ANTI-CTLA-4 BINDING PROTEINS AND METHODS OF USE THEREOF

1. CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of U.S. Provisional Application No. 63/218,198, filed on July 2, 2021, the entire contents of which are incorporated by reference herein.

2. SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing with 12088 sequences which has been submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 29, 2022, is named "49446_WO_Sequence_Listing_Final", and is 1.86 megabytes in size.

3. FIELD

[0003] Provided herein are antigen-binding proteins (ABPs) with binding specificity for CTLA-4 and compositions comprising such ABPs, including pharmaceutical compositions, diagnostic compositions, and kits. Also provided are methods of making CTLA-4 ABPs, and methods of using CTLA-4 ABPs, for example, for therapeutic purposes, diagnostic purposes, and research purposes.

4. BACKGROUND

[0004] CTLA-4, also known as cytotoxic T-lymphocyte associated protein 4 and CD152 (cluster of differentiation 152), is a cell surface receptor that suppresses T cell inflammatory activity, T cell co-stimulation, activation, and proliferation. CTLA-4 is constitutively expressed by regulatory T cells (Tregs) and upregulated in stimulated T cells. CD80 and CD86, also expressed in antigen presenting cells (APCs) such as dendritic cells (DCs), are the primary ligands of CTLA-4. The interaction between CTLA-4 and its ligands is vitally important for downregulating the immune responses and promoting self-tolerance by suppressing T cell inflammatory activity. This activity prevents autoimmune diseases, as well as prevents the immune system from killing cancer cells.

[0005] CTLA-4 is a member of the immunoglobulin superfamily that is expressed by activated T cells and transmits an inhibitory signal to T cells. CTLA-4 binds CD80 and CD86 with greater affinity and avidity than CD28 thus enabling it to outcompete CD28 for its ligands. CTLA-4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. CTLA-4 is also found in regulatory T cells (Tregs) and contributes to their inhibitory function. T cell activation through the T cell receptor and CD28 leads to increased expression of CTLA-4. The mechanism by which CTLA-4 acts in T cells remains somewhat controversial. Biochemical evidence suggested that CTLA-4 recruits a phosphatase to the T cell receptor (TCR), thus attenuating the signal. This work remains unconfirmed in the literature since its first publication. More recent work has suggested that CTLA-4 may function in vivo by capturing and removing

B7-1 and B7-2 from the membranes of antigen-presenting cells, thus making these unavailable for triggering of CD28.

[0006] Variants in CTLA-4 have been associated with insulin-dependent diabetes mellitus, Graves' disease, Hashimoto's thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, primary biliary cirrhosis and other autoimmune diseases. The comparatively high binding affinity of CTLA-4 for CD80 and CD86 has made it a potential therapeutic target for autoimmune diseases. Soluble fusion proteins of CTLA-4 and antibodies (CTLA-4-Ig) have been developed for clinical use.

[0007] Recently, CTLA-4 antibodies have been used with varying success to treat some types of cancer. CTLA-4 inhibitors have been shown to antagonize binding of CTLA-4 to its ligands, thereby activating the immune system to attack tumors. The current mechanism of action of known anti-CTLA-4 therapies is to block the interaction between CTLA-4 and its ligands for checkpoint inhibition. For example, CTLA-4 monoclonal antibodies (mAbs) such as ipilimumab were originally intended to block the binding of CTLA-4 to its ligands, the B7 proteins CD80 and CD86, i.e., "checkpoint inhibition". Blocking CTLA-4 binding to B7 proteins frees B7 proteins to bind to CD28, inducing T cell co-stimulation and activation. CTLA-4 antibodies have also been used to induce antibody-dependent cell-mediated cytotoxicity (ADCC) of Tregs specific to the tumor microenvironment, thus reducing immune tolerance to the tumor. Thus, in addition to blocking the interaction of CTLA-4 with its B7 ligands, anti-CTLA-4 mAbs are also able to induce antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) of intratumoral $FOXP3^+$ regulatory T cells (Tregs), which express comparatively high levels of surface CTLA-4.

[0008] Thus, inhibition of CTLA-4 function is currently one of the most promising systemic therapeutic approach for various diseases. There is a need for developing CTLA-4 ABPs that can be used for treatment, diagnosis, and research of various diseases, including cancer and autoimmune disease.

[0009] PCT Application PCT/US2019/068820, filed on December 27, 2019 and published as Publication No. WO2020140084A1, describes CTLA-4 ABPs, which application is incorporated by reference in its entirety herein.

5. SUMMARY

[0010] Provided herein are ABPs (e.g, GIGA-564, GIGA-2328) with binding specificity for CTLA-4 and methods of using the ABPs. The ABPs specifically bind a human CTLA-4 (SEQ ID: 7001) or a fragment of the human CTLA-4.

[0011] In particular, in one aspect, the present disclosure provides a CTLA-4 monoclonal antibody, GIGA-564, with minimal ability to block CTLA-4 binding to its CD80/CD86 ligands

but has superior anti-tumor activity with reduced toxicity. The anti-CTLA-4 antibody was demonstrated to induce less peripheral Treg proliferation, and more efficient intratumoral Treg depletion, in murine models expressing human CTLA-4. The anti-tumor activity of the anti-CTLA-4 antibody was further enhanced when it was more afucosylated (GIGA-2328).

[0012] The present disclosure also provides that the anti-CTLA-4 monoclonal antibodies bind to CTLA-4 at an epitope that differs from other, known anti-CTLA-4 antibodies (e.g., Ipilimumab), and has limited checkpoint inhibitor activity and thus is a weak checkpoint inhibitor. Surprisingly, efficacy of the anti-CTLA-4 antibodies presented herein was found to be associated with FcR-mediated Treg depletion in the tumor microenvironment and reduced proliferation of the remaining Tregs. The anti-CTLA-4 antibody also induces less Treg proliferation and has increased ability to induce *in vitro* FcR signaling and *in vivo* depletion of intratumoral Tregs. Experimental results described herein suggest that the enhanced FcR activity of the weak checkpoint inhibitor likely contributes to its enhanced anti-tumor activity. They also show that weak checkpoint inhibition was associated with lower toxicity in murine models.

[0013] It was further demonstrated that the anti-CTLA-4 monoclonal antibodies provided herein can enhance anti-tumor effects in combination with anti-PD-1 antibody, suggesting that the anti-CTLA-4 antibodies can work against tumors resistant to the anti-PD-1 antibody. Based on the studies, the present disclosure provides methods of treating cancer resistant to anti-PD-1 or anti-PD-L1 treatment. Further provided include dose regimens and pharmaceutical formulations that can be used in the treatment methods.

[0014] Accordingly, the present disclosure provides a method of treating cancer comprising the step of: administering a cancer patient an effective amount of an antigen binding protein (anti-CTLA-4 ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4, wherein the anti-CTLA-4 ABP comprises a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID NO:12080, a CDR1-H consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077.

[0015] In some embodiments, the cancer is resistant to anti-PD-1 or anti-PD-L1 treatment. In some embodiments, the cancer is resistant to treatment of anti-PD-1 antibody or anti-PD-L1 antibody. In some embodiments, the cancer patient has progressed or relapsed after anti-PD-1 or anti-PD-L1 treatment. In some embodiments, the method further comprises the step of deciding whether the cancer is resistant to anti-PD-1 treatment or anti-PD-L1 treatment.

[0016] In some embodiments, the cancer patient has melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, hepatocellular carcinoma, esophageal cancer, breast cancer, sarcoma, MSI-Hi/dMMR colorectal cancer, ovarian cancer, or cervical cancer, bladder, prostate, TMB-HI tumors of any origin, a tumor which is MSI, a tumor that is dMMR, a T cell leukemia/lymphoma, NHL, a tumor expressing CTLA-4 by the cancer cell.

[0017] In some embodiments, the method further comprises the step of administering an antigen binding protein (anti-PD-1 ABP or anti-PD-L1 ABP) that specifically binds a human PD-1 or anti-PD-L1. In some embodiments, the anti-PD-1 ABP is pembrolizumab. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio selected from 3:1, 3:10, 1:3, 1:10, 10:1, 10:3, 9:1, and 1:1. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio from 2:1 to 10:1. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio from 1:1 to 1:10. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio of 3:1.

[0018] In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on the same day. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on different days. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on the same day. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on different days.

[0019] In some embodiments, the effective amount of the anti-CTLA-4 ABP is less than 30mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is at least 0.01mg/kg, 0.03mg/kg, 0.1mg/kg, 0.3mg/kg, or 1mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is from 0.5mg/kg to 30mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is from 1mg/kg to 18mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is from 1mg/kg to 10mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is 1mg/kg, 3mg/kg, or 30mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is 9 mg/kg or 27 mg/kg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is from 50mg to 2500mg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is from 50mg to 1000mg. In some embodiments, the effective amount of the anti-CTLA-4 ABP is from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg. In some embodiments, the effective amount of

the anti-CTLA-4 ABP is 80mg, 240mg, 720mg, 800mg, 1440mg or 2160mg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose of 50mg, 100mg, 150mg, 250mg, 700mg, 800mg, 900mg, 1000mg, 1500mg, 2000mg, or 2500mg in each administration.

[0020] In some embodiments, the anti-CTLA-4 ABP comprises a variable light chain (VL) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (VH) comprising a sequence at least 97% identical to SEQ ID NO:114. In some embodiments, the anti-CTLA-4 ABP comprises a variable light chain (VL) comprising the sequence of SEQ ID NO:14 and a variable heavy chain (VH) comprising the sequence of SEQ ID NO:114.

[0021] In some embodiments, the anti-CTLA-4 ABP comprises an scFv or a full length monoclonal antibody. In some embodiments, the anti-CTLA-4 ABP comprises an immunoglobulin constant region. In some embodiments, the anti-CTLA-4 ABP is a IgG1 ABP. In some embodiments, the anti-CTLA-4 ABP comprises an IGHG1*01 human heavy chain constant region gene segment. In some embodiments, the anti-CTLA-4 ABP comprises a lysine at amino acid position 97 (R97) according to IMGT exon numbering. In some embodiments, the anti-CTLA-4 ABP comprises a lysine at amino acid position 97 (R214) according to EU numbering.

[0022] In some embodiments, the anti-CTLA-4 ABP comprises an afucosylated Fc region. In some embodiments, the anti-CTLA-4 ABP is produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof. In some embodiments, the cell is cultured in the absence of fucose. In some embodiments, the anti-CTLA-4 ABP is produced from a cell lacking or with reduced expression of Fut8. In some embodiments, the anti-CTLA-4 ABP is produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF). In some embodiments, the anti-CTLA-4 ABP is produced from a cell overexpressing glycosyltransferase (GnTIII). In some embodiments, the anti-CTLA-4 ABP has been isolated based on its fucosylation status. In some embodiments, the anti-CTLA-4 ABP comprises an Fc region lacking core fucosylation of the N-glycan of the Fc portion. In some embodiments, the ABP is an afucosylated monoclonal antibody.

[0023] In some embodiments, the anti-CTLA-4 ABP is administered in a pharmaceutical composition. In some embodiments, the pharmaceutical composition has pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition has pH from 6.0 to 6.5. In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 20mM of histidine. In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments,

the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises 0.1-1 mg/ml Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 0.2 mg/ml Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 20mM histidine, 270mM sucrose, and 0.2 mg/ml Polysorbate 20, and has pH 6.2.

[0024] In some embodiments, the pharmaceutical composition comprises from 5 mg/mL to 20 mg/mL of the anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises 20 mg/mL of the anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises 10 mg/mL of the anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises 5 mg/mL of the anti-CTLA-4 ABP.

[0025] In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated. In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated at least twice, three times, four times, or more. In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated every day, every two days, every three days, every four days, every five days or every six days. In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated every week, every two weeks, every three weeks, every four weeks, every five weeks, every six weeks or every seven weeks. In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated every 1-2 weeks, every 2-3 weeks, every 3-4 weeks, every 4-5 weeks, every 5-6 weeks, every 6-7 weeks, every 7-8 weeks, every 8-9 weeks, every 9-10 weeks, every 10-11 weeks, every 11-12 weeks, every 12-13 weeks, every 13-14 weeks, or every 14-15 weeks. In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated every month, every two months, every three months, every four months, every five months, or less frequent. In some embodiments, the step of administering the anti-CTLA-4 ABP is repeated every 1-2 months, every 2-3 months, every 3-4 months, every 4-5 months, or every 5-6 months. In some embodiments, the anti-PD-1 antibody or anti-PD-L1 antibody is administered in combination with the anti-CTLA-4 ABP in each of the repeated administrations. In some embodiments, the anti-PD-1 antibody or anti-PD-L1 antibody is administered in combination with the anti-CTLA-4 ABP in some but not all of the repeated administrations.

[0026] In one aspect, the present disclosure provides a pharmaceutical composition comprising an anti-CTLA-4 ABP and a pharmaceutically acceptable excipient, wherein the anti-CTLA-4 ABP is an isolated antigen binding protein (ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and comprises a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID

NO:12080, a CDR1-H consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077.

[0027] In some embodiments, the anti-CTLA-4 ABP comprises a variable light chain (VL) comprising the sequence of SEQ ID NO:14 and a variable heavy chain (VH) comprising the sequence of SEQ ID NO:114.

[0028] In some embodiments, the pharmaceutical composition has pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition has pH from 6.0 to 6.5. In some embodiments, the pharmaceutical composition has pH 6.2.

[0029] In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 20mM of histidine. In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises 0.1-1 mg/ml Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 0.2 mg/ml Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 20mM histidine, 270mM sucrose, and 0.02% PS-20, and has pH 6.2.

[0030] In some embodiments, the pharmaceutical composition comprises 5 mg/mL to 20 mg/mL of the anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises 20 mg/mL of the anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises 10 mg/mL of the anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises 5 mg/mL of the anti-CTLA-4 ABP.

[0031] In some embodiments, less than 50% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, less than 40% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, less than 30% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, less than 20% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, less than 10% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, 3% to 30% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, 10% to 30% of the anti-CTLA-4 ABP is fucosylated. In some embodiments, 15% to 25% of the anti-CTLA-4 ABP is fucosylated.

[0032] In some embodiments, the pharmaceutical composition is formulated for injection. In some embodiments, the pharmaceutical composition is formulated for iv infusion.

[0033] The present disclosure further provides a unit dose form of the pharmaceutical composition. In some embodiments, the unit dose comprises 50mg to 5000mg of the anti-CTLA-

4 ABP. In some embodiments, the unit dose comprises 50mg to 2500mg of the anti-CTLA-4 ABP. In some embodiments, the unit dose comprises 10mg to 2000mg of the anti-CTLA-4 ABP. In some embodiments, the unit dose comprises the anti-CTLA-4 ABP at an amount from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg. In some embodiments, the unit dose comprises the anti-CTLA-4 ABP at an amount of 80mg, 240mg, 720mg, 800mg, 1440mg or 2160mg. In some embodiments, the unit dose comprises the anti-CTLA-4 ABP at an amount of 50mg, 100mg, 150mg, 250mg, 700mg, 800mg, 900mg, 1000mg, 1500mg, 2000mg, or 2500mg in each administration.

[0034] In some embodiments, when bound to CTLA-4, the ABP contacts amino acids K130, Y139, L141, I143 but does not contact amino acid R70 of the CTLA-4, or R70 is not energetically a major contributor to the interaction between CTLA-4 and the ABP; and/or the CTLA-4 can associate with CD80/CD86 when bound to the ABP; and/or an interaction between the ABP and amino acid L74A and/or E68 of the CTLA-4 is greater than an interaction between Ipilimumab and amino acid L74A of CTLA-4.

[0035] In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO:12078 or SEQ ID NO: 1014, a CDR2-L consisting of SEQ ID NO:12079 or SEQ ID NO: 2014, a CDR3-L consisting of SEQ ID NO:12080 or SEQ ID NO: 3014, a CDR1-H consisting of SEQ ID NO:12075 or SEQ ID NO: 4014, a CDR2-H consisting of SEQ ID NO:12076 or SEQ ID NO: 5014 and a CDR3-H consisting of SEQ ID NO:12077 or SEQ ID NO: 6014. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12004, a CDR2-L consisting of SEQ ID NO: 12014, a CDR3-L consisting of SEQ ID NO: 12024, a CDR1-H consisting of SEQ ID NO: 12039, a CDR2-H consisting of SEQ ID NO: 12049, and a CDR3-H consisting of SEQ ID NO: 12059. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12005, a CDR2-L consisting of SEQ ID NO: 12015, a CDR3-L consisting of SEQ ID NO: 12025, a CDR1-H consisting of SEQ ID NO: 12040, a CDR2-H consisting of SEQ ID NO: 12050, and a CDR3-H consisting of SEQ ID NO: 12060. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12006, a CDR2-L consisting of SEQ ID NO: 12016, a CDR3-L consisting of SEQ ID NO: 12026, a CDR1-H consisting of SEQ ID NO: 12041, a CDR2-H consisting of SEQ ID NO: 12051, and a CDR3-H consisting of SEQ ID NO: 12061. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12007, a CDR2-L consisting of SEQ ID NO: 12017, a CDR3-L consisting of SEQ ID NO: 12027, a CDR1-H consisting of SEQ ID NO: 12042, a CDR2-H consisting of SEQ ID NO: 12052, and a CDR3-H consisting of SEQ ID NO: 12062. In some

embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12008, a CDR2-L consisting of SEQ ID NO: 12018, a CDR3-L consisting of SEQ ID NO: 12028, a CDR1-H consisting of SEQ ID NO: 12043, a CDR2-H consisting of SEQ ID NO: 12053, and a CDR3-H consisting of SEQ ID NO: 12063.

[0036] In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:114.

[0037] In some embodiments, the ABP comprises an scFv or a full length monoclonal antibody. In some embodiments, the ABP comprises an immunoglobulin constant region.

[0038] In some embodiments, the ABP binds human CTLA-4 with a K_D of less than 500nM, as measured by surface plasmon resonance; or the ABP binds human CTLA-4 with a K_D of less than 200nM, as measured by surface plasmon resonance; or the ABP binds human CTLA-4 with a K_D of less than 25nM, as measured by surface plasmon resonance; or the ABP binds to human CTLA-4 on a cell surface with a K_D of less than 25nM.

[0039] In some embodiments, the ABP is a IgG1 ABP. In some embodiments, the ABP comprises an IGHG1*01 human heavy chain constant region gene segment. In some embodiments, the ABP comprises a lysine at amino acid position 97 (R97) according to IMGT exon numbering. In some embodiments, the ABP comprises a lysine at amino acid position 97 (R214) according to EU numbering.

[0040] In some embodiments, the ABP comprises an afucosylated Fc region.

[0041] In some embodiments, the ABP is produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof. In some embodiments, the cell is cultured in the absence of fucose.

[0042] In some embodiments, the ABP is produced from a cell lacking or with reduced expression of Fut8. In some embodiments, the ABP is produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF). In some embodiments, the ABP is produced from a cell overexpressing glycosyltransferase (GnTIII). In some embodiments, the ABP has been isolated based on its fucosylation status.

[0043] In some embodiments, the ABP comprises an Fc region lacking core fucosylation of the N-glycan of the Fc portion. In some embodiments, the ABP is an afucosylated monoclonal antibody.

[0044] Aspects of the present disclosure also include a pharmaceutical composition comprising the ABP of the present disclosure and a pharmaceutically acceptable excipient.

[0045] In some embodiments, less than 50% of the ABP is fucosylated. In some embodiments, less than 40% of the ABP is fucosylated. In some embodiments, less than 30% of the ABP is fucosylated. In some embodiments, less than 20% of the ABP is fucosylated. In some embodiments, less than 10% of the ABP is fucosylated. In some embodiments, more than 30% of the ABP is fucosylated. In some embodiments, more than 40% of the ABP is fucosylated. In some embodiments, more than 50% of the ABP is fucosylated. In some embodiments, more than 60% of the ABP is fucosylated. In some embodiments, more than 70% of the ABP is fucosylated. In some embodiments, more than 80% of the ABP is fucosylated. In some embodiments, more than 90% of the ABP is fucosylated.

[0046] In some embodiments, the pharmaceutical composition has a pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises 170mM or 270mM of sucrose. In some embodiments, the pharmaceutical composition comprises 5 mg/mL to 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 5 mg/mL of the ABP.

[0047] Aspects of the present disclosure provide a method of treating a disease comprising the step of: administering to a subject in need thereof an effective amount of the ABP of any of the ABPs of the present disclosure or the pharmaceutical composition thereof.

[0048] In some embodiments, the disease is selected from the group consisting of cancer, AIDS, Alzheimer's disease and viral or bacterial infection. In some embodiments, the disease is selected from the group consisting of autoimmune disease, autoinflammatory disease, and inflammation.

[0049] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, additional therapeutic agent is selected from an anti-PD-L1, an anti-PD1, a LAG-3 inhibitor, a CD47 inhibitor, a TIGIT inhibitor, a chemotherapy agent, an immune-stimulatory agent, radiation, a BRAF inhibitor, a MEK inhibitor, a PI3K inhibitor, a cytokine, a polynucleotide encoding a cytokine, an oncolytic virus encoding a cytokine, and a combination thereof. In some embodiments, the method further

comprises the step of adoptive cell therapy, or treatment with cancer vaccine, oncolytic virus, or anti-CD40 inhibitor.

[0050] Aspects of the present disclosure include an isolated polynucleotide encoding the ABP. Aspects of the present disclosure include a vector comprising the isolated polynucleotide. Aspects of the present disclosure provide a host cell comprising the isolated polynucleotide or the vector of the present disclosure. In some embodiments, the host cell further comprises a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase). In some embodiments, the host cell is cultured in the absence of fucose. In some embodiments, the host cell is lacking or having reduced expression of Fut8. In some embodiments, the host cell is cultured in the presence of a fucosylation inhibitor 2-Fluorfucose (2FF). In some embodiments, the host cell is overexpressing glycosyltransferase (GnTIII). In some embodiments, the host cell is a cell line from CHOZN GS using the 2G UNic translational enhancer element.

[0051] Aspects of the present disclosure provide a method of producing an isolated antigen binding protein (ABP) that specifically binds human CTLA-4, comprising inducing expression of the ABP in the host cell of the present disclosure, and isolating the ABP.

[0052] In some embodiments, the method further comprises the step of isolating the ABP based on its fucosylation status. In some embodiments, the host cell is cultured in a cultured medium comprising a fucosylation inhibitor. In some embodiments, the fucosylation inhibitor is 2-Fluorfucose (2FF).

[0053] A method of reducing CTLA-4^{HI} Tregs in a subject with limited proliferation of remaining Tregs comprising administering an effective dose of the ABP or the pharmaceutical composition described in the present disclosure.

[0054] In some embodiments, the subject is a human subject, optionally, a human subject with RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, MSI colorectal cancer, ovarian cancer, or cervical cancer.

[0055] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, the additional therapeutic agent is an anti-PD-L1 or an anti-PD1 or a combination thereof.

[0056] In another aspect, the present disclosure provides an isolated antigen binding protein (ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4 (CTLA-4), wherein the ABP comprises: (a) a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID NO:12080, a CDR1-H

consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077; (b) a CDR1-L consisting of SEQ ID NO: 1014, a CDR2-L consisting of SEQ ID NO: 2014, a CDR3-L consisting of SEQ ID NO: 3014, a CDR1-H consisting of SEQ ID NO: 4014, a CDR2-H consisting of SEQ ID NO: 5014 and a CDR3-H consisting of SEQ ID NO: 6014; (c) a CDR1-L consisting of SEQ ID NO: 12004, a CDR2-L consisting of SEQ ID NO: 12014, a CDR3-L consisting of SEQ ID NO: 12024, a CDR1-H consisting of SEQ ID NO: 12039, a CDR2-H consisting of SEQ ID NO: 12049, and a CDR3-H consisting of SEQ ID NO: 12059; (d) a CDR1-L consisting of SEQ ID NO: 12005, a CDR2-L consisting of SEQ ID NO: 12015, a CDR3-L consisting of SEQ ID NO: 12025, a CDR1-H consisting of SEQ ID NO: 12040, a CDR2-H consisting of SEQ ID NO: 12050, and a CDR3-H consisting of SEQ ID NO: 12060; (e) a CDR1-L consisting of SEQ ID NO: 12006, a CDR2-L consisting of SEQ ID NO: 12016, a CDR3-L consisting of SEQ ID NO: 12026, a CDR1-H consisting of SEQ ID NO: 12041, a CDR2-H consisting of SEQ ID NO: 12051, and a CDR3-H consisting of SEQ ID NO: 12061; (f) a CDR1-L consisting of SEQ ID NO: 12007, a CDR2-L consisting of SEQ ID NO: 12017, a CDR3-L consisting of SEQ ID NO: 12027, a CDR1-H consisting of SEQ ID NO: 12042, a CDR2-H consisting of SEQ ID NO: 12052, and a CDR3-H consisting of SEQ ID NO: 12062; or (g) a CDR1-L consisting of SEQ ID NO: 12008, a CDR2-L consisting of SEQ ID NO: 12018, a CDR3-L consisting of SEQ ID NO: 12028, a CDR1-H consisting of SEQ ID NO: 12043, a CDR2-H consisting of SEQ ID NO: 12053, and a CDR3-H consisting of SEQ ID NO: 12063.

