As used herein, “ipilimumab analogue” refers to a monoclonal antibody, which binds specifically to CTLA-4, comprising a heavy chain with an amino acid sequence of SEQ ID NO.: 199 and a light chain with an amino acid sequence of SEQ ID NO.: 200.

[0073] As used herein, unless otherwise indicated, any nonproprietary or generic name of a biological product includes the biological product and any biosimilar product thereof. For example, the nonproprietary name, ipilimumab, refers to the biological product sold under the trade name YERVOY; it also includes any biosimilar product of the biological product.

[0074] As used herein, unless otherwise indicated, the term “biosimilar product” refers to 1) a biological product having an amino acid sequence that is identical to a reference product; 2) a biological product having a different amino acid sequence (e.g., N- or C-terminal

truncations) from a reference product; or 3) a biological product having a different posttranslational modification (e.g., glycosylation or phosphorylation) from a reference product, wherein the biosimilar product and the reference product utilize the same mechanism or mechanisms of action for the prevention, treatment, or cure of a disease or condition.

[0075] As used herein, the term "derivative" refers to a modification of one or more amino acid residues of a peptide by chemical or biological means, either with or without an enzyme, *e.g.*, by glycosylation, alkylation, acylation, ester formation, or amide formation.

[0076] As used herein, a polypeptide or amino acid sequence "derived from" a designated polypeptide or protein refers to the origin of the polypeptide. In certain embodiments, the polypeptide or amino acid sequence which is derived from a particular sequence (sometimes referred to as the "starting" or "parent" or "parental" sequence) has an amino acid sequence that is essentially identical to the starting sequence or a portion thereof, wherein the portion consists of at least 10-20 amino acids, at least 20-30 amino acids, or at least 30-50 amino acids, or at least 50-150 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the starting sequence. For example, a binding domain can be derived from an antibody, *e.g.*, a Fab, F(ab')₂, Fab', scFv, single domain antibody (sdAb), etc.

[0077] Polypeptides derived from another polypeptide can have one or more mutations relative to the starting polypeptide, *e.g.*, one or more amino acid residues which have been substituted with another amino acid residue or which has one or more amino acid residue insertions or deletions. The polypeptide can comprise an amino acid sequence which is not naturally occurring. Such variations necessarily have less than 100% sequence identity or similarity with the starting polypeptide. In one embodiment, the variant will have an amino acid sequence from about 60% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the starting polypeptide. In another embodiment, the variant will have an amino acid sequence from about 75% to less than 100%, from about 80% to less than 100%, from about 85% to less than 100%, from about 90% to less than 100%, from about 95% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the starting polypeptide.

[0078] As used herein, unless otherwise provided, a position of an amino acid residue in a variable region of an immunoglobulin molecule is numbered according to the IMGT numbering convention (Brochet, X, et al, Nucl. Acids Res. (2008) 36, W503-508) and a position of an amino acid residue in a constant region of an immunoglobulin molecule is numbered according to EU nomenclature (Ward *et al.*, 1995 *Therap. Immunol.* 2:77-94). Other

numbering conventions are known in the art (e.g., the Kabat numbering convention (Kabat, Sequences of Proteins of Immunological Interest, 5th ed. Bethesda, MD: Public Health Service, National Institutes of Health (1991)).

[0079] As used herein, the term “human” antibody refers to an antibody of human origin or a humanized antibody.

[0080] As used herein, the term “humanized” refers to a process of making an antibody or immunoglobulin binding proteins and polypeptides derived from a non-human species (e.g., mouse or rat) less immunogenic to humans, while still retaining antigen-binding properties of the original antibody, using genetic engineering techniques. In some embodiments, the binding domain(s) of an antibody or immunoglobulin binding proteins and polypeptides (e.g., light and heavy chain variable regions, Fab, scFv) are humanized. Non-human binding domains can be humanized using techniques known as CDR grafting (Jones *et al.*, *Nature* 321:522 (1986)) and variants thereof, including “reshaping” (Verhoeyen, *et al.*, 1988 *Science* 239:1534-1536; Riechmann, *et al.*, 1988 *Nature* 332:323-337; Tempest, *et al.*, *Bio/Technol* 1991 9:266-271), “hyperchimerization” (Queen, *et al.*, 1989 *Proc Natl Acad Sci USA* 86:10029-10033; Co, *et al.*, 1991 *Proc Natl Acad Sci USA* 88:2869-2873; Co, *et al.*, 1992 *J Immunol* 148:1149-1154), and “veneering” (Mark, *et al.*, “Derivation of therapeutically active humanized and veneered anti-CD18 antibodies.” In: Metcalf BW, Dalton BJ, eds. Cellular adhesion: molecular definition to therapeutic potential. New York: Plenum Press, 1994: 291-312). If derived from a non-human source, other regions of the antibody or immunoglobulin binding proteins and polypeptides, such as the hinge region and constant region domains, can also be humanized.

[0081] As used herein, the term “patient in need” or “subject in need” refers to a patient or a subject at risk of, or suffering from, a disease, disorder or condition that is amenable to treatment or amelioration with a CD152-binding antibody or a composition thereof provided herein.

[0082] As used herein, the term “pharmaceutically acceptable” refers to molecular entities and compositions that do not generally produce allergic or other serious adverse reactions when administered using routes well known in the art. Molecular entities and compositions approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans are considered to be “pharmaceutically acceptable.”

[0083] As used herein, the term “treatment,” “treating,” or “ameliorating” refers to either a therapeutic treatment or prophylactic/preventative treatment. A treatment is therapeutic if at

least one symptom of disease in an individual receiving treatment improves or a treatment can delay worsening of a progressive disease in an individual, or prevent onset of additional associated diseases.

[0084] As used herein, the term “therapeutically effective amount (or dose)” or “effective amount (or dose)” of a specific binding molecule or compound refers to that amount of the compound sufficient to result in amelioration of one or more symptoms of the disease being treated in a statistically significant manner or a statistically significant improvement in organ function. When referring to an individual active ingredient, administered alone, a therapeutically effective dose refers to that ingredient alone. When referring to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered serially or simultaneously (in the same formulation or concurrently in separate formulations).

[0085] As used herein, the terms, “Antibody-dependent cell-mediated cytotoxicity” and “ADCC,” refer to a cell-mediated process in which nonspecific cytotoxic cells that express FcγRs (e.g., monocytic cells such as Natural Killer (NK) cells and macrophages) recognize bound antibody (or other protein capable of binding FcγRs) on a target cell and subsequently cause lysis of the target cell. In principle, any effector cell with an activating FcγR can be triggered to mediate ADCC. The primary cells for mediating ADCC are NK cells, which express only FcγRIII, whereas monocytes, depending on their state of activation, localization, or differentiation, can express FcγRI, FcγRII, and FcγRIII. For a review of FcγR expression on hematopoietic cells, see, e.g., Ravetch et al., 1991, *Annu. Rev. Immunol.*, 9:457-92.

[0086] As used herein, the term “promoter” refers to a region of DNA involved in binding RNA polymerase to initiate transcription.

[0087] As used herein, the terms “nucleic acid,” “nucleic acid molecule,” or “polynucleotide” refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the terms encompass nucleic acids containing analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or

deoxyinosine residues (Batzer *et al.* (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka *et al.* (1985) *J. Biol. Chem.* 260:2605-2608; Cassol *et al.* (1992); Rossolini *et al.* (1994) *Mol. Cell. Probes* 8:91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene. As used herein, the terms "nucleic acid," "nucleic acid molecule," or "polynucleotide" are intended to include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs thereof.

[0088] The term "expression" refers to the biosynthesis of a product encoded by a nucleic acid. For example, in the case of nucleic acid segment encoding a polypeptide of interest, expression involves transcription of the nucleic acid segment into mRNA and the translation of mRNA into one or more polypeptides.

[0089] The terms "expression unit" and "expression cassette" are used interchangeably herein and denote a nucleic acid segment encoding a polypeptide of interest and capable of providing expression of the nucleic acid segment in a host cell. An expression unit typically comprises a transcription promoter, an open reading frame encoding the polypeptide of interest, and a transcription terminator, all in operable configuration. In addition to a transcriptional promoter and terminator, an expression unit can further include other nucleic acid segments such as, *e.g.*, an enhancer or a polyadenylation signal.

[0090] The term "expression vector," as used herein, refers to a nucleic acid molecule, linear or circular, comprising one or more expression units. In addition to one or more expression units, an expression vector can also include additional nucleic acid segments such as, for example, one or more origins of replication or one or more selectable markers. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both.

[0091] As used herein, the term "sequence identity" refers to a relationship between two or more polynucleotide sequences or between two or more polypeptide sequences. When a position in one sequence is occupied by the same nucleic acid base or amino acid residue in the corresponding position of the comparator sequence, the sequences are said to be "identical" at that position. The percentage "sequence identity" is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of "identical" positions. The number of "identical" positions is then divided by the total number of positions in the comparison window and multiplied by 100 to yield the percentage of "sequence identity." Percentage of "sequence identity" is determined

by comparing two optimally aligned sequences over a comparison window. The comparison window for nucleic acid sequences can be, for instance, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 or more nucleic acids in length. The comparison window for polypeptide sequences can be, for instance, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300 or more amino acids in length. In order to optimally align sequences for comparison, the portion of a polynucleotide or polypeptide sequence in the comparison window can comprise additions or deletions termed gaps while the reference sequence is kept constant. An optimal alignment is that alignment which, even with gaps, produces the greatest possible number of “identical” positions between the reference and comparator sequences. Percentage “sequence identity” between two sequences can be determined using the version of the program “BLAST 2 Sequences” which was available from the National Center for Biotechnology Information as of September 1, 2004, which program incorporates the programs BLASTN (for nucleotide sequence comparison) and BLASTP (for polypeptide sequence comparison), which programs are based on the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90(12):5873-5877, 1993). When utilizing “BLAST 2 Sequences,” parameters that were default parameters as of September 1, 2004, can be used for word size (3), open gap penalty (11), extension gap penalty (1), gap dropoff (50), expect value (10) and any other required parameter including but not limited to matrix option. Two nucleotide or amino acid sequences are considered to have “substantially similar sequence identity” or “substantial sequence identity” if the two sequences have at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity relative to each other.

Antibodies

[0092] Disclosed herein are human monoclonal antibodies comprising a CD152-binding domain. The antibodies can be heavy-chain-only antibodies. The antibodies can consist of only two heavy chains. The antibodies can comprise no light chains. The antibodies can bind specifically to CD152. The antibodies can be isolated monoclonal antibodies that bind specifically to CD152 with high affinity.

[0093] The anti-CD152 antibodies disclosed herein can bind specifically to human CD152. In some cases, the anti-CD152 antibodies can bind to human CD152 with high affinity (*e.g.*, $K_D < 6.0 \times 10^{-11} \text{M}$). The anti-CD152 antibodies can have a comparable or higher affinity to CTLA-4 when compared to an ipilimumab analogue. The anti-CD152 antibodies can also

block the binding of CD152 to its ligands B7.1. The anti-CD152 antibodies can have enhanced tumor/peripheral serum ratio than the ipilimumab analogue. The anti-CD152 antibodies can induce a higher ADCC, for example, the antibodies can induce at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 15-fold, or at least about 20-fold increase of lysis activity by NK cells.

[0094] The anti-CD152 antibodies can comprise a CD152-binding domain, which comprises an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3. The anti-CD152 antibodies can also comprise CDR1, CDR2, and CDR3 that differ from those of anti-CD152 antibodies disclosed herein by one or more conservative modifications. It is understood in the art that certain conservative sequence modification can be made which do not remove antigen binding. See, e.g., Brummell *et al.*, 1993, *Biochem* 32:1180-8; de Wildt *et al.*, 1997, *Prot. Eng.* 10:835-41; Komissarov *et al.*, 1997, *J. Biol. Chem.* 272:26864-26870; Hall *et al.*, 1992, *J. Immunol.* 149:1605-12; Kelley and O'Connell, 1993, *Biochem.* 32:6862-35; Adib-Conquy *et al.*, 1998, *Int. Immunol.* 10:341-6 and Beers *et al.*, 2000, *Clin. Can. Res.* 6:2835-43.

Pharmaceutical compositions and formulations

[0095] A pharmaceutical composition can comprise one or more anti-CD152 antibodies disclosed herein formulated together with a pharmaceutically acceptable excipient. An excipient is said to be a “pharmaceutically acceptable excipient” if its administration can be tolerated by a recipient patient. Excipients that can be used include carriers, surface active agents, thickening or emulsifying agents, solid binders, dispersion or suspension aids, solubilizers, colorants, flavoring agents, coatings, disintegrating agents, lubricants, sweeteners, preservatives, isotonic agents, and combinations thereof. The selection and use of suitable excipients is taught in Gennaro, ed., Remington: *The Science and Practice of Pharmacy*, 20th Ed. (Lippincott Williams & Wilkins 2003), and in Gennaro, ed., *Remington's Pharmaceutical Sciences* (Mack Publishing Company, 19th ed. 1995). Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable excipient. Formulations can further include one or more carriers, diluents, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, *etc.*

[0096] The amount of active ingredient which can be combined with a carrier material to produce a single dosage form can vary depending upon the subject being treated and the particular mode of administration and can generally be that amount of the pharmaceutical

composition which produces a therapeutic effect. Generally, the amount of active ingredient can range from about 0.01% to about 99% (w/w) of the composition, for example, can be about 0.1%-1%, about 0.1%-5%, about 0.1-10%, about 0.1%-20%, about 0.5%-1%, about 0.5%-5%, about 0.5%-10%, about 0.5%-20%, about 1%-5%, about 1%-10%, about 1%-20%, about 5%-10%, about 5%-20%, about 10%-20%, about 10%-30%, about 20%-30%, about 20%-40%, about 30%-40%, about 30%-50%, about 40%-50%, about 40%-60%, about 50%-60%, about 50%-70%, about 60%-70%, about 60%-80%, about 70%-80%, about 70%-90%, about 80%-90%, about 80%-95%, or 95%-99% of the pharmaceutical composition. Preferably, the amount of active ingredient can be from about 0.1% to about 70%, and most preferably from about 1% to about 30% of the pharmaceutical composition.

[0097] The pharmaceutical composition can be suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active ingredient can be coated in a material to protect it from the action of acids and other natural conditions that may inactivate it. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Alternatively, an antibody of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, *e.g.*, intranasally, orally, vaginally, rectally, sublingually or topically. The pharmaceutical composition can be in the form of sterile aqueous solutions or dispersions. The pharmaceutical composition can also be formulated in a microemulsion, liposome, or other ordered structure suitable to high drug concentration.

[0098] The pharmaceutical composition may be formulated in a dosage form selected from the group consisting of: an oral unit dosage form, an intravenous unit dosage form, an intranasal unit dosage form, a suppository unit dosage form, an intradermal unit dosage form, an intramuscular unit dosage form, an intraperitoneal unit dosage form, a subcutaneous unit dosage form, an epidural unit dosage form, a sublingual unit dosage form, and an intracerebral unit dosage form. The oral unit dosage form may be selected from the group consisting of: tablets, pills, pellets, capsules, powders, lozenges, granules, solutions, suspensions, emulsions, syrups, elixirs, sustained-release formulations, aerosols, and sprays.

[0099] The pharmaceutical composition can be a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. See, e.g., Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

[00100] The monoclonal antibodies disclosed herein can be formulated to ensure proper distribution *in vivo*. For example, to ensure that the therapeutic antibody of the invention cross the blood-brain barrier, they can be formulated in liposomes, which may additionally comprise targeting moieties to enhance selective transport to specific cells or organs. See, e.g. U.S. Pat. Nos. 4,522,811; 5,374,548; 5,416,016; and 5,399,331; V. V. Ranade, 1989, *J. Clin. Pharmacol.* 29:685; Umezawa *et al.*, 1988, *Biochem. Biophys. Res. Commun.* 153:1038; Bloeman *et al.*, 1995, *FEBS Lett.* 357:140; M. Owais *et al.*, 1995, *Antimicrob. Agents Chemother.* 39:180; Briscoe *et al.*, 1995, *Am. J. Physiol.* 1233:134; Schreier *et al.*, 1994, *J. Biol. Chem.* 269:9090; Keinänen and Laukkanen, 1994, *FEBS Lett.* 346:123; and Killion and Fidler, 1994, *Immunomethods* 4:273.

[00101] The pharmaceutical composition may optionally contain one or more additional pharmaceutically active ingredients, such as another antibody or a drug. The pharmaceutical compositions of the invention also can be administered in a combination therapy with, for example, another anti-cancer agent, another anti-inflammatory agent, or a vaccine.

[00102] Pharmaceutical compositions can be supplied as a kit comprising a container that comprises the pharmaceutical composition as described herein. A pharmaceutical composition can be provided, for example, in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Alternatively, such a kit can include a dry-powder disperser, liquid aerosol generator, or nebulizer for administration of a pharmaceutical composition. Such a kit can further comprise written information on indications and usage of the pharmaceutical composition.

Methods of treatment

[00103] Further disclosed herein is a method of treating a disorder by administering a subject a therapeutically effective amount of the antibody or the pharmaceutical composition disclosed herein. The anti-CD152 antibodies disclosed herein may be used in a method for treating a subject (for example, a human or a non-human primate) or for manufacture of a

medicament for treating a subject. Generally, such methods include administering to a subject in need of such treatment an anti-CD152 antibodies as described herein.

[00104] The anti-CD152 antibodies disclosed herein may be used in a method for treating a subject (for example, a human or a non-human primate) or for manufacture of a medicament for treating a subject. Generally, such methods include administering to a subject in need of such treatment an anti-CD152 antibody as described herein. In some embodiments, the anti-CD152 antibody comprises at least one effector function selected from antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), such that the anti-CD152 antibody induces ADCC and/or CDC against CD152-expressing cells in the subject.

[00105] Also disclosed herein is a method for treating a disorder characterized by overexpression of a tumor antigen, such as cancer. Examples of tumor antigens that may be recognized by a bispecific anti-CD152 antibody may include PSMA, CD19, CD20, CD37, CD38, CD123, Her2, ROR1, RON, glycoprotein A33 antigen (gpA33) and CEA. Generally, such methods include administering to a subject in need of such treatment a therapeutically effective amount of an anti-CD152 antibody comprising a second binding domain that binds a tumor antigen as described herein. The anti-CD152 antibody can induce redirected T-cell cytotoxicity (RTCC) against tumor antigen-expressing cells in the subject.

[00106] The method can be used for treating cancers such as, prostate cancer, colorectal cancer, renal cell carcinoma, bladder cancer, salivary gland cancer, pancreatic cancer, ovarian cancer, non-small cell lung cancer, melanoma, breast cancer (e.g., triple negative breast cancer), adrenal cancer, mantle cell lymphoma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, Non-Hodgkin's lymphoma, acute myeloid leukemia (AML), B-lymphoid leukemia, blastic plasmacytoid dendritic neoplasm (BPDCN), and hairy cell leukemia.

[00107] Also disclosed herein is a method for treating an autoimmune disorder comprising administering a therapeutically effective amount of the pharmaceutical compositions or anti-CD152 antibody described herein to a patient in need thereof.

[00108] Subjects for administration of the anti-CD152 antibodies as described herein include patients at high risk for developing a particular disorder as well as patients presenting with an existing such disorder. Typically, the subject has been diagnosed as having the disorder for which treatment is sought. Further, subjects can be monitored during the course of treatment for any change in the disorder (e.g., for an increase or decrease in clinical symptoms

of the disorder). Also, in some variations, the subject does not suffer from another disorder requiring treatment that involves targeting CD152-expressing cells.

[00109] In prophylactic applications, pharmaceutical compositions can be administered to a patient susceptible to, or otherwise at risk of, a particular disorder in an amount sufficient to eliminate or reduce the risk or delay the onset of the disorder. In therapeutic applications, compositions can be administered to a patient suspected of, or already suffering from such a disorder in an amount sufficient to cure, or at least partially arrest, the symptoms of the disorder and its complications. An amount adequate to accomplish this is referred to as a therapeutically effective dose or amount. In both prophylactic and therapeutic regimes, agents can be administered in several dosages until a sufficient response has been achieved. Typically, the response is monitored and repeated dosages are given if the desired response starts to fade.

[00110] To identify subject patients for treatment according to the methods of the disclosure, accepted screening methods can be employed to determine risk factors associated with specific disorders or to determine the status of an existing disorder identified in a subject. Such methods can include, for example, determining whether an individual has relatives who have been diagnosed with a particular disorder. Screening methods can also include, for example, conventional work-ups to determine familial status for a particular disorder known to have a heritable component. For example, various cancers are also known to have certain inheritable components. Inheritable components of cancers include, for example, mutations in multiple genes that are transforming (*e.g.*, Ras, Raf, EGFR, cMet, and others), the presence or absence of certain HLA and killer inhibitory receptor (KIR) molecules, or mechanisms by which cancer cells are able to modulate immune suppression of cells like NK cells and T-cells, either directly or indirectly (*see, e.g.*, Ljunggren and Malmberg, *Nature Rev. Immunol.* 7:329-339, 2007; Boyton and Altmann, *Clin. Exp. Immunol.* 149:1-8, 2007). Toward this end, nucleotide probes can be routinely employed to identify individuals carrying genetic markers associated with a particular disorder of interest. In addition, a wide variety of immunological methods are known in the art that are useful to identify markers for specific disorder. For example, various ELISA immunoassay methods are available and well-known in the art that employ monoclonal antibody probes to detect antigens associated with specific tumors. Screening can be implemented as indicated by known patient symptomology, age factors, related risk factors, etc. These methods allow the clinician to routinely select patients in need of the methods described herein for treatment. In accordance with these methods, targeting

pathological, tumor antigen-expressing cells can be implemented as an independent treatment program or as a follow-up, adjunct, or coordinate treatment regimen to other treatments.

[00111] Determination of effective dosages in this context is typically based on animal model studies followed up by human clinical trials and is guided by determining effective dosages and administration protocols that significantly reduce the occurrence or severity of the subject disorder in model subjects. Effective doses of the compositions of the present disclosure vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, whether treatment is prophylactic or therapeutic, as well as the specific activity of the composition itself and its ability to elicit the desired response in the individual. Usually, the patient is a human, but in some diseases, the patient can be a nonhuman mammal. Typically, dosage regimens are adjusted to provide an optimum therapeutic response, *i.e.*, to optimize safety and efficacy. Accordingly, a therapeutically effective amount is also one in which any undesired collateral effects are outweighed by the beneficial effects of administering an anti-CD152 antibody as described herein. For administration of an anti-CD152 antibody, a dosage may range from about 0.1 µg to 100 mg/kg or 1 µg/kg to about 50 mg/kg, and more usually 10 µg to 5 mg/kg of the subject's body weight. In more specific embodiments, an effective amount of the agent is between about 1 µg/kg and about 20 mg/kg, between about 10 µg/kg and about 10 mg/kg, or between about 0.1 mg/kg and about 5 mg/kg. Dosages within this range can be achieved by single or multiple administrations, including, *e.g.*, multiple administrations per day or daily, weekly, bi-weekly, or monthly administrations. For example, in certain variations, a regimen consists of an initial administration followed by multiple, subsequent administrations at weekly or bi-weekly intervals. Another regimen consists of an initial administration followed by multiple, subsequent administrations at monthly or bi-monthly intervals. Alternatively, administrations can be on an irregular basis as indicated by monitoring clinical symptoms of the disorder.

[00112] Dosage of the pharmaceutical composition can be varied by the attending clinician to maintain a desired concentration at a target site. For example, if an intravenous mode of delivery is selected, local concentration of the agent in the bloodstream at the target tissue can be between about 0.01-50 nanomoles of the composition per liter, sometimes between about 1.0 nanomole per liter and 10, 15, or 25 nanomoles per liter depending on the subject's status and projected measured response. Higher or lower concentrations can be selected based on the mode of delivery, *e.g.*, trans-epidermal delivery versus delivery to a mucosal surface. Dosage

should also be adjusted based on the release rate of the administered formulation, *e.g.*, nasal spray versus powder, sustained release oral or injected particles, transdermal formulations, *etc.* To achieve the same serum concentration level, for example, slow-release particles with a release rate of 5 nanomolar (under standard conditions) would be administered at about twice the dosage of particles with a release rate of 10 nanomolar.

[00113] The anti-CD152 therapeutic (*e.g.*, anti-CD152 antibody) may also be administered at a daily dosage of from about 0.001 to about 10 milligrams (mg) per kilogram (mpk) of body weight, preferably given as a single daily dose or in divided doses about two to six times a day. For administration to a human adult patient, the therapeutically effective amount may be administered in doses in the range of 0.2 mg to 800 mg per dose, including but not limited to 0.2 mg per dose, 0.5 mg per dose, 1 mg per dose, 5 mg per dose, 10 mg per dose, 25 mg per dose, 100 mg per dose, 200 mg per dose, and 400 mg per dose, and multiple, usually consecutive daily doses may be administered in a course of treatment. The anti-CD152 therapeutic can be administered at different times of the day. In one embodiment the optimal therapeutic dose can be administered in the evening. In another embodiment the optimal therapeutic dose can be administered in the morning. The total daily dosage of the anti-CD152 therapeutic thus can in one embodiment range from about 1 mg to about 2 g, and often ranges from about 100 mg to about 1.5 g, and most often ranges from about 200 mg to about 1200 mg. In the case of a typical 70 kg adult human, the total daily dose of the anti-CD152 therapeutic can range from about 2 mg to about 1200 mg and will often range, as noted above, from about 0.2 mg to about 800 mg.

[00114] Dosage regimens can also be adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required.

[00115] For administration of the antibody, the dosage may range from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can

be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months.

Preferred dosage regimens for an anti-CD152 antibody of the invention include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the antibody being given using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks. In some methods, dosage is adjusted to achieve a plasma antibody concentration of about 1-1000 $\mu\text{g/ml}$ and in some methods about 25-300 $\mu\text{g/ml}$.

[00116] A "therapeutically effective dosage" of an anti-CD152 antibody of the invention preferably results in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. For example, for the treatment of tumor-bearing subjects, a "therapeutically effective dosage" preferably inhibits tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. A therapeutically effective amount of a therapeutic antibody can decrease tumor size, or otherwise ameliorate symptoms in a subject, which is typically a human or can be another mammal.

[00117] With particular regard to treatment of solid tumors, protocols for assessing endpoints and anti-tumor activity are well-known in the art. While each protocol may define tumor response assessments differently, the RECIST (Response evaluation Criteria in solid tumors) criteria is currently considered to be the recommended guidelines for assessment of tumor response by the National Cancer Institute (*see Therasse et al., J. Natl. Cancer Inst.* 92:205-216, 2000). According to the RECIST criteria tumor response means a reduction or elimination of all measurable lesions or metastases. Disease is generally considered measurable if it comprises lesions that can be accurately measured in at least one dimension as $\geq 20\text{mm}$ with conventional techniques or $\geq 10\text{mm}$ with spiral CT scan with clearly defined margins by medical photograph or X-ray, computerized axial tomography (CT), magnetic resonance imaging (MRI), or clinical examination (if lesions are superficial). Non-measurable disease means the disease comprises of lesions $< 20\text{mm}$ with conventional techniques or $< 10\text{mm}$ with spiral CT scan, and truly non-measurable lesions (too small to accurately measure).

Non-measurable disease includes pleural effusions, ascites, and disease documented by indirect evidence.

[00118] The criteria for objective status are required for protocols to assess solid tumor response. Representative criteria include the following: (1) Complete Response (CR), defined as complete disappearance of all measurable disease; no new lesions; no disease related symptoms; no evidence of non-measurable disease; (2) Partial Response (PR) defined as 30% decrease in the sum of the longest diameter of target lesions (3) Progressive Disease (PD), defined as 20% increase in the sum of the longest diameter of target lesions or appearance of any new lesion; (4) Stable or No Response, defined as not qualifying for CR, PR, or Progressive Disease. (*See Therasse et al., supra.*)

[00119] Additional endpoints that are accepted within the oncology art include overall survival (OS), disease-free survival (DFS), objective response rate (ORR), time to progression (TTP), and progression-free survival (PFS) (*see Guidance for Industry: Clinical Trial Endpoints for the Approval of Cancer Drugs and Biologics*, April 2005, Center for Drug Evaluation and Research, FDA, Rockville, MD.)

[00120] The anti-CD152 antibodies can be used to suppress CTLA-4-mediated signaling pathways that negatively-regulate immune responses, and to therefore enhance tumor-specific immune responses, either as a monotherapy or in combination with anti-PD-L1 monoclonal antibodies or other anticancer drugs.

Methods of preparing antibodies

[00121] The antibodies disclosed herein can be a human heavy-chain-only antibody (HCAb) generated from Harbour humanized mice (U.S. Pat. Nos. 9,353,179, 9,346,877 and 8,921, 522, and European Patent Nos. 1776383 and 1864998). The molecules produced by the HCAb mice can be soluble and can have affinities, diversity and/or physicochemical properties comparable to traditional human IgG antibodies.

[00122] The preparation of HCABs from the HCAb mice can facilitate generation of soluble human VH domains, the minimal immunoglobulin recognition unit, and thus the construction of novel multi-functional molecules comprising either multiple VH domains or VH domain(s) coupled to other molecules, such as bi-specifics, Antibody Drug Conjugates or VH domain-derived diagnostic or therapeutic molecules.

[00123] The anti-CD152 antibodies can also be prepared using an antibody having one or more of the V_H sequences of the anti-CD152 antibody disclosed herein as starting material to engineer a modified antibody. An antibody can be engineered by modifying one or more

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

[00124] Polynucleotide molecules comprising a desired polynucleotide sequence can be propagated by placing the molecule in a vector. Viral and non-viral vectors can be used, including plasmids. The choice of plasmid will depend on the type of cell in which propagation is desired and the purpose of propagation. Certain vectors are useful for amplifying and making large amounts of the desired DNA sequence. Other vectors are suitable for expression in cells in culture. Still other vectors are suitable for transfer and expression in cells in a whole animal or person. The choice of appropriate vector is well within the skill of the art. Many such vectors are available commercially. The partial or full-length polynucleotide is inserted into a vector typically by means of DNA ligase attachment to a cleaved restriction enzyme site in the vector. Alternatively, the desired nucleotide sequence can be inserted by homologous recombination *in vivo*. Typically this is accomplished by attaching regions of homology to the vector on the flanks of the desired nucleotide sequence. Regions of homology are added by ligation of oligonucleotides, or by polymerase chain reaction using primers comprising both the region of homology and a portion of the desired nucleotide sequence, for example.

[00125] For expression, an expression cassette or system may be employed. To express a nucleic acid encoding a polypeptide disclosed herein, a nucleic acid molecule encoding the polypeptide, operably linked to regulatory sequences that control transcriptional expression in an expression vector, is introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector. The gene product encoded by a polynucleotide of the disclosure is expressed in any convenient expression system, including, for example, bacterial, yeast, insect, amphibian and mammalian systems. In the expression vector, the polypeptide-encoding polynucleotide is linked to a regulatory sequence as appropriate to obtain the desired expression properties. These can include promoters, enhancers, terminators, operators, repressors, and inducers. The promoters can be regulated (*e.g.*, the promoter from the steroid inducible pIND vector (Invitrogen)) or constitutive (*e.g.*, promoters from CMV, SV40, Elongation Factor, or LTR sequences). These are linked to the desired nucleotide sequence

using the techniques described above for linkage to vectors. Any techniques known in the art can be used. Accordingly, the expression vector will generally provide a transcriptional and translational initiation region, which can be inducible or constitutive, where the coding region is operably linked under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination region.

[00126] An expression cassette can be introduced into a variety of vectors, *e.g.*, plasmid, BAC, YAC, bacteriophage such as lambda, P1, M13, *etc.*, plant or animal viral vectors (*e.g.*, retroviral-based vectors, adenovirus vectors), and the like, where the vectors are normally characterized by the ability to provide selection of cells comprising the expression vectors. The vectors can provide for extrachromosomal maintenance, particularly as plasmids or viruses, or for integration into the host chromosome. Where extrachromosomal maintenance is desired, an origin sequence is provided for the replication of the plasmid, which can be low- or high copy-number. A wide variety of markers are available for selection, particularly those which protect against toxins, more particularly against antibiotics. The particular marker that is chosen is selected in accordance with the nature of the host, where, in some cases, complementation can be employed with auxotrophic hosts. Introduction of the DNA construct can use any convenient method, including, *e.g.*, conjugation, bacterial transformation, calcium-precipitated DNA, electroporation, fusion, transfection, infection with viral vectors, biolistics, and the like. The disclosure relates to an expression vector comprising a nucleic acid segment, wherein said nucleic acid segment may comprise a nucleotide sequence set forth in SEQ ID NO: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 163, 169, 175, 181, or 187.

[00127] Accordingly, proteins for use within the present disclosure can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells (including cultured cells of multicellular organisms), particularly cultured mammalian cells. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001), and Ausubel *et al.*, *Short Protocols in Molecular Biology* (4th ed., John Wiley & Sons, 1999).