[0057] In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:114.

[0058] In some embodiments, the ABP comprises an scFv or a full length monoclonal antibody. In some embodiments, the ABP comprises an immunoglobulin constant region.

[0059] In some embodiments, the ABP binds human CTLA-4 with a K_D of less than 500nM, as measured by surface plasmon resonance; or the ABP binds human CTLA-4 with a K_D of less than 200nM, as measured by surface plasmon resonance; or the ABP binds human CTLA-4 with a K_D of less than 25nM, as measured by surface plasmon resonance; or the ABP binds to human CTLA-4 on a cell surface with a K_D of less than 25nM.

[0060] In some embodiments, the ABP is a IgG1 ABP. In some embodiments, the ABP comprises an IGHG1*01 human heavy chain constant region gene segment. In some embodiments, the ABP comprises a lysine at amino acid position 97 (R97) according to IMGT

exon numbering. In some embodiments, the ABP comprises a lysine at amino acid position 97 (R214) according to EU numbering.

[0061] In some embodiments, the ABP comprises an afucosylated Fc region.

[0062] In some embodiments, the ABP is produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof. In some embodiments, the cell is cultured in the absence of fucose. In some embodiments, the ABP is produced from a cell lacking or with reduced expression of Fut8. In some embodiments, the ABP is produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF). In some embodiments, the ABP is produced from a cell overexpressing glycosyltransferase (GnTIII). In some embodiments, the ABP has been isolated based on its fucosylation status.

[0063] In some embodiments, the ABP comprising an Fc region lacking core fucosylation of the N-glycan of the Fc portion. In some embodiments, the ABP is an afucosylated monoclonal antibody. In some embodiments, the afucosylated Fc region has less than 30% fucosylation, and wherein less than 30% fucosylation enhances FcgRIII (Fc Gamma Receptor III) signaling. In some embodiments, the afucosylated Fc region has less than 30% fucosylation, and wherein less than 30% fucosylation enhances FcgRIIIa (Fc Gamma Receptor IIIa) signaling.

[0064] Aspects of the present disclosure include a pharmaceutical composition comprising the ABP of the present disclosure, and a pharmaceutically acceptable excipient.

[0065] In some embodiments, less than 50% of the ABP is fucosylated. In some embodiments, less than 40% of the ABP is fucosylated. In some embodiments, less than 30% of the ABP is fucosylated. In some embodiments, less than 20% of the ABP is fucosylated. In some embodiments, less than 10% of the ABP is fucosylated. In some embodiments, more than 30% of the ABP is fucosylated. In some embodiments, more than 40% of the ABP is fucosylated. In some embodiments, more than 50% of the ABP is fucosylated. In some embodiments, more than 60% of the ABP is fucosylated. In some embodiments, more than 70% of the ABP is fucosylated. In some embodiments, more than 80% of the ABP is fucosylated. In some embodiments, more than 90% of the ABP is fucosylated.

[0066] In some embodiments, the pharmaceutical composition has a pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises 170mM or 270mM of sucrose. In some embodiments, the pharmaceutical composition comprises 5 mg/mL

to 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 5 mg/mL of the ABP.

[0067] Aspects of the present disclosure provide a method of treating a disease comprising the step of: administering to a subject in need thereof an effective amount of the ABP of any of the ABPs of the present disclosure or the pharmaceutical composition thereof.

[0068] In some embodiments, the disease is selected from the group consisting of cancer, AIDS, Alzheimer's disease and viral or bacterial infection. In some embodiments, the disease is selected from the group consisting of autoimmune disease, autoinflammatory disease, and inflammation.

[0069] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, additional therapeutic agent is selected from an anti-PD-L1, an anti-PD1, a LAG-3 inhibitor, a CD47 inhibitor, a TIGIT inhibitor, a chemotherapy agent, an immune-stimulatory agent, radiation, a BRAF inhibitor, a MEK inhibitor, a PI3K inhibitor, a cytokine, a polynucleotide encoding a cytokine, an oncolytic virus encoding a cytokine, and a combination thereof. In some embodiments, the method further comprises the step of adoptive cell therapy, or treatment with cancer vaccine, oncolytic virus, or anti-CD40 inhibitor.

[0070] Aspects of the present disclosure include an isolated polynucleotide encoding the ABP. Aspects of the present disclosure include a vector comprising the isolated polynucleotide. Aspects of the present disclosure provide a host cell comprising the isolated polynucleotide or the vector of the present disclosure. In some embodiments, the host cell further comprises a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase). In some embodiments, the host cell is cultured in the absence of fucose. In some embodiments, the host cell is lacking or having reduced expression of Fut8. In some embodiments, the host cell is cultured in the presence of a fucosylation inhibitor 2-Fluorfucose (2FF). In some embodiments, the host cell is overexpressing glycosyltransferase (GnTIII).

[0071] Aspects of the present disclosure provide a method of treating cancer comprising the step of: administering to a subject in need thereof an effective amount of the ABP or the pharmaceutical composition of the present disclosure. In some embodiments, the subject has a malignant tumor. In some embodiments, when administered, the ABP comprises increased Fc receptor (FcR) signaling as compared to ipilimumab, and wherein said administering reduces the amount of CTLA-4^{HI} Tregs in the subject. In some embodiments, said administering reduces

proliferation of peripheral Tregs in the subject as compared to ipilimumab. In some embodiments, said administering reduces tumors more effectively than ipilimumab.

[0072] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, the additional therapeutic agent is selected from an anti-PD-L1, an anti-PD1, a TIGIT inhibitor, a LAG-3 inhibitor, a CD47 inhibitor, a BRAF inhibitor, a MEK inhibitor, a PI3K inhibitor, a chemotherapy agent, an immune-stimulatory agent, radiation, a cytokine, a polynucleotide encoding a cytokine, an oncolytic virus encoding a cytokine, and a combination thereof. In some embodiments, the method further comprises the step of adoptive cell therapy, or treatment with cancer vaccine, oncolytic virus, or anti-CD40 inhibitor.

[0073] In some embodiments, the ABP comprises an afucosylated Fc region that has less than 30% fucosylation, and wherein less than 30% fucosylation enhances FcgRIII signaling. In some embodiments, the ABP comprises an afucosylated Fc region that has less than 30% fucosylation, and wherein less than 30% fucosylation enhances FcgRIIIa signaling. In some embodiments, the ABP comprises a fucosylated Fc region that has more than 70% fucosylation.

[0074] Aspects of the present disclosure include an isolated polynucleotide encoding the ABP. Aspects of the present disclosure include a vector comprising the isolated polynucleotide. Aspects of the present disclosure provide a host cell comprising the isolated polynucleotide or the vector of the present disclosure. In some embodiments, the host cell further comprises a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase). In some embodiments, the host cell is cultured in the absence of fucose. In some embodiments, the host cell is lacking or having reduced expression of Fut8. In some embodiments, the host cell is cultured in the presence of a fucosylation inhibitor 2-Fluorfucose (2FF). In some embodiments, the host cell is overexpressing glycosyltransferase (GnTIII).

[0075] Aspects of the present disclosure provide a method of producing an isolated antigen binding protein (ABP) that specifically binds human CTLA-4, comprising inducing expression of the ABP in the host cell of the present disclosure, and isolating the ABP, wherein the ABP comprises an afucosylated Fc.

[0076] In some embodiments, the method further comprises the step of isolating the ABP based on its fucosylation status. In some embodiments, the host cell is cultured in a cultured medium comprising a fucosylation inhibitor. In some embodiments, the fucosylation inhibitor is 2-Fluorfucose (2FF).

[0077] In another aspect, the present disclosure provides an isolated antigen binding protein (ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4 (CTLA-4), comprising an IGHG1*01 human heavy chain constant region gene segment.

[0078] In some embodiments, the ABP comprises: (a) a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID NO:12080, a CDR1-H consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077; (b) a CDR1-L consisting of SEQ ID NO: 1014, a CDR2-L consisting of SEQ ID NO: 2014, a CDR3-L consisting of SEQ ID NO: 3014, a CDR1-H consisting of SEQ ID NO: 4014, a CDR2-H consisting of SEQ ID NO: 5014 and a CDR3-H consisting of SEQ ID NO: 6014; (c) a CDR1-L consisting of SEQ ID NO: 12004, a CDR2-L consisting of SEQ ID NO: 12014, a CDR3-L consisting of SEQ ID NO: 12024, a CDR1-H consisting of SEQ ID NO: 12039, a CDR2-H consisting of SEQ ID NO: 12049, and a CDR3-H consisting of SEQ ID NO: 12059; (d) a CDR1-L consisting of SEQ ID NO: 12005, a CDR2-L consisting of SEQ ID NO: 12015, a CDR3-L consisting of SEQ ID NO: 12025, a CDR1-H consisting of SEQ ID NO: 12040, a CDR2-H consisting of SEQ ID NO: 12050, and a CDR3-H consisting of SEQ ID NO: 12060; (e) a CDR1-L consisting of SEQ ID NO: 12006, a CDR2-L consisting of SEQ ID NO: 12016, a CDR3-L consisting of SEQ ID NO: 12026, a CDR1-H consisting of SEQ ID NO: 12041, a CDR2-H consisting of SEQ ID NO: 12051, and a CDR3-H consisting of SEQ ID NO: 12061; (f) a CDR1-L consisting of SEQ ID NO: 12007, a CDR2-L consisting of SEQ ID NO: 12017, a CDR3-L consisting of SEQ ID NO: 12027, a CDR1-H consisting of SEQ ID NO: 12042, a CDR2-H consisting of SEQ ID NO: 12052, and a CDR3-H consisting of SEQ ID NO: 12062; or (g) a CDR1-L consisting of SEQ ID NO: 12008, a CDR2-L consisting of SEQ ID NO: 12018, a CDR3-L consisting of SEQ ID NO: 12028, a CDR1-H consisting of SEQ ID NO: 12043, a CDR2-H consisting of SEQ ID NO: 12053, and a CDR3-H consisting of SEQ ID NO: 12063. In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:114.

[0079] In some embodiments, the ABP comprises: (a) a CDR1-L consisting of any one of SEQ ID NOs:, a CDR2-L consisting of any one of SEQ ID NOs: 1001-1028, a CDR3-L consisting of any one of SEQ ID NOs: 2001-2028, a CDR1-H consisting of any one of SEQ ID NOs:, a CDR2-H consisting of consisting of any one of SEQ ID NOs: 3001-3028. In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to any one of SEQ ID NOs:1-28 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:1-128.

[0080] In some embodiments, the ABP comprises a lysine at amino acid position 97 (R97) according to IMGT exon numbering. In some embodiments, the ABP comprises a lysine at amino acid position 97 (R214) according to EU numbering. In some embodiments, the ABP comprises an afucosylated Fc region. In some embodiments, produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof. In some embodiments, the cell is cultured in the absence of fucose. In some embodiments, produced from a cell lacking or with reduced expression of Fut8. In some embodiments, produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF). In some embodiments, produced from a cell overexpressing glycosyltransferase (GnTIII). In some embodiments, having been isolated based on its fucosylation status. In some embodiments, the ABP comprises an Fc region lacking core fucosylation of the N-glycan of the Fc portion. In some embodiments, the ABP is an afucosylated monoclonal antibody.

[0081] Aspects of the present disclosure include a pharmaceutical composition comprising the ABP of the present disclosure, and a pharmaceutically acceptable excipient.

[0082] In some embodiments, less than 50% of the ABP is fucosylated. In some embodiments, less than 40% of the ABP is fucosylated. In some embodiments, less than 30% of the ABP is fucosylated. In some embodiments, less than 20% of the ABP is fucosylated. In some embodiments, less than 10% of the ABP is fucosylated. In some embodiments, more than 30% of the ABP is fucosylated. In some embodiments, more than 40% of the ABP is fucosylated. In some embodiments, more than 50% of the ABP is fucosylated. In some embodiments, more than 60% of the ABP is fucosylated. In some embodiments, more than 70% of the ABP is fucosylated. In some embodiments, more than 80% of the ABP is fucosylated. In some embodiments, more than 90% of the ABP is fucosylated.

[0083] In some embodiments, the pharmaceutical composition has a pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises 170mM or 270mM of sucrose. In some embodiments, the pharmaceutical composition comprises 5 mg/mL to 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 5 mg/mL of the ABP.

[0084] Aspects of the present disclosure provide a method of treating a disease comprising the step of: administering to a subject in need thereof an effective amount of the ABP or the pharmaceutical composition.

[0085] In some embodiments, the disease is selected from the group consisting of cancer, AIDS, Alzheimer's disease and viral or bacterial infection. In some embodiments, the disease is selected from the group consisting of autoimmune disease, autoinflammatory disease, and inflammation.

[0086] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, the additional therapeutic agent is selected from an anti-PD-L1, an anti-PD1, a TIGIT inhibitor, a LAG-3 inhibitor, a CD47 inhibitor, a BRAF inhibitor, a MEK inhibitor, a PI3K inhibitor, a chemotherapy agent, an immune-stimulatory agent, radiation, a cytokine, a polynucleotide encoding a cytokine, an oncolytic virus encoding a cytokine, and a combination thereof. In some embodiments, the method further comprises the step of adoptive cell therapy, or treatment with cancer vaccine, oncolytic virus, or anti-CD40 inhibitor.

[0087] Aspects of the present disclosure include an isolated polynucleotide encoding the ABP. Aspects of the present disclosure include a vector comprising the isolated polynucleotide. Aspects of the present disclosure provide a host cell comprising the isolated polynucleotide or the vector of the present disclosure. In some embodiments, the host cell further comprises a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase). In some embodiments, the host cell is cultured in the absence of fucose. In some embodiments, the host cell is lacking or having reduced expression of Fut8. In some embodiments, the host cell is cultured in the presence of a fucosylation inhibitor 2-Fluorfucose (2FF). In some embodiments, the host cell is overexpressing glycosyltransferase (GnTIII).

[0088] Aspects of the present disclosure provide a method of producing an isolated antigen binding protein (ABP) that specifically binds human CTLA-4, comprising inducing expression of the ABP in the host cell, and isolating the ABP.

[0089] In some embodiments, the method further comprises the step of isolating the ABP based on its fucosylation status. In some embodiments, the host cell is cultured in a cultured medium comprising a fucosylation inhibitor. In some embodiments, the fucosylation inhibitor is 2-Fluorfucose (2FF).

[0090] Aspects of the present disclosure provide a method of reducing CTLA-4^{HI} Tregs in a subject with limited proliferation of remaining Tregs comprising administering an effective dose of the ABP or the pharmaceutical composition.

[0091] In some embodiments, the subject is a human subject, optionally, a human subject with melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, MSI colorectal cancer, ovarian cancer, or cervical cancer. In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, the additional therapeutic agent is an anti-PD-L1 or an anti-PD1 or a combination thereof. In some embodiments, the subject has a tumor with high levels of Tregs, high levels of CTLA-4, high levels of NK cells, or high levels of activating FcRs.

[0092] In another aspect, the present disclosure provides an isolated antigen binding protein (ABP) that specifically binds to an antigen, comprising an IGHG1*01 human heavy chain constant region gene segment.

[0093] In some embodiments, the ABP comprises a lysine at amino acid position 97 (R97) according to IMGT exon numbering. In some embodiments, the ABP comprises a lysine at amino acid position 97 (R214) according to EU numbering.

[0094] In some embodiments, the ABP comprises an afucosylated Fc region. In some embodiments, the ABP is produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof. In some embodiments, the cell is cultured in the absence of fucose. In some embodiments, produced from a cell lacking or with reduced expression of Fut8. In some embodiments, produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF).

[0095] In some embodiments, the ABP is produced from a cell overexpressing glycosyltransferase (GnTIII). In some embodiments, the ABP has been isolated based on its fucosylation status. In some embodiments, the ABP comprises an Fc region lacking core fucosylation of the N-glycan of the Fc portion. In some embodiments, the ABP is an afucosylated monoclonal antibody.

[0096] In some embodiments, the ABP is selected from an anti-CTLA-4 antibody or antigen-binding fragment thereof, anti-PD-L1 antibody or antigen-binding fragment thereof, an anti-PD1 antibody or antigen-binding fragment thereof, a TIGIT antibody or antigen-binding fragment thereof, a LAG-3 antibody or antigen-binding fragment thereof, a CD47 antibody or antigen-binding fragment thereof, a BRAF antibody or antigen-binding fragment thereof, a MEK antibody or antigen-binding fragment thereof, an OX40 antibody or antigen-binding fragment thereof, a 41BB antibody or antigen-binding fragment thereof, and a PI3K antibody or antigen-binding fragment thereof.

[0097] In some embodiments, the ABP comprises: (a) a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID NO:12080, a CDR1-H consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077; (b) a CDR1-L consisting of SEQ ID NO: 1014, a CDR2-L consisting of SEQ ID NO: 2014, a CDR3-L consisting of SEQ ID NO: 3014, a CDR1-H consisting of SEQ ID NO: 4014, a CDR2-H consisting of SEQ ID NO: 5014 and a CDR3-H consisting of SEQ ID NO: 6014; (c) a CDR1-L consisting of SEQ ID NO: 12004, a CDR2-L consisting of SEQ ID NO: 12014, a CDR3-L consisting of SEQ ID NO: 12024, a CDR1-H consisting of SEQ ID NO: 12039, a CDR2-H consisting of SEQ ID NO: 12049, and a CDR3-H consisting of SEQ ID NO: 12059; (d) a CDR1-L consisting of SEQ ID NO: 12005, a CDR2-L consisting of SEQ ID NO: 12015, a CDR3-L consisting of SEQ ID NO: 12025, a CDR1-H consisting of SEQ ID NO: 12040, a CDR2-H consisting of SEQ ID NO: 12050, and a CDR3-H consisting of SEQ ID NO: 12060; (e) a CDR1-L consisting of SEQ ID NO: 12006, a CDR2-L consisting of SEQ ID NO: 12016, a CDR3-L consisting of SEQ ID NO: 12026, a CDR1-H consisting of SEQ ID NO: 12041, a CDR2-H consisting of SEQ ID NO: 12051, and a CDR3-H consisting of SEQ ID NO: 12061; (f) a CDR1-L consisting of SEQ ID NO: 12007, a CDR2-L consisting of SEQ ID NO: 12017, a CDR3-L consisting of SEQ ID NO: 12027, a CDR1-H consisting of SEQ ID NO: 12042, a CDR2-H consisting of SEQ ID NO: 12052, and a CDR3-H consisting of SEQ ID NO: 12062; or (g) a CDR1-L consisting of SEQ ID NO: 12008, a CDR2-L consisting of SEQ ID NO: 12018, a CDR3-L consisting of SEQ ID NO: 12028, a CDR1-H consisting of SEQ ID NO: 12043, a CDR2-H consisting of SEQ ID NO: 12053, and a CDR3-H consisting of SEQ ID NO: 12063.

[0098] In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:114.

[0099] In some embodiments, the ABP comprises: (a) a CDR1-L consisting of any one of SEQ ID NO: 12081:, a CDR2-L consisting of SEQ ID NO: 12082, a CDR3-L consisting of SEQ ID NO: 12083, a CDR1-H consisting of SEQ ID NO: 12084, a CDR2-H consisting of consisting of SEQ ID NO: 12085, and a CDR3-H consisting of SEQ ID NO: 12086.

[00100] In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO: 12088 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO: 12087.

[00101] In some embodiments, the pharmaceutical composition has a pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises 170mM or 270mM of sucrose. In some embodiments, the pharmaceutical composition comprises 5 mg/mL to 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 20 mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 5 mg/mL of the ABP.

[00102] Aspects of the present disclosure include a method of treating a disease comprising the step of: administering to a subject in need thereof an effective amount of the ABP or the pharmaceutical composition.

[00103] In some embodiments, the disease is selected from the group consisting of cancer, AIDS, Alzheimer's disease and viral or bacterial infection. In some embodiments, the disease is selected from the group consisting of autoimmune disease, autoinflammatory disease, and inflammation. In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject.

[00104] In some embodiments, the additional therapeutic agent is selected from a chemotherapy agent, an immune-stimulatory agent, radiation, a cytokine, a polynucleotide encoding a cytokine, an oncolytic virus encoding a cytokine, and a combination thereof.

[00105] Aspects of the present disclosure include an isolated polynucleotide encoding the ABP. Aspects of the present disclosure include a vector comprising the isolated polynucleotide. Aspects of the present disclosure provide a host cell comprising the isolated polynucleotide or the vector of the present disclosure. In some embodiments, the host cell further comprises a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase). In some embodiments, the host cell is cultured in the absence of fucose. In some embodiments, the host cell is lacking or having reduced expression of Fut8. In some embodiments, the host cell is cultured in the presence of a fucosylation inhibitor 2-Fluorfucose (2FF). In some embodiments, the host cell is overexpressing glycosyltransferase (GnTIII).

[00106] Aspects of the present disclosure provide a method of producing an isolated antigen binding protein (ABP) that specifically binds human CTLA-4, comprising inducing expression of the ABP in the host cell, and isolating the ABP.

[00107] In some embodiments, the method further comprises the step of isolating the ABP based on its fucosylation status. In some embodiments, the host cell is cultured in a cultured medium comprising a fucosylation inhibitor. In some embodiments, the fucosylation inhibitor is 2-Fluorfucose (2FF).

[00108] Aspects of the present disclosure provide a method of reducing CTLA-4^{HI} Tregs in a subject with limited proliferation of remaining Tregs comprising administering an effective dose of the ABP or the pharmaceutical composition.

[00109] In some embodiments, the subject is a human subject, optionally, a human subject with melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, MSI colorectal cancer, ovarian cancer, or cervical cancer.

[00110] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject. In some embodiments, the subject has a tumor with high levels of Tregs, high levels of CTLA-4, high levels of NK cells, or high levels of activating FcRs.

[00111] Aspects of the present disclosure provide a method of reducing CTLA-4^{HI} Tregs in a subject with limited proliferation of remaining Tregs comprising administering to the subject an effective dose of an antigen binding protein (ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4 (CTLA-4).

[00112] In some embodiments, the subject is a human subject, optionally, a human subject with melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, MSI colorectal cancer, ovarian cancer, or cervical cancer. In some embodiments, the cancer patient has melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, hepatocellular carcinoma, esophageal cancer, breast cancer, sarcoma, MSI-Hi/dMMR colorectal cancer, ovarian cancer, or cervical cancer, bladder, prostate, TMB-HI tumors of any origin, a tumor which is MSI, a tumor that is dMMR, a T cell leukemia/lymphoma, NHL, a tumor expressing CTLA-4 by the cancer cell.

[00113] In some embodiments, the method further comprises the step of administering one or more additional therapeutic agents to the subject.

[00114] In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:114.

[00115] In some embodiments, the ABP comprises: (a) a CDR1-L consisting of any one of SEQ ID NOs:, a CDR2-L consisting of any one of SEQ ID NOs: 1001-1028, a CDR3-L consisting of any one of SEQ ID NOs: 3001-3028, a CDR1-H consisting of any one of SEQ ID NOs:, a CDR2-H consisting of consisting of any one of SEQ ID NOs: 3001-3028.

[00116] In some embodiments, the ABP comprises a variable light chain (V_L) comprising a sequence at least 97% identical to any one of SEQ ID NOs:1-28 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:101-128.

6. BRIEF DESCRIPTION OF THE DRAWINGS

[00117] **FIG. 1** summarizes the method of generating scFv libraries from B cells isolated from mice in which the antibody variable regions are fully human and selecting for a yeast expressing an scFv having affinity to the antigen derived from a B cell expressing an antibody having affinity to the antigen. FIG. 1 discloses SEQ ID NOS 11971-11998, respectively, in order of appearance.

[00118] **FIG. 2** illustrates scFv amplification procedure. First, a mixture of primers directed against the IgK C region, the IgG C region, and all V regions is used to separately amplify IgK and IgH. Second, the V-H and C-K primers contain a region of complementarity that results in the formation of an overlap extension amplicon that is a fusion product between IgK and IgH. The region of complementarity comprises a DNA sequence that encodes a Gly-Ser rich scFv linker sequence. Third, semi-nested PCR is performed to add adapters for Illumina sequencing or yeast display.