[00128] To direct a recombinant protein into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence) is provided in the expression vector. The

secretory signal sequence can be that of the native form of the recombinant protein, or can be derived from another secreted protein or synthesized *de novo*. The secretory signal sequence is operably linked to the polypeptide-encoding DNA sequence, *i.e.*, the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences can be positioned elsewhere in the DNA sequence of interest (*see, e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

[00129] Cultured mammalian cells are suitable hosts for production of recombinant proteins for use within the present disclosure. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler *et al.*, *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann *et al.*, *EMBO J.* 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel *et al.*, *supra*), and liposome-mediated transfection (Hawley-Nelson *et al.*, *Focus* 15:73, 1993; Ciccarone *et al.*, *Focus* 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed by, for example, Levinson *et al.*, U.S. Patent No. 4,713,339; Hagen *et al.*, U.S. Patent No. 4,784,950; Palmiter *et al.*, U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Examples of suitable mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21, BHK-570; ATCC CRL 8544, ATCC CRL 10314), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61; CHO DG44; CHO DXB11 (Hyclone, Logan, UT); *see also, e.g.*, Chasin *et al.*, *Som. Cell. Molec. Genet.* 12:555, 1986)), rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658). Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Manassas, Virginia. Strong transcription promoters can be used, such as promoters from SV-40 or cytomegalovirus. *See, e.g.*, U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patents Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

[00130] Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as “transfectants.” Cells

that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as “stable transfectants.” Exemplary selectable markers include a gene encoding resistance to the antibiotic neomycin, which allows selection to be carried out in the presence of a neomycin-type drug, such as G-418 or the like; the gpt gene for xanthine-guanine phosphoribosyl transferase, which permits host cell growth in the presence of mycophenolic acid/xanthine; and markers that provide resistance to zeocin, bleomycin, blastocidin, and hygromycin (*see, e.g., Gatignol et al., Mol. Gen. Genet.* 207:342, 1987; Drocourt *et al., Nucl. Acids Res.* 18:4009, 1990). Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as “amplification.” Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. An exemplary amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase*) can also be used.

[00131] Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar *et al., J. Biosci. (Bangalore)* 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino *et al., US 5,162,222* and WO 94/06463.

[00132] Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV). *See King and Possee, The Baculovirus Expression System: A Laboratory Guide* (Chapman & Hall, London); O'Reilly *et al., Baculovirus Expression Vectors: A Laboratory Manual* (Oxford University Press., New York 1994); and *Baculovirus Expression Protocols. Methods in Molecular Biology* (Richardson ed., Humana Press, Totowa, NJ, 1995). Recombinant baculovirus can also be produced through the use of a transposon-based system described by Luckow *et al. (J. Virol.* 67:4566-4579, 1993). This system, which utilizes transfer vectors, is commercially available in kit form (BAC-TO-BAC kit; Life Technologies, Gaithersburg, MD). The transfer vector (*e.g., PFASTBAC1*; Life Technologies) contains a Tn7 transposon to move the DNA encoding the protein of interest into a baculovirus genome maintained in *E. coli* as a large plasmid called a “bacmid.” *See Hill-Perkins and Possee, J. Gen. Virol.* 71:971-976, 1990; Bonning *et al., J. Gen. Virol.* 75:1551-1556, 1994; and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543-1549,

1995. In addition, transfer vectors can include an in-frame fusion with DNA encoding a polypeptide extension or affinity tag as disclosed above. Using techniques known in the art, a transfer vector containing a protein-encoding DNA sequence is transformed into *E. coli* host cells, and the cells are screened for bacmids which contain an interrupted lacZ gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, such as Sf9 cells. Recombinant virus that expresses the protein of interest is subsequently produced. Recombinant viral stocks are made by methods commonly used in the art.

[00133] For protein production, the recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda* (e.g., Sf9 or Sf21 cells) or *Trichoplusia ni* (e.g., HIGH FIVE cells; Invitrogen, Carlsbad, CA). See generally Glick and Pasternak, *Molecular Biotechnology, Principles & Applications of Recombinant DNA* (ASM Press, Washington, D.C., 1994). See also U.S. Patent No. 5,300,435. Serum-free media are used to grow and maintain the cells. Suitable media formulations are known in the art and can be obtained from commercial suppliers. The cells are grown up from an inoculation density of approximately $2-5 \times 10^5$ cells to a density of $1-2 \times 10^6$ cells, at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally described in available laboratory manuals (see, e.g., King and Possee, *supra*; O'Reilly *et al.*, *supra*; Richardson, *supra*).

[00134] Fungal cells, including yeast cells, can also be used within the present disclosure. Yeast species of in this regard include, e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki *et al.*, U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch *et al.*, U.S. Patent No. 5,037,743; and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). An exemplary vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman *et al.*, U.S. Patent No. 4,615,974; and Bitter, U.S. Patent

No. 4,977,092) and alcohol dehydrogenase genes. *See also* U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936; and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii*, and *Candida maltosa* are known in the art. *See, e.g.*, Gleeson *et al.*, *J. Gen. Microbiol.* 132:3459-3465, 1986; Cregg, U.S. Patent No. 4,882,279; and Raymond *et al.*, *Yeast* 14:11-23, 1998. *Aspergillus* cells can be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533. Production of recombinant proteins in *Pichia methanolica* is disclosed in U.S. Patents Nos. 5,716,808; 5,736,383; 5,854,039; and 5,888,768.

[00135] Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus*, and other genera are also useful host cells within the present disclosure. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well-known in the art (*see, e.g.*, Sambrook and Russell, *supra*). When expressing a recombinant protein in bacteria such as *E. coli*, the protein can be retained in the cytoplasm, typically as insoluble granules, or can be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured protein can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the alternative, the protein can be recovered from the cytoplasm in soluble form and isolated without the use of denaturants. The protein is recovered from the cell as an aqueous extract in, for example, phosphate buffered saline. To capture the protein of interest, the extract is applied directly to a chromatographic medium, such as an immobilized antibody or heparin-Sepharose column. Secreted proteins can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding. Antibodies, including single-chain antibodies, can be produced in bacterial host cells according to known methods. *See, e.g.*, Bird *et al.*, *Science* 242:423-426, 1988; Huston *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988; and Pantoliano *et al.*, *Biochem.* 30:10117-10125, 1991.

[00136] Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media can also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

[00137] Anti-CD152 antibodies may be purified by conventional protein purification methods, typically by a combination of chromatographic techniques. *See generally Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988); Scopes, *Protein Purification: Principles and Practice* (Springer-Verlag, New York 1994). Proteins comprising an immunoglobulin Fc region can be purified by affinity chromatography on immobilized protein A or protein G. Additional purification steps, such as gel filtration, can be used to obtain the desired level of purity or to provide for desalting, buffer exchange, and the like.

[00138] The following examples of the invention are to further illustrate the nature of the invention. It should be understood that the following examples do not limit the invention and that the scope of the invention is to be determined by the appended claims.

Examples

Example 1 – Generation of anti-CTLA-4 antibodies

[00139] Human CTLA-4-ECD protein (Acro Bio) was used as an immunogen to generate anti-CTLA-4 antibodies. The uses of human immunoglobulin transgenic mouse technology for the development and preparation of human antibodies was first described by Abgenix (xeno mouse and Medarex (HuMab "mouse")); Lonberg et al., 1994, *Nature*, 368: 856-859; Lonberg and Huszar, 1995, *Internal Rev. Immunol.*, 13:65-93; Harding and Lonberg, 1995, *Ann. N.Y. Acad. Sci.*, 764:536-546).

[00140] HCAb mice were immunized with the human CTLA-4-ECD protein at 20 mg/per mouse every two weeks for three times, and six of them were immunized for additional five times at 44 mg/per mouse. Except for the first injection, where Stimune (Prionics) was used as an adjuvant, all boosts were done with the Ribi adjuvant (Sigma adjuvant system S6322-1VL). After immunization, single cell suspensions were isolated from the mouse bone marrow, spleen

and lymph nodes. Then mouse plasma cells were isolated using plasma cell isolation kit (Miltenyi, Cat.No.130-092-530). Briefly, total RNAs from mouse plasma cells were prepared, and reversely transcribed to cDNAs in a large pool. Human VH regions were amplified from the cDNAs using primers as follows.

Forward primer:

lib-3-23/53-S: 5'-GTGTCCAGTGTGAGGTGCAGCTG (SEQ ID NO: 193) and

lib-3-11-S: 5'-GTGTCCAGTGTCAGGTGCAGCTG (SEQ ID NO:194)

Reversed primer:

mG1hrv: 5'-GGCTTACAACCACAATCCCTGGGC (SEQ ID NO:195)

[00141] All of the amplified VH domain-containing PCR fragments were cloned into a mammalian expression vector pTT5. The obtained plasmids were transformed into bacteria (DH5 α) by electroporation. Plasmids were duplicated and purified, and then transfected into HEK 293 cells for antibody production.

[00142] The 293 cells were incubated in 293 FreeStyle medium (12338018, Thermo) for 10 days, and supernatants were screened with an ELISA assay. Recombinant human CTLA4-his proteins(Acro Bio) were diluted in PBS with a concentration of 2 μ g/mL, and 100 μ L of the diluted CTLA-4-his proteins were added per well to ELISA microplates, which were incubated overnight at 4°C to coat the plates with the recombinant proteins. The plates were then blocked with ELISA blocking solution (containing 2% BSA, 0.05% (v/v) Tween-20, pH 7.4 PBS buffer, w/v) at 37°C for two hours and then incubated with supernatants for 1 hour at 37°C. The plates were washed and incubated with horseradish peroxidase (HRP) conjugated goat anti-human IgG (H+L) antibody (A18805, Life technologies) at 37°C for one hour. 100 μ L of tetramethylbenzidine (TMB) were added, and the plates were incubated at room temperature for 15 minutes. 50 μ L of 1N HCl were added to terminate the reaction. Thirty-five positive clones showing significant staining were picked out for further tests.

[00143] The 35 clones were sequenced, and 9 clones out of the 35 were chosen with unique CDR3 sequences. Nucleic acid and amino acid sequences of these 9 anti- CTLA-4 antibodies were summarized in Table 1. The HCAb antibodies contained two heavy chains only.

Table 1. Nucleic acid and amino acid sequences of human anti-CTLA-4 antibodies

Clone ID	SEQ ID Nos					
	na-heavy chain	aa-heavy chain	aa-heavy chain variable region	aa-heavy chain CDR1	aa-heavy chain CDR2	aa-heavy chain CDR3
CL3	1	2	3	4	5	6
CL5	7	8	9	10	11	12
CL11	13	14	15	16	17	18
CL20	19	20	21	22	23	24
CL22	25	26	27	28	29	30
CL24	31	32	33	34	35	36
CL25	37	38	39	40	41	42
CL30	43	44	45	46	47	48
CL34	49	50	51	52	53	54

na: nucleic acid; aa: amino acid

Example 2 – Preparation and purification of anti-CTLA-4 antibodies

[00144] Step 1. Preparation of HEK 293F cells overexpressing hCTLA-4

[00145] The nucleotide sequence encoding human CTLA-4 (SEQ ID NO: 196, encoding an amino acid sequence of SEQ ID NO: 197) was subcloned into a pcDNA3.1 vector (Clontech) to obtain a plasmid. HEK293 and CHO-K1 cells (Invitrogen) were transiently transfected with the plasmids using PEI, and transformants were cultured in DMEM culture media containing 0.5g/mL penicillin/streptomycin and 10% (w/w) fetal bovine serum (FBS) for 2 weeks. A limited dilution into a 96-well culture plate was carried out, and the plate was incubated at 37°C with 5% (v/v) CO₂ for approximately 2 weeks. Monoclones were expanded in 6-well plates, and the expanded clones were screened by flow cytometry using commercially available anti-hCTLA-4 antibodies (R&D Systems). Clones exhibiting higher growth rates and higher fluorescence intensity as measured by FACS were further expanded and cryopreserved in liquid nitrogen.

[00146] Step 2. Determining binding activity of anti-CTLA-4 antibodies in HEK 293F cell medium by ELISA and cell based FACS binding assay

[00147] Recombinant human CTLA4-his proteins (Acro Bio) were diluted in PBS with a concentration of 2 µg/mL, and 100 µL of the diluted CTLA-4-his proteins were added per well to ELISA microplates, which were incubated overnight at 4°C to coat the plates with the

recombinant proteins. The plates were then blocked with ELISA blocking solution (containing 2% BSA, 0.05% (v/v) Tween-20, pH 7.4 PBS buffer, w/v) at 37°C for two hours and then incubated with 293F cell medium containing anti-CTLA-4 antibodies (see Example 1) for 1 hour at 37°C. The plates were washed and incubated with horseradish peroxidase (HRP) conjugated goat anti-human IgG (H+L) antibody (A18805, Life technologies) at 37°C for one hour. 100µL of tetramethylbenzidine (TMB) were added, and the plates were incubated at room temperature for 15 minutes. 50µL of 1N HCl were added to terminate the reaction, and the OD450nm was determined by an ELISA plate reader.

[00148] Meanwhile, 293-hCTLA-4 cells prepared in step 1 were cultured and used to measure the antibody binding activity. The cells were treated with enzyme-free cell dissociation solution (Versene solution, Invitrogen) and then collected. BSA was added to the cell suspension to a final concentration of 1%, and the cells were blocked for 30 minutes on ice and then washed twice with HBSS. The cells were collected after centrifugation and resuspended in FACS buffer (HBSS + 1%BSA, v/v) at 2×10^6 cells/mL. 100µL of the cell suspension was then added to each well of a 96-well plate. 100µL of 293F cell medium containing anti-CTLA-4 antibodies (see Example 1) were added to each well of the 96-well plate and incubated for 1 hour on ice. Cells were washed twice with FACS buffer, and 100µL of Alexa 488-labeled anti-human (H+L) antibody (Invitrogen) were added to the 96-well plate and incubated for 1 hour on ice. The samples were washed three times with FACS buffer, and 100µL of fixation buffer (4% paraformaldehyde v/v) were added to each well and incubated for 10 minutes. The cells were then washed twice with FACS buffer and resuspended in 100µL of FACS buffer. The mean fluorescence intensity (MFI) was determined using FACS Calibur (BD).

[00149] *Step 3 Production and purification of leading candidate antibodies*

[00150] The concentration of antibodies from the HEK293 cells were about 1-10µg/mL, and varied widely. In addition, the FBS and the components of the culture medium could interfere with the analysis. Therefore, it was necessary to perform small scale (1-5 mg) antibody production and purification.

[00151] The constructs containing nucleotide sequences encoding the anti-CTLA-4 antibodies (as listed in Table 1) were introduced into 293 cells. Supernatant containing target antibodies were harvested 6-7 days post transfection by centrifugation and filtration. Monoclonal antibodies were purified by passing them through 2mL Protein G columns (GE Healthcare). Protein G columns were first equilibrated with PBS buffer (pH7.2), and the

hybridoma culture supernatants were then applied to the equilibrated Protein G columns with a constant flow rate of 3mL/minute. The columns were each then washed with PBS buffer having a volume 3 times larger than that of the column. The anti-CTLA-4 antibodies were then eluted with elution buffer (0.1M acetate buffer, pH2.5), and the UV absorbance of the eluates were monitored using a UV detector (A280 UV absorption peak). 10% of 1.0M Tris-HCL buffer was added to the eluates to neutralize the pH, and the samples were sterile-filtered by passing them through 0.22 micron filters. Sterile-filtered purified anti-CTLA-4 antibodies were obtained.

[00152] The concentrations of purified anti-CTLA-4 antibodies were determined by UV absorbance (A280/1.4), and the purity and endotoxin level (Lonza kit) were measured. The purified anti-CTLA-4 antibodies had endotoxin concentrations less than 1.0 EU/mg.

Example 3 – Characterization of leading candidate antibodies

[00153] 293 cells were stably transfected with pTT5 plasmids containing the nucleic acid sequence encoding human CTLA-4 (SEQ ID NO: 196) to generate 293F cells stably expressing human CTLA-4 (herein referred to as 293-hCTLA-4 cells). Additional 293 cells were stably transfected with pIRES plasmids containing the nucleic acid sequence encoding full length cyno CTLA-4 (SEQ ID NO: 198) to generate 293 cells stably expressing cyno CTLA-4 (herein referred to as 293-cynoCTLA-4 cells). 293-hCTLA-4 and 293-cynoCTLA-4 cells were cultured and expanded in T-75 culture flasks to 90% confluence. The culture medium was aspirated, and the cells were washed twice with HBSS (Hanks Balanced Salt Solution, Invitrogen). The cells were treated with enzyme-free cell dissociation solution (Versene solution, Invitrogen) and collected. The cells were then washed twice with HBSS, cell counts were determined, and cells were resuspended with HBSS at 2×10^6 cells/mL. BSA was added to the cell suspension to a final concentration of 1%, and the cells were blocked for 30 minutes on ice and then washed twice with HBSS. The cells were collected after centrifugation and resuspended in FACS buffer (HBSS + 1%BSA, v/v) at 2×10^6 cells/mL. 100μL of the cell suspension were then added to each well of a 96-well plate. 100μL of purified anti-CTLA-4 antibodies from Example 2 or control antibodies were added to each well of the 96-well plate and incubated for 1 hour on ice, wherein the heavy chain and light chain of the Ipilimumab analogue had amino acid sequences of SEQ ID NO.: 199 and SEQ ID NO.: 200, respectively. Cells were washed twice with FACS buffer, and 100μL of Alexa 488-labeled anti-human (H+L) antibody (Invitrogen) were added to the 96-well plate and incubated for 1 hour on ice. The samples were washed three times with FACS buffer, and 100μL of

fixation buffer (containing 4% paraformaldehyde, v/v) were added to each well and incubated for 10 minutes. The cells were then washed twice with FACS buffer and resuspended in 100µL of FACS buffer.

[00154] The mean fluorescence intensity (MFI) was determined using FACS Calibur (BD), and the results were shown in Fig. 1 and Fig. 2. The antibodies from Example 2 had binding activity to human or cyno CTLA on 293F cells comparable to the Ipilimumab analogue.

Example 4 – Determination of anti-CTLA-4 antibodies' ability to block binding of CTLA-4 to B7.1

[00155] Cell-based receptor-ligand binding assay was performed to determine the ability of the anti-CTLA-4 antibodies to block the binding of CTLA-4 to its ligands B7.1. Recombinant B7.1^{ECD}-Fc protein (B71-H5259, Acro Bio) was biotinylated using EZ-LINK NHS-PEG12-Biotin (Thermo Scientific#21312) according to the manufacturer's instruction. Biotinylated B7.1^{ECD}-Fc protein was concentrated and free label removed by using Amicon centrifugal filter (10kDa cut off). The extracellular domain of B7.1 corresponded to amino acids Val35-Asn242 of Uniprot database protein P33681.

[00156] 293-hCTLA-4 cells prepared in Example 3 were cultured and expanded in T-75 culture flasks to 60-80% confluence. The culture medium was aspirated, and the cells were washed twice with PBS. The cells were treated with enzyme-free cell dissociation solution (Versene solution, Invitrogen) and collected. Dissociation solution was neutralized by the addition of 8mL of culture medium, and cell counts were determined. Cells were centrifuged at 300g for 5 minutes and resuspended in blocking buffer (containing 2% BSA, pH 7.4 PBS buffer, w/v) at 1×10^6 cells/mL. The cells were blocked for 15 minutes at 37°C. Meanwhile, the wells of 96-well round-bottom plates were blocked with 200µL of blocking buffer for 1 hour at 37°C. The blocking buffer was discarded, and 200µL of cells were dispensed to each well of the 96-well plates (2×10^5 cells/well). The plates were centrifuged at 500g for 5 minutes, and the supernatants were discarded. The cells were resuspended in 100µL of anti-CTLA-4 antibodies prepared in blocking buffer with varying concentrations. 100µL of biotinylated B7.1^{ECD}-Fc (60µg/mL in blocking buffer) were added to each well of 96-well plate and mixed by shaking gently. The plates were incubated at 4°C for 90 minutes and washed twice with 200µL blocking buffer. The blocking buffer was discarded, and the cells were resuspended in 100µL of streptavidin-Alexa 488 solution (Invitrogen, 1:500 in blocking buffer) and incubated at 4°C for 1 hour. The plates were washed three times with blocking buffer and added with 200µL of blocking buffer.

The mean fluorescence intensity (MFI) was determined using FACS Calibur (BD). The results, as shown in Fig. 3, demonstrated that the anti-CTLA-4 antibodies can block the binding of cell-expressed CTLA-4 to its ligand B7.1 at a level comparable to the Ipilimumab analogue.

Example 5 – Anti-CTLA-4 HCAb antibodies promoted IL-20 release

[00157] *Step 1 PBMC stimulation test*

[00158] 100µL of PBMC (containing 1×10^5 cells) were added to the wells of a 96-well plate, and 50µL of each test antibody having various concentrations was then added to the 96-well plate and incubated for 15 minutes at room temperature. 50µL of 100ng/ml SEB were added to each well and cultured at 37°C, 5% CO₂ for 72 hours. The supernatants were collected and stored at -20°C until analysis.

[00159] *Step 2 Detection of interleukin IL-2 secretion by ELISA*

[00160] Quantification of the levels of IL-2 in culture supernatant was carried out using Human IL-2 Quantikine ELISA Kit (DY202, R&D Systems) following the manufacturer-provided operating instructions. Briefly, the anti-IL-2 polyclonal antibodies were coated onto the ELISA microplates, and 100µL of the culture supernatant as well as the standard were added to each well and incubated at room temperature for 2 hours. The plates were washed 4 times with wash buffer, followed by the addition of HRP-conjugated anti-human IL-2 antibodies, and incubated at room temperature for 2 hours. After washes, a chromogenic substrate (5120-0077, SeraCare) was added and incubated in the dark at room temperature for 30 minutes, and the reaction was terminated by the addition of a stop solution (E661006-0200, BBI Life sciences).

[00161] The absorbance at 450nm was determined using an ELISA plate reader, and the results, as shown in Fig. 4A and 4B, demonstrated that some of the anti-CTLA-4 antibodies can increase IL-2 secretion at low concentrations compared to human IgG (AB170090, Crown Bio) or the Ipilimumab analogue.

Example 6 – Anti-CTLA-4 HCAb antibodies bound to human CTLA-4 but not to murine CTLA-4 in ELISA assay

[00162] Recombinant human or mouse CTLA4-his protein (CT4-H5229 for human protein, CT4-M52H5 for mouse protein, Acro Bio) was diluted in PBS to a concentration of 2 µg/mL, and 100 µL of the diluted CTLA-4-his protein was added per well to ELISA microplates, which were incubated overnight at 4°C to coat the plates with the recombinant proteins. The plates were then blocked with ELISA blocking solution (containing 2% BSA, 0.05% (v/v)

Tween-20, pH7.4 PBS buffer, w/v) at 37°C for two hours and then incubated with anti-CTLA-4 antibodies with various concentrations for 1 hour at 37°C. The plates were washed and incubated with horseradish peroxidase (HRP) conjugated goat anti-human IgG (H+L) antibody (A18805, Life technologies) at 37°C for one hour. 100μL of tetramethylbenzidine (TMB) were added, and the plates were incubated at room temperature for 15 minutes. 50μL of 1N HCl were added to terminate the reaction, and the OD450nm was determined by an ELISA plate reader.

[00163] Data was shown in Fig. 5A and Fig. 5B, suggesting all clones bound to human CTLA-4 but almost did not bind to murine CTLA-4.

Example 7 - Anti-CTLA-4 antibody mutants promoted IL-20 release

[00164] A T cell stimulation assay was performed on some antibodies mentioned above and their mutants to examine the effect of these antibodies on T cell stimulation by blocking the binding of CTLA-4 to its ligands B7.1 and B7.2.

[00165] The antibody mutants were prepared by applying S239D and I332E mutations in the Fc constant domain of HCAb clone 5, clone 11, clone 22, clone25 and clone 30 by PCR. The mutated antibodies were expressed in HEK293 cells and purified as described in Example 2. The nucleic acid and amino acid sequences of the antibody mutants were determined using standard molecular biology methods and are summarized in Table 2.

Table 2. Nucleic acid and amino acid sequences of anti-CTLA-4 antibody mutants

Clone ID	SEQ ID Nos					
	na-heavy chain	aa-heavy chain	aa-heavy chain variable region	aa-heavy chain CDR1	aa-heavy chain CDR2	aa-heavy chain CDR3
CL5-eA	139	140	141	142	143	144
CL11-eA	115	116	117	118	119	120
CL22-eA	121	122	123	124	125	126
CL25-eA	127	128	129	130	131	132
CL30-eA	133	134	135	136	137	138

na: nucleic acid; aa: amino acid

[00166] PBMC stimulation test and Detection of interleukin IL-2 secretion by ELISA were performed as in Example 5.

[00167] The results in Fig. 6A and 6B demonstrated that the anti-CTLA-4 antibody mutants promoted more IL-2 secretion when compared to human IgG1 isotype control and their parental clones.

Example 8 – Anti-CTLA-4 antibody variants with PTM removed

[00168] The amino acid sequences of 7 HCAb clones (CL22, CL25, CL5, CL3, CL11, CL30, CL24) were aligned to gene IGHV3-53*01 and displayed in Table 3. The differences to germline gene and PTM sites were highlighted. The sequences were numbered in Chothia numbering scheme.

[00169] The germline gene IGHV3-53 does not have N-glycosylation motif natively, and those N-glycosylation motifs carried in the 7 HCAb clones were formed by somatic mutations. Therefore, one approach to remove this motif was to substitute it by the corresponding counterpart residues in germline, for example, to replace the NVS motif in CDR1 of CL11 by TVS of germline. Alternative approach was also explored by combining PTM removal with “germlining” based on the concept of CDR-grafting used for humanization. In the second approach, CDRs of each HCAb were grafted to germline IGHV3-53 frameworks and key framework residues from parental HCAb were also retained. For some antibodies, the amino acid mutations located in special residues, which may affect the binding activity between CTLA-4 and antibodies, were restored. The nucleic acid and amino acid sequences of human anti-CTLA-4 HCAb antibody variants with PTM removal were listed in Table 4.

Table 3. Differences of HCAb amino acid sequence compared to the germline sequence

Clone#	Germline		Critical PTM						
	IGHV	Ident%	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
CL22	IGHV3-53*02	89.7%					NxS/T		
CL25	IGHV3-53*02	96.9%					NxS/T		
CL5	IGHV3-53*02	86.6%		NxS/T					
CL3	IGHV3-53*02	88.7%		NxS/T			NxS/T		
CL11	IGHV3-53*02	86.6%		NxS/T					
CL30	IGHV3-53*02	87.6%		NxS/T					
CL24	IGHV3-53*02	84.5%		NxS/T			NxS/T		
CL20	IGHV3-74*03	98.0%				NxS/T, NS, DG			
CL34	IGHV3-74*03	88.8%				NxS/T, NS, DG		DG	

Table 4. Nucleic acid and amino acid sequences of anti-CTLA-4 antibody variants with PTM removal

Clone ID	SEQ ID Nos					
	na-heavy chain	aa-heavy chain	aa-heavy chain variable region	aa-heavy chain CDR1	aa-heavy chain CDR2	aa-heavy chain CDR3
CL5-dPTM	103	104	105	106	107	108
CL5-dPTM'	109	110	111	112	113	114
CL5-eA-dPTM	145	146	147	148	149	150
CL5-eA-dPTM'	151	152	153	154	155	156
CL5'-dPTM'	169	170	171	172	173	174
CL5'-eA-dPTM'	181	182	183	184	185	186
CL11-dPTM	55	56	57	58	59	60
CL11-dPTM'	61	62	63	64	65	66
CL11'-dPTM'	163	164	165	166	167	168
CL11'-eA-dPTM'	175	176	177	178	179	180
CL20'-eA	187	188	189	190	191	192
CL22-dPTM	67	68	69	70	71	72
CL22-dPTM'	73	74	75	76	77	78
CL25-dPTM	79	80	81	82	83	84
CL25-dPTM'	85	86	87	88	89	90
CL30-dPTM	91	92	93	94	95	96
CL30-dPTM'	97	98	99	100	101	102

na: nucleic acid; aa: amino acid

Example 9 - Cell-based binding activity of anti-CTLA-4 antibody variants with PTM removal

[00170] Cell-based binding assay was performed to determine the binding ability of the anti-CTLA-4 antibody variants with PTM removal to human CTLA-4. The assay procedure was similar to that described in Example 3. Briefly speaking, 293F-hCTLA-4 cells were harvested using enzyme-free cell dissociation solution (Versene solution, Invitrogen) and then neutralized by culture medium, and cell counts were determined. Cells were centrifuged and blocked in blocking buffer (containing 2% BSA, pH 7.4 PBS buffer, w/v) at 1×10^6 cells/mL for 15 minutes at 37°C. 200μL of cells were dispensed to each well of the 96-well plates (2 x

10⁵ cells/well). The plates were centrifuged and the supernatants were discarded. The cells were resuspended in 100µL of anti-CTLA-4 antibodies prepared in blocking buffer. The plates were incubated at 4°C for 90 minutes and washed twice. After the blocking buffer was discarded, the cells were resuspended in 100µL of Alexa 488-labeled anti-human (H+L) antibody (1:500, Invitrogen) and incubated at 4°C for 1 hour. The cells were washed and resuspended in 200µL of blocking buffer. The mean fluorescence intensity (MFI) was determined using FACS Calibur (BD).

[00171] The results, as shown in Fig. 7A and 7B, demonstrated that the anti-CTLA-4 antibody variants with the PTM removal bound cell-expressed human CTLA-4.

Example 10 - Blocking activity of anti-CTLA-4 antibody variants with PTM removal on CTLA-4 - B7.1 and B7.2 interaction

[00172] Cell-based receptor ligand binding assay was performed as described in Example 4 to determine the ability of the anti-CTLA-4 antibody variants with PTM removal to block the binding of CTLA-4 to its ligands B7.1.

[00173] The results, as shown in Fig. 8A, 8B and 8C, demonstrated that the anti-CTLA-4 antibody variants with PTM removal blocked the binding of cell-expressed CTLA-4 to its ligand B7.1 and B7.2 at a level comparable to the Ipilimumab analogue.

Example 11 - Anti-CTLA-4 antibody variants with PTM removal promoted IL-20 release

[00174] PBMC stimulation and IL-20 level quantification were carried out as described in Example 5.

[00175] The results, as shown in Fig. 9, demonstrated that the anti-CTLA-4 antibody variants with PTM removal can still promote IL-2 secretion. The anti-CTLA-4 antibodies with S239D and I332E mutations and PTM removal induced increased IL-2 secretion than those with S239D and I332E mutations but no PTM removal.

Example 12 – Binding affinity and dissociation constant of anti-CTLA-4 antibody Variants with PTM removal

[00176] Dissociation constants were determined by Biacore T200 (GE Healthcare), following the specifications of the instrument provided by the manufacturer. Briefly, 1 µg/mL diluted anti-CTLA-4 antibodies in 10 mM NaOAc (pH 5.0, sigma) were immobilized on flow cell of a Series S CM5 sensor chip. Remaining active ester groups were blocked with 1 M ethanolamine (pH 8.5). With HBS-EP+ as the running buffer, recombinant human CTLA-4-his (CT4-H5229, AcroBio) and cynoCTLA-4-his proteins (CT4-C5227, AcroBio) with five serial diluted concentrations were injected over flow cells at 30 µL/min with the association

time of 180s. Buffer flow was maintained for dissociation for 600s. The KD value for each interaction between antibody and antigen was evaluated using Biacore T200 evaluation software 1.0 and the fitting model of 1:1 binding.

[00177] The results were shown in Fig. 10 and Table 5. The binding affinity of two clones were similar to that of the Ipilimumab analogue.

Table 5. Binding kinetics and affinities of human anti-CTLA-4 Abs to human CTLA-4^{ECD}-his protein and cynoCTLA-4^{ECD}-his protein as determined by Biacore T200

Clone ID	Proteins	KD (M)	ka (1/Ms)	kd (1/s)
CL5'-dPTM'	Human CTLA-4 ^{ECD} -his	4.28E-11	5.35E+06	2.29E-04
CL5'-dPTM'	cyno CTLA-4 ^{ECD} -his	5.91E-11	5.21E+06	3.08E-04
CL5'-eA-dPTM'	Human CTLA-4 ^{ECD} -his	1.40E-11	5.40E+06	7.58E-05
CL5'-eA-dPTM'	cyno CTLA-4 ^{ECD} -his	2.43E-11	4.55E+06	1.10E-04
Ipilimumab analogue	Human CTLA-4 ^{ECD} -his	7.32E-11	1.23E+06	8.98E-05
Ipilimumab analogue	cyno CTLA-4 ^{ECD} -his	3.47E-10	3.73E+06	1.29E-03

Example 13 - In vitro ADCC Function Analysis

[00178] To confirm the presumed NK dependent cytotoxic activity of human anti-CTLA-4 antibodies, antibody-dependent cell-mediated cytotoxicity (ADCC) assay was performed both on CTLA-4-expressing CHO-K1 cells and CTLA-4 expressing in vitro stimulated Treg cells.