[00119] **FIG. 3** includes a schematic for the monoclonal antibodies sorted in their epitope bins as determined by high-throughput Array SPR.

[00120] **FIG. 4A-4E** include plots from the histopathological staining of hCTLA-4 KI mice bearing MC38 tumors treated with PBS or an anti-CTLA-4 ABP. The plots show scoring of H&E (FIG. 4A), immunoglobulin (Ig) (FIG. 4B and FIG. 4C), and C3 stains (FIG. 4D and FIG. 4E) from the right kidney. ipi is Ipilimumab, and CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone.

[00121] **FIG. 5** includes a plot showing the alkaline phosphatase levels in treated hCTLA4 KI mice bearing MC38 tumors. IPI is Ipilimumab, and CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone. U/L is units per liter.

[00122] **FIG. 6** includes plots for the percentages of intratumoral regulatory T cells (Treg) cells and intratumoral natural killer (NK) cells after the indicated treatments.

[00123] **FIG. 7** includes a plot showing the changes in body weight of the hCTLA4 mice receiving the indicated treatments. Ipi is Ipilimumab, and CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone. Error bars represent +/- standard error of the mean.

[00124] **FIG. 8A-8F** include plots showing the influence of the control, Ipi and the anti-CTLA4 (CTLA4.A2, CTLA4.A14, CTLA4.A14.2a) treatments on percentage of the indicated cell populations, including CD3+ cells (FIG. 8A), CD4+ cells (FIG. 8B), CD69+ cells (FIG. 8C), ICOS+ cells (FIG. 8D), PD1+ cells (FIG. 8E), and FOXP3+ cells (FIG. 8F) in hCTLA-4 KI mice implanted with MC38 tumor cell. Ipi is Ipilimumab, and CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone.

[00125] **FIG. 9A-9D** include plots showing the influence of the control, Ipi and the anti-CTLA-4 (CTLA4.A2, CTLA4.A14, CTLA4.A14.2a) treatments on percentage of the indicated cell populations, including CD8+ cells (FIG. 9A), CD69+ cells (FIG. 9B), ICOS+ cells (FIG. 9C) and PD1+ cells (FIG. 9D) in hCTLA-4 KI mice implanted with MC38 tumor cell. Ipi is Ipilimumab, and CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone.

[00126] **FIG. 10** includes plots showing the influence of the control, Ipi and the anti-CTLA-4 treatments on percentage of dendritic cells (DCs) and activated dendritic cells (CD86+). Ipi is Ipilimumab, and CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone.

[00127] **FIG. 11** includes a plot showing the mean tumor volume after treatment with 0.3 mg/kg of the indicated anti-CTLA-4s.

[00128] **FIG. 12A-12B** shows that FcR effector function is required for the anti-tumor efficacy of ipilimumab in a murine model. **FIG. 12A**. The plot shows the frequency of CD4+FOXP3- T cells from the LN of individual control (C57BL/6) or hCTLA-4 KI mice that are CD44LoCD62L+ as determined by flow cytometry (mean +/- SEM). **FIG. 12B**. hCTLA-4 KI mice bearing MC38 tumors were randomized when tumors were 50 – 151 mm3 (day 0) and treated bi-weekly with the indicated antibody at 5 mg/kg for 5 doses. The plot shows the tumor volume (mean ± SEM) of MC38 tumors over time that were treated with the indicated antibody. Tumor volume from mice euthanized due to tumor burden above 3000 mm^3 was carried forward. Thin vertical lines indicate censored data: Thin gray vertical lines indicate animals likely lost post dosing due to anti-drug antibody (ADA)-induced hypersensitivity, thin black vertical lines refer to animals euthanized due to tumor burden and for which the data was carried forward. Ipi analog indicates an ipilimumab mAb produced and purified by Applicant; N297Q indicates that the antibody contains an N297Q mutation in the Fc domain of the antibody to abrogate Fc effector function. n = 8 for isotype, aCTLA-4.28, Ipi-N297Q; n=7 for ipi analog and aCTLA-

4.28. p = 0.0004 when comparing Ipi analog to isotype and p = 0.0006 when comparing aCTLA-4.28 to isotype for change in tumor volume between groups (linear mixed effects model).

[00129] **FIG. 13A-13D** show that GIGA-564 has little ability to block the interaction of CTLA-4 and CD80/CD86 *in vitro*. **FIG. 13A.** The ability of CTLA-4 mAbs to block binding of CD80 or CD86 was measured using a plate-based ELISA method. CTLA-4 was used to coat the plate, then after the antibody samples were incubated, His-tagged CD80 or CD86 was added, and the amount of ligand able to bind to CTLA-4 was measured. Blocking mAbs that prevent CD80 or CD86 from binding to CTLA-4 reduce the absorbance signal due to lack of CD80 or CD86 binding to CTLA-4. Weak-blocking mAbs still allow CD80 or CD86 to bind, preventing the loss of all absorbance signal. **FIG. 13B**. Plots show the ability of GIGA-564 to block the interaction between CTLA-4 and the B7 ligands CD80 and CD86 compared to ipilimumab and CTLA-4.28, as assessed by ELISA as described in (**FIG. 13A**). Absorbance values were normalized to an anti-PD-1 control (pembrolizumab) and displayed as the average of two technical replicates. **FIG. 13C-13D**. The key residues mediating CTLA-4 binding were identified for GIGA-564 and ipilimumab by shotgun mutagenesis of CTLA-4, followed by staining and flow cytometry assessment of binding. **FIG. 13C.** Shown is the crystal structure (Protein Database [PDB] 1I8L) of the complex between CD80 and CTLA-4 on which the CTLA-4 epitope residues shared between ipilimumab and GIGA-564 and the key differentiating residue R70 are highlighted (visualized with Pymol). Additionally, G142 was identified as a secondary residue for the epitope of ipilimumab but not GIGA-564. **FIG. 13D**. Table showing key amino acids on CTLA-4 of interest for these epitopes; those found by mutational analysis to be important for binding of CTLA-4 to CD80 or CD86 in a cell-based assay are marked in gray to indicate the epitope residues for those proteins.

[00130] **FIG. 14A-14C** show that GIGA-564 inhibits tumor growth in murine models. **FIG. 14A.** hCTLA-4 KI mice bearing MC38 tumors were randomized when tumors were 50 – 150 mm^3 (day 0) and treated bi-weekly with the indicated antibody at 5 mg/kg for 5 doses. Plots show the tumor volume (mean ± SEM) of MC38 tumors over time that were treated with the indicated antibody. Tumor volume from mice euthanized due to tumor burden above 3000 mm^3 was carried forward. Thin gray vertical lines indicate censored data (animals likely lost post dosing due to ADA-induced hypersensitivity). This experiment is also described in **FIG. 12B**. n = 7 for GIGA-564 and n = 6 for vehicle and commercial ipilimumab. **FIG. 14B.** hCTLA-4 KI mice bearing RM-1 tumors were randomized when tumors were 40 – 125 mm^3 (day 0) and treated on day 0, 3, and 6 with 5 mg/kg of the indicated antibody. Plots show the tumor volume (mean ± SEM) of RM-1 tumors over time that were treated with the indicated antibody. Tumor

volume from mice euthanized due to tumor burden above 3000 mm^3 was carried forward until no mice from that group were alive (thin black vertical lines). Thin vertical lines indicate censored data. The thin gray vertical line indicates one mouse from the ipilimumab treated group was euthanized due to tumor ulceration. n = 11 for ipilimumab and GIGA-564 treatment and n = 7 for isotype treatment. **FIG. 14C** (as is also shown in FIG. 11). hCTLA-4 KI mice bearing MC38 tumors were randomized when tumors were 65 – 125 mm^3 (day 0) and treated on days 0, 3, and 6 with 0.3 mg/kg of the indicated antibody. Plots show the tumor volume (mean ± SEM) of MC38 tumors over time that were treated with the indicated antibody. Tumor volume from mice euthanized due to tumor burden above 3000 mm^3 was carried forward until no mice from that group remained alive (thin black vertical lines). n = 13 for ipilimumab and GIGA-564 and n = 8 for isotype treated animals. P-values are shown for statistically significant differences in changes in tumor volume measured longitudinally (linear mixed effects model).

[00131] **FIG. 15A-15E** shows that GIGA-564 induces less peripheral Treg proliferation but potently mediates depletion of intratumoral Tregs. **FIG. 15A.** hCTLA-4 KI mice (n = 12) were treated with 5 mg/kg hIgG1 isotype control, ipilimumab, or GIGA-564 on days 0, 3, and 6, and euthanized for flow cytometry analysis on day 7. Two samples in the GIGA-564 group were excluded from analysis due to low cell count. The percent of CD8 T cells (Live, $CD45^+TCR\beta^+CD8^+$), CD4 Tconv (Live, $CD45^+TCR\beta^+CD4^+FOXP3^-$), and Tregs (Live, $CD45^+TCR\beta^+CD4^+FOXP3^+$) within the non-draining (left anterior axillary) lymph node (LN) expressing Ki67 was determined by flow cytometry (first 3 panels). The right panel shows the fold-change in the percent of cells of each subtype expressing Ki67 in ipilimumab or GIGA-564 treated mice relative to the mean frequency of that cell type expressing in the isotype control treated group. In this right panel, p-values were calculated using the Mann-Whitney (Wilcoxon) test without adjustment for multiple pairwise comparison. Fewer proliferating Tregs may further enhance efficacy as compared to ipilimumab in patients. **FIG. 15B-15E.** hCTLA-4 KI mice bearing established MC38 tumors were randomized (n = 6) and treated once with 5 mg/kg hIgG1 isotype control, ipilimumab, or GIGA-564 and cells from the non-draining LN and tumor were analyzed the following day by flow cytometry. **FIG. 15B.** Frequency of Tregs (as percentage of $CD45^+$ cells) in LN (left) and tumor (right) in each treatment group. **FIG. 15C.** CTLA-4 geometric mean fluorescence intensity (MFI) in Tregs in LN (left) and tumor (right). **FIG. 15D.** Representative contour plots of CD4 T cells in each treatment group. X-axis and Y-axis correspond to FOXP3 and CTLA-4, respectively. **FIG. 15E.** Ratio of CD8 T cells relative to Tregs in LN (left) and tumor (right). Unless otherwise indicated, p-values were calculated using Wilcoxon rank sum test and horizontal lines indicate means. Flow cytometry showed that GIGA-

564 is more efficient than ipilimumab at depleting CTLA-4+ Tregs in the tumor microenvironment. Data from **FIG. 15D** is reshown in FIG. 32.

[00132] **FIGs. 16A-16D** shows that GIGA-564 induces more FcR signaling than ipilimumab. **FIGs. 16A-16D.** Target CHO cells expressing human CTLA-4 with the Y201G mutation to enhance surface expression were incubated with a titration series of ipilimumab (black squares), GIGA-564 (black stars), or a variant of GIGA-564 with LALA-PG mutations to disrupt FcγR binding (GIGA-564_LALA-PG, gray circles). Jurkat/NFAT-Luc effector cells with (**FIG. 16A**) mouse FcγRIV or FcγRIII, (**FIG. 16B**) human FcγRIIIa (high-affinity V158 or low-affinity F158 variant), (**FIG. 16C**) human FcγRIIa (high-affinity H131 or low-affinity R131 variant), or (**FIG. 16D**) human FcγRIIb were then added. Cells were incubated for 6 hours at 37°C then luciferase activity was measured. Data shown are relative luminescence units (RLU) emitting from the effectors cells and are plotted as the average of two technical replicates.

[00133] **FIGs. 17A-17B** shows GIGA-564 provides protection in a murine tumor re-challenge model. **FIG. 17A.** hCTLA-4 KI mice bearing MC38 tumors were randomized when tumors were 60 – 120 mm^3 (day 8) and then treated every 3 days for 3 doses with the indicated antibody at 1 mg/kg. Plots show the tumor volume (mean ± SEM) of MC38 tumors over time that were treated with the indicated antibody. Tumor volume from mice euthanized due to tumor burden above 3000 mm^3 was carried forward (thin vertical black lines). n = 8 for isotype and commercial ipilimumab, n = 9 for GIGA-564. **FIG. 17B.** MC38 cells were implanted on the opposite flank of naïve C57BL/6 mice and mice from (**FIG. 17A**) that were previously treated with ipilimumab or GIGA-564 and had a durable complete response on day 43 (0 mm^3 tumor volume). Plots show the tumor volume (mean ± SEM) of MC38 tumors over time on mice that were previously treated with the indicated antibody or naïve mice (n = 5). Tumor volume (in mm^3) was analyzed using a linear mixed effects model including treatment group and day as fixed effects and animal identifier (ID) as a random effect to account for repeated measures. Statistical comparisons were made using the Wald test against the isotype control group in the initial challenge model and against the naïve control group in the re-challenge model. Compared to the naïve group, previous treatment with ipilimumab ($p = 0.0089$) or GIGA-564 ($p = 0.0088$) limited tumor growth.

[00134] **FIGs. 18A-18C** shows that GIGA-564 plus pembrolizumab induces less toxicity than ipilimumab plus pembrolizumab in a murine model. hCTLA-4/hPD-1 double KI mice on the BALB/c background between 4-5 weeks of age were treated with vehicle, pembrolizumab, pembrolizumab plus ipilimumab (Ipi), or pembrolizumab plus GIGA-564 every 3 days for 9

doses. One week after the last dose mice were euthanized and tissues collected for pathology analysis. **FIG. 18A.** Plot shows percent change in body weight over time in mice treated with the indicated therapy (mean ± SEM, n = 10). Four mice from the pembrolizumab plus ipilimumab group died on day 12 and from the pembrolizumab plus GIGA-564 treated group three mice died on day 12 and one mouse died on day 15, all likely due to post dosing ADA-induced hypersensitivity. A mixed effect model found no statistical differences in the percent body weight change between groups. **FIGs. 18B-18C.** Plots show skin inflammation (**FIG. 18B**) or colonic epithelial damage (colitis; **FIG. 18C**) scores (mean +/- SEM) induced by each treatment regimen. Horizontal lines indicate the median. Adjusted p-values were calculated using the Benjamini-Hochberg step-down procedure to account for multiple comparisons.

[00135] **FIG. 19** shows a Model depicting ipilimumab and GIGA-564 mechanisms of action. Top panel: Ipilimumab blocks CTLA-4 interaction with CD80/CD86, which allows antigen presenting cells (APCs) to co-stimulate peripheral Tregs enhancing their proliferation. GIGA-564 weakly blocks CTLA-4 interaction with CD80/CD86 and thus induces less Treg proliferation. Bottom panel: Ipilimumab and GIGA-564 bind CTLA-4 on intratumoral Tregs to induce Treg killing via interactions with Fc receptor (FcR) on effector cells. GIGA-564 induces stronger FcR signaling and thus more efficiently depletes intratumoral Tregs than ipilimumab.

[00136] **FIGs. 20A-20E** shows in vitro characterization of scFvs reformatted as full-length antibodies. **FIG. 20A**. Clonal cluster analysis for the FACS-enriched anti-CTLA-4 scFv clones. Each node represents an scFv clone (full-length IgK+IgH). The total number of amino acid differences were computed between each pairwise alignment of scFv sequences. Edges indicate pairwise alignments with $\leq$9 amino acid differences (Clustergram modified from Fig. 7 of Asensio et al., 2019). The scFv sequence for ipilimumab (ipi) was included for comparison. scFv clones for full-length antibodies described in this study are labeled with ID numbers. **FIG. 20B**. The affinity of the indicated antibody to soluble CTLA-4 was determined by SPR (Carterra). Plots show association and dissociation signals with a 5-fold dilution series of antigen starting at 500 nM. **FIG. 20C**. A 50:50 mixture of CTLA-4$^+$ and CD27$^+$ (CTLA-4$^-$) CHO cells were stained with 10 μg/ml of the indicated antibody. An anti-human IgG secondary antibody conjugated to PE was used to detect cells labeled with the indicated anti-CTLA-4 antibody, while anti-CD27-FITC identified CD27$^+$ CHO cells. Histograms show staining of CTLA-4$^+$ (CD27-FITC$^-$) or CTLA-4$^-$ (CD27-FITC$^+$) cells by the indicated antibody as determine by flow cytometry. **FIG. 20D**. The cell-based CTLA-4 Blockade Bioassay (Promega) involved co-culturing CTLA-4-expressing Jurkat cells with Raji cells, which naturally express CD80 and CD86, in the presence of the indicated mAbs. mAbs that bind to CTLA-4 and block the ability of CTLA-4 to interact

with CD80/CD86 lead to CD28 pathway-activated luciferase expression. Plot shows the amount of luciferase expression induced (relative luciferase units; RLU) when cells were cultured with a titration series of the indicated antibody. Due to constraints on sample size in each Promega bioassay kit, this set of aCTLA-4 mAbs was analyzed using multiple plates. To control for plate-to-plate variability in maximum signal, the ipilimumab analog was run on each plate, and a representative sample was used to calculate the EC50 and maximum signal for ipilimumab in **Table 23**. The plate in which each antibody was tested is indicated in **TABLE 23**. Plate A and B were run at the same time, while Plate C and Plate D were run separately at later times. **FIG. 20E.** Correlation between antibody affinity and cell-based assay activity. Antibody affinity (KD) for CTLA-4 was compared to the blocking EC50 and the RLU maximum signal; no correlation was found for either when the data was fit by linear regression. Each data point represents a single antibody, and the following antibodies are distinguished as follows: ipilimumab analog (red square), GIGA-564 (inverted triangle), and aCTLA-4.28 (star). Data is from (**FIG. 20D**) and **TABLE 23**.

[00137] **FIG. 21** provides a validation of N297Q mutants binding to cell-surface CTLA-4. CHO cells with and without human CTLA-4 expression were incubated with 10 μg/mL of the indicated antibody, then an anti-human IgG secondary antibody conjugated to FITC was used to detect cells bound by the indicated CTLA-4 antibody. Histograms show staining of CTLA-4$^+$ (lighter gray) or CTLA-4$^-$ (darker gray) cells by the indicated antibody as determine by flow cytometry.

[00138] **FIGs. 22A-22D** shows that co-stimulation enhances Treg proliferation. Histograms show CellTrace Violet signal (gated on Live, CD3$^+$CD4$^+$ cells) of **FIG. 22A.** Treg or **FIG. 22B.** Tconv cells cultured in the presence of M-450 Tosyl activated beads coated with anti-CD3 antibody with and without CD80, or **FIG. 22C.** Treg or **FIG. 22D.** Tconv cells activated with M-450 Tosyl activated beads coated with anti-CD3 antibody plus CD80 in the presence of rhCTLA-4 (Abatacept) and/or anti-CTLA-4 mAb (aCTLA-4.28).

[00139] **FIGs. 23A-23D** show anti-CTLA-4s deplete intratumoral Tregs in hCTLA-4 KI mice. Flow cytometry analysis of cells from MC38 tumor bearing hCTLA-4 KI mice receiving CTLA-4 mAbs. **FIGs. 23A** and **23C.** 6 mice per group were treated with 5 mg/kg hIgG1 isotype control, ipilimumab, or GIGA-564 on day 0 and 3, and cells were analyzed on day 4. **FIGs. 23B** and **23D.** 12 mice per group were treated with 5 mg/kg hIgG1 isotype control, ipilimumab, or GIGA-564 on day 0, 3, and 6, and cells were analyzed on day 7. Two lymph node samples in the GIGA-564 group were excluded from analysis due to low cell count. **FIGs. 23A-23B.** Frequency

of Tregs (Live, $CD45^+TCR\beta^+CD4^+FOXP3^+$, as percentage of $CD45^+$ cells) in lymph nodes (left) and tumor (right) in each treatment group. **FIGs. 23C-23D.** Geometric mean fluorescence intensity (MFI) of intracellular CTLA-4 in Tregs in LN (left) and tumor (right). P-values were calculated using Wilcoxon rank sum test. Lines depict mean +/- SEM.

[00140] **FIG. 24A-24E** show GIGA-564 induces more FcR signaling than ipilimumab. **FIG. 24A.** Purified CTLA-4 antibodies were diluted to a starting concentration of 5 μg/mL for an 8-point, 5-fold titration series in different pH buffers, then added to wells coated with rhCTLA-4-Fc. Bound antibodies were detected with anti-constant kappa-HRP and measured for absorbance at 450 nm. **FIG. 24B.** CHO cells expressing wildtype hCTLA-4 were incubated with titrations of ipilimumab or GIGA-564 at 37°C to allow for internalization. The amount of antibody remaining on the surface was then determined by staining with anti-human IgG Fc. **FIGs. 24C-24D**. GIGA-564 from three different productions was tested for fucosylation levels (bar graphs each depict a single data point) (**FIG. 24C**) and human FcγRIIIA signaling (**FIG. 24D**). PN-2758.01 and PN-4088.01 were generated from transient transfection of ExpiCHO cells, while PN-4261.01 was from a stably-expressing pool of CHOZN clones (EP-1, enriched pool 1). **FIG. 24C.** The fucosylation level, as determined by UPLC analysis, is shown for each sample. **FIG. 24D-24E**. Graph shows human FcγRIIIA (V variant) signaling as determined in a cell-based assay for the indicated three preparations of GIGA-564 (**FIG. 24D**) or GIGA-564 and ipilimumab with the indicated amount of fucosylation (**FIG. 24E**). A negative control protein with mutations to disrupt Fc receptor binding (GIGA-564_LALAPG) was also tested in the reporter bioassays. Data shown are RLU emitting from effectors cells.

[00141] **FIGs 25A-25E** shows GIGA-564 results in less toxicity than ipilimumab in murine models. **FIGs. 25A-25D**. hCTLA-4/hPD-1 double KI mice on the BALB/c background between 4 to 5 weeks of age were treated with vehicle, pembrolizumab, pembrolizumab plus ipilimumab, or pembrolizumab plus GIGA-564 every 3 days for 9 doses. One week after the last dose mice were euthanized and tissues collected for pathology analysis. Graphs show colon length (**FIG. 25A**) or spleen weight (**FIG. 25B**) from mice of each group at time of euthanasia. The number of $CD45^+$ cells/mm^2 counted on randomly selected CD45 stained heart FFPE sections (**FIG. 25C**) or the heart pathology score (**FIG. 25D**) as determined by a pathologist are shown. **FIG. 25E.** hCTLA-4 KI mice bearing MC38 tumors were treated with vehicle (PBS) or 5 mg/kg ipilimumab or GIGA-564 twice a week for 5 doses (**FIG. 14A**). On day 20 post initiation of treatment, mice were euthanized and kidneys were processed into formalin fixed paraffin embedded blocks (FFPE). FFPE sections were stained for anti-mouse immunoglobin or C3 and

scored by a board-certified veterinary pathologist blinded from the study. Positive staining in glomeruli was along the capillary basement membranes. Graphs show percent of glomeruli positive for anti-murine IgG or C3 and the relative intensity of positive glomeruli; at least 5 glomeruli were examined in each section on 40x/high power. Data from **FIG. 25E** is also shown in **FIGs. 4A-4E.** Lines indicate mean +/- SEM. Adjusted p-value were calculated using the Benjamini-Hochberg step-down procedure to account for multiple comparisons.

[00142] **FIG. 26** shows that checkpoint inhibitor ipilimumab increases the percentage of proliferating Tregs in mice expressing humanized CTLA-4. Data from **FIG. 26** is from the same experiment as **FIG.15A**.

[00143] **FIG. 27** shows that intratumoral Treg depletion is the mechanism of action for anti-CTLA-4, not checkpoint inhibition. FIG. 27 (left) shows that Fc effector function in anti-CTLA-4 is necessary for robust anti-tumor responses. As shown, tumor volume decreased with ipilimumab and GIGA-577 as compared to ADCC-deficient ipilimumab and ADCC-deficient GIGA-577. Data from **FIG. 27** includes data as shown in **FIG. 12B and 14A.**

[00144] **FIG. 28** represents that GIGA-564 anti-CTLA-4 antibody has weak checkpoint inhibitor activity, but has a strong affinity for CTLA-4. A schematic showing the conventional mechanism versus the current mechanism of action as described in the present application is compared. Depletion of Tregs in the tumor after treatment with GIGA-564 is through ADCC/ADCP binding, rather than the interaction of CTLA-4 and its ligands.

[00145] **FIG. 29** shows that GIGA-564 weakly blocks CD80/CD86 binding interactions to CTLA-4 as compared to ipilimumab. **FIG. 29** may reshow data shown in **FIG. 20D** (plate A).

[00146] **FIG. 30** shows that GIGA-564 has superior anti-tumor activity compared to ipilimumab. Humanized CTLA-4 knock-in mice bearing MC38 tumors (n = 8-13) were dosed with GIGA-564 or ipilimumab at 0.3 mpk on days 0, 3, and 6 of the study. Following administration of GIGA-564, human CTLA-4 knock-in mice having MC38 tumors showed reduced tumor volume and an increased probability of survival, as compared to ipilimumab and the isotype. Experiments described in **FIG. 30** is also described in **FIGs. 11** and **14C**. Tumor growth data from **FIG. 30** includes data as shown in **FIGs. 11** and **14C**.