[00179] CTLA-4-expressing CHO-K1 cells as described in Example 2, step 1, were adjusted to a concentration of 2×10^5 cells/mL with ADCC medium (containing RPMI 1640 without phenol red, 10% FBS and 1% penicillin/streptomycin). 50 μ L of cell suspensions (1×10^4 viable cells) were added to each well of a v-bottom 96-well plate. The test antibodies were serially diluted in ADCC medium (without phenol red) and 50 μ L of each resulting solution was added to the wells in triplicate. The final antibody concentrations were: 0.087pM,

0.44pM, 2.2pM, 10.9pM, 54pM, 272pM, 1.36nM, and 6.8nM. The plate was incubated at 37°C for 30 minutes. NK92 cells stably transfected with FcγRIII158V were adjusted with ADCC medium (without phenol red) so that by adding 100μL of NK92 cells stably transfected with FcγRIII158V to the target cells, the ratio of effector to target cells was 5:1. The plate was then incubated at 37°C for 6 hours. After 6 hours of incubation, the plate was centrifuged and 50μL of each supernatant was transferred into a new plate. The supernatant was incubated with 50 μL LDH detection buffer at room temperature for 30min and measured for the absorbance at 490nm. For maximum cell lysis control, 50μL of CTLA-4-expressing CHO-K1 cells, 50μL of ADCC medium and 100μL of 1% triton-X100 buffer were added for LDH detection. For minimum cell lysis control, 50μL of CTLA-4-expressing CHO-K1 cells and 150μL of ADCC medium were added for LDH detection. The absorbance at 492/650nm was measured. The percentage of Cell lysis was calculated as $100 \times (\text{absorbance of samples} - \text{absorbance of background}) / (\text{absorbance of maximum release} - \text{absorbance of minimum release})$. All the percentage of cell lysis values were calculated using GraphPad Prism 5.0.

[00180] The results, as shown in Fig. 11, showed that clone CL5-dPTM' antibody induced ADCC effect on CTLA-4-expressing CHO-K1 cells, and clone CL5-eA-dTPM' with additional S239D and I332E mutation showed a higher ADCC activity than Ipilimumab analogue.

[00181] CTLA-4-expressing in vitro stimulated Treg cells were derived from in vitro isolated naïve CD4⁺ T cells. First, naïve CD4⁺ T cells were isolated from primary PBMC according to the manufacturers instruction (Miltenyi, 130-094-131). Then naïve CD4⁺ T cells were activated by incubated with Dynabeads human T-activator CD3/CD28 (1:1) (Thermo, 11131D), 10ng/ml IL-2 (PeproTech, 200-02-B) and 20ng/ml TGF-β1 (PeproTech, 100-21) for three days. On the day of ADCC killing experiment, stimulated Treg cells were adjusted to a concentration of 1×10^6 cells/mL with ADCC medium (containing RPMI 1640 without phenol red, 10% FBS and 1% penicillin/streptomycin). 1×10^6 Cells were stained by 5ul calcein AM (Therom, C34851, stock prepared as 50ug per 50ul DMSO) for 1h at 37°C. Treg cells were washed three times by ADCC medium. 50μL of Treg cell suspensions (5×10^3 viable cells) were added to each well of a v-bottom 96-well plate. The test antibodies were serially diluted in ADCC medium (without phenol red) and 50μL of each resulting solution was added to the wells in triplicate. The final antibody concentrations were: 1pM, 10pM, 100pM, 1nM, 10nM, and 100nM. The plate was incubated at room temperature for 30 minutes. Fresh PBMC (Miaotong) were adjusted 5×10^6 cells/mL with ADCC medium (without phenol red). By adding 50μL of fresh PBMC to the stained Treg cells, the ratio of

effector to target cells was 50:1. The plate was then incubated at 37°C for 2 hours. After 2 hours of incubation, the plate was centrifuged and 100µL of each supernatant was transferred into a new plate. The supernatant was measured by Enspire instrument. For maximum cell lysis control, 50µL of calcein AM stained Treg cells, 50µL of ADCC medium and 100µL of 1% triton-X100 buffer were added for released calcein AM detection. For minimum cell lysis control, 50µL of calcein AM stained cells and 150µL of ADCC medium were added for released calcein AM detection. The absorbance at 520/650nm was measured. The percentage of specific killing is calculated as $100 \times (\text{absorbance of samples} - \text{absorbance of background}) / (\text{absorbance of maximum release} - \text{absorbance of minimum release})$. All the percentage of specific killing values were calculated using GraphPad Prism 5.0.

[00182] The results, as shown in Fig. 11B, showed that the human anti-CTLA-4 antibodies induced ADCC effect on CTLA-4-expressing Treg cells, and the antibody with S239D and I332E mutation showed a higher ADCC activity than the unmutated one.

Example 14 - Pharmacokinetic Study of anti-CTLA-4 antibodies

[00183] *Step 1 Single dose anti-CTLA-4 antibody treatment in mice*

[00184] Male C57BL/6 mice were injected with 3 mg/kg anti-CTLA-4 antibodies via tail vein to measure the serum concentration of anti-CTLA-4 antibodies. The animals were restrained manually and approximately 100 µL blood/time point was collected via retro-orbital puncture, the time point being Pre-dose, 0.167, 1, 4, 8, 24 hr, 2, 4, 7, 14 days post antibody injection. The terminal collection was via cardiac puncture. Blood samples were stayed at room temperature and centrifuged at 2,000Xg for 5 min at 4°C to obtain serum samples.

[00185] *Step 2 Serum concentration of anti-CTLA-4 antibodies as measured by ELISA*

[00186] All serum samples were diluted at 20 folds in Assay Diluent first. Additional dilution was made in 5% mouse serum (PBS, v/v). Recombinant human CTLA4-his protein (Acro Bio) was diluted in PBS to a concentration of 0.5 µg/mL, and 50 µL of the diluted CTLA-4-his protein sample were added per well to ELISA microplates, which were incubated overnight at 4°C to coat the plates with the recombinant proteins. The plates were then blocked with ELISA blocking solution (containing 2% BSA, 0.05% (v/v) Tween-20, pH7.4 PBS buffer, w/v) at 37°C for two hours. The blocking buffer was aspirated away and plates were incubated with diluted serum samples for 1 hour at 37°C. The plates were washed three times with wash buffer (PBS + 0.01% (v/v) Tween 20) and incubated with horseradish peroxidase (HRP) conjugated goat anti-human IgG(Fc) antibody (A0170, Sigma) at 37°C for one hour. 100µL of tetramethylbenzidine (TMB) were added, and the plates were incubated at room temperature

for 15 minutes. 100 μ L of 0.1N HCl was added to each well to stop the reaction. The absorbance at 450nm was measured with an ELISA plate reader (SpectraMax M2).

[00187] The corresponding serum concentration of anti-CTLA-4 antibodies were shown in Fig. 12, with detailed data listed in Table 6.

Table 6. Mean serum concentration of antibodies after an IV dose at 3 mg/kg in mice

Individual and mean serum concentration-time data of Ipilimumab analogue after an IV dose of 3 mg/kg in male C57BL/6 mice								
Dose (mg/kg)	Dose route	Sampling time (Day)	Concentration (µg/mL)			Mean (µg/mL)	SD	CV(%)
			Individual (#1~#12)					
3	IV	0	BQL	BQL	BQL	BQL	NA	NA
		0.00694	67.3	69.8	60.2	65.8	4.99	7.59
		0.0417	52.8	58.4	54.0	55.1	2.92	5.29
		0.167	42.1	55.6	52.7	50.2	7.09	14.14
		0.333	37.3	42.0	38.1	39.2	2.54	6.50
		1	21.7	26.0	24.0	23.9	2.163	9.04
		2	19.8	22.8	17.6	20.1	2.59	12.9
		4	17.7	17.5	17.0	17.4	0.389	2.24
		7	18.3	13.3	13.4	15.03	2.829	18.83
		14	10.8	9.8	10.3	10.30	0.531	5.15
PK parameters		Unit	Estimated Value					
CL		mL/day/kg	7.12					
Vss		mL/kg	127					
V1		mL/kg	47.2					
Alpha t _{1/2}		day	0.271					
Beta t _{1/2}		day	12.8					
AUC		day*µg/mL	421					
MRT		day	17.8					

Individual and mean serum concentration-time data of CL5 after an IV dose at 3 mg/kg in								
Dose (mg/kg)	Dose route	Sampling time (Day)	Concentration (µg/mL)			Mean (µg/mL)	SD	CV(%)
			Individual					
3	IV	0	BQL	BQL	BQL	BQL	NA	NA
		0.00694	44.3	49.7	50.9	48.3	3.54	7.33
		0.0417	37.5	39.6	33.4	36.8	3.20	8.67
		0.167	26.0	26.0	22.5	24.8	2.00	8.07
		0.333	22.1	18.7	21.0	20.6	1.70	8.27
		1	12.8	14.1	14.0	13.6	0.697	5.12
		2	12.7	13.6	11.0	12.4	1.32	10.6
		4	11.0	10.5	9.62	10.4	0.690	6.66
		7	9.94	9.09	10.2	9.75	0.594	6.09
		14	4.99	5.42	5.89	5.43	0.449	8.26
PK parameters		Unit	Estimated Values					
CL		mL/day/kg	13.9					
Vss		mL/kg	194					
V ₁		mL/kg	64.0					
Alpha t _{1/2}		day	0.113					
Beta t _{1/2}		day	9.92					
AUC		day*µg/mL	216					
MRT		day	14.0					

[00188] Additionally, the tumor to serum ratio of anti-CTLA4 HCAb concentration was measured in C57BL/6 mice bearing MC38 tumors. Mice were injected with 3 mg/kg anti-CTLA-4 antibodies via tail vein to measure the serum and tumor resident concentration of anti-CTLA-4 antibodies. The animals were restrained manually and approximately 100 µL of blood/time point was collected via retro-orbital puncture, the time point being 8 and 24 hr post injection. The terminal collection was via cardiac puncture. Blood samples were kept at room temperature and centrifuged at 2,000Xg for 5 min at 4°C to obtain serum samples. The serum and tumor concentrations of anti-CTLA-4 antibodies were also tested by ELISA as in step2.

[00189] The tumor to serum ratio of anti-CTLA4 HCAb concentration in CL5-eA-dPTM' group was about one-fold higher than that in the Ipi analogue group, as shown in Fig. 13, which may be due to the unique feature of heavy-chain-only HCAb antibodies. The higher tumor to serum distribution may lead to a higher tumor tissue penetration of HCAb antibodies.

Example 15 - Mice bearing MC38 tumor better survived with human anti-CTLA-4 antibodies

[00190] Cryopreserved murine colon carcinoma MC-38 cell line was recovered and cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin at 37°C to get enough cells for tumor implantation. The cultured MC-38 cells were harvested, re-suspended in PBS at a density of 1x10⁷ cells/ml with viability >90% and subcutaneously implanted into the right flank of 120 hCTLA-4 knock in mice (GempharmaTech). Five days

after tumor inoculation, 81 mice with tumor size ranging from 26-64 mm³ (average tumor size was 40 mm³) were selected and assigned into 9 groups using stratified randomization with 9 mice per group based upon their tumor volumes. The treatments were started from the day of randomization (defined as D0). Group 1 was treated with hlgG1 i.p. at 10mpk on D0, D3, D6, D10, D13, D16; Group 2 was treated with CL20'-eA (sequences in Table 7) i.p. at 5.4mpk on D0, D3, D6, D10, D13, D16; Group 3 was treated with Ipilimumab analogue i.p. at 10mpk on D0, D3, D6, D10, D13, D16; Group 4 was treated with Ipilimumab analogue i.p. at 1mpk on D0, D3, D6, D10, D13, D16; Group 5 was treated with CL5'-dPTM' i.p. at 5.4mpk on D0, D3, D6, D10, D13, D16; Group 6 was treated with CL5'-dPTM' i.p. at 0.54mpk on D0, D3, D6, D10, D13, D16; Group 7 was treated with CL5'-eA-dPTM' i.p. at 5.4mpk on D0, D3, D6, D10, D13, D16; Group 8 was treated with CL5'-eA-dPTM' i.p. at 1.5mpk on D0, D3, D6, D10, D13, D16; and Group 9 was treated with CL5'-eA-dPTM' i.p. at 0.54mpk on D0, D3, D6, D10, D13, D16.

Table 7. Nucleic acid and amino acid sequences of clone CL20'-eA

Clone ID	SEQ ID Nos					
	na-heavy chain	aa-heavy chain	aa-heavy chain variable region	aa-heavy chain CDR1	aa-heavy chain CDR2	aa-heavy chain CDR3
CL20'-eA	157	158	159	160	161	162

na: nucleic acid; aa: amino acid

[00191] The tumor sizes were measured three times per week during the treatment. When an individual animal reached to the termination endpoint (TV>2000 mm³), it was euthanized. The time from treatment initiation to the termination was deemed as its survival time. Survival curve was plotted by Kaplan-Meier method. Median survival time (MST) was calculated for each group. Increase of life span (ILS) was calculated according to the following formula:

$$ILS (\%) = (MST_{Treatment} - MST_{Vehicle}) / MST_{Vehicle} \times 100\%$$

[00192] ILS(%) > 25% will be considered as biologically significant survival benefit according to National Cancer Institute Criteria.

[00193] Relative change of body weight (RCBW) of each mouse were calculated according to the following formula: $RCBW (\%) = (BW_i - BW_0) / BW_0 \times 100\%$, wherein BW_i referred to average body weight on Day i, and BW₀ referred to average bodyweight on Day 0.

[00194] Tumor volumes (TV) were calculated based on the following formula: tumor volume = (length × width²)/2.

[00195] Tumor growth inhibition rate (TGI%) of each dosing group was calculated according to the following formula: $TGI\% = [1 - TV_i / TV_{vi}] * 100\%$, wherein TV_i referred to average tumor volume of a dosing group on Day i , and TV_{vi} referred to average tumor volume of the vehicle group on Day i .

[00196] Mean and standard error of the mean (SEM) of mice body weight, RCBW and tumor volume of each group were calculated using Microsoft Excel 2007. Figures of body weight, relative change of body weight, tumor growth curve, and tumor growth inhibition were plotted using GraphPad Prism 5. Tumor growth between different groups was analyzed using Two-way RM ANOVA. Kaplan-Meier survival curves were analyzed using Log-Rank test. A P-value of <0.05 was considered statistically significant.

[00197] The entire study was terminated on D65. Individual tumor growth curves of each group were shown in Fig. 14A. Animal time-to-end point Kaplan-Meier survival curves were shown in Fig. 14B. All the treatments were tolerated without any adverse effect, as observed in Fig. 14C. In most of these groups, mice were all sacrificed when the tumor volume reached 2000mm^3 . In Group 7, 3 out of 9 mice treated with CL5'-eA-dPTM' at 5.4mpk survived with tumor free on D65. In Group 9, 2 out of 9 mice administered with CL5'-eA-dPTM' at 0.54mpk survived with tumor free on D65.

[00198] MST was calculated for each group and shown in Table 8. The MST of vehicle group hIgG1 at 5.4mpk and CL20'-eA at 5.4mpk were both 14 days. The MSTs of the treatment groups with ipilimumab analogue at 10mpk and 1mpk, CL5'-dPTM' at 5.4mpk and 0.54mpk, CL5'-eA-dPTM' at 5.4mpk, 1.5mpk and 0.54mpk were 14, 23, 19, 21, 14, 40, 21 and 28 days, respectively. Also can be seen in Table 8, ILS in the treatment groups were 64.3%, 35.7%, 50%, 0%, 185.7%, 50% and 100% compared with vehicle treatment group hIgG1. Ipilimumab analogue at 10mpk, CL5'-dPTM' at 5.4mpk, CL5'-eA-dPTM' at 5.4mpk, 1.5mpk and 0.54mpk significantly increased median survival time. Mice treated with CL5'-eA-dPTM' at 5.4mpk appeared a better ILS than those with the Ipilimumab analogue at 10mpk.

Table 8. Survival Analysis

Group		MST	ILS(%)	p value
G1	hlgG1, 10mpk	14	-	--
G2	CL20'-eA, 5.4mpk	14	0.0	ns
G3	Ipilimumab analogue, 10mpk	23	64.3	<0.01
G4	Ipilimumab analogue, 1mpk	19	35.7	ns
G5	CL5'-dPTM', 5.4mpk	21	50.0	<0.05
G6	CL5'-dPTM', 0.54mpk	14	0.0	ns
G7	CL5'-eA-dPTM', 5.4mpk	40	185.7	<0.05
G8	CL5'-eA-dPTM', 1.5mpk	21	50.0	<0.05
G9	CL5'-eA-dPTM', 0.54mpk	28	100.0	<0.05

Note : $ILS = (MST_{G1} - MST_{Gx}) / MST_{G1} * 100\%$; P value are all groups compared to G1 group.

Example 16 - Inhibition of MC38 tumor growth in hCTLA-4 knock in mice by anti- CTLA-4 HCAb

[00199] Cryopreserved murine colon carcinoma MC-38 cell line was recovered and cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin at 37°C to get enough cells for tumor implantation. The cultured MC-38 cells were harvested, re-suspended in PBS at a density of 5×10^6 cells/ml with viability >90% and subcutaneously implanted into the right flank of 60 hCTLA-4 knock in mice. Seven days after tumor inoculation, 30 tumor -bearing mice with mean tumor size of 102 mm³ were selected and randomized into 5 groups (n=6) based on their tumor sizes. The treatments were started at the day of the randomization (defined as D0). Enrolled mice were treated with hIgG1(0.5 mpk), the Ipilimumab analogue (0.5 and 0.2 mpk) and CL5'-eA-dPTM' (0.27 and 0.1 mpk) respectively, by intraperitoneally (i.p.) on D0, D6, D9, D13, D16 and D19. Mice were monitored daily and body weights were recorded on the work days. The tumor sizes were measured twice per week during the treatment.