[00147] **FIG. 31** shows that GIGA-564 induces less Treg proliferation than Ipilimumab. Human CTLA-4 knock-in mice bearing MC38 tumors were treated with 5 mpk of ipilimumab or GIGA-564 on days 0, 3, and 6 and sacrificed on Day 7. GIGA-564 treatment resulted in fewer proliferating Tregs in the periphery. Experiments described here was also described in **FIG. 15A**. **FIG. 31** includes data previously shown in **FIG. 15A**.

[00148] **FIG. 32** shows representative contour plots of CD4 T cells in each treatment group. X-axis and Y-axis correspond to FOXP3 and CTLA-4, respectively. **FIG. 32** reshows results from **FIGs. 15D**.

[00149] **FIG. 33** shows that GIGA-564 induces more FcR signaling than Ipilimumab when co-cultured with hCTLA-4+ cells. Cellular signaling via CTLA-4-Fc-FcR interaction was assessed *in vitro* with human CTLA-4+ cells. GIGA-564 showed increased FcR signaling over ipilimumab. Further experiments ruled out that the difference is due to Fc-FcR affinity or CTLA-4 cells surface cycling. **FIG. 33** may reshow some data from **FIG. 16B**.

[00150] **FIG. 34** provides the development and validation of CHO cells expressing cynomolgus (cyno) CTLA-4 on the cell surface. Flow histograms show that the cyno CTLA-4 and hCTLA-4 CHO cell lines express antigen on the cell surface bound by anti-CTLA-4 clone L3D10 or BNI3, this validates expression of cyno or human CTLA-4 on the surface of these cell lines, respectively.

[00151] **FIG. 35** shows that GIGA-564 has reduced ability to bind surface expressed cyno CTLA-4 compared to Ipilimumab. **FIG. 35** shows flow cytometric analysis of binding of mAbs to cyno or human CTLA-4+ CHO cells. The data shows that while GIGA-564 and Ipi have relatively similar ability to bind hCTLA-4; compared to Ipi, GIGA-564 has a greatly reduced ability to bind to cyno CTLA-4 expressed on the cell surface. Data from atezo binding (negative control) is reshown on multiple plots for reference.

[00152] **FIG. 36A-36E** show that GIGA-564 has reduced binding to some presentations of cyno CTLA-4 compared to Ipilimumab as tested using an ELISA assay. In these ELISAs binding of GIGA-564 to various CTLA-4 proteins sourced from multiple manufacturers, including Fc chimera and his-tagged formats, was tested. To test the binding of GIGA-564 to Fc chimera CTLA-4 proteins, rcmCTLA-4-Fc from R&D systems (9336-CT-200, **FIG. 36A**), Sino Biological (90213-C02H, **FIG. 36B**), or ACROBiosystems (CT4-C5256, **FIG. 36C**) were coated at 1 ug/mL on one half of separate ELISA plates. Recombinant human CTLA-4-Fc (rhCTLA-4) from R&D Systems (7268-CT-100, **FIGs. 36A-36C**) was coated at 1 ug/mL, on the other half of each of the plates. Similarly, to test the binding of GIGA-564 to his-tagged CTLA-4, rcmCTLA-4-His from Sino Biological (90213-C08H, **FIGs. 36D**) or ACROBiosystems (CT4-C5227, **FIGs. 36E**) were coated at 1ug/mL on one half of separate ELISA plates. RhCTLA-4-Fc (ACROBiosystems, CT4-H5229, **FIGs. 36D-E**) was coated at 1 ug/mL, on the other half of each of these plates. After coating, the plates were incubated overnight at 4°C. The following day, the plates were blocked with 5% milk in PBST for 1 hour on a plate shaker at room temperature.

Titration series of ipilimumab, atezolizumab (negative control), and GIGA-564 (GG-564) (starting at 5 ug/mL) were added to the plates and incubated on a plate shaker for 1 hour at room temperature to allow mAb binding. Excess, unbound mAbs were removed by washing with PBST. Bound mAbs were then detected with an HRP-conjugated anti-kappa light chain antibody (0.5 ug/ml, Southern Biotech 2060-50). After incubation on a plate shaker for 1 hour at room temperature and washing, the plates were developed with TMB substrate. After sufficient signal was achieved, 1 N hydrochloric acid was added to stop development. Absorbance at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices). EC50 values were calculated by plotting absorbance vs. the log of concentration using Prism (GraphPad). As Ipi has similar binding to human and cyno CTLA-4 but GIGA-564 in most cases has reduced ability to bind cyno CTLA-4 compared to human CTLA-4 the results suggest that the Ipi and GIGA-564 epitopes are different in practice.

[00153] **FIG. 37** provides response plots generated to determine an optimized formulation for the GIGA-564. Response plots showed the impact of pH and sucrose concentration when NaCl and buffer concentration are fixed at 100mM and 30mM, respectively

[00154] **FIG. 38** shows theoretical response plots generated to determine the buffer concentration and NaCl amount expected to result in the highest Tm for the formulation of GIGA-564 antibody as shown.

[00155] **FIG. 39** shows a pareto analysis that provided which variables had the most impact on the formulation for GIGA-564.

[00156] **FIG. 40** provides a conformational analysis of a formulation for GIGA-564. The plots show that high NaCl is the most important for conformation, pH has some effect, with lower pH being better, and sucrose has some effect, with higher being better.

[00157] **FIG. 41** shows that GIGA-2328 is designed to have low fucosylation for the purpose of enhancing FcγRIIIa signaling. An ADCC reporter bioassay for human FcγRIIIa, V variant (G7011) from Promega Corporation was carried out following the manufacturer's instructions. Briefly, CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were suspended in RPMI 1640 + 4% FBS media with the indicated antibody and incubated at 37°C for 30 minutes. Jurkat/NFAT-Luc effector cells expressing human FcγRIIIa, V variant were added to each well at an effector:target ratio of 5:1 and incubated at 37°C for 6 hours. Luciferase activity was measured by using the included Bio-Glo Luciferase Assay Reagent with the SpectraMax i3x reader. Luciferase activity measured in relative luminescence units (RLU) were plotted against the concentration of antibody.

[00158] **FIGs. 42A-42B** shows that GIGA-564 induces antibody-dependent cellular cytotoxicity (ADCC) and/or antibody-dependent cellular phagocytosis (ADCP) by human PBMCs against a target cell line expressing CTLA-4. Cryopreserved human PBMCs from two donors were thawed and recovered overnight in RMPI media containing 100 U/mL IL-2 and 10% FBS. CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were stained with CellTrace Violet (Thermo Fisher), then incubated with either GIGA-564 or a human IgG1 isotype control at the indicated concentrations at 37°C for 30 minutes. PMBC effector cells were then added at a 20:1 effector to target ratio and the samples were incubated at 37°C for 4 hours to allow for ADCC/ADCP. The samples were then washed with MACS buffer, stained with the viability dye 7-Aminoactinomycin D (7-AAD) to label dead cells, and analyzed by flow cytometry. (FIG. 42A) Representative gating strategy to determine ADCC/ADCP. Samples were first gated for target cells (CellTrace Violet+), then single cells, then dead (7-AAD+) cells. (FIG. 42B) Plots of percent dead cells at each concentration of GIGA-564 and human IgG1 isotype. GIGA-564 leads to higher percent dead CTLA-4+ target cells than the isotype control.

[00159] **FIGs. 43A-43B** show that IgG1 allotype may impact signaling via the FcγRIIIa receptor. **FIG. 43A** shows sequence information of IgG1 allotype IGHG1*08 and IGHG1*01, which differ by one amino acid in the CH1 domain. The differing residue between these two allotypes is highlighted, and surrounding residues are provided for reference. Position given in both IMGT and EU numbering. **FIG. 43B** shows clinical ipilimumab (Yervoy) uses the IGHG1*08 allotype, and results in lower FcγRIIIa signaling than ipilimumab and GIGA-564 produced by GigaGen, which use the IGHG1*01. These results suggest that allotype may impact FcγRIIIa signaling. ADCC reporter bioassays for human FcγRIIIa, V variant (G7011) were purchased from Promega Corporation. The assays were carried out following the manufacturer's instructions. Briefly, CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were suspended in RPMI 1640 + 4% FBS media with the indicated antibody and incubated at 37°C for 30 minutes. Jurkat/NFAT-Luc effector cells expressing human FcγRIIIa, V variant were added to each well at an effector:target ratio of 5:1 and incubated at 37°C for 6 hours. Luciferase activity was measured by using the included Bio-Glo Luciferase Assay Reagent with the SpectraMax i3x reader. Luciferase activity measured in relative luminescence units (RLU) were plotted against the concentration of antibody.

[00160] **FIGs. 44A-44B** show differential scanning fluorimetry (DSF) analysis results of nine formulations containing GIGA-564 (G1-G9) under four different conditions, (A) 5°C

storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks. Compositions of G1 to G9 are provided in the below table.

form.	mg/mL	pH	[buffer]	type	[NaCl]	[sucrose]	PS-20	Osmo
1	20	6.5	20mM	histidine	---	270mM	0.02%	290 mOsm/kg
2	20	6.5	20mM	histidine	50mM	270mM	0.02%	390 mOsm/kg
3	20	6.5	20mM	histidine	50mM	170mM	0.02%	290 mOsm/kg
4	20	6	20mM	histidine	---	270mM	0.02%	290 mOsm/kg
5	20	6	20mM	histidine	50mM	270mM	0.02%	390 mOsm/kg
6	20	6	20mM	histidine	50mM	170mM	0.02%	290 mOsm/kg
7	20	5.5	20mM	citrate	---	270mM	0.02%	290 mOsm/kg
8	20	5.5	20mM	citrate	50mM	270mM	0.02%	390 mOsm/kg
9	20	5.5	20mM	citrate	50mM	170mM	0.02%	290 mOsm/kg

[00161] **FIG. 45** shows dynamic light scattering (DLS) analysis results with cumulant radius for nine formulations containing GIGA-564 (G1-G9) under four different conditions, (A) 5°C storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks.

[00162] **FIGs. 46A-46B** show SE-HPLC analysis results of nine formulations containing GIGA-564 (G1-G9) under four different conditions, (A) 5°C storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks.

[00163] **FIGs. 47A-47B** show BioAnalyzer results for nine formulations containing GIGA-564 (G1-G9) under four different conditions, (A) 5°C storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks.

[00164] **FIG. 48A** and **FIG. 48B** show CTLA-4 binding affinities of GIGA-564 in formulations 1 (G1) (FIG. 48A) or 4 (G4) (FIG. 48B) tested by ELISA.

[00165] **FIG. 49A** and **FIG. 49B** show human FcgammaRIIIa-V variant signaling induced by GIGA-564 in formulations 1 (G1 or Buffer 1) (FIG. 49A) or 4 (G4 or Buffer 4) (FIG. 49B) tested by ELISA as determined by cell-based assay.

[00166] **FIG. 50A** and **FIG. 50B** show MC38 tumor sizes in hCTLA-4 KI mice over time after treatment with PBS, or the indicated amount of ipilimumab (commercial Yervoy), GIGA-564, afucosylated ipilimumab (next-generation), or GIGA-2328 (2328, GIGA-564 with more afucosylation) on days 0, 3, and 6. Data is median +/- 95% CI.

[00167] **FIG. 51A** and **FIG. 51B** provide human FcRIIIa-V activity induced by GIGA-564, GIGA-564_XF (afucosylated GIGA-564, also known as GIGA-2328, produced in a stable cell line), GIGA-2328 produced transiently, GIGA-564 with the LALA-PG mutation to eliminate Fc function, ipilimumab, ipilimumab_XF (afucosylated ipilimumab), and GIGA-564_AEX

(GIGA-564 purified by protein A and then also purified with anion exchange) as determined by cell based assay.

[00168] **FIG. 52A** provides serum concentration of GIGA-564 in NHPs after a single IV bolus administration of GIGA-564 at two different doses (3 mg/kg and 30 mg/kg). **FIG. 52B** provides dose normalized serum concentration of GIGA-564 in NHPs after a single IV bolus administration of GIGA-564 at two different doses (3 mg/kg and 30 mg/kg).

[00169] **FIG. 53** provides serum concentration of GIGA-564 in individual NHPs after a single IV bolus administration of GIGA-564 at two different doses (3 mg/kg and 30 mg/kg).

[00170] **FIG. 54A** provides serum concentration of GIGA-564 in human subjects after administration of GIGA-564 at two different doses (3 mg/kg and 30 mg/kg), as projected based on PK study in NHPs. **FIG. 54B** provides dose normalized serum concentration of GIGA-564 in human subjects after administration of GIGA-564 at two different doses (3 mg/kg and 30 mg/kg), as projected based on PK study in NHPs.

[00171] **FIG. 55A** provides serum concentration of GIGA-564 in individual human subjects after administration of GIGA-564 at two different doses (3 mg/kg and 30 mg/kg), as projected based on PK study in NHPs. **FIG. 55B** provides time profiles of serum concentration of GIGA-564 after monthly dosing of GIGA-564 in human subjects projected based on PK study in NHPs.

[00172] **FIG. 56** provides cytokine/chemokine release from human peripheral blood mononuclear cells (PBMCs) in response to GIGA-564 or controls under wet bound conditions.

[00173] **FIG. 57** provides cytokine/chemokine release from human peripheral blood mononuclear cells (PBMCs) in response to ipilimumab or controls under wet bound conditions.

[00174] **FIG. 58** provides cytokine/chemokine release from human peripheral blood mononuclear cells (PBMCs) in response to soluble GIGA-564 or controls.

[00175] **FIG. 59** provides cytokine/chemokine release from human peripheral blood mononuclear cells (PBMCs) in response to soluble ipilimumab or controls.

[00176] **FIG. 60** shows cytotoxicity effects of soluble or wet-bound GIGA-564 (top) and ipilimumab (bottom) in PBMCs.

[00177] **FIG. 61** provides a time course of tumor growth in the hCTLA-4 KI mice bearing established MC38 tumors treated with PBS or GIGA-564 (1 mg/kg, 0.3 mg/kg, 0.1 mg/kg, 0.03 mg/kg, 0.01 mg/kg) on days 0, 3, and 6.

7. DETAILED DESCRIPTION

7.1. Definitions

[00178] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. *See, e.g.*, Sambrook *et al. Molecular Cloning: A Laboratory Manual, 2d ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) and Ausubel *et al., Current Protocols in Molecular Biology*, Greene Publishing Associates (1992), and Harlow and Lane *Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990), which are incorporated herein by reference. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The terminology used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques can be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[00179] The following terms, unless otherwise indicated, shall be understood to have the following meanings:

[00180] The terms "CTLA-4," "CTLA-4 protein," and "CTLA-4 antigen" are used interchangeably herein to refer to human CTLA-4, or any variants (*e.g.*, splice variants and allelic variants), isoforms, and species homologs of human CTLA-4 that are naturally expressed by cells, or that are expressed by cells transfected with a *ctla4* gene. In some embodiments, the CTLA-4 protein is a CTLA-4 protein naturally expressed by a primate (*e.g.*, a monkey or a human), a rodent (*e.g.*, a mouse or a rat), a dog, a camel, a cat, a cow, a goat, a horse, or a sheep. In some embodiments, the CTLA-4 protein is human CTLA-4 (hCTLA-4; SEQ ID NO: 7001).

[00181] The term "immunoglobulin" refers to a class of structurally related proteins generally comprising two pairs of polypeptide chains: one pair of light (L) chains and one pair of heavy (H) chains. In an "intact immunoglobulin," all four of these chains are interconnected by

disulfide bonds. The structure of immunoglobulins has been well characterized. *See, e.g.,* Paul, *Fundamental Immunology* 7th ed., Ch. 5 (2013) Lippincott Williams & Wilkins, Philadelphia, PA. Briefly, each heavy chain typically comprises a heavy chain variable region (V_H) and a heavy chain constant region (C_H). The heavy chain constant region typically comprises three domains, abbreviated C_{H1}, C_{H2}, and C_{H3}. Each light chain typically comprises a light chain variable region (V_L) and a light chain constant region. The light chain constant region typically comprises one domain, abbreviated C_L.

[00182] The term "antigen-binding protein" (ABP) refers to a protein comprising one or more antigen-binding domains that specifically bind to an antigen or epitope. In some embodiments, the antigen-binding domain binds the antigen or epitope with specificity and affinity similar to that of naturally occurring antibodies. In some embodiments, the ABP comprises an antibody. In some embodiments, the ABP consists of an antibody. In some embodiments, the ABP consists essentially of an antibody. In some embodiments, the ABP comprises an alternative scaffold. In some embodiments, the ABP consists of an alternative scaffold. In some embodiments, the ABP consists essentially of an alternative scaffold. In some embodiments, the ABP comprises an antibody fragment. In some embodiments, the ABP consists of an antibody fragment. In some embodiments, the ABP consists essentially of an antibody fragment. A "CTLA-4 ABP," "anti- CTLA-4 ABP," or "CTLA-4-specific ABP" is an ABP, as provided herein, which specifically binds to the antigen CTLA-4. In some embodiments, the ABP binds the extracellular domain of CTLA-4. In certain embodiments, a CTLA-4 ABP provided herein binds to an epitope of CTLA-4 that is conserved between or among CTLA-4 proteins from different species.

[00183] The term "antibody" is used herein in its broadest sense and includes certain types of immunoglobulin molecules comprising one or more antigen-binding domains that specifically bind to an antigen or epitope. An antibody specifically includes intact antibodies (*e.g.*, intact immunoglobulins), antibody fragments, and multi-specific antibodies. One example of an antigen-binding domain is an antigen-binding domain formed by a V_H -V_L dimer. An antibody is one type of ABP.

[00184] The term "afucosylation" or "afucosylated" in the context of an Fc refers to a substantial lack of core fucosylation of the N-glycan covalently attached, directly or indirectly, to the N-glycosylation site, e.g., amino acid residue position 297 of the human IgG1 Fc region, numbered according to the EU index (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)), or the corresponding residue in non-IgG1 or non-human IgG1 immunoglobulins.

[00185] When the fucosylation rate is indicated in the context of a composition comprising antibodies, the rate indicates the proportion of focusylated antibodies among the total antibodies in the composition. For example, 70% of fucosylation indicates that 70% of the antibodies in the composition are fucosylated and 30% of the antibodies in the composition are afucosylated.

[00186] The term "alternative scaffold" refers to a molecule in which one or more regions may be diversified to produce one or more antigen-binding domains that specifically bind to an antigen or epitope. In some embodiments, the antigen-binding domain binds the antigen or epitope with specificity and affinity similar to that of naturally occurring antibodies. Exemplary alternative scaffolds include those derived from fibronectin (*e.g.*, Adnectins™), the β-sandwich (*e.g.*, iMab), lipocalin (*e.g.*, Anticalins®), EETI-II/AGRP, BPTI/LACI-D1/ITI-D2 (*e.g.*, Kunitz domains), thioredoxin peptide aptamers, protein A (*e.g.*, Affibody®), ankyrin repeats (*e.g.*, DARPins), gamma-B-crystallin/ubiquitin (*e.g.*, Affilins), $CTLD_3$ (*e.g.*, Tetranectins), Fynomers, and (LDLR-A module) (*e.g.*, Avimers). Additional information on alternative scaffolds is provided in Binz et al., *Nat. Biotechnol.*, 2005 23:1257-1268; Skerra, *Current Opin. in Biotech.*, 2007 18:295-304; and Silacci et al., *J. Biol. Chem.*, 2014, 289:14392-14398; each of which is incorporated by reference in its entirety. An alternative scaffold is one type of ABP.

[00187] The term "antigen-binding domain" means the portion of an ABP that is capable of specifically binding to an antigen or epitope.

[00188] The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a naturally occurring antibody structure and having heavy chains that comprise an Fc region.

[00189] The term "Fc region" means the C-terminal region of an immunoglobulin heavy chain that, in naturally occurring antibodies, interacts with Fc receptors and certain proteins of the complement system. The structures of the Fc regions of various immunoglobulins, and the glycosylation sites contained therein, are known in the art. *See* Schroeder and Cavacini, *J. Allergy Clin. Immunol.*, 2010, 125:S41-52, incorporated by reference in its entirety. The Fc region may be a naturally occurring Fc region, or an Fc region modified as described elsewhere in this disclosure.

[00190] The V_H and V_L regions may be further subdivided into regions of hypervariability ("hypervariable regions (HVRs);" also called "complementarity determining regions" (CDRs)) interspersed with regions that are more conserved. The more conserved regions are called framework regions (FRs). Each V_H and V_L generally comprises three CDRs and four FRs, arranged in the following order (from N-terminus to C-terminus): FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. The CDRs are involved in antigen binding, and influence antigen specificity

and binding affinity of the antibody. *See* Kabat et al., *Sequences of Proteins of Immunological Interest* 5th ed. (1991) Public Health Service, National Institutes of Health, Bethesda, MD, incorporated by reference in its entirety.

[00191] The light chain from any vertebrate species can be assigned to one of two types, called kappa (κ) and lambda (λ), based on the sequence of its constant domain.

[00192] The heavy chain from any vertebrate species can be assigned to one of five different classes (or isotypes): IgA, IgD, IgE, IgG, and IgM. These classes are also designated α, δ, ε, γ, and μ, respectively. The IgG and IgA classes are further divided into subclasses on the basis of differences in sequence and function. Humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

[00193] The amino acid sequence boundaries of a CDR can be determined by one of skill in the art using any of a number of known numbering schemes, including those described by Kabat et al., *supra* ("Kabat" numbering scheme); Al-Lazikani et al., 1997, *J. Mol. Biol.*, 273:927-948 ("Chothia" numbering scheme); MacCallum et al., 1996, *J. Mol. Biol.* 262:732-745 ("Contact" numbering scheme); Lefranc et al., *Dev. Comp. Immunol.*, 2003, 27:55-77 ("IMGT" numbering scheme); and Honegge and Plückthun, *J. Mol. Biol.*, 2001, 309:657-70 ("AHo" numbering scheme); each of which is incorporated by reference in its entirety.

[00194] TABLE 1 provides exemplary positions of CDR1-L (CDR1 of V_L), CDR2-L (CDR2 of V_L), CDR3-L (CDR3 of V_L), CDR1-H (CDR1 of V_H), CDR2-H (CDR2 of V_H), and CDR3-H (CDR3 of V_H), as identified by the Kabat and Chothia schemes. For CDR1-H, residue numbering is provided using both the Kabat and Chothia numbering schemes.

[00195] CDRs may be assigned, for example, using antibody numbering software, such as Abnum, available at www.bioinf.org.uk/abs/abnum/, and described in Abhinandan and Martin, *Immunology*, 2008, 45:3832-3839, incorporated by reference in its entirety.

TABLE 1
Residues in CDRs according to Kabat and Chothia numbering schemes.

CDR	Kabat	Chothia
CDR1-L	24-34	24-34
CDR2-L	50-56	50-56
CDR3-L	89-97	89-97
CDR1-H (Kabat Numbering)	31-35B	26-32 or 34*
CDR1-H (Chothia Numbering)	31-35	26-32
CDR2-H	50-65	52-56
CDR3-H	95-102	95-102

* The C-terminus of CDR1-H, when numbered using the Kabat numbering convention, varies between 32 and 34, depending on the length of the CDR.

[00196] The "EU numbering scheme" is generally used when referring to a residue in an antibody heavy chain constant region (*e.g.*, as reported in Kabat et al., *supra*).

[00197] An "antibody fragment" comprises a portion of an intact antibody, such as the antigen-binding or variable region of an intact antibody. Antibody fragments include, for example, Fv fragments, Fab fragments, $F(ab')_2$ fragments, Fab' fragments, scFv (sFv) fragments, and scFv-Fc fragments.

[00198] "Fv" fragments comprise a non-covalently-linked dimer of one heavy chain variable domain and one light chain variable domain.

[00199] "Fab" fragments comprise, in addition to the heavy and light chain variable domains, the constant domain of the light chain and the first constant domain (C_{H1}) of the heavy chain. Fab fragments may be generated, for example, by recombinant methods or by papain digestion of a full-length antibody.

[00200] "$F(ab')_2$" fragments contain two Fab' fragments joined, near the hinge region, by disulfide bonds. $F(ab')_2$ fragments may be generated, for example, by recombinant methods or by pepsin digestion of an intact antibody. The F(ab') fragments can be dissociated, for example, by treatment with ß-mercaptoethanol.