[00200] Relative change of body weight (RCBW) , tumor volumes (TV), and tumor growth inhibition rate (TGI%) were calculated as in Example 16. Mean, standard error of the mean (SEM) of mice body weight, RCBW and tumor volume of each group were calculated using Microsoft Excel 2007. Figures of body weight, relative change of body weight, tumor growth curve, and tumor growth inhibition were plotted using GraphPad Prism 5. Tumor growth between different groups was analyzed using Two-way RM ANOVA. Kaplan-Meier survival

curves were analyzed using Log-Rank test. A P-value of <0.05 was considered statistically significant.

[00201] Tumor growth curves were shown in Fig. 15A. All the treatments were tolerated without any adverse effect, as observed in Fig. 15B. Mice in groups of CL5'-eA-dPTM' at 0.1 mpk and 0.27 mpk, ipilimumab analogue at 0.5mpk showed significant tumor growth inhibition from D20 to D30 compared with mice in group of hIgG1 at 0.2mpk. While, ipilimumab analogue treatment at 0.2mg/kg did not show significant tumor growth inhibition compared to hIgG1 at 0.2mpk. CL5'-eA-dPTM' promoted a better tumor growth inhibition than the Ipilimumab analogue at the same dose.

[00202] While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

Claims:

1. An isolated monoclonal antibody, comprising a CD152-binding domain, wherein the CD152-binding domain comprises an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, wherein the CDR1, CDR2, and CDR3 comprise amino acid sequences having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to

- (1) SEQ ID NOs: 4, 5 and 6, respectively;
- (2) SEQ ID NOs: 10, 11 and 12, respectively;
- (3) SEQ ID NOs: 16, 17 and 18, respectively;
- (4) SEQ ID NOs: 22, 23 and 24, respectively;
- (5) SEQ ID NOs: 28, 29 and 30, respectively;
- (6) SEQ ID NOs: 34, 35 and 36, respectively;
- (7) SEQ ID NOs: 40, 41 and 42, respectively;
- (8) SEQ ID NOs: 46, 47 and 48, respectively;
- (9) SEQ ID NOs: 52, 53 and 54, respectively;
- (10) SEQ ID NOs: 58, 59 and 60, respectively;
- (11) SEQ ID NOs: 64, 65 and 66, respectively;
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- (13) SEQ ID NOs: 76, 77 and 78, respectively;
- (14) SEQ ID NOs: 82, 83 and 84, respectively;
- (15) SEQ ID NOs: 88, 89 and 90, respectively;
- (16) SEQ ID NOs: 94, 95 and 96, respectively;
- (17) SEQ ID NOs: 100, 101 and 102, respectively;
- (18) SEQ ID NOs: 106, 107 and 108, respectively;
- (19) SEQ ID NOs: 112, 113 and 114, respectively;
- (20) SEQ ID NOs: 118, 119 and 120, respectively;
- (21) SEQ ID NOs: 124, 125 and 126, respectively;
- (22) SEQ ID NOs: 130, 131 and 132, respectively;
- (23) SEQ ID NOs: 136, 137 and 138, respectively;
- (24) SEQ ID NOs: 142, 143 and 144, respectively;
- (25) SEQ ID NOs: 148, 149 and 150, respectively;

- (26) SEQ ID NOs: 154, 155 and 156, respectively;
 - (27) SEQ ID NOs: 160, 161 and 162, respectively;
 - (28) SEQ ID NOs: 166, 167 and 168, respectively;
 - (29) SEQ ID NOs: 172, 173 and 174, respectively;
 - (30) SEQ ID NOs: 178, 179 and 180, respectively;
 - (31) SEQ ID NOs: 184, 185 and 186, respectively; or
 - (32) SEQ ID NOs: 190, 191 and 192, respectively.
2. The isolated monoclonal antibody of claim 0, wherein the antibody is a heavy-chain-only antibody.
3. The isolated monoclonal antibody of claim 1 or 2, wherein the antibody comprises two immunoglobulin heavy chains.
4. The isolated monoclonal antibody of any one of claims 0 to 3, wherein the antibody consists of two immunoglobulin heavy chains.
5. The isolated monoclonal antibody of claim 3 or 4, wherein at least one of the two immunoglobulin heavy chains comprises an amino acid sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NOs: 2, 8, 14, 20, 26, 32, 38, 44, 50, 56, 62, 68, 74, 80, 86, 92, 98, 104, 110, 116, 122, 128, 134, 140, 146, 152, 158, 164, 170, 176, 182, or 188.
6. The isolated monoclonal antibody of any one of claims 0 to 5, wherein the immunoglobulin heavy chain variable region comprises an amino acid sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NOs: 3, 9, 15, 21, 27, 33, 39, 45, 51, 57, 63, 69, 75, 81, 87, 93, 99, 105, 111, 117, 123, 129, 135, 141, 147, 153, 159, 165, 171, 177, 183, or 189.
7. The isolated monoclonal antibody of any one of claims 0 to 6, which exhibits one or a combination of the following properties: (a) binding to human CD152; (b) binds specifically to monkey CD152; (c) not binding to mouse CD152; (d) blocking the binding of CD152 to CD80, CD86, or both; (e) promoting secretion of IL-2 by immune cells; (f) inducing T-cell activation; (g) stimulating an anti-tumor immune response by immune cells.

8. The isolated monoclonal antibody of any one of claims 0 to 7, which exhibits one or a combination of the following properties: (a) binding to human CD152 with an affinity higher than that of an ipilimumab analogue; (b) binding to human CD152 with the affinity that is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold higher than that of an ipilimumab analogue; (c) dissociating from human CD152 with a K_d of 1.0×10^{-9} M or less; (d) dissociating from human CD152 with the K_d that is lower than that of an ipilimumab analogue; (e) dissociating from human CD152 with the K_d that is at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, or at least 100-fold lower than that of an ipilimumab analogue.
9. The isolated monoclonal antibody of any one of claims 1 to 8, wherein the antibody dissociates from human CD152 with a K_d of 6.0×10^{-11} M or less.
10. The isolated monoclonal antibody of any one of claims 1 to 9, wherein the antibody is a human, humanized, or chimeric antibody.
11. A pharmaceutical composition, comprising the isolated monoclonal antibody of claim 0, and a pharmaceutically acceptable excipient.
12. The pharmaceutical composition of claim 11, wherein the pharmaceutically acceptable excipient is selected from the group consisting of carriers, surface active agents, thickening or emulsifying agents, solid binders, dispersion or suspension aids, solubilizers, colorants, flavoring agents, coatings, disintegrating agents, lubricants, sweeteners, preservatives, isotonic agents, and a combination thereof.
13. The pharmaceutical composition of claim 11 or 12, further comprising a second antibody, wherein the second antibody is an immunostimulatory antibody or costimulatory antibody.
14. The pharmaceutical composition of claim 13, wherein the immunostimulatory antibody is selected from the group consisting of an anti-PD-1 antibody, an anti-PD-L1 antibody, an anti-LAG-3 antibody, an anti-TIM 3 antibody, an anti-STAT3 antibody, and an anti-ROR1 antibody.
15. The pharmaceutical composition of claim 13 or 14, wherein the costimulatory antibody is an anti-CD137 antibody or an anti-GITR antibody.

16. A nucleic acid molecule encoding the isolated antibody of any one of claims 0 to **Error!**
Reference source not found.

17. The nucleic acid molecule of claim 16, wherein the nucleic acid molecule comprises a nucleotide sequence having at least 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 1, 7, 13, 19, 25, 31, 37, 43, 49, 55, 61, 67, 73, 79, 85, 91, 97, 103, 109, 115, 121, 127, 133, 139, 145, 151, 163, 169, 175, 181, or 187.

18. An expression vector comprising the nucleic acid molecule of claim 06 or 17, wherein the nucleic acid molecule is operatively linked to regulatory sequences suitable for expression of the nucleic acid segment in a host cell.

19. A host cell comprising the expression vector of claim 18.

16. A method of inducing an antibody-dependent cell-mediated cytotoxicity (ADCC) against a cell expressing a tumor associated antigen, the method comprising contacting a T cell with the isolated antibody of any one of claims 0 to 10, wherein said contacting is under conditions whereby the ADCC against the cell expressing the tumor associated antigen is induced.

17. Use of the isolated monoclonal antibody of any one of claims 0 to 10 or the pharmaceutical composition of any one of claims 11 to 15 in preparation of a medicament for treating a disorder, wherein the disorder is a cancer or an autoimmune disease.

18. The use of claim 17, wherein the disorder is a cancer.

19. The use of claim 18, wherein the cancer is selected from the group consisting of leukemia, lymphoma, CLL, small lymphocytic lymphoma, marginal cell B-Cell lymphoma, Burkett's Lymphoma, renal cell carcinoma, colon cancer, colorectal cancer, breast cancer, epithelial squamous cell cancer, melanoma, myeloma, stomach cancer, brain cancer, lung cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, prostate cancer, testicular cancer, thyroid cancer, and head and neck cancer.

20. The use of any one of claims 17 to 19, wherein the medicament further contains an additional therapeutic agent.

21. The use of claim 20, wherein the additional therapeutic agent is an anti-cancer agent.

22. The use of claim 20, wherein the additional therapeutic agent is Ipilimumab and an biosimilar product thereof.
23. The use of claim 17, wherein the disorder is an autoimmune disease.

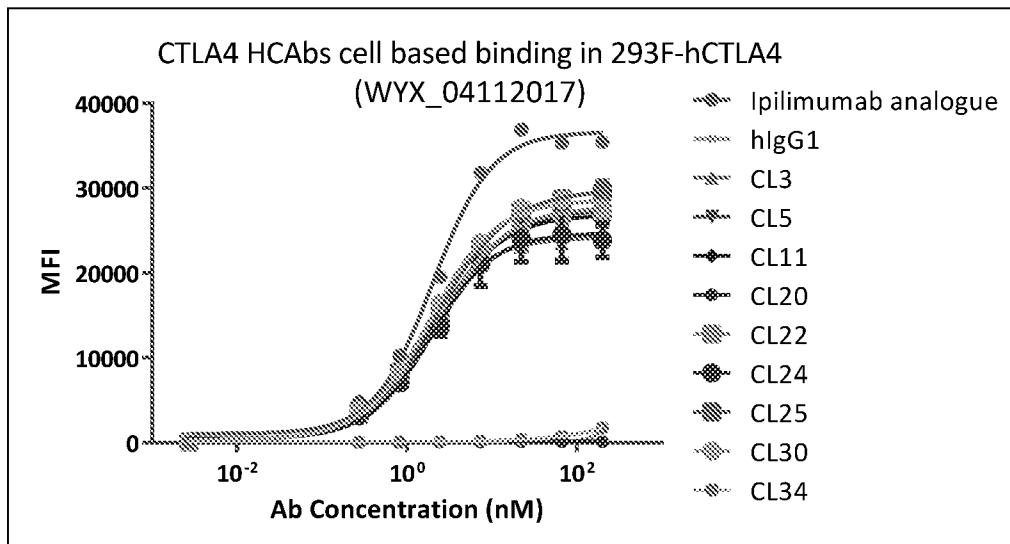


FIG. 1

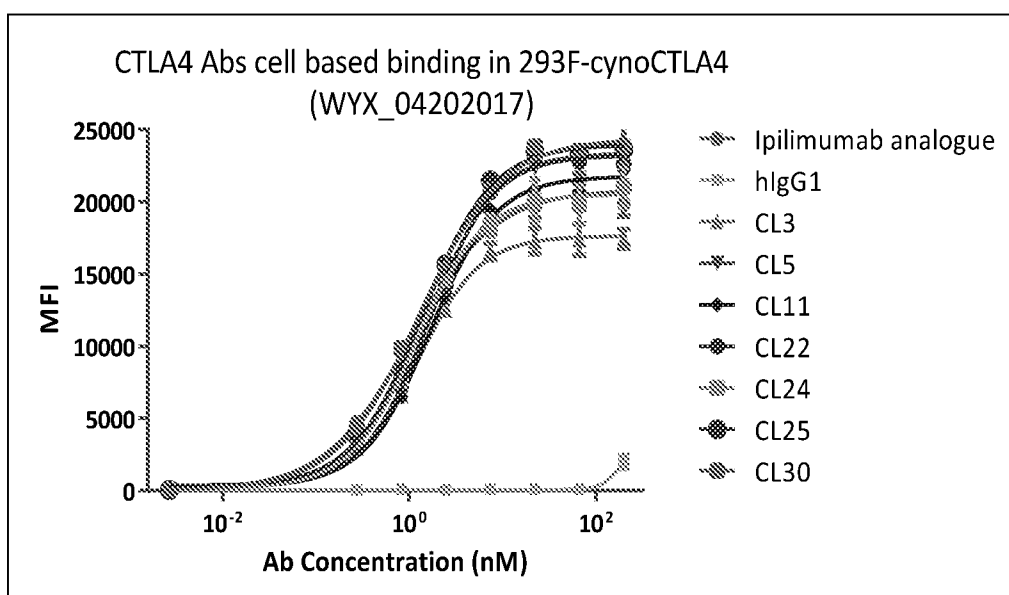


FIG. 2

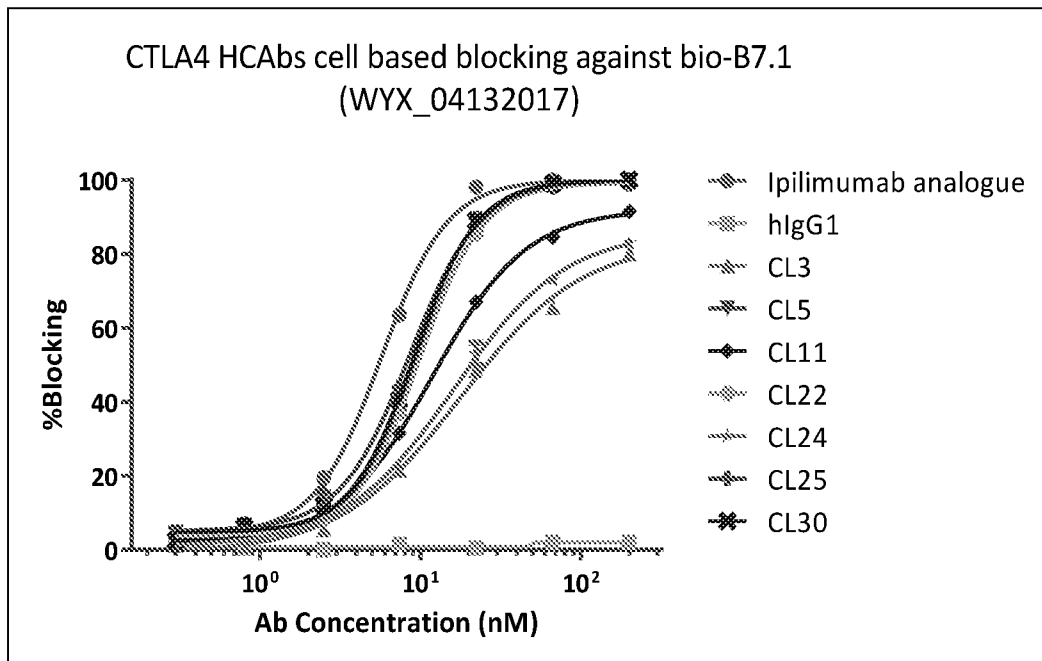
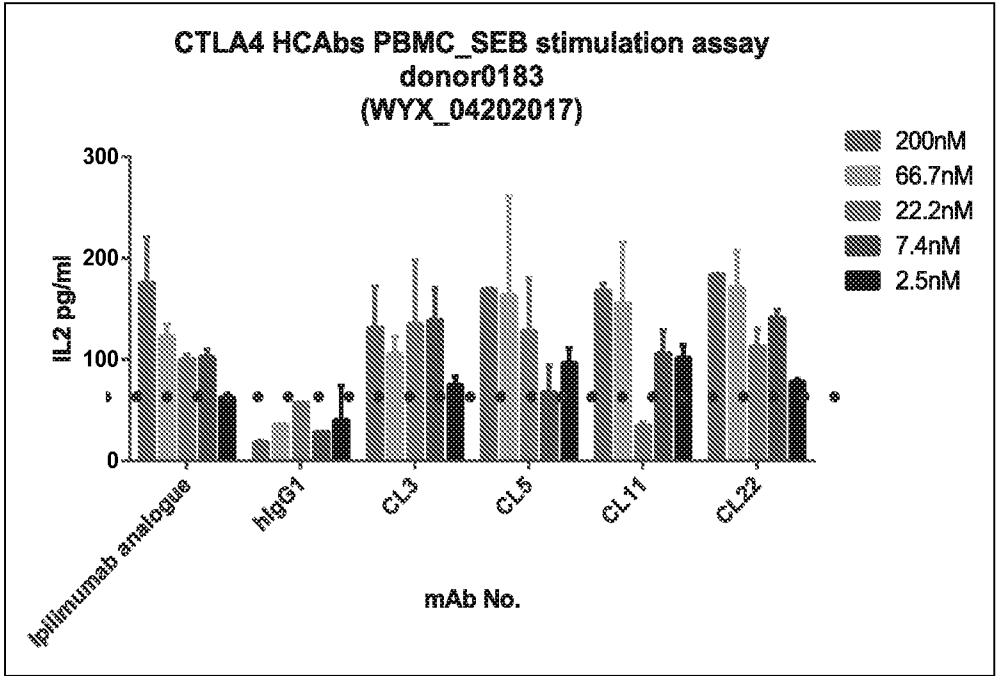