[00201] "Single-chain Fv" or "sFv" or "scFv" antibody fragments comprise a V_H domain and a V_L domain in a single polypeptide chain. The V_H and V_L are generally linked by a peptide linker. *See* Plückthun A. (1994). In some embodiments, the linker is a $(GGGGS)_n$ (SEQ ID NO: 11968). In some embodiments, n = 1, 2, 3, 4, 5, or 6. *See* Antibodies from *Escherichia coli*. In Rosenberg M. & Moore G.P. (Eds.), *The Pharmacology of Monoclonal Antibodies* vol. 113 (pp. 269-315). Springer-Verlag, New York, incorporated by reference in its entirety.

[00202] "scFv-Fc" fragments comprise an scFv attached to an Fc domain. For example, an Fc domain may be attached to the C-terminal of the scFv. The Fc domain may follow the V_H or V_L, depending on the orientation of the variable domains in the scFv (i.e., V_H -V_L or V_L -V_H). Any suitable Fc domain known in the art or described herein may be used. In some cases, the Fc domain comprises an IgG4 Fc domain.

[00203] The term "single domain antibody" refers to a molecule in which one variable domain of an antibody specifically binds to an antigen without the presence of the other variable domain. Single domain antibodies, and fragments thereof, are described in Arabi Ghahroudi et al., *FEBS Letters*, 1998, 414:521-526 and Muyldermans et al., *Trends in Biochem. Sci.*, 2001, 26:230-245, each of which is incorporated by reference in its entirety.

[00204] A "monospecific ABP" is an ABP that comprises a binding site that specifically binds to a single epitope. An example of a monospecific ABP is a naturally occurring IgG

molecule which, while divalent, recognizes the same epitope at each antigen-binding domain. The binding specificity may be present in any suitable valency.

[00205] The term "monoclonal antibody" refers to an antibody from a population of substantially homogeneous antibodies. A population of substantially homogeneous antibodies comprises antibodies that are substantially similar and that bind the same epitope(s), except for variants that may normally arise during production of the monoclonal antibody. Such variants are generally present in only minor amounts. A monoclonal antibody is typically obtained by a process that includes the selection of a single antibody from a plurality of antibodies. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, yeast clones, bacterial clones, or other recombinant DNA clones. The selected antibody can be further altered, for example, to improve affinity for the target ("affinity maturation"), to humanize the antibody, to improve its production in cell culture, and/or to reduce its immunogenicity in a subject.

[00206] The term "chimeric antibody" refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

[00207] "Humanized" forms of non-human antibodies are chimeric antibodies that contain minimal sequence derived from the non-human antibody. A humanized antibody is generally a human antibody (recipient antibody) in which residues from one or more CDRs are replaced by residues from one or more CDRs of a non-human antibody (donor antibody). The donor antibody can be any suitable non-human antibody, such as a mouse, rat, rabbit, chicken, or non-human primate antibody having a desired specificity, affinity, or biological effect. In some instances, selected framework region residues of the recipient antibody are replaced by the corresponding framework region residues from the donor antibody. Humanized antibodies may also comprise residues that are not found in either the recipient antibody or the donor antibody. Such modifications may be made to further refine antibody function. For further details, *see* Jones et al., *Nature*, 1986, 321:522-525; Riechmann et al., *Nature*, 1988, 332:323-329; and Presta, *Curr. Op. Struct. Biol.*, 1992, 2:593-596, each of which is incorporated by reference in its entirety.

[00208] A "human antibody" is one which possesses an amino acid sequence corresponding to that of an antibody produced by a human or a human cell, or derived from a non-human source that utilizes a human antibody repertoire or human antibody-encoding sequences (*e.g.*, obtained from human sources or designed *de novo*). Human antibodies specifically exclude humanized antibodies. In some embodiments, rodents are genetically engineered to replace their rodent antibody sequences with human antibodies.

[00209] An "isolated ABP" or "isolated nucleic acid" is an ABP or nucleic acid that has been separated and/or recovered from a component of its natural environment. Components of the natural environment may include enzymes, hormones, and other proteinaceous or nonproteinaceous materials. In some embodiments, an isolated ABP is purified to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence, for example by use of a spinning cup sequenator. In some embodiments, an isolated ABP is purified to homogeneity by gel electrophoresis (*e.g.*, SDS-PAGE) under reducing or nonreducing conditions, with detection by Coomassie blue or silver stain. An isolated ABP includes an ABP *in situ* within recombinant cells, since at least one component of the ABP's natural environment is not present. In some embodiments, an isolated ABP or isolated nucleic acid is prepared by at least one purification step. In some embodiments, an isolated ABP or isolated nucleic acid is purified to at least 80%, 85%, 90%, 95%, or 99% by weight. In some embodiments, an isolated ABP or isolated nucleic acid is purified to at least 80%, 85%, 90%, 95%, or 99% by volume. In some embodiments, an isolated ABP or isolated nucleic acid is provided as a solution comprising at least 85%, 90%, 95%, 98%, 99% to 100% ABP or nucleic acid by weight. In some embodiments, an isolated ABP or isolated nucleic acid is provided as a solution comprising at least 85%, 90%, 95%, 98%, 99% to 100% ABP or nucleic acid by volume.

[00210] "Affinity" refers to the strength of the total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an ABP) and its binding partner (*e.g.*, an antigen or epitope). Unless indicated otherwise, as used herein, "affinity" refers to intrinsic binding affinity, which reflects a 1:1 interaction between members of a binding pair (*e.g.*, ABP and antigen or epitope). The affinity of a molecule X for its partner Y can be represented by the dissociation equilibrium constant (K_D). The kinetic components that contribute to the dissociation equilibrium constant are described in more detail below. Affinity can be measured by common methods known in the art, including those described herein. Affinity can be determined, for example, using surface plasmon resonance (SPR) technology (*e.g.*, BIACORE®) or biolayer interferometry (*e.g.*, FORTEBIO®).

[00211] With regard to the binding of an ABP to a target molecule, the terms "bind," "specific binding," "specifically binds to," "specific for," "selectively binds," and "selective for" a particular antigen (*e.g.*, a polypeptide target) or an epitope on a particular antigen mean binding that is measurably different from a non-specific or non-selective interaction (*e.g.*, with a non-target molecule). Specific binding can be measured, for example, by measuring binding to a target molecule and comparing it to binding to a non-target molecule. Specific binding can also be determined by competition with a control molecule that mimics the epitope recognized on the

target molecule. In that case, specific binding is indicated if the binding of the ABP to the target molecule is competitively inhibited by the control molecule. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 50% of the affinity for CTLA-4. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 40% of the affinity for CTLA-4. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 30% of the affinity for CTLA-4. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 20% of the affinity for CTLA-4. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 10% of the affinity for CTLA-4. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 1% of the affinity for CTLA-4. In some embodiments, the affinity of a CTLA-4 ABP for a non-target molecule is less than about 0.1% of the affinity for CTLA-4.

[00212] The term "k_d" (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular ABP -antigen interaction. This value is also referred to as the k_{off} value.

[00213] The term "k_a" ($M^{-1} \times sec^{-1}$), as used herein, refers to the association rate constant of a particular ABP -antigen interaction. This value is also referred to as the k_{on} value.

[00214] The term "K_D" (M), as used herein, refers to the dissociation equilibrium constant of a particular ABP -antigen interaction. $K_D = k_d/k_a$.

[00215] The term "K_A" (M^{-1}), as used herein, refers to the association equilibrium constant of a particular ABP -antigen interaction. $K_A = k_a/k_d$.

[00216] An "affinity matured" ABP is one with one or more alterations (*e.g.*, in one or more CDRs or FRs) that result in an improvement in the affinity of the ABP for its antigen, compared to a parent ABP which does not possess the alteration(s). In one embodiment, an affinity matured ABP has nanomolar or picomolar affinity for the target antigen. Affinity matured ABPs may be produced using a variety of methods known in the art. For example, Marks et al. (*Bio/Technology*, 1992, 10:779-783, incorporated by reference in its entirety) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR and/or framework residues is described by, for example, Barbas et al. (*Proc. Nat. Acad. Sci. U.S.A.*, 1994, 91:3809-3813); Schier et al., *Gene*, 1995, 169:147-155; Yelton et al., *J. Immunol.*, 1995, 155:1994-2004; Jackson et al., *J. Immunol.*, 1995, 154:3310-33199; and Hawkins et al, *J. Mol. Biol.*, 1992, 226:889-896; each of which is incorporated by reference in its entirety.

[00217] An "immunoconjugate" is an ABP conjugated to one or more heterologous molecule(s).

[00218] "Effector functions" refer to those biological activities mediated by the Fc region of an antibody, which activities may vary depending on the antibody isotype. Examples of antibody effector functions include C1q binding to activate complement dependent cytotoxicity (CDC), Fc receptor binding to activate antibody-dependent cellular cytotoxicity (ADCC), and antibody dependent cellular phagocytosis (ADCP).

[00219] When used herein in the context of two or more ABPs, the term "competes with" or "cross-competes with" indicates that the two or more ABPs compete for binding to an antigen (*e.g.*, CTLA-4). In one exemplary assay, CTLA-4 is coated on a surface and contacted with a first CTLA-4 ABP, after which a second CTLA-4 ABP is added. In another exemplary assay, a first CTLA-4 ABP is coated on a surface and contacted with CTLA-4, and then a second CTLA-4 ABP is added. If the presence of the first CTLA-4 ABP reduces binding of the second CTLA-4 ABP, in either assay, then the ABPs compete. The term "competes with" also includes combinations of ABPs where one ABP reduces binding of another ABP, but where no competition is observed when the ABPs are added in the reverse order. However, in some embodiments, the first and second ABPs inhibit binding of each other, regardless of the order in which they are added. In some embodiments, one ABP reduces binding of another ABP to its antigen by at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%. A skilled artisan can select the concentrations of the antibodies used in the competition assays based on the affinities of the ABPs for CTLA-4 and the valency of the ABPs. The assays described in this definition are illustrative, and a skilled artisan can utilize any suitable assay to determine if antibodies compete with each other. Suitable assays are described, for example, in Cox et al., "Immunoassay Methods," in *Assay Guidance Manual [Internet]*, Updated December 24, 2014 (www(dot)ncbi(dot)nlm(dot)nih(dot)gov/books/NBK92434/; accessed September 29, 2015); Silman et al., *Cytometry*, 2001, 44:30-37; and Finco et al., *J. Pharm. Biomed. Anal.*, 2011, 54:351-358; each of which is incorporated by reference in its entirety.

[00220] The term "epitope" means a portion of an antigen the specifically binds to an ABP. Epitopes frequently consist of surface-accessible amino acid residues and/or sugar side chains and may have specific three-dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter may be lost in the presence of denaturing solvents. An epitope may comprise amino acid residues that are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. The epitope to which an ABP binds can be determined using known techniques for epitope determination such as, for example,

testing for ABP binding to CTLA-4 variants with different point-mutations, or to chimeric CTLA-4 variants.

[00221] Percent "identity" between a polypeptide sequence and a reference sequence, is defined as the percentage of amino acid residues in the polypeptide sequence that are identical to the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, MEGALIGN (DNASTAR), CLUSTALW, CLUSTAL OMEGA, or MUSCLE software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[00222] A "conservative substitution" or a "conservative amino acid substitution," refers to the substitution an amino acid with a chemically or functionally similar amino acid. Conservative substitution tables providing similar amino acids are well known in the art. By way of example, the groups of amino acids provided in TABLES 2-4 are, in some embodiments, considered conservative substitutions for one another.

TABLE 2: Selected groups of amino acids that are considered conservative substitutions for one another, in certain embodiments.	
Acidic Residues	D and E
Basic Residues	K, R, and H
Hydrophilic Uncharged Residues	S, T, N, and Q
Aliphatic Uncharged Residues	G, A, V, L, and I
Non-polar Uncharged Residues	C, M, and P
Aromatic Residues	F, Y, and W

TABLE 3: Additional selected groups of amino acids that are considered conservative substitutions for one another, in certain embodiments.	
Group 1	A, S, and T
Group 2	D and E
Group 3	N and Q
Group 4	R and K
Group 5	I, L, and M
Group 6	F, Y, and W

TABLE 4: Further selected groups of amino acids that are considered conservative substitutions for one another, in certain embodiments.	
Group A	A and G
Group B	D and E
Group C	N and Q
Group D	R, K, and H

Group E	I, L, M, V
Group F	F, Y, and W
Group G	S and T
Group H	C and M

[00223] Additional conservative substitutions may be found, for example, in Creighton, *Proteins: Structures and Molecular Properties* 2nd ed. (1993) W. H. Freeman & Co., New York, NY. An ABP generated by making one or more conservative substitutions of amino acid residues in a parent ABP is referred to as a "conservatively modified variant."

[00224] The term "treating" (and variations thereof such as "treat" or "treatment") refers to clinical intervention in an attempt to alter the natural course of a disease or condition in a subject in need thereof. Treatment can be performed both for prophylaxis and during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminish of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

[00225] As used herein, the term "therapeutically effective amount" or "effective amount" refers to an amount of an ABP or pharmaceutical composition provided herein that, when administered to a subject, is effective to treat a disease or disorder.

[00226] As used herein, the term "subject" means a mammalian subject. Exemplary subjects include humans, monkeys, dogs, cats, mice, rats, cows, horses, camels, goats, rabbits, and sheep. In certain embodiments, the subject is a human. In some embodiments the subject has a disease or condition that can be treated with an ABP provided herein. In some embodiments, the disease or condition is a cancer. In some embodiments, the disease or condition is a viral infection.

[00227] The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic or diagnostic products (*e.g.*, kits) that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic or diagnostic products.

[00228] The term "cytotoxic agent," as used herein, refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction.

[00229] A "chemotherapeutic agent" refers to a chemical compound useful in the treatment of cancer. Chemotherapeutic agents include "anti-hormonal agents" or "endocrine therapeutics" which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer.

[00230] The term "cytostatic agent" refers to a compound or composition which arrests growth of a cell either *in vitro* or *in vivo*. In some embodiments, a cytostatic agent is an agent that reduces the percentage of cells in S phase. In some embodiments, a cytostatic agent reduces the percentage of cells in S phase by at least about 20%, at least about 40%, at least about 60%, or at least about 80%.

[00231] The term "tumor" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer," "cancerous," "cell proliferative disorder," "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein. The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In some embodiments, the cell proliferative disorder is a cancer.

[00232] The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective in treating a subject, and which contains no additional components which are unacceptably toxic to the subject.

[00233] The terms "modulate" and "modulation" refer to reducing or inhibiting or, alternatively, activating or increasing, a recited variable.

[00234] The terms "increase" and "activate" refer to an increase of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, or greater in a recited variable.

[00235] The terms "reduce" and "inhibit" refer to a decrease of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, or greater in a recited variable.

[00236] The term "agonize" refers to the activation of receptor signaling to induce a biological response associated with activation of the receptor. An "agonist" is an entity that binds to and agonizes a receptor.

[00237] The term "antagonize" refers to the inhibition of receptor signaling to inhibit a biological response associated with activation of the receptor. An "antagonist" is an entity that binds to and antagonizes a receptor.

[00238] The term "effector T cell" includes T helper (i.e., CD4+) cells and cytotoxic (i.e., CD8+) T cells. CD4+ effector T cells contribute to the development of several immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. CD8+ effector T cells destroy virus-infected cells and

tumor cells. *See* Seder and Ahmed, *Nature Immunol.*, 2003, 4:835-842, incorporated by reference in its entirety, for additional information on effector T cells.

[00239] The term "regulatory T cell" includes cells that regulate immunological tolerance, for example, by suppressing effector T cells. In some embodiments, the regulatory T cell has a CD4+CD25+Foxp3+ phenotype. In some embodiments, the regulatory T cell has a CD8+CD25+ phenotype. *See* Nocentini et al., *Br. J. Pharmacol.*, 2012, 165:2089-2099, incorporated by reference in its entirety, for additional information on regulatory T cells.

[00240] The term "dendritic cell" refers to an antigen-presenting cell capable of activating a naïve T cell and stimulating growth and differentiation of a B cell.

[00241] A "variant" of a polypeptide (*e.g.*, an antibody) comprises an amino acid sequence wherein one or more amino acid residues are inserted into, deleted from and/or substituted into the amino acid sequence relative to the native polypeptide sequence, and retains essentially the same biological activity as the native polypeptide. The biological activity of the polypeptide can be measured using standard techniques in the art (for example, if the variant is an antibody, its activity may be tested by binding assays, as described herein). Variants of the present disclosure include fragments, analogs, recombinant polypeptides, synthetic polypeptides, and/or fusion proteins.

[00242] A "derivative" of a polypeptide is a polypeptide (*e.g.*, an antibody) that has been chemically modified, *e.g.*, via conjugation to another chemical moiety such as, for example, polyethylene glycol, albumin (*e.g.*, human serum albumin), phosphorylation, and glycosylation. Unless otherwise indicated, the term "antibody" includes, in addition to antibodies comprising two full-length heavy chains and two full-length light chains, derivatives, variants, fragments, and muteins thereof, examples of which are described below.

[00243] A nucleotide sequence is "operably linked" to a regulatory sequence if the regulatory sequence affects the expression (*e.g.*, the level, timing, or location of expression) of the nucleotide sequence. A "regulatory sequence" is a nucleic acid that affects the expression (*e.g.*, the level, timing, or location of expression) of a nucleic acid to which it is operably linked. The regulatory sequence can, for example, exert its effects directly on the regulated nucleic acid, or through the action of one or more other molecules (*e.g.*, polypeptides that bind to the regulatory sequence and/or the nucleic acid). Examples of regulatory sequences include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Further examples of regulatory sequences are described in, for example, Goeddel, 1990, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA and Baron et al., 1995, Nucleic Acids Res. 23:3605–06. [0078]

[00244] A "host cell" is a cell that can be used to express a nucleic acid, *e.g.*, a nucleic acid of the present disclosure. A host cell can be a prokaryote, for example, *E. coli*, or it can be a eukaryote, for example, a single-celled eukaryote (*e.g.*, a yeast or other fungus), a plant cell (*e.g.*, a tobacco or tomato plant cell), an animal cell (*e.g.*, a human cell, a monkey cell, a hamster cell, a rat cell, a mouse cell, or an insect cell) or a hybridoma. Examples of host cells include CS-9 cells, the COS-7 line of monkey kidney cells (ATCC CRL 1651) (*see* Gluzman et al., 1981, Cell 23:175), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells or their derivatives such as Veggie CHO and related cell lines which grow in serum-free media (*see* Rasmussen et al., 1998, Cytotechnology 28:31), HeLa cells, BHK (ATCC CRL 10) cell lines, the CV1/EBNA cell line derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) (*see* McMahan et al., 1991, EMBO J. 10:2821), human embryonic kidney cells such as 293, 293 EBNA or MSR 293, human epidermal A431 cells, human Colo205 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, primary explants, HL-60, U937, HaK or Jurkat cells. Typically, a host cell is a cultured cell that can be transformed or transfected with a polypeptide-encoding nucleic acid, which can then be expressed in the host cell.

[00245] The phrase "recombinant host cell" can be used to denote a host cell that has been transformed or transfected with a nucleic acid to be expressed. A host cell also can be a cell that comprises the nucleic acid but does not express it at a desired level unless a regulatory sequence is introduced into the host cell such that it becomes operably linked with the nucleic acid. It is understood that the term host cell refers not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to, *e.g.*, mutation or environmental influence, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[00246] In some embodiments, the host cell is used in adoptive cell therapy for delivery of the ABP to the subject.

7.2. Other interpretational conventions

[00247] Ranges recited herein are understood to be shorthand for all of the values within the range, inclusive of the recited endpoints. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50.

[00248] Unless otherwise indicated, reference to a compound that has one or more stereocenters intends each stereoisomer, and all combinations of stereoisomers, thereof.

7.3. Antigen binding protein

[00249] In one aspect, the present disclosure provides antigen binding proteins ("ABP") (*e.g.*, antibodies, antibody fragments, antibody derivatives, antibody muteins, and antibody variants). In some embodiments, the ABP bind to CTLA-4.

[00250] In some embodiments, the present disclosure provides ABPs that bind to an epitope of CTLA-4, which is different from Ipilimumab. In some embodiments, the epitope comprises K130, Y139, L141, and I143, but not R70. In some embodiments, the ABP contacts amino acids K130, Y139, L141, I143 but does not contact amino acid R70 of the CTLA-4. In some embodiments, the ABP can bind CTLA-4 even while the CTPA-4 interacts with CD80/CD86. In some embodiments, an interaction between the ABP and amino acid L74A and/or E68 of the CTLA-4 is greater than an interaction between Ipilimumab and amino acid L74A of CTLA-4.

[00251] In some embodiments, the present disclosure provides antigen binding proteins that comprise a light chain variable region selected from the group consisting of A1LC-A28LC or a heavy chain variable region selected from the group consisting of A1HC-A28HC, and fragments, derivatives, muteins, and variants thereof. Such an antigen binding protein can be denoted using the nomenclature "LxHy," wherein "x" corresponds to the number of the light chain variable region and "y" corresponds to the number of the heavy chain variable region as they are labeled in the sequences below. That is to say, for example, that "A1HC" denotes the heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 101; "A1LC" denotes the light chain variable region comprising the amino acid sequence of SEQ ID NO:1, and so forth. More generally speaking, "L2H1" refers to an antigen binding protein with a light chain variable region comprising the amino acid sequence of L2 (SEQ ID NO:2) and a heavy chain variable region comprising the amino acid sequence of H1 (SEQ ID NO:101). For clarity, all ranges denoted by at least two members of a group include all members of the group between and including the end range members. Thus, the group range A1-A28, includes all members between A1 and A28, as well as members A1 and A28 themselves. The group range A4-A6 includes members A4, A5, and A6, etc. In a particular embodiment, the ABP is A14. In some embodiments, the ABP comprises six CDR sequences of A14 (GIGA-564). In some embodiments, the ABP comprises the heavy chain and the light chain sequences of A14 (GIGA-564). In some embodiments, the ABP comprises a heavy chain variable domain and a light chain variable domain of A14 (GIGA-564).

[00252] In some embodiments, the ABP comprises a heavy chain variable domain having a sequence at least 95% identical to the heavy chain variable domain of A14 (GIGA-564). In some embodiments, the ABP comprises a heavy chain variable domain having a sequence at least

96% identical to the heavy chain variable domain of A14 (GIGA-564). In some embodiments, the ABP comprises a heavy chain variable domain having a sequence at least 97% identical to the heavy chain variable domain of A14 (GIGA-564), respectively. In some embodiments, the ABP comprises a heavy chain variable domain having a sequence at least 98% identical to the heavy chain variable domain of A14 (GIGA-564), respectively. In some embodiments, the ABP comprises a heavy chain variable domain having a sequence at least 99% identical to the heavy chain variable domain of A14 (GIGA-564), respectively.

[00253] In some embodiments, the ABP comprises a light chain variable domain having a sequence at least 95% identical to the light chain variable domain of A14 (GIGA-564), respectively. In some embodiments, the ABP comprises a light chain variable domain having a sequence at least 96% identical to the light chain variable domain of A14 (GIGA-564), respectively. In some embodiments, the ABP comprises a light chain variable domain having a sequence at least 957% identical to the light chain variable domain of A14 (GIGA-564), respectively. In some embodiments, the ABP comprises a light chain variable domain having a sequence at least 98% identical to the light chain variable domain of A14 (GIGA-564), respectively. In some embodiments, the ABP comprises a light chain variable domain having a sequence at least 99% identical to the light chain variable domain of A14 (GIGA-564), respectively.

[00254] In some embodiments, antigen binding proteins comprise variable (V(D)J) regions of both heavy and light chain sequences identical to one of the clones in the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512. In some embodiments, antigen binding proteins comprise variable (V(D)J) regions of either heavy or light chain sequence identical to one of the clones in the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512. In some embodiments, antigen binding proteins are expressed from the expression vector in one of the clones in the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512.