FIG. 3

A



B

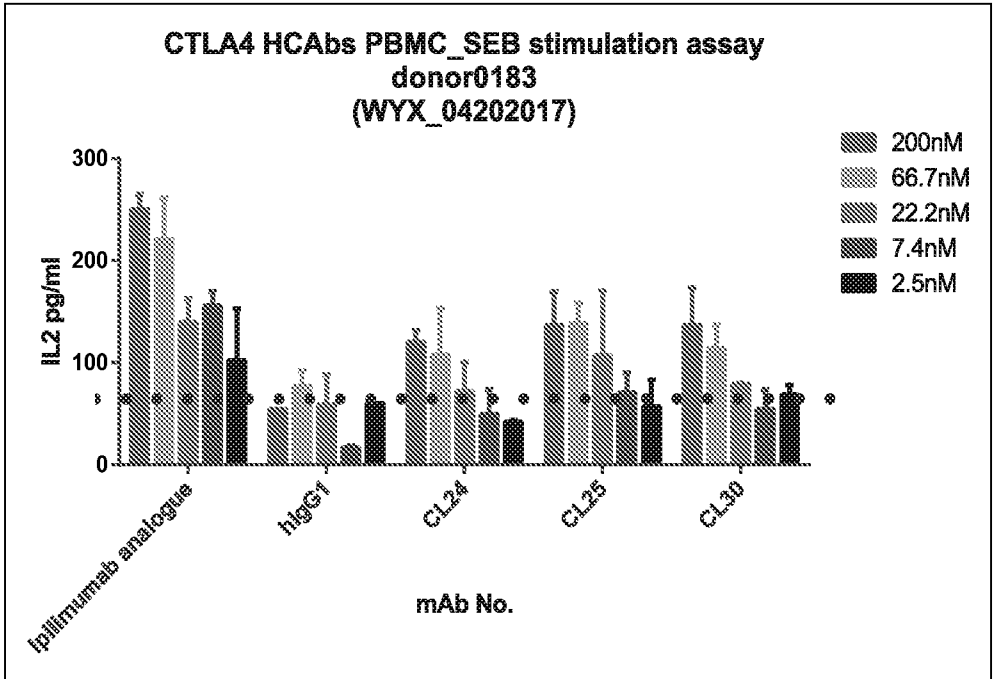
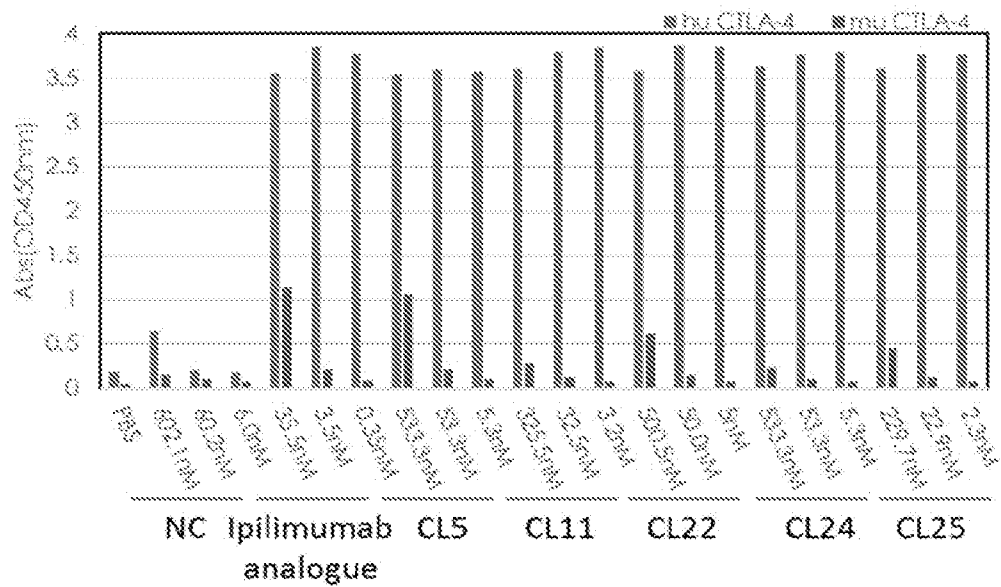


FIG. 4

A



B

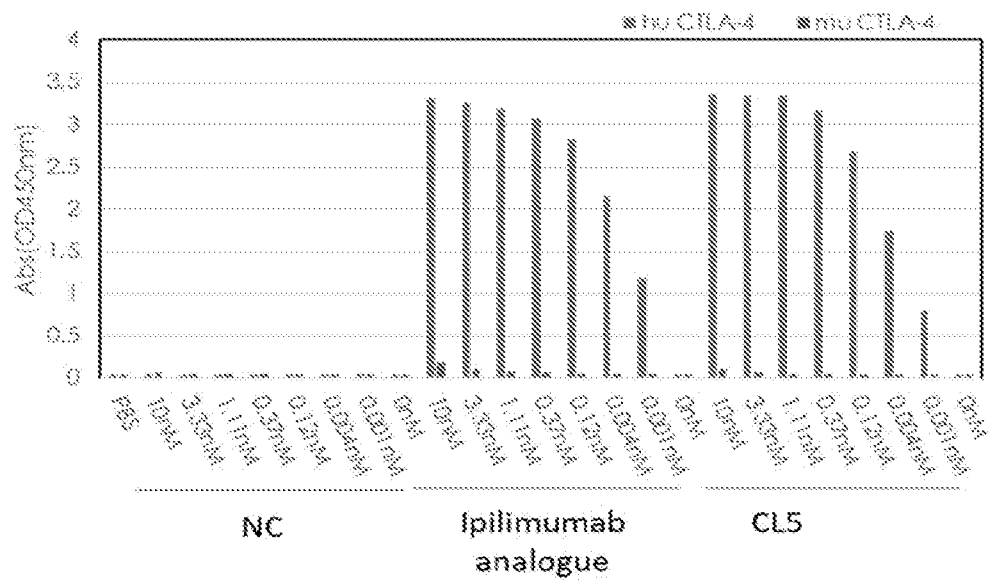
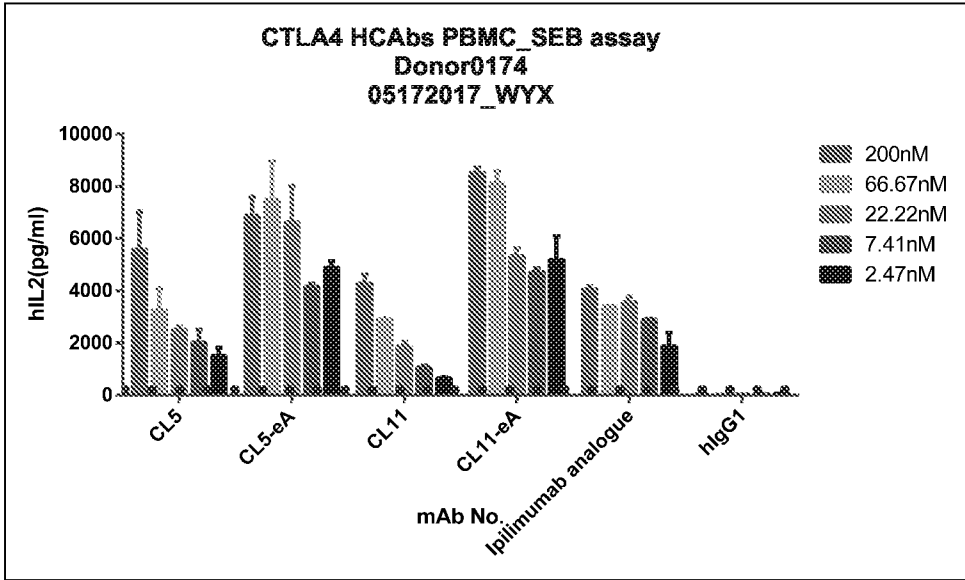