[00255] Also shown below are the locations of the CDRs (underlined) that create part of the antigen-binding site, while the Framework Regions (FRs) are the intervening segments of these variable domain sequences. In both light chain variable regions and heavy chain variable regions there are three CDRs (CDR1-3) and four FRs (FR 1-4). The CDR regions of each light and heavy chain also are grouped by antibody type (A1, A2, A3, etc.). Antigen binding proteins of the present disclosure include, for example, antigen binding proteins having a combination of light chain and heavy chain variable domains selected from the group of combinations consisting of L1H1 (antibody A1; used interchangeably herein as "aCTLA-4.9"), L2H2 (antibody A2; used

interchangeably herein as "aCTLA-4.4"), L3H3 (antibody A3; used interchangeably herein as "aCTLA-4.2"), L4H4 (antibody A4; used interchangeably herein as "aCTLA-4.29"), L5H5 (antibody A5; used interchangeably herein as "aCTLA-4.28"), L6H6 (antibody A6; used interchangeably herein as "aCTLA-4.26"), L7H7 (antibody A7; used interchangeably herein as "aCTLA-4.3"), L8H8 (antibody A8; used interchangeably herein as "aCTLA-4.1"), L9H9 (antibody A9; used interchangeably herein as "aCTLA-4.24"), L10H10 (antibody A10; used interchangeably herein as "aCTLA-4.22"), L11H11 (antibody A11; used interchangeably herein as "aCTLA-4.31), L12H12 (antibody A12; used interchangeably herein as "aCTLA-4.12"), L13H13 (antibody A13; used interchangeably herein as "aCTLA-4.14"), L13H13 (antibody A13; used interchangeably herein as "aCTLA-4.14") ... and L28H28 (antibody A28). Antigen binding proteins of the present disclosure include, for example, antigen binding proteins having a light chain and heavy chain variable domain selected from the group consisting of L18H18 (antibody A18; used interchangeably herein as "aCTLA-4.11"), L15H15 (antibody A15; used interchangeably herein as "aCTLA-4.18"), L16H16 (antibody A16; used interchangeably herein as "aCTLA-4.5"), and L17H17 (antibody A17; used interchangeably herein as "aCTLA-4.17"). In some embodiments, ABPs of the present disclosure comprise L14H14 (antibody A14; used interchangeably herein as "aCTLA-4.15" or GIGA-564).

[00256] Antigen binding proteins of the present disclosure include, for example, antigen binding proteins having a light chain and heavy chain variable domain selected from the group consisting of L19H19 (antibody A19; used interchangeably herein as "aCTLA-4.7"), L20H20 (antibody A20; used interchangeably herein as "aCTLA-4.25"), L21H21 (antibody A21; used interchangeably herein as "aCTLA-4.10"), L22H22 (antibody A22; used interchangeably herein as "aCTLA-4.21"), L23H23 (antibody A23; used interchangeably herein as "aCTLA-4.23"), L24H24 (antibody A24; used interchangeably herein as "aCTLA-4.27"), L25H25 (antibody A25; used interchangeably herein as "aCTLA-4.32"), L26H26 (antibody A26; used interchangeably herein as "aCTLA-4.20"), and L27H27 (antibody A27; used interchangeably herein as "aCTLA-4.8"). In some embodiments, ABPs of the present disclosure comprise a light chain and heavy chain variable domain of L14H14 (antibody A14; used interchangeably herein as "aCTLA-4.15" or GIGA-564).

[00257] In some embodiments, antigen binding proteins comprise all six CDR sequences (three CDRs of light chain and three CDRs of heavy chain) identical to one of the clones in the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512. In some embodiments, antigen binding proteins comprise three out of six CDR sequences (three CDRs of light chain or three CDRs of heavy chain) identical to one of the clones in the library of CTLA-4

binding clones, deposited under ATCC Accession NO. PTA-125512. In some embodiments, antigen binding proteins comprise one, two, three, four, or five out of six CDR sequences identical to one of the clones in the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512.

[00258] In one embodiment, the present disclosure provides an antigen binding protein comprising a light chain variable domain comprising a sequence of amino acids that differs from the sequence of a light chain variable domain selected from the group consisting of L1 through L28 only at 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 residues, wherein each such sequence difference is independently either a deletion, insertion, or substitution of one amino acid residue. In another embodiment, the light-chain variable domain comprises a sequence of amino acids that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of a light chain variable domain selected from the group consisting of L1-L28. In another embodiment, the light chain variable domain comprises a sequence of amino acids that is encoded by a nucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleotide sequence that encodes a light chain variable domain selected from the group consisting of L1-L28 (which includes L1, L2, L3, L4, L5, L6, L7, L8, L9, L10, L11, L12, L13, L14, ... and L28). In another embodiment, the light chain variable domain comprises a sequence of amino acids that is encoded by a polynucleotide that hybridizes under moderately stringent conditions to the complement of a polynucleotide that encodes a light chain variable domain selected from the group consisting of L1-L28. In another embodiment, the light chain variable domain comprises a sequence of amino acids that is encoded by a polynucleotide that hybridizes under moderately stringent conditions to the complement of a polynucleotide that encodes a light chain variable domain selected from the group consisting of L1-L28. In another embodiment, the light chain variable domain comprises a sequence of amino acids that is encoded by a polynucleotide that hybridizes under moderately stringent conditions to a complement of a light chain polynucleotide of L1-L28.

[00259] In one embodiment, the present disclosure provides an antigen binding protein comprising a light chain variable domain comprising a sequence of amino acids that differs from the sequence of a light chain variable domain encoded by one of the clones of the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512, only at 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 residues, wherein each such sequence difference is independently either a deletion, insertion, or substitution of one amino acid residue. In another embodiment, the light-chain variable domain comprises a sequence of amino acids that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of a light

chain variable domain encoded by one of the clones of the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512. In another embodiment, the light chain variable domain comprises a sequence of amino acids that is encoded by a nucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleotide sequence of one of the clones of the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512.

[00260] In another embodiment, the present disclosure provides an antigen binding protein comprising a heavy chain variable domain comprising a sequence of amino acids that differs from the sequence of a heavy chain variable domain selected from the group consisting of H1-H28 only at 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 residue(s), wherein each such sequence difference is independently either a deletion, insertion, or substitution of one amino acid residue. In another embodiment, the heavy chain variable domain comprises a sequence of amino acids that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of a heavy chain variable domain selected from the group consisting of H1-H28. In another embodiment, the heavy chain variable domain comprises a sequence of amino acids that is encoded by a nucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleotide sequence that encodes a heavy chain variable domain selected from the group consisting of H1-H28. In another embodiment, the heavy chain variable domain comprises a sequence of amino acids that is encoded by a polynucleotide that hybridizes under moderately stringent conditions to the complement of a polynucleotide that encodes a heavy chain variable domain selected from the group consisting of H1-H28. In another embodiment, the heavy chain variable domain comprises a sequence of amino acids that is encoded by a polynucleotide that hybridizes under moderately stringent conditions to the complement of a polynucleotide that encodes a heavy chain variable domain selected from the group consisting of H1-H28. In another embodiment, the heavy chain variable domain comprises a sequence of amino acids that is encoded by a polynucleotide that hybridizes under moderately stringent conditions to a complement of a heavy chain polynucleotide disclosed herein.

[00261] In one embodiment, the present disclosure provides an antigen binding protein comprising a heavy chain variable domain comprising a sequence of amino acids that differs from the sequence of a heavy chain variable domain encoded by one of the clones of the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512, only at 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 residues, wherein each such sequence difference is independently either a deletion, insertion, or substitution of one amino acid residue. In another

embodiment, the heavy chain variable domain comprises a sequence of amino acids that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of a heavy chain variable domain encoded by one of the clones of the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512. In another embodiment, the heavy chain variable domain comprises a sequence of amino acids that is encoded by a nucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to a nucleotide sequence of one of the clones of the library of CTLA-4 binding clones, deposited under ATCC Accession NO. PTA-125512.

[00262] Particular embodiments of antigen binding proteins of the present disclosure comprise one or more amino acid sequences that are identical to the amino acid sequences of one or more of the CDRs and/or FRs referenced herein. In one embodiment, the antigen binding protein comprises a light chain CDR1 sequence illustrated above. In another embodiment, the antigen binding protein comprises a light chain CDR2 sequence illustrated above. In another embodiment, the antigen binding protein comprises a light chain CDR3 sequence illustrated above. In another embodiment, the antigen binding protein comprises a heavy chain CDR1 sequence illustrated above. In another embodiment, the antigen binding protein comprises a heavy chain CDR2 sequence illustrated above. In another embodiment, the antigen binding protein comprises a heavy chain CDR3 sequence illustrated above.

[00263] In one embodiment, the present disclosure provides an antigen binding protein that comprises one or more CDR sequences that differ from a CDR sequence shown above by no more than 5, 4, 3, 2, or 1 amino acid residues.

[00264] In some embodiments, at least one of the antigen binding protein's CDR1 sequences is a CDR1 sequence from A1-A28, CDR1-L1 to 28, or CDR1-H1 to 28 as shown in TABLE 5. In some embodiments, at least one of the antigen binding protein's CDR2 sequences is a CDR2 sequence from A1-A28, CDR2-L1 to 28, or CDR2-H1 to 28 as shown in TABLE 5. In some embodiments, at least one of the antigen binding protein's CDR3 sequences is a CDR3 sequence from A1-A28, CDR3-L1 to 28, or CDR3-H1 to 28 as shown in TABLE 5.

[00265] In another embodiment, the antigen binding protein's light chain CDR3 sequence is a light chain CDR3 sequence from A1-A28 or CDR3-L1 to 28, as shown in TABLE 5, and the antigen binding protein's heavy chain CDR3 sequence is a heavy chain sequence from A1-A28 or CDR-H1 to 28, as shown in TABLE 5.

[00266] In some embodiments, at least one of the antigen binding protein's CDR1 sequences is a light chain CDR1 sequence of QSVSSSYLA (SEQ ID NO: 12078 or 1014). In some embodiments, at least one of the antigen binding protein's CDR2 sequences is a light chain

CDR2 sequence of GASSRAT (SEQ ID NO: 12079 or 2014). In some embodiments, at least one of the antigen binding protein's CDR3 sequences is a light chain CDR3 sequence of QQYGSSPWT (SEQ ID NO: 12080 or 3014).

[00267] In some embodiments, at least one of the antigen binding protein's CDR1 sequences is a heavy chain CDR1 sequence of GFTFSSY (SEQ ID NO: 12075 or 4014). In some embodiments, at least one of the antigen binding protein's CDR2 sequences is a heavy chain CDR2 sequence of WYEGRN (SEQ ID NO: 12076 or 5014). In some embodiments, at least one of the antigen binding protein's CDR3 sequences is a heavy chain CDR3 sequence of AGDLGAFDI (SEQ ID NO: 12077 or 6014).

[00268] In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12004, a CDR2-L consisting of SEQ ID NO: 12014, a CDR3-L consisting of SEQ ID NO: 12024, a CDR1-H consisting of SEQ ID NO: 12039, a CDR2-H consisting of SEQ ID NO: 12049, and a CDR3-H consisting of SEQ ID NO: 12059. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12005, a CDR2-L consisting of SEQ ID NO: 12015, a CDR3-L consisting of SEQ ID NO: 12025, a CDR1-H consisting of SEQ ID NO: 12040, a CDR2-H consisting of SEQ ID NO: 12050, and a CDR3-H consisting of SEQ ID NO: 12060. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12006, a CDR2-L consisting of SEQ ID NO: 12016, a CDR3-L consisting of SEQ ID NO: 12026, a CDR1-H consisting of SEQ ID NO: 12041, a CDR2-H consisting of SEQ ID NO: 12051, and a CDR3-H consisting of SEQ ID NO: 12061. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12007, a CDR2-L consisting of SEQ ID NO: 12017, a CDR3-L consisting of SEQ ID NO: 12027, a CDR1-H consisting of SEQ ID NO: 12042, a CDR2-H consisting of SEQ ID NO: 12052, and a CDR3-H consisting of SEQ ID NO: 12062. In some embodiments, the ABP comprises a CDR1-L consisting of SEQ ID NO: 12008, a CDR2-L consisting of SEQ ID NO: 12018, a CDR3-L consisting of SEQ ID NO: 12028, a CDR1-H consisting of SEQ ID NO: 12043, a CDR2-H consisting of SEQ ID NO: 12053, and a CDR3-H consisting of SEQ ID NO: 12063.

[00269] In another embodiment, the antigen binding protein comprises 1, 2, 3, 4, or 5 CDR sequence(s) that each independently differs by 6, 5, 4, 3, 2, 1, or 0 single amino acid additions, substitutions, and/or deletions from a CDR sequence of A1-A23, and the antigen binding protein further comprises 1, 2, 3, 4, or 5 CDR sequence(s) that each independently differs by 6, 5, 4, 3, 2, 1, or 0 single amino acid additions, substitutions, and/or deletions from a CDR sequence. In some embodiments, the antigen binding protein comprises 1, 2, 3, 4, or 5 CDR sequence(s) that each

has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to a CDR sequence of A1-A28.

[00270] The nucleotide sequences of A1-A28, or the amino acid sequences of A1-A28, can be altered, for example, by random mutagenesis or by site-directed mutagenesis (*e.g.*, oligonucleotide-directed site-specific mutagenesis) to create an altered polynucleotide comprising one or more particular nucleotide substitutions, deletions, or insertions as compared to the non-mutated polynucleotide.

[00271] Other derivatives of anti-CTLA-4 antibodies within the scope of this disclosure include covalent or aggregative conjugates of anti-CTLA-4 antibodies, or fragments thereof, with other proteins or polypeptides, such as by expression of recombinant fusion proteins comprising heterologous polypeptides fused to the N-terminus or C-terminus of an anti-CTLA-4 antibody polypeptide.

[00272] One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum *et al.*, 1994, EMBO J. 13:3992-4001. The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

[00273] In other embodiments, the variable portion of the heavy and/or light chains of an anti-CTLA-4 antibody may be substituted for the variable portion of an antibody heavy and/or light chain.

[00274] Oligomers that contain one or more antigen binding proteins may be employed as CTLA-4 antagonists or agonists. Oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers. Oligomers comprising two or more antigen binding protein are contemplated for use, with one example being a homodimer. Other oligomers include heterodimers, homotrimers, heterotrimers, homotetramers, heterotetramers, *etc.*

[00275] One embodiment is directed to oligomers comprising multiple antigen binding proteins joined *via* covalent or non-covalent interactions between peptide moieties fused to the antigen binding proteins. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived

from antibodies are among the peptides that can promote oligomerization of antigen binding proteins attached thereto, as described in more detail below.

[00276] In particular embodiments, the oligomers comprise from two to four antigen binding proteins. The antigen binding proteins of the oligomer may be in any form, such as any of the forms described above, *e.g.*, variants or fragments. Preferably, the oligomers comprise antigen binding proteins that have CTLA-4 binding activity.

[00277] One embodiment of the present disclosure is directed to a dimer comprising two fusion proteins created by fusing a CTLA-4 binding fragment of an anti-CTLA-4 antibody to the Fc region of an antibody.

[00278] Alternatively, the oligomer is a fusion protein comprising multiple antigen binding proteins, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233.

[00279] Another method for preparing oligomeric antigen binding proteins involves use of a leucine zipper.

[00280] In one aspect, the present disclosure provides antigen binding proteins that interfere with the binding of CTLA-4 to its ligands. Such antigen binding proteins can be made against CTLA-4, or a fragment, variant or derivative thereof, and screened in conventional assays for the ability to interfere with binding of CTLA-4 to its ligands. Examples of suitable assays are assays that test the antigen binding proteins for the ability to inhibit binding of CTLA-4 ligands to cells expressing CTLA-4, or that test antigen binding proteins for the ability to reduce a biological or cellular response that results from the binding of CTLA-4 ligands to cell surface CTLA-4. For example, antibodies can be screened according to their ability to bind to immobilized antibody surfaces (CTLA-4). Antigen binding proteins that block the binding of CTLA-4 to a ligand can be employed in treating any CTLA-4-related condition, including but not limited to cancer. In an embodiment, a human anti-CTLA-4 monoclonal antibody generated by procedures involving immunization of transgenic mice is employed in treating such conditions.

[00281] Antigen-binding fragments of antigen binding proteins of the present disclosure can be produced by conventional techniques. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques also are contemplated.

[00282] Additional embodiments include chimeric antibodies, *e.g.*, humanized versions of non-human (*e.g.*, murine) monoclonal antibodies.

[00283] Procedures have been developed for generating human or partially human antibodies in non-human animals. In one embodiment, a non-human animal, such as a transgenic

mouse, is immunized with a CTLA-4 polypeptide, such that antibodies directed against the CTLA-4 polypeptide are generated in the animal.

[00284] One example of a suitable immunogen is a soluble human CTLA-4, such as a polypeptide comprising the extracellular domain of the protein having the following sequence: SEQ ID: 7001 or other immunogenic fragment of the protein.

[00285] Antigen binding proteins (*e.g.*, antibodies, antibody fragments, and antibody derivatives) of the present disclosure can comprise any constant region known in the art. The light chain constant region can be, for example, a kappa- or lambda-type light chain constant region, *e.g.*, a human kappa- or lambda-type light chain constant region. The heavy chain constant region can be, for example, an alpha-, delta-, epsilon-, gamma-, or mu-type heavy chain constant regions, *e.g.*, a human alpha-, delta-, epsilon-, gamma-, or mu-type heavy chain constant region. In one embodiment, the light or heavy chain constant region is a fragment, derivative, variant, or mutein of a naturally occurring constant region.

[00286] Techniques are known for deriving an antibody of a different subclass or isotype from an antibody of interest, *i.e.*, subclass switching. Thus, IgG antibodies may be derived from an IgM antibody, for example, and *vice versa*. Such techniques allow the preparation of new antibodies that possess the antigen-binding properties of a given antibody (the parent antibody), but also exhibit biological properties associated with an antibody isotype or subclass different from that of the parent antibody. Recombinant DNA techniques may be employed. Cloned DNA encoding particular antibody polypeptides may be employed in such procedures, *e.g.*, DNA encoding the constant domain of an antibody of the desired isotype. *See* also Lantto *et al.*, 2002, *Methods Mol. Biol.* 178:303-16.

[00287] In one embodiment, an antigen binding protein of the present disclosure comprises the IgG1 heavy chain domain of any of A1-A28 (H1-H28) or a fragment of the IgG1 heavy chain domain of any of A1-A28 (H1-H28). In another embodiment, an antigen binding protein of the present disclosure comprises the kappa light chain constant chain region of A1-A28 (L1-L28), or a fragment of the kappa light chain constant region of A1-A28 (L1-L28). In another embodiment, an antigen binding protein of the present disclosure comprises both the IgG1 heavy chain domain, or a fragment thereof, of A1-A28 (H1-H28) and the kappa light chain domain, or a fragment thereof, of A1-A28 (L1-L28).

[00288] In another embodiment, an antigen binding protein of the present disclosure comprises both the IgG1 heavy chain domain or a fragment thereof, of A1-A28 (H1-H28). In some embodiments, the IgG1 heavy chain domain comprises a lysine at amino acid position 97 (K97) according to IMGT exon numbering system. In another embodiment, the IgG1 heavy

chain domain comprises a lysine at amino acid position 214 (K214) according to EU numbering system. In some embodiments, the IgG1 heavy chain domain comprises an Arginine at amino acid position 97 (R97) according to IMGT exon numbering system. In another embodiment, the IgG1 heavy chain domain comprises an Arginine at amino acid position 214 (R214) according to EU numbering system.

[00289] In some embodiments, the antigen binding protein of the present disclosure comprises the IgG1 heavy chain domain of A14 (H14) (SEQ ID NO: 114) or a fragment of the IgG1 heavy chain domain, and the IgG1 light chain domain of A14 (H14) (SEQ ID NO: 14) or fragment of the IgG1 light chain domain.

[00290] Accordingly, the antigen binding proteins of the present disclosure include those comprising, for example, the variable domain combinations L1H1, L2H2, L3H3, L4H4, L5H5, L6H6, L7H7, L8H8, L9H9, L10H10, L11H11, L12H12, L13H13, ... and L28H28, having a desired isotype (for example, IgA, IgG1, IgG2, IgG3, IgG4, IgM, IgE, and IgD) as well as Fab or $F(ab')_2$ fragments thereof. Moreover, if an IgG4 is desired, it may also be desired to introduce a point mutation (CPSCP (SEQ ID NO: 11969) -> CPPCP (SEQ ID NO: 11970)) in the hinge region as described in Bloom *et al.*, 1997, *Protein Science* 6:407, incorporated by reference herein) to alleviate a tendency to form intra-H chain disulfide bonds that can lead to heterogeneity in the IgG4 antibodies.

[00291] In one embodiment, the antigen binding protein has a K_{off} of $1x10^{-4}$ s^{-1} or lower. In another embodiment, the K_{off} is $5x10^{-5}$ s^{-1} or lower. In another embodiment, the K_{off} is substantially the same as an antibody having a combination of light chain and heavy chain variable domain sequences selected from the group of combinations consisting of L1H1, L2H2, L3H3, L4H4, L5H5, L6H6, ... and L28H28. In another embodiment, the antigen binding protein binds to CTLA-4 with substantially the same K_{off} as an antibody that comprises one or more CDRs from an antibody having a combination of light chain and heavy chain variable domain sequences selected from the group of combinations consisting of L1H1, L2H2, L3H3, L4H4, L5H5, L6H6, ... and L23H28. In another embodiment, the antigen binding protein binds to CTLA-4 with substantially the same K_{off} as an antibody that comprises one of the amino acid sequences illustrated above. In another embodiment, the antigen binding protein binds to CTLA-4 with substantially the same K_{off} as an antibody that comprises one or more CDRs from an antibody that comprises one of the amino acid sequences illustrated above.

[00292] In one aspect, the present disclosure provides antigen-binding fragments of an anti-CTLA-4 antibody of the present disclosure. Such fragments can consist entirely of antibody-derived sequences or can comprise additional sequences. Examples of antigen-binding

fragments include Fab, F(ab')2, single chain antibodies, diabodies, triabodies, tetrabodies, and domain antibodies. Other examples are provided in Lunde *et al.*, 2002, Biochem. Soc. Trans. 30:500-06.

[00293] Single chain antibodies (scFv) may be formed by linking heavy and light chain variable domain (Fv region) fragments via an amino acid bridge (short peptide linker, *e.g.*, a synthetic sequence of amino acid residues), resulting in a single polypeptide chain. ScFvs comprising the variable domain combinations L1H1, L2H2, L3H3, L4H4, L5H5, L6H6, ..., and L28H28 are encompassed by the present disclosure.

[00294] ABPs provided herein can be anti-CTLA-4 antibodies purified from host cells that have been transfected by a gene encoding the antibodies by elution of filtered supernatant of host cell culture fluid using a Heparin HP column, using a salt gradient.

[00295] In some embodiments, the host cell is used in adoptive cell therapy for delivery of the ABP to the subject. In some embodiments, the methods described herein can administer the host cell that has been transfected by a gene encoding the anti-CTLA-4 antibodies.

[00296] An antigen binding protein can have, for example, the structure of a naturally occurring immunoglobulin.

[00297] In one aspect, the present disclosure provides an ABP comprising a human heavy chain constant region gene segment of an IGHG1*01. In some embodiments, the IGHG1*01 Fc antibody comprises an scFv. In some embodiments, the ABP is specific to CTLA-4. In some embodiments, the ABP comprises an antigen binding domain of an antibody therapeutic approved or in regulatory review. In some embodiments, the ABP comprises an antigen binding domain of Ipilimumab, Toripalimab, Amivantamab, Dostarlimab, Cemiplimab, Durvalumab, Atezolizumab, or Pembrolizumab.

[00298] In some embodiments, the ABP has an enhanced FcR signaling or Fc effector function by comprising a human heavy chain constant region gene segment an IGHG1*01. Accordingly, the present disclosure further provides a method of inducing FcR-mediated Treg depletion in the tumor microenvironment, comprising the step of administering the ABP comprising a human heavy chain constant region gene segment of an IGHG1*01. The present disclosure also provides a method of improving FcR signaling or Fc effector function of an ABP by introducing a human heavy chain constant region gene segment of an IGHG1*01 to the ABP.

[00299] In one aspect, antigen binding proteins in accordance with the present disclosure include antigen binding proteins that inhibit a biological activity of CTLA-4.

[00300] In some embodiments, the antigen binding proteins in accordance with the present disclosure include an IGHG1*01 Fc anti-CTLA-4 antibody or antigen-binding fragment thereof

that enhances FcR signaling or Fc effector function. In some embodiments, the IGHG1*01 Fc anti-CTLA-4 antibody comprises an scFvs.

[00301] Different antigen binding proteins may bind to different domains of CTLA-4 or act by different mechanisms of action. As indicated herein *inter alia*, the domain region is designated such as to be inclusive of the group, unless otherwise indicated. For example, amino acids 4-12 refers to nine amino acids: amino acids at positions 4, and 12, as well as the seven intervening amino acids in the sequence. Other examples include antigen binding proteins that inhibit binding of CTLA-4 to its ligands. An antigen binding protein need not completely inhibit a CTLA-4-induced activity to find use in the present disclosure; rather, antigen binding proteins that reduce a particular activity of CTLA-4 are contemplated for use as well. (Discussions herein of particular mechanisms of action for CTLA-4-binding antigen binding proteins in treating particular diseases are illustrative only, and the methods presented herein are not bound thereby.)

[00302] A Fab fragment is a monovalent fragment having the V_L, V_H, C_L and C_{H1} domains; a $F(ab')_2$ fragment is a bivalent fragment having two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment has the V_H and C_{H1} domains; an Fv fragment has the V_L and V_H domains of a single arm of an antibody; and a dAb fragment has a V_H domain, a V_L domain, or an antigen-binding fragment of a V_H or V_L domain (US Pat. No. 6,846,634, 6,696,245, US App. Pub. No. 05/0202512, 04/0202995, 04/0038291, 04/0009507, 03/0039958, Ward et al., Nature 341:544-546, 1989).

[00303] Polynucleotide and polypeptide sequences of particular light and heavy chain variable domains are described below. Antibodies comprising a light chain and heavy chain are designated by combining the name of the light chain and the name of the heavy chain variable domains. For example, "L4H7," indicates an antibody comprising the light chain variable domain of L4 (comprising a sequence of SEQ ID NO:4) and the heavy chain variable domain of H7 (comprising a sequence of SEQ ID NO:107). Light chain variable sequences are provided in SEQ ID Nos: 1-28, and heavy chain variable sequences are provided in SEQ ID Nos:101-128.

[00304] In other embodiments, an antibody may comprise a specific heavy or light chain, while the complementary light or heavy chain variable domain remains unspecified. In particular, certain embodiments herein include antibodies that bind a specific antigen (such as CTLA-4) by way of a specific light or heavy chain, such that the complementary heavy or light chain may be promiscuous, or even irrelevant, but may be determined by, for example, screening combinatorial libraries. Portolano et al., J. Immunol. V. 150 (3), pp. 880-887 (1993); Clackson et al., Nature v. 352 pp. 624-628 (1991); Adler *et al.*, A natively paired antibody library yields drug leads with higher sensitivity and specificity than a randomly paired antibody library, MAbs

(2018)); Adler *et al.*, Rare, high-affinity mouse anti-CTLA-4 antibodies that function in checkpoint blockade, discovered using microfluidics and molecular genomics, MAbs (2017).

[00305] Naturally occurring immunoglobulin chains exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. From N-terminus to C-terminus, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat et al. in Sequences of Proteins of Immunological Interest, 5th Ed., US Dept. of Health and Human Services, PHS, NIH, NIH Publication no. 91-3242, 1991.

[00306] The term "human antibody," also referred to as "fully human antibody," includes all antibodies that have one or more variable and constant regions derived from human immunoglobulin sequences. In one embodiment, all of the variable and constant domains are derived from human immunoglobulin sequences (a fully human antibody). These antibodies may be prepared in a variety of ways, examples of which are described below, including through the immunization with an antigen of interest of a mouse that is genetically modified to express antibodies derived from human heavy and/or light chain-encoding genes.

[00307] A humanized antibody has a sequence that differs from the sequence of an antibody derived from a non-human species by one or more amino acid substitutions, deletions, and/or additions, such that the humanized antibody is less likely to induce an immune response, and/or induces a less severe immune response, as compared to the non-human species antibody, when it is administered to a human subject. In one embodiment, certain amino acids in the framework and constant domains of the heavy and/or light chains of the non-human species antibody are mutated to produce the humanized antibody. In another embodiment, the constant domain(s) from a human antibody are fused to the variable domain(s) of a non-human species. In another embodiment, one or more amino acid residues in one or more CDR sequences of a non-human antibody are changed to reduce the likely immunogenicity of the non-human antibody when it is administered to a human subject, wherein the changed amino acid residues either are not critical for immunospecific binding of the antibody to its antigen, or the changes to the amino acid sequence that are made are conservative changes, such that the binding of the humanized antibody to the antigen is not significantly worse than the binding of the non-human antibody to the antigen. Examples of how to make humanized antibodies may be found in U.S. Pat. Nos. 6,054,297, 5,886,152 and 5,877,293.

[00308] The term "chimeric antibody" refers to an antibody that contains one or more regions from one antibody and one or more regions from one or more other antibodies. In one

embodiment, one or more of the CDRs are derived from a human anti-CTLA-4 antibody. In another embodiment, all of the CDRs are derived from a human anti-CTLA-4 antibody. In another embodiment, the CDRs from more than one human anti-CTLA-4 antibodies are mixed and matched in a chimeric antibody. For instance, a chimeric antibody may comprise a CDR1 from the light chain of a first human anti-CTLA-4 antibody, a CDR2 and a CDR3 from the light chain of a second human anti-CTLA-4 antibody, and the CDRs from the heavy chain from a third anti-CTLA-4 antibody. Further, the framework regions may be derived from one of the same anti-CTLA-4 antibodies, from one or more different antibodies, such as a human antibody, or from a humanized antibody. In one example of a chimeric antibody, a portion of the heavy and/or light chain is identical with, homologous to, or derived from an antibody from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is/are identical with, homologous to, or derived from an antibody (-ies) from another species or belonging to another antibody class or subclass. Also included are fragments of such antibodies that exhibit the desired biological activity (i.e., the ability to specifically bind CTLA-4).

[00309] Fragments or analogs of antibodies can be readily prepared by those of ordinary skill in the art following the teachings of this specification and using techniques well-known in the art.

[00310] Another form of an antibody fragment is a peptide comprising one or more complementarity determining regions (CDRs) of an antibody. CDRs (also termed "minimal recognition units", or "hypervariable region") can be incorporated into a molecule either covalently or noncovalently to make it an antigen binding protein. CDRs can be obtained by constructing polynucleotides that encode the CDR of interest. Such polynucleotides are prepared, for example, by using the polymerase chain reaction to synthesize the variable region using mRNA of antibody producing cells as a template (*see*, for example, Larrick et al., Methods: A Companion to Methods in Enzymology 2:106, 1991; Courtenay Luck, "Genetic Manipulation of Monoclonal Antibodies," in Monoclonal Antibodies: Production, Engineering and Clinical Application, Ritter et al. (eds.), page 166 (Cambridge University Press 1995); and Ward et al., "Genetic Manipulation and Expression of Antibodies," in Monoclonal Antibodies: Principles and Applications, Birch et al., (eds.), page 137 (Wiley Liss, Inc. 1995).

[00311] Thus, in one embodiment, the binding agent comprises at least one CDR as described herein. The binding agent may comprise at least two, three, four, five or six CDR's as described herein. The binding agent may further comprise at least one variable region domain of an antibody described herein. The variable region domain may be of any size or amino acid

composition and will generally comprise at least one CDR sequence responsible for binding to human CTLA-4, for example CDR1-H, CDR2-H, CDR3-H, CDR1-L, CDR2-L, and CDR3-L, specifically described herein and which is adjacent to or in frame with one or more framework sequences. In general terms, the variable (V) region domain may be any suitable arrangement of immunoglobulin heavy (V_H) and/or light (V_L) chain variable domains. Thus, for example, the V region domain may be monomeric and be a V_H or V_L domain, which is capable of independently binding human CTLA-4 with an affinity at least equal to 1×10^{7}M or less as described below. Alternatively, the V region domain may be dimeric and contain V_H V_H, V_H V_L, or V_L V_L, dimers. The V region dimer comprises at least one V_H and at least one V_L chain that may be non-covalently associated (hereinafter referred to as Fv). If desired, the chains may be covalently coupled either directly, for example via a disulfide bond between the two variable domains, or through a linker, for example a peptide linker, to form a single chain Fv (scFV).

[00312] The variable region domain may be any naturally occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain that has been created using recombinant DNA engineering techniques. Such engineered versions include those created, for example, from a specific antibody variable region by insertions, deletions, or changes in or to the amino acid sequences of the specific antibody. Particular examples include engineered variable region domains containing at least one CDR and optionally one or more framework amino acids from a first antibody and the remainder of the variable region domain from a second antibody.

[00313] The variable region domain may be covalently attached at a C terminal amino acid to at least one other antibody domain or a fragment thereof. Thus, for example, a V_H domain that is present in the variable region domain may be linked to an immunoglobulin CH1 domain, or a fragment thereof. Similarly a V_L domain may be linked to a CK domain or a fragment thereof. In this way, for example, the antibody may be a Fab fragment wherein the antigen binding domain contains associated V_H and V_L domains covalently linked at their C termini to a CH1 and CK domain, respectively. The CH1 domain may be extended with further amino acids, for example to provide a hinge region or a portion of a hinge region domain as found in a Fab' fragment, or to provide further domains, such as antibody CH2 and CH3 domains.

[00314] In some embodiments, the ABP comprises an Fc region lacking a fucose sugar unit on the N glycan.

[00315] In some embodiments, the ABP comprises Glycine at the amino acid position 201.

[00316] In some embodiments, the ABP is produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof. In certain

embodiments, the cell is cultured in the absence of fucose. In some embodiments, the ABP is produced from a cell lacking or with reduced expression of Fut8. In some embodiments, the ABP is produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF).

[00317] In some embodiments, the ABP is produced from a cell overexpressing glycosyltransferase (GnTIII). In some embodiments, the ABP is isolated based on its fucosylation status.

[00318] In some embodiments, the ABP is an afucosylated monoclonal antibody.

[00319] As described herein, antibodies comprise at least one of these CDRs. For example, one or more CDR may be incorporated into known antibody framework regions (IgG1, IgG2, *etc.*), or conjugated to a suitable vehicle to enhance the half-life thereof. Suitable vehicles include, but are not limited to Fc, polyethylene glycol (PEG), albumin, transferrin, and the like. These and other suitable vehicles are known in the art. Such conjugated CDR peptides may be in monomeric, dimeric, tetrameric, or other form. In one embodiment, one or more water-soluble polymer is bonded at one or more specific position, for example at the amino terminus, of a binding agent.

[00320] In another example, individual V_L or V_H chains from an antibody (i.e. CTLA-4 antibody) can be used to search for other V_H or V_L chains that could form antigen-binding fragments (or Fab), with the same specificity. Thus, random combinations of V_H and V_L chain Ig genes can be expressed as antigen-binding fragments in a bacteriophage library (such as fd or lambda phage). For instance, a combinatorial library may be generated by utilizing the parent V_L or V_H chain library combined with antigen-binding specific V_L or V_H chain libraries, respectively. The combinatorial libraries may then be screened by conventional techniques, for example by using radioactively labeled probe (such as radioactively labeled CTLA-4). *See*, for example, Portolano et al., J. Immunol. V. 150 (3) pp. 880-887 (1993).

[00321] Diabodies are bivalent antibodies comprising two polypeptide chains, wherein each polypeptide chain comprises V_H and V_L domains joined by a linker that is too short to allow for pairing between two domains on the same chain, thus allowing each domain to pair with a complementary domain on another polypeptide chain (*see, e.g.*, Holliger *et al.*, 1993, *Proc. Natl. Acad. Sci.* USA 90:6444-48, and Poljak *et al.*, 1994, *Structure* 2:1121-23). If the two polypeptide chains of a diabody are identical, then a diabody resulting from their pairing will have two identical antigen binding sites. Polypeptide chains having different sequences can be used to make a diabody with two different antigen binding sites. Similarly, tribodies and

tetrabodies are antibodies comprising three and four polypeptide chains, respectively, and forming three and four antigen binding sites, respectively, which can be the same or different.

[00322] Antibody polypeptides are also disclosed in U. S. Patent No. 6,703,199, including fibronectin polypeptide monobodies. Other antibody polypeptides are disclosed in U.S. Patent Publication 2005/0238646, which are single-chain polypeptides.

[00323] In certain embodiments, an antibody comprises one or more water soluble polymer attachments, including, but not limited to, polyethylene glycol, polyoxyethylene glycol, or polypropylene glycol. *See, e.g.*, U.S. Pat. Nos. 4,640,835, 4,496,689, 4,301,144, 4,670,417, 4,791,192 and 4,179,337. In certain embodiments, a derivative binding agent comprises one or more of monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol) and polyvinyl alcohol, as well as mixtures of such polymers. In certain embodiments, one or more water-soluble polymer is randomly attached to one or more side chains. In certain embodiments, PEG can act to improve the therapeutic capacity for a binding agent, such as an antibody. Certain such methods are discussed, for example, in U.S. Pat. No. 6,133,426, which is hereby incorporated by reference for any purpose.

[00324] In some embodiments, the ABPs of the present disclosure are monoclonal antibodies that bind to CTLA-4.

[00325] Monoclonal antibodies may be produced using any technique known in the art, *e.g.*, by immortalizing spleen cells harvested from the transgenic animal after completion of the immunization schedule. The spleen cells can be immortalized using any technique known in the art, *e.g.*, by fusing them with myeloma cells to produce hybridomas. Hybridoma cell lines are identified that produce an antibody that binds a CTLA-4 polypeptide. Such hybridoma cell lines, and anti-CTLA-4 monoclonal antibodies produced by them, are encompassed by the present disclosure. Myeloma cells for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas). Examples of suitable cell lines for use in mouse fusions include Sp-20, P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; examples of cell lines used in rat fusions include R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210. Other cell lines useful for cell fusions are U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6. Hybridomas or mAbs may be further

screened to identify mAbs with particular properties, such as the ability to block a CTLA-4-induced activity.

[00326] An antibody of the present disclosure may also be a fully human monoclonal antibody. An isolated fully human antibody is provided that specifically binds to the CTLA-4, wherein the antigen binding protein possesses at least one *in vivo* biological activity of a human anti-CTLA-4 antibody.

7.4. Nucleic acids

[00327] In one aspect, the present disclosure provides isolated nucleic acid molecules. The nucleic acids comprise, for example, polynucleotides that encode all or part of an antigen binding protein, for example, one or both chains of an antibody of the present disclosure, or a fragment, derivative, mutein, or variant thereof, polynucleotides sufficient for use as hybridization probes, PCR primers or sequencing primers for identifying, analyzing, mutating or amplifying a polynucleotide encoding a polypeptide, anti-sense nucleic acids for inhibiting expression of a polynucleotide, and complementary sequences of the foregoing. The nucleic acids can be any length. They can be, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 750, 1,000, 1,500, 3,000, 5,000 or more nucleotides in length, and/or can comprise one or more additional sequences, for example, regulatory sequences, and/or be part of a larger nucleic acid, for example, a vector. The nucleic acids can be single-stranded or double-stranded and can comprise RNA and/or DNA nucleotides, and artificial variants thereof (*e.g.*, peptide nucleic acids).

[00328] Nucleic acids encoding antibody polypeptides (*e.g.*, heavy or light chain, variable domain only, or full length) can be isolated from B-cells of mice that have been immunized with CTLA-4.

[00329] Nucleic acid sequences encoding the variable regions of the heavy and light chain variable regions are shown herein. The skilled artisan will appreciate that, due to the degeneracy of the genetic code, each of the polypeptide sequences disclosed herein is encoded by a large number of other nucleic acid sequences. The present disclosure provides each degenerate nucleotide sequence encoding each antigen binding protein of the present disclosure. In some embodiments, the nucleic acid sequences have been codon optimized. In some embodiments, the nucleic acid sequences have been codon optimized for expression in a mammalian cell.

[00330] The present disclosure further provides nucleic acids that hybridize to other nucleic acids (*e.g.*, nucleic acids comprising a nucleotide sequence of any of CTLA-4 gene) under particular hybridization conditions.

[00331] Changes can be introduced by mutation into a nucleic acid, thereby leading to changes in the amino acid sequence of a polypeptide (*e.g.*, an antigen binding protein) that it encodes. Mutations can be introduced using any technique known in the art.

[00332] Mutations can be introduced into a nucleic acid without significantly altering the biological activity of a polypeptide that it encodes. For example, one can make nucleotide substitutions leading to amino acid substitutions at non-essential amino acid residues. In one embodiment, a nucleotide sequence provided herein for CTLA-4, or a desired fragment, variant, or derivative thereof, is mutated such that it encodes an amino acid sequence comprising one or more deletions or substitutions of amino acid residues that are shown herein for CTLA-4 to be residues where two or more sequences differ. Alternatively, one or more mutations can be introduced into a nucleic acid that selectively change the biological activity (*e.g.*, binding of CTLA-4) of a polypeptide that it encodes. For example, the mutation can quantitatively or qualitatively change the biological activity. Examples of quantitative changes include increasing, reducing or eliminating the activity. Examples of qualitative changes include changing the antigen specificity of an antigen binding protein.

[00333] In another aspect, the present disclosure provides nucleic acid molecules that are suitable for use as primers or hybridization probes for the detection of nucleic acid sequences of the present disclosure. A nucleic acid molecule of the present disclosure can comprise only a portion of a nucleic acid sequence encoding a full-length polypeptide of the present disclosure, for example, a fragment that can be used as a probe or primer or a fragment encoding an active portion (*e.g.*, a CTLA-4 binding portion) of a polypeptide of the present disclosure.

7.5. Expression vectors

[00334] The present disclosure provides vectors comprising a nucleic acid encoding a polypeptide of the present disclosure or a portion thereof. Examples of vectors include, but are not limited to, plasmids, viral vectors, non-episomal mammalian vectors and expression vectors, for example, recombinant expression vectors.

[00335] In another aspect of the present disclosure, expression vectors containing the nucleic acid molecules and polynucleotides of the present disclosure are also provided, and host cells transformed with such vectors, and methods of producing the polypeptides are also provided. The term "expression vector" refers to a plasmid, phage, virus or vector for expressing (e.g. or inducing expression of) a polypeptide from a polynucleotide sequence. Vectors for the expression of the polypeptides contain at a minimum sequence required for vector propagation and for expression of the cloned insert. An expression vector comprises a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a sequence that encodes polypeptides and

proteins to be transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. These sequences may further include a selection marker. Vectors suitable for expression in host cells are readily available and the nucleic acid molecules are inserted into the vectors using standard recombinant DNA techniques. Such vectors can include promoters which function in specific tissues, and viral vectors for the expression of polypeptides in targeted human or animal cells.

[00336] The recombinant expression vectors of the present disclosure can comprise a nucleic acid of the present disclosure in a form suitable for expression of the nucleic acid in a host cell. Accordingly, in one aspect, the present disclosure provides a host cell comprising the polynucleotide or the vector encoding the ABP of the present disclosure. The host cell can be used to produce the ABP *ex vivo*. In some embodiments, the host cell is administered to a subject to induce expression of the ABP *in vivo*. In some embodiments, the host cells are used as a therapeutic for treatment of a disease.

[00337] In some embodiments, the expression vector is an expression vector purified from one of the clones of the library of CTLA-4 binding clones deposited under ATCC Accession No. PTA-125512. In some embodiments, the expression vector is generated by genetic modification of one of an expression vector in one of the clones purified from the library of CTLA-4 binding clones deposited under ATCC Accession No. PTA-125512. In some embodiments, the expression vector is generated by using variable region sequences of heavy and light chains of one of the clones of the library of CTLA-4 binding clones deposited under ATCC Accession No. PTA-125512.

[00338] The present disclosure further provides methods of making polypeptides. A variety of other expression/host systems may be utilized.

[00339] In some embodiments, the mammalian cells used in recombinant protein production include engineered cells that produce a reduced amount of core fucosylation (e.g., relative to the amount of core fucosylation of a non-engineered cell). In some embodiments, the mammalian cells used in recombinant protein production include engineered cells that produce a reduced amount of fucose (e.g., relative to the amount of fucose of a non-engineered cell). In some embodiments, the mammalian cells used in recombinant protein production comprise CHO cells. In some embodiments, the mammalian cell is a GlymaxX® cell line. In certain embodiments, the cell line produces afucosylated recombinant proteins. In certain embodiments, the mammalian cell has less or reduced fucosylation, e.g., where the mammalian cell produces a reduced amount of fucose. In certain embodiments, during cell culture, the method includes adding a fucosylation inhibitor to the media that the cells are grown in. Non-limiting examples of

fucosylation inhibitors include: fucosyltransferase (FUT) inhibitor, 2- fluoro peracetylated fucose (2FF), 2-fluorofucose (SGN-2FF), Fucotrim I (P-D-Rha6F2-1P), Fucotrim II (P-D-Rha6F3-1P), A2FF1P, and B2FF1. In some embodiments, the mammalian cells used in recombinant protein production are engineered to overexpress glycosyltransferase. In certain embodiments, the glycosyltransferase is Beta-1,4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase.

[00340] In some embodiments, the ABP comprises an afucosylated Fc region. In some embodiments, the antibody is afucosylated (e.g., the N-glycan of the Fc region of the antibody does not have core fucose sugar units).

[00341] In certain embodiments, the glycosyltransferase competes with FuT8. In some embodiments, the mammalian cell line used in recombinant protein production produces an ABP that has reduced fucosylation (e.g. less than 70% fucosylation, less than 65% fucosylation, less than 60% fucosylation, less than 55% fucosylation, less than 50% fucosylation, less than 45% fucosylation, less than 40% fucosylation, less than 35% fucosylation, less than 30% fucosylation, less than 25% fucosylation, less than 20% fucosylation, less than 15% fucosylation, less than 10% fucosylation, less than 5% fucosylation, or less than 2.5% fucosylation).

[00342] In some embodiments, the mammalian cell line used in recombinant protein production produces an ABP that has increased fucosylation (e.g. more than 99% fucosylation, more than 95% fucosylation, more than 90% fucosylation, more than 85% fucosylation, more than 80% fucosylation, more than 75% fucosylation, more than 70% fucosylation, more than 65% fucosylation, more than 60% fucosylation, more than 55% fucosylation, more than 50% fucosylation, more than 45% fucosylation, more than 40% fucosylation, more than 35% fucosylation, more than 30% fucosylation, more than 25% fucosylation, more than 20% fucosylation, more than 15% fucosylation, more than 10% fucosylation, more than 5% fucosylation, or more than 2.5% fucosylation).

[00343] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Once such cells are transformed with vectors that contain selectable markers as well as the desired expression cassette, the cells can be allowed to grow in an enriched media before they are switched to selective media, for example. The selectable marker is designed to allow growth and recovery of cells that successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using

tissue culture techniques appropriate to the cell line employed. An overview of expression of recombinant proteins is found in Methods of Enzymology, v. 185, Goeddell, D.V., ed., Academic Press (1990). Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die), among other methods.

[00344] The transformed cells can be cultured under conditions that promote expression of the polypeptide, and the polypeptide recovered by conventional protein purification procedures (as defined above). One such purification procedure includes the use of affinity chromatography, *e.g.*, over a matrix having all or a portion (*e.g.*, the extracellular domain) of CTLA-4 bound thereto. Polypeptides contemplated for use herein include substantially homogeneous recombinant mammalian anti- CTLA-4 antibody polypeptides substantially free of contaminating endogenous materials.

[00345]

[00346] The polypeptides and proteins of the present disclosure can be purified according to protein purification techniques well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the proteinaceous and non-proteinaceous fractions. Having separated the peptide polypeptides from other proteins, the peptide or polypeptide of interest can be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). The term "purified polypeptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the polypeptide is purified to any degree relative to its naturally-obtainable state. A purified polypeptide therefore also refers to a polypeptide that is free from the environment in which it may naturally occur. Generally, "purified" will refer to a polypeptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a peptide or polypeptide composition in which the polypeptide or peptide forms the major component of the composition, such as constituting about 50 %, about 60 %, about 70 %, about 80 %, about 85 %, or about 90 % or more of the proteins in the composition.

[00347] Various techniques suitable for use in purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies (immunoprecipitation) and the like or by heat denaturation, followed by centrifugation; chromatography such as affinity chromatography (Protein-A columns), ion

exchange, gel filtration, reverse phase, hydroxylapatite, hydrophobic interaction chromatography, isoelectric focusing, gel electrophoresis, and combinations of these techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified polypeptide. Exemplary purification steps are provided in the Examples below.

[00348] Various methods for quantifying the degree of purification of polypeptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific binding activity of an active fraction, or assessing the amount of peptide or polypeptide within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a polypeptide fraction is to calculate the binding activity of the fraction, to compare it to the binding activity of the initial extract, and to thus calculate the degree of purification, herein assessed by a "-fold purification number." The actual units used to represent the amount of binding activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the polypeptide or peptide exhibits a detectable binding activity.