FIG. 5

A



B

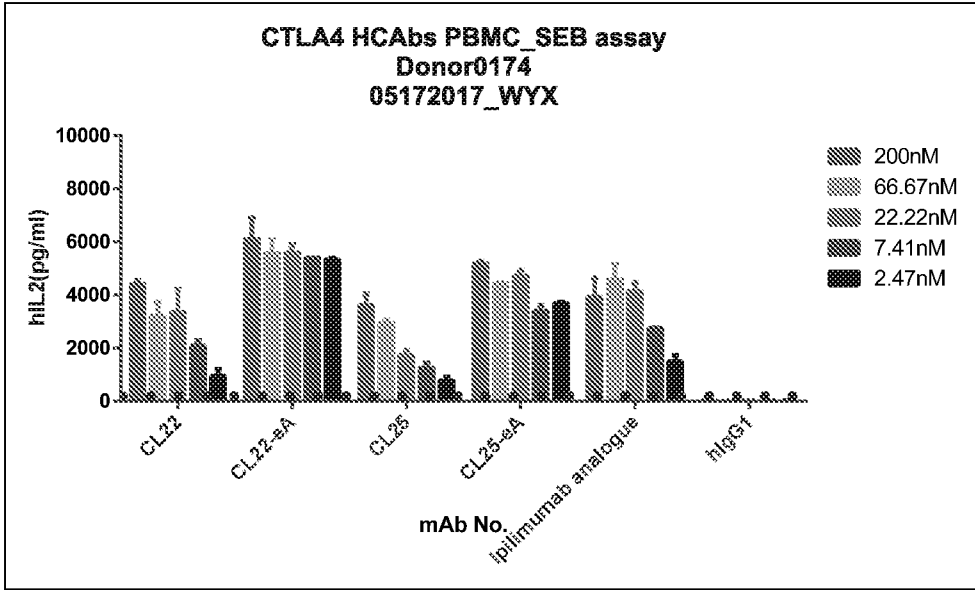


FIG. 6

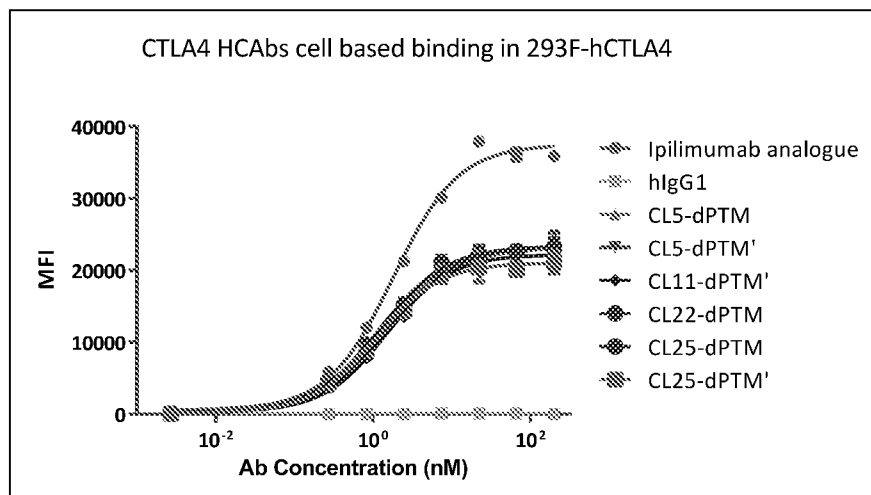


FIG. 7

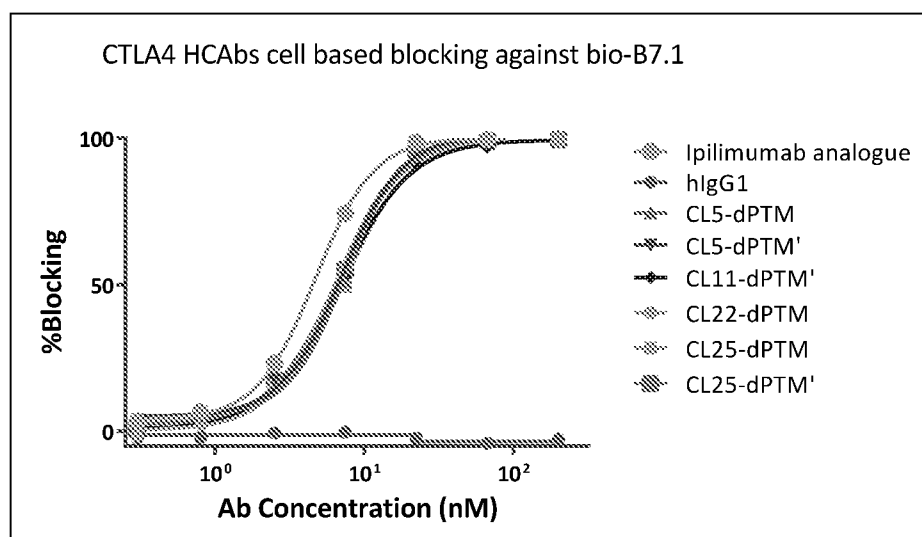


FIG. 8

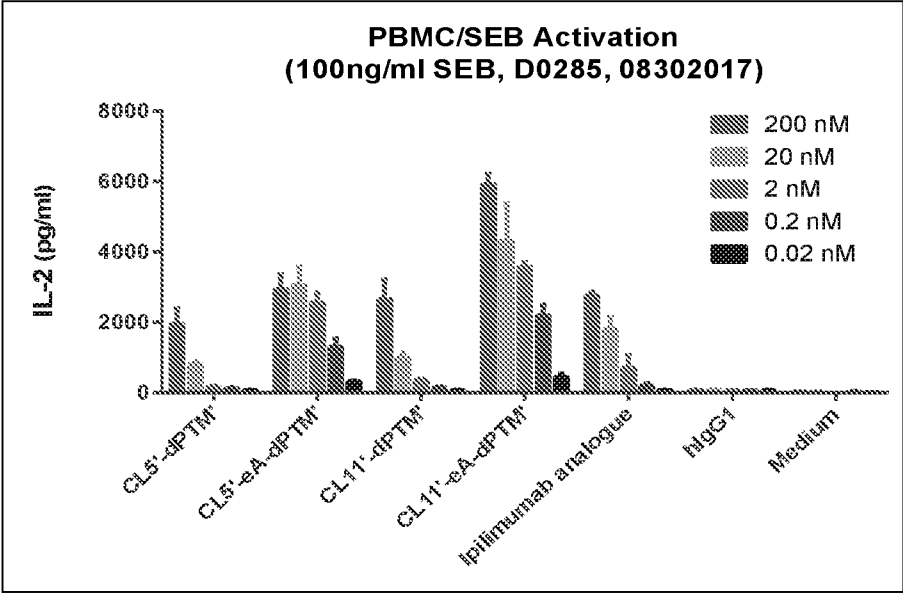


FIG. 9

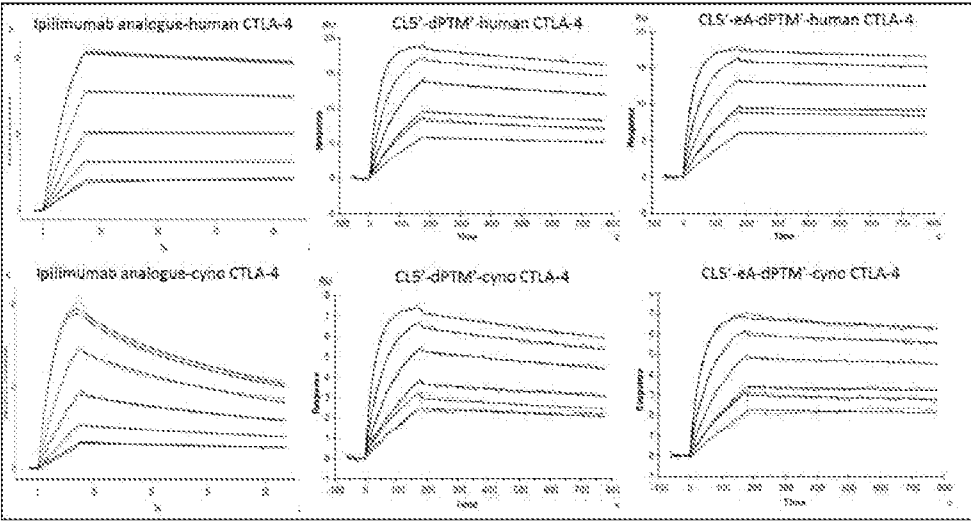


FIG. 10

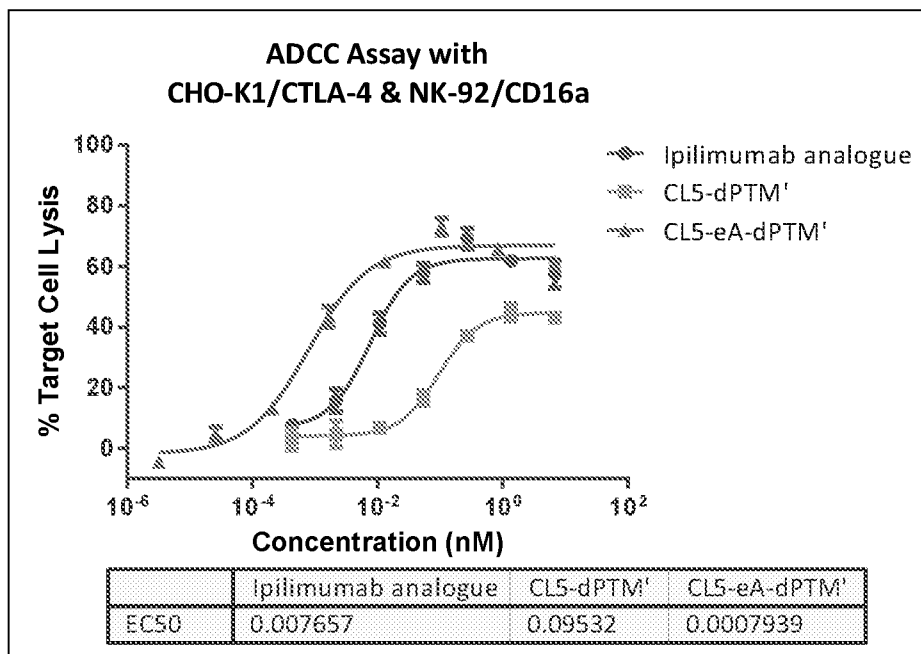


FIG. 11

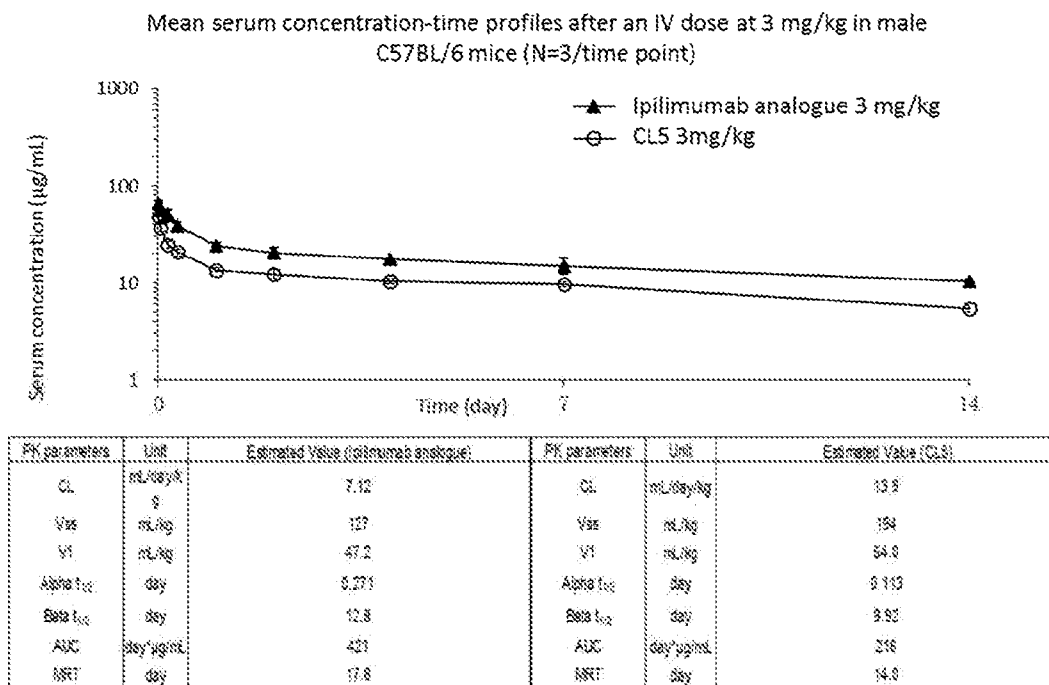


FIG. 12

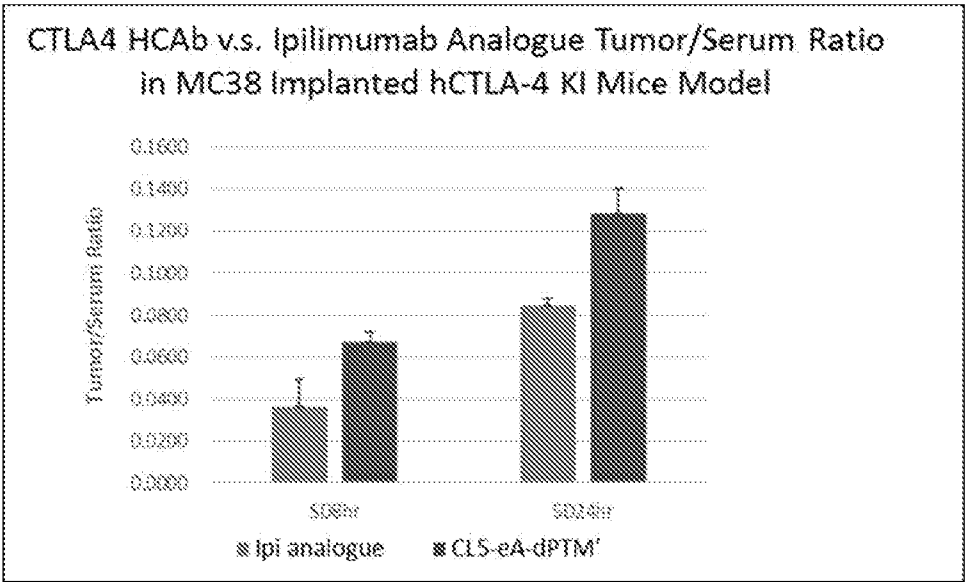


FIG. 13

A

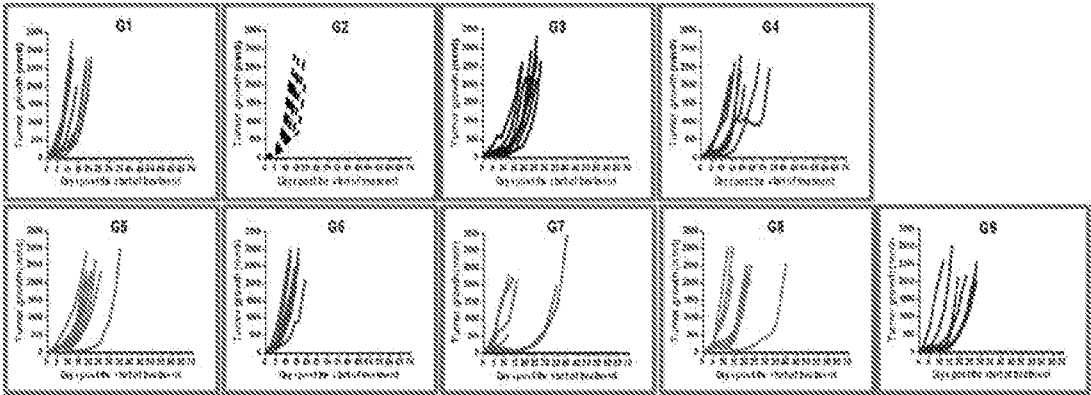
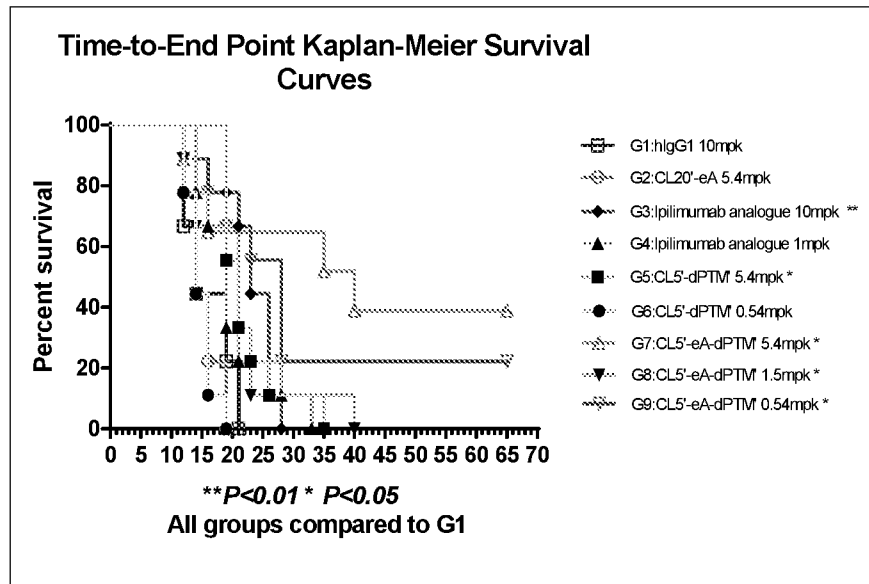


FIG. 14

B



C

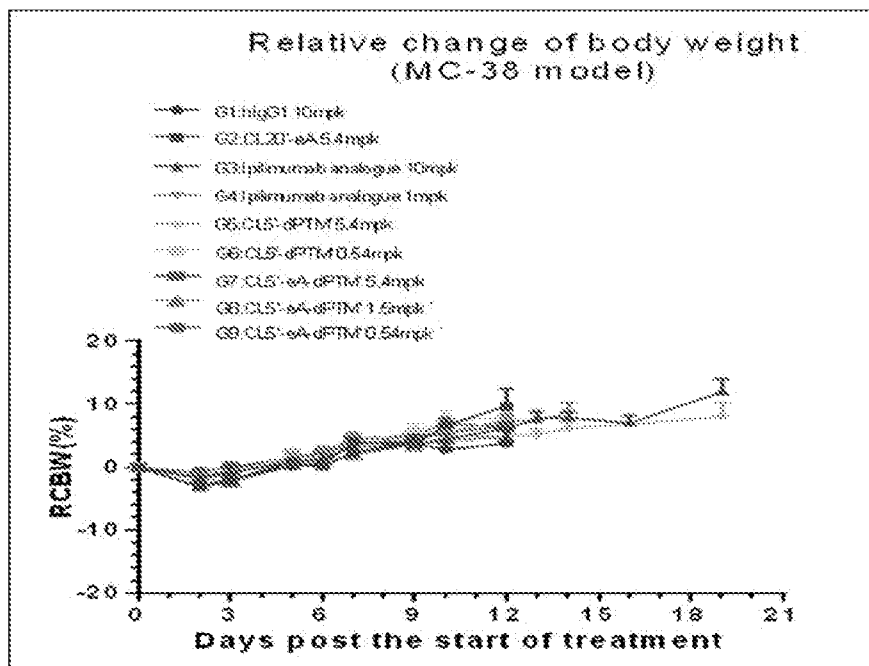
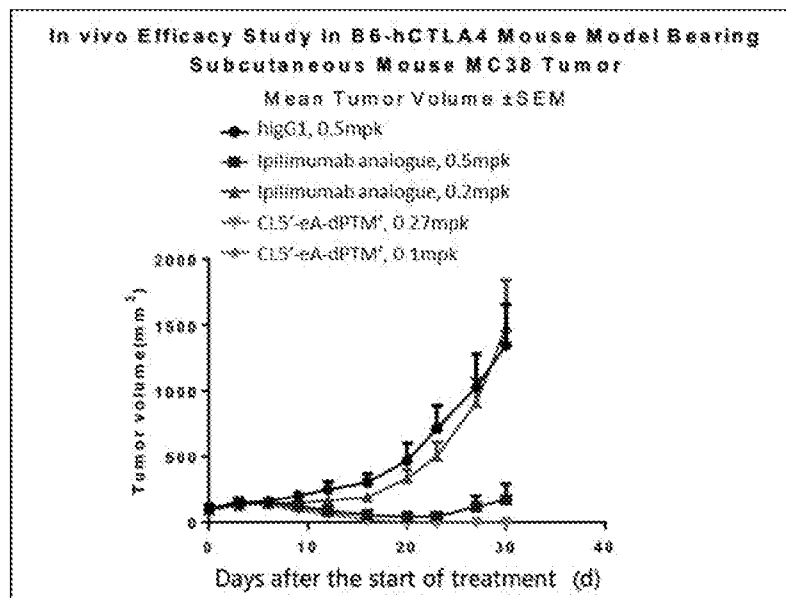


FIG. 14 (Continued)

A



B

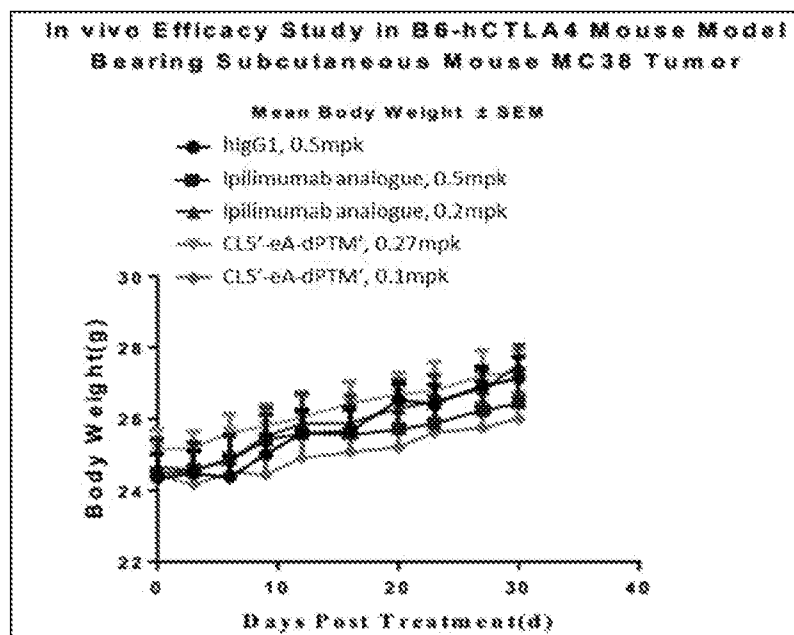


FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/122190

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/28(2006.01)i; A61K 39/395(2006.01)i; A61P 35/00(2006.01)n

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K,A61K,A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS,DWPI,SIPOABS,CNXTXT,WOTXT,JPTXT,USTXT,EPTXT,CNKI,Wanfang,PubMed,GenBank,ISI web of knowledge:CD152, CTLA-4, antagonist, heavy-chain-only antibody,antibody,nanoantibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 106220732 A (GUANGXI MEDICAL UNIVERSITY) 14 December 2016 (2016-12-14) abstract, claim 1, 9, paragraphs 0004, 0079	1-27
X	CN 106046164 A (GUANGXI MEDICAL UNIVERSITY) 26 October 2016 (2016-10-26) abstract, claim 1, 9, paragraphs 0004, 0079	1-27
X	CN 106046165 A (GUANGXI MEDICAL UNIVERSITY) 26 October 2016 (2016-10-26) abstract, claim 1, 9, paragraphs 0004, 0079	1-27
X	CN 106188297 A (GUANGXI MEDICAL UNIVERSITY) 07 December 2016 (2016-12-07) abstract, claim 1, 9, paragraphs 0004, 0079	1-27
A	CN 103547595 A (ANTITOPE LTD.) 29 January 2014 (2014-01-29) the whole document	1-27
A	CN 102766210 A (MEDAREX, INC.) 07 November 2012 (2012-11-07) the whole document	1-27
A	US 2006240006 A1 (CHU CHISHIH ET AL.) 26 October 2006 (2006-10-26) the whole document	1-27



Further documents are listed in the continuation of Box C.



See patent family annex.

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“A” document defining the general state of the art which is not considered to be of particular relevance

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“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

23 February 2019

Date of mailing of the international search report

25 March 2019

Name and mailing address of the ISA/CN

National Intellectual Property Administration, PRC
6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing
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Facsimile No. (86-10)62019451

Telephone No. 86-(10)-53962097

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2018/122190

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CN	106046165	A	26 October 2016	None			
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

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