7.6. Method of generating antibodies

[00349] Fully human monoclonal antibodies may be generated by any number of techniques with which those having ordinary skill in the art will be familiar. Such methods include, but are not limited to, Epstein Barr Virus (EBV) transformation of human peripheral blood cells (*e.g.*, containing B lymphocytes), *in vitro* immunization of human B-cells, fusion of spleen cells from immunized transgenic mice carrying inserted human immunoglobulin genes, isolation from human immunoglobulin V region phage libraries, or other procedures as known in the art and based on the disclosure herein. For example, fully human monoclonal antibodies may be obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. Methods for obtaining fully human antibodies from transgenic mice are described, for example, by Green *et al.*, *Nature Genet.* 7:13, 1994; Lonberg *et al.*, *Nature* 368:856, 1994; Taylor *et al.*, *Int. Immun.* 6:579, 1994; U.S. Patent No. 5,877,397; Bruggemann *et al.*, 1997 *Curr. Opin. Biotechnol.* 8:455-58; Jakobovits *et al.*, 1995 *Ann. N. Y. Acad. Sci.* 764:525-35. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci (*see also* Bruggemann *et al.*, *Curr. Opin. Biotechnol.* 8:455-58 (1997)). For example, human immunoglobulin transgenes may be mini-gene constructs, or transloci on yeast artificial chromosomes, which undergo B-cell-specific DNA rearrangement and hypermutation in the mouse lymphoid tissue. Fully human

monoclonal antibodies may be obtained by immunizing the transgenic mice, which may then produce human antibodies specific for CTLA-4. Lymphoid cells of the immunized transgenic mice can be used to produce human antibody-secreting hybridomas according to the methods described herein. Polyclonal sera containing fully human antibodies may also be obtained from the blood of the immunized animals.

[00350] Another method for generating human antibodies of the present disclosure includes immortalizing human peripheral blood cells by EBV transformation. *See, e.g.*, U.S. Patent No. 4,464,456. Such an immortalized B-cell line (or lymphoblastoid cell line) producing a monoclonal antibody that specifically binds to CTLA-4 can be identified by immunodetection methods as provided herein, for example, an ELISA, and then isolated by standard cloning techniques. The stability of the lymphoblastoid cell line producing an anti-CTLA-4 antibody may be improved by fusing the transformed cell line with a murine myeloma to produce a mouse-human hybrid cell line according to methods known in the art (*see, e.g.*, Glasky *et al.*, *Hybridoma* 8:377-89 (1989)). Still another method to generate human monoclonal antibodies is *in vitro* immunization, which includes priming human splenic B-cells with human CTLA-4, followed by fusion of primed B-cells with a heterohybrid fusion partner. *See, e.g.*, Boerner *et al.*, 1991 *J. Immunol.* 147:86-95.

[00351] In certain embodiments, a B-cell that is producing an anti-human CTLA-4 antibody is selected and the light chain and heavy chain variable regions are cloned from the B-cell according to molecular biology techniques known in the art (WO 92/02551; U.S. Patent 5,627,052; Babcook *et al.*, *Proc. Natl. Acad. Sci. USA* 93:7843-48 (1996)) and described herein.

[00352] In some embodiments, specific antibody-producing B-cells are selected by using a method that allows identification natively paired antibodies. For example, a method described in Adler *et al.*, A natively paired antibody library yields drug leads with higher sensitivity and specificity than a randomly paired antibody library, MAbs (2018), which is incorporated by reference in its entirety herein, can be employed.

[00353] After the B-cells producing the desired antibody are selected, the specific antibody genes may be cloned by isolating and amplifying DNA or mRNA according to methods known in the art and described herein.

[00354] The methods for obtaining antibodies of the present disclosure can also adopt various phage display technologies known in the art. *See, e.g.*, Winter *et al.*, 1994 *Annu. Rev. Immunol.* 12:433-55; Burton *et al.*, 1994 *Adv. Immunol.* 57:191-280. Human or murine immunoglobulin variable region gene combinatorial libraries may be created in phage vectors that can be screened to select Ig fragments (Fab, Fv, sFv, or multimers thereof) that bind

specifically to CTLA-4 binding protein or variant or fragment thereof. *See, e.g.*, U.S. Patent No. 5,223,409; Huse *et al.*, 1989 *Science* 246:1275-81; Sastry *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5728-32 (1989); Alting-Mees *et al.*, *Strategies in Molecular Biology* 3:1-9 (1990); Kang *et al.*, 1991 *Proc. Natl. Acad. Sci. USA* 88:4363-66; Hoogenboom *et al.*, 1992 *J. Molec. Biol.* 227:381-388; Schlebusch *et al.*, 1997 *Hybridoma* 16:47-52 and references cited therein.

[00355] Antibody fragments fused to another protein, such as a minor coat protein, can be also used to enrich phage with antigen. Then, using a random combinatorial library of rearranged heavy (V_H) and light (V_L) chains from mice immune to the antigen (*e.g.* CTLA-4), diverse libraries of antibody fragments are displayed on the surface of the phage. These libraries can be screened for complementary variable domains, and the domains purified by, for example, affinity column. *See* Clackson et al., Nature, V. 352 pp. 624-628 (1991).

[00356] Heavy and light chain immunoglobulin cDNA expression libraries may also be prepared in lambda phage, for example, using λImmunoZap^{TM}(H) and λImmunoZap^{TM}(L) vectors (Stratagene, La Jolla, California).

[00357] In one embodiment, in a hybridoma the variable regions of a gene expressing a monoclonal antibody of interest are amplified using nucleotide primers. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. (*See, e.g.*, Stratagene (La Jolla, California), which sells primers for mouse and human variable regions including, among others, primers for V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions.) These primers may be used to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP^{TM}H or ImmunoZAP^{TM}L (Stratagene), respectively.

[00358] Once cells producing antibodies according to the disclosure have been obtained using any of the above-described immunization and other techniques, the specific antibody genes may be cloned by isolating and amplifying DNA or mRNA therefrom according to standard procedures as described herein. The antibodies produced therefrom may be sequenced and the CDRs identified and the DNA coding for the CDRs may be manipulated as described previously to generate other antibodies according to the disclosure.

[00359] CTLA-4 binding agents of the present disclosure preferably modulate CTLA-4 function in the cell-based assay described herein and/or the *in vivo* assay described herein and/or bind to one or more of the domains described herein and/or cross-block the binding of one of the antibodies described in this application and/or are cross-blocked from binding CTLA-4 by one of the antibodies described in this application. Accordingly such binding agents can be identified using the assays described herein.

[00360] In certain embodiments, antibodies are generated by first identifying antibodies that bind to one or more of the domains provided herein and/or neutralize in the cell-based and/or *in vivo* assays described herein and/or cross-block the antibodies described in this application and/or are cross-blocked from binding CTLA-4 by one of the antibodies described in this application. The CDR regions from these antibodies are then used to insert into appropriate biocompatible frameworks to generate CTLA-4 binding agents. The non-CDR portion of the binding agent may be composed of amino acids, or may be a non-protein molecule. The assays described herein allow the characterization of binding agents. Preferably the binding agents of the present disclosure are antibodies as defined herein.

[00361] Other antibodies according to the disclosure may be obtained by conventional immunization and cell fusion procedures as described herein and known in the art.

[00362] Molecular evolution of the complementarity determining regions (CDRs) in the center of the antibody binding site also has been used to isolate antibodies with increased affinity, for example, antibodies having increased affinity for c-erbB-2, as described by Schier *et al.*, 1996, *J. Mol. Biol.* 263:551. Accordingly, such techniques are useful in preparing antibodies to CTLA-4. Antigen binding proteins directed against a CTLA-4 can be used, for example, in assays to detect the presence of CTLA-4 polypeptides, either *in vitro* or *in vivo*. The antigen binding proteins also may be employed in purifying CTLA-4 proteins by immunoaffinity chromatography.

[00363] Although human, partially human, or humanized antibodies will be suitable for many applications, particularly those involving administration of the antibody to a human subject, other types of antigen binding proteins will be suitable for certain applications. Non-human antibodies of the present disclosure can be, for example, derived from any antibody-producing animal, such as mouse, rat, rabbit, goat, donkey, or non-human primate (such as monkey (*e.g.*, cynomolgus or rhesus monkey) or ape (*e.g.*, chimpanzee)). An antibody from a particular species can be made by, for example, immunizing an animal of that species with the desired immunogen (*e.g.*, a CTLA-4 polypeptide) or using an artificial system for generating antibodies of that species (*e.g.*, a bacterial or phage display-based system for generating antibodies of a particular species), or by converting an antibody from one species into an antibody from another species by replacing, *e.g.*, the constant region of the antibody with a constant region from the other species, or by replacing one or more amino acid residues of the antibody so that it more closely resembles the sequence of an antibody from the other species. In one embodiment, the antibody is a chimeric antibody comprising amino acid sequences derived from antibodies from two or more different species.

[00364] Antigen binding proteins may be prepared, and screened for desired properties, by any of a number of conventional techniques. Certain of the techniques involve isolating a nucleic acid encoding a polypeptide chain (or portion thereof) of an antigen binding protein of interest (*e.g.*, an anti- CTLA-4 antibody), and manipulating the nucleic acid through recombinant DNA technology. The nucleic acid may be fused to another nucleic acid of interest, or altered (*e.g.*, by mutagenesis or other conventional techniques) to add, delete, or substitute one or more amino acid residues, for example. Furthermore, the antigen binding proteins may be purified from cells that naturally express them (*e.g.*, an antibody can be purified from a hybridoma that produces it), or produced in recombinant expression systems, using any technique known in the art. *See*, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet *et al.* (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988).

[00365] Any expression system known in the art can be used to make the recombinant polypeptides of the present disclosure. Expression systems are detailed comprehensively above. In general, host cells are transformed with a recombinant expression vector that comprises DNA encoding a desired polypeptide. Among the host cells that may be employed are prokaryotes, yeast or higher eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *Bacilli*. Higher eukaryotic cells include insect cells and established cell lines of mammalian origin. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman *et al.*, 1981, Cell 23:175), L cells, 293 cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, BHK (ATCC CRL 10) cell lines, and the CVI/EBNA cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan *et al.*, 1991, EMBO J. 10: 2821. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels *et al.* (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985).

[00366] It will be appreciated that an antibody of the present disclosure may have at least one amino acid substitution, providing that the antibody retains binding specificity. Therefore, modifications to the antibody structures are encompassed within the scope of the present disclosure. These may include amino acid substitutions, which may be conservative or non-conservative that do not destroy the CTLA-4 binding capability of an antibody. Conservative amino acid substitutions may encompass non-naturally occurring amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics and other reversed or inverted forms of amino acid

moieties. A conservative amino acid substitution may also involve a substitution of a native amino acid residue with a normative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position.

[00367] Non-conservative substitutions may involve the exchange of a member of one class of amino acids or amino acid mimetics for a member from another class with different physical properties (*e.g.* size, polarity, hydrophobicity, charge). Such substituted residues may be introduced into regions of the human antibody that are homologous with non-human antibodies, or into the non-homologous regions of the molecule.

[00368] Moreover, one skilled in the art may generate test variants containing a single amino acid substitution at each desired amino acid residue. The variants can then be screened using activity assays known to those skilled in the art. Such variants could be used to gather information about suitable variants. For example, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, variants with such a change may be avoided. In other words, based on information gathered from such routine experiments, one skilled in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

[00369] A skilled artisan will be able to determine suitable variants of the polypeptide as set forth herein using well-known techniques. In certain embodiments, one skilled in the art may identify suitable areas of the molecule that may be changed without destroying activity by targeting regions not believed to be important for activity. In certain embodiments, one can identify residues and portions of the molecules that are conserved among similar polypeptides. In certain embodiments, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

[00370] Additionally, one skilled in the art can review structure-function studies identifying residues in similar polypeptides that are important for activity or structure. In view of such a comparison, one can predict the importance of amino acid residues in a protein that correspond to amino acid residues which are important for activity or structure in similar proteins. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted important amino acid residues.

[00371] One skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In view of such information, one skilled in the art may predict the alignment of amino acid residues of an antibody with respect to its three dimensional structure. In certain embodiments, one skilled in the art may

choose not to make radical changes to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules.

[00372]

[00373] In certain embodiments, variants of antibodies include glycosylation variants wherein the number and/or type of glycosylation site has been altered compared to the amino acid sequences of a parent polypeptide. In certain embodiments, variants comprise a greater or a lesser number of N-linked glycosylation sites than the native protein. An N-linked glycosylation site is characterized by the sequence: Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X can be any amino acid residue except proline. The substitution of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions which eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created. Additional preferred antibody variants include cysteine variants wherein one or more cysteine residues are deleted from or substituted for another amino acid (*e.g.*, serine) as compared to the parent amino acid sequence. Cysteine variants can be useful when antibodies must be refolded into a biologically active conformation such as after the isolation of insoluble inclusion bodies. Cysteine variants generally have fewer cysteine residues than the native protein, and typically have an even number to minimize interactions resulting from unpaired cysteines.

[00374] Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. In certain embodiments, amino acid substitutions can be used to identify important residues of antibodies to CTLA-4, or to increase or decrease the affinity of the antibodies to CTLA-4 described herein.

[00375] According to certain embodiments, preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and/or (4) confer or modify other physiochemical or functional properties on such polypeptides. According to certain embodiments, single or multiple amino acid substitutions (in certain embodiments, conservative amino acid substitutions) may be made in the naturally-occurring sequence (in certain embodiments, in the portion of the polypeptide outside the domain(s) forming intermolecular contacts). In certain embodiments, a conservative amino acid substitution typically may not substantially change the structural characteristics of the parent sequence (*e.g.*, a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other

types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton *et al. Nature* 354:105 (1991), which are each incorporated herein by reference.

[00376] In certain embodiments, antibodies of the present disclosure may be chemically bonded with polymers, lipids, or other moieties.

[00377] The binding agents may comprise at least one of the CDRs described herein incorporated into a biocompatible framework structure. In one example, the biocompatible framework structure comprises a polypeptide or portion thereof that is sufficient to form a conformationally stable structural support, or framework, or scaffold, which is able to display one or more sequences of amino acids that bind to an antigen (*e.g.*, CDRs, a variable region, etc.) in a localized surface region. Such structures can be a naturally occurring polypeptide or polypeptide "fold" (a structural motif), or can have one or more modifications, such as additions, deletions or substitutions of amino acids, relative to a naturally occurring polypeptide or fold. These scaffolds can be derived from a polypeptide of any species (or of more than one species), such as a human, other mammal, other vertebrate, invertebrate, plant, bacteria or virus.

[00378] Typically the biocompatible framework structures are based on protein scaffolds or skeletons other than immunoglobulin domains. For example, those based on fibronectin, ankyrin, lipocalin, neocarzinostain, cytochrome b, CP1 zinc finger, PST1, coiled coil, LACI-D1, Z domain and tendamistat domains may be used (*See e.g.*, Nygren and Uhlen, 1997, *Curr. Opin. in Struct. Biol.*, 7, 463-469).

[00379] Humanized antibodies can be produced using techniques known to those skilled in the art (Zhang, W., *et al.*, *Molecular Immunology. 42(12)*:1445-1451, 2005; Hwang W. *et al.*, *Methods. 36(1)*:35-42, 2005; Dall'Acqua WF, *et al.*, *Methods 36(1)*:43-60, 2005; and Clark, M., I*mmunology Today. 21(8)*:397-402, 2000).

[00380] Additionally, one skilled in the art will recognize that suitable binding agents include portions of these antibodies, such as one or more of CDR1-L1 to 28 with SEQ ID NOS 1001-1028; CDR2-L1 to 28 with SEQ ID NOS 2001-2028; CDR3-L1 to 28 with SEQ ID NOS 3001-3028; CDR1-H1to 28 with SEQ ID NOS 4001-4028; CDR2-H1to 28 with SEQ ID NOS 5001-5028; and CDR3-H1to 28 with SEQ ID NOS 6001-6028, as specifically disclosed herein. At least one of the regions of CDR regions may have at least one amino acid substitution from the sequences provided here, provided that the antibody retains the binding specificity of the non-substituted CDR. The non-CDR portion of the antibody may be a non-protein molecule, wherein

the binding agent cross-blocks the binding of an antibody disclosed herein to CTLA-4 and/or neutralizes CTLA-4. The non-CDR portion of the antibody may be a non-protein molecule in which the antibody exhibits a similar binding pattern to human CTLA-4 peptides in a competition binding assay as that exhibited by at least one of antibodies A1-A28, and/or neutralizes CTLA-4. The non-CDR portion of the antibody may be composed of amino acids, wherein the antibody is a recombinant binding protein or a synthetic peptide, and the recombinant binding protein cross-blocks the binding of an antibody disclosed herein to CTLA-4 and/or neutralizes CTLA-4. The non-CDR portion of the antibody may be composed of amino acids, wherein the antibody is a recombinant antibody, and the recombinant antibody exhibits a similar binding pattern to human CTLA-4 peptides in the human CTLA-4 peptide epitope competition binding assay (described hereinbelow) as that exhibited by at least one of the antibodies A1-A28, and/or neutralizes CTLA-4.

[00381] Where an antibody comprises one or more of CDR1-H, CDR2-H, CDR3-H, CDR1-L, CDR2-L and CDR3-L as described above, it may be obtained by expression from a host cell containing DNA coding for these sequences. A DNA coding for each CDR sequence may be determined on the basis of the amino acid sequence of the CDR and synthesized together with any desired antibody variable region framework and constant region DNA sequences using oligonucleotide synthesis techniques, site-directed mutagenesis and polymerase chain reaction (PCR) techniques as appropriate. DNA coding for variable region frameworks and constant regions is widely available to those skilled in the art from genetic sequences databases such as GenBank®.

[00382] Once synthesized, the DNA encoding an antibody of the present disclosure or fragment thereof may be propagated and expressed according to any of a variety of well-known procedures for nucleic acid excision, ligation, transformation, and transfection using any number of known expression vectors. Thus, in certain embodiments expression of an antibody fragment may be preferred in a prokaryotic host, such as *Escherichia coli* (*see, e.g.*, Pluckthun *et al.*, 1989 *Methods Enzymol.* 178:497-515). In certain other embodiments, expression of the antibody or a fragment thereof may be preferred in a eukaryotic host cell, including yeast (*e.g.*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Pichia pastoris*), animal cells (including mammalian cells) or plant cells. Examples of suitable animal cells include, but are not limited to, myeloma (such as a mouse NSO line), COS, CHO, or hybridoma cells. Examples of plant cells include tobacco, corn, soybean, and rice cells.

[00383] One or more replicable expression vectors containing DNA encoding an antibody variable and/or constant region may be prepared and used to transform an appropriate cell line,

for example, a non-producing myeloma cell line, such as a mouse NSO line or a bacteria, such as *E. coli*, in which production of the antibody will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory sequences, particularly a promoter and leader sequence operatively linked to the variable domain sequence. Particular methods for producing antibodies in this way are generally well-known and routinely used. For example, basic molecular biology procedures are described by Maniatis *et al.* (*Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, New York, 1989; *see also* Maniatis *et al*, 3rd ed., Cold Spring Harbor Laboratory, New York, (2001)). DNA sequencing can be performed as described in Sanger *et al.* (PNAS 74:5463, (1977)) and the Amersham International plc sequencing handbook, and site directed mutagenesis can be carried out according to methods known in the art (Kramer *et al., Nucleic Acids Res.* 12:9441, (1984); Kunkel *Proc. Natl. Acad. Sci. USA* 82:488-92 (1985); Kunkel *et al., Methods in Enzymol.* 154:367-82 (1987); the Anglian Biotechnology Ltd. handbook). Additionally, numerous publications describe techniques suitable for the preparation of antibodies by manipulation of DNA, creation of expression vectors, and transformation and culture of appropriate cells (Mountain A and Adair, J R in *Biotechnology and Genetic Engineering Reviews* (ed. Tombs, M P, 10, Chapter 1, 1992, Intercept, Andover, UK); "Current Protocols in Molecular Biology", 1999, F.M. Ausubel (ed.), Wiley Interscience, New York).

[00384] Where it is desired to improve the affinity of antibodies according to the disclosure containing one or more of the above-mentioned CDRs can be obtained by a number of affinity maturation protocols including maintaining the CDRs (Yang *et al., J. Mol. Biol.*, 254, 392-403, 1995), chain shuffling (Marks *et al., Bio/Technology*, 10, 779-783, 1992), use of mutation strains of *E. coli.* (Low *et al., J. Mol. Biol.*, 250, 350-368, 1996), DNA shuffling (Patten *et al., Curr. Opin. Biotechnol.*, 8, 724-733, 1997), phage display (Thompson *et al., J. Mol. Biol.*, 256, 7-88, 1996) and sexual PCR (Crameri, *et al., Nature*, 391, 288-291, 1998). All of these methods of affinity maturation are discussed by Vaughan *et al.* (*Nature Biotech.*, 16, 535-539, 1998).

[00385] It will be understood by one skilled in the art that some proteins, such as antibodies, may undergo a variety of posttranslational modifications. The type and extent of these modifications often depends on the host cell line used to express the protein as well as the culture conditions. Such modifications may include variations in glycosylation, methionine oxidation, diketopiperizine formation, aspartate isomerization and asparagine deamidation. A frequent modification is the loss of a carboxy-terminal basic residue (such as lysine or arginine)

due to the action of carboxypeptidases (as described in Harris, R.J. *Journal of Chromatography* 705:129-134, 1995).

7.7. Sequences

[00386] Antibodies A1-A28 comprise heavy and light chain V(J)D polynucleotides (also referred to herein as L1-L28 and H1-H28, respectively). Antibodies A1-A28 comprise the sequences listed in TABLE 5. For example, antibody A1 comprises light chain L1 (SEQ ID NO:1) and heavy chain H1 (SEQ ID NO:101). CDR sequences in the light chain (L1-L28) and heavy chain (H1-H28) are also provided with a specific SEQ ID NOs. For example, three CDR sequences (CDR1, CDR 2 and CDR3) for L1 are CDR1-L1 (SEQ ID NO:1001), CDR2-L1 (SEQ ID NO:2001) and CDR3-L1 (SEQ ID NO:3001), respectively and three CDR sequences (CDR1, CDR 2 and CDR3) for H1 are CDR1-H1 (SEQ ID NO:4001), CDR2-H1 (SEQ ID NO:5001) and CDR3-H1 (SEQ ID NO:6001).

TABLE 5

Antibodies	Light Chain	Heavy Chain
A1	L1 (SEQ ID NO:1) L1 comprises CDR1-L1 (SEQ ID NO:1001), CDR2-L1 (SEQ ID NO:2001) and CDR3-L1 (SEQ ID NO:3001)	H1 (SEQ ID NO: 101) H1 comprises CDR1-H1 (SEQ ID NO: 4001), CDR2-H1 (SEQ ID NO: 5001) and CDR3-H1 (SEQ ID NO: 6001)
A2	L2 (SEQ ID NO:2) L2 comprises CDR1-L2 (SEQ ID NO:1002), CDR2-L2 (SEQ ID NO:2002) and CDR3-L2 (SEQ ID NO:3002)	H2 (SEQ ID NO: 102) He comprises CDR1-H2 (SEQ ID NO: 4002), CDR2-H2 (SEQ ID NO: 5002) and CDR3-H2 (SEQ ID NO: 6002)
A3	L3 (SEQ ID NO:3) L3 comprises CDR1-L3 (SEQ ID NO:1003), CDR2-L3 (SEQ ID NO:2003) and CDR3-L3 (SEQ ID NO:3003)	H3 (SEQ ID NO: 103) H3 comprises CDR1-H3 (SEQ ID NO:4003), CDR2-H3 (SEQ ID NO:5003) and CDR3-H3 (SEQ ID NO:6003)
A4	L4 (SEQ ID NO:4) L4 comprises CDR1-L4 (SEQ ID NO:1004), CDR2-L4 (SEQ ID NO:2004) and CDR3-L4 (SEQ ID NO:3004)	H4 (SEQ ID NO:104) H4 comprises CDR1-H4 (SEQ ID NO:4004), CDR2-H4 (SEQ ID NO:5004) and CDR3-H4 (SEQ ID NO:6004)
A5	L5 (SEQ ID NO:5) L5 comprises CDR1-L5 (SEQ ID NO:1005), CDR2-L5 (SEQ ID NO:2005) and CDR3-L5 (SEQ ID NO:3005)	H5 (SEQ ID NO:105) H4 comprises CDR1-H5 (SEQ ID NO:4005), CDR2-H5 (SEQ ID NO:5005) and CDR3-H5 (SEQ ID NO:6005)
A6	L6 (SEQ ID NO:6) L6 comprises CDR1-L6 (SEQ ID NO:1006), CDR2-L6 (SEQ ID NO:2006) and CDR3-L6 (SEQ ID NO:3006)	H6 (SEQ ID NO:106) H6 comprises CDR1-H6 (SEQ ID NO:4006), CDR2-H6 (SEQ ID NO:5006) and CDR3-H6 (SEQ ID NO:6006)
A7	L7 (SEQ ID NO:7) L7 comprises CDR1-L7 (SEQ ID NO:1007), CDR2-L7 (SEQ ID NO:2007) and CDR3-L7 (SEQ ID NO:3007)	H7 (SEQ ID NO:107) H7 comprises CDR1-H7 (SEQ ID NO:4007), CDR2-H7 (SEQ ID NO:5007) and CDR3-H7 (SEQ ID NO:6007)
A8	L8 (SEQ ID NO:8) L8 comprises CDR1-L8 (SEQ ID NO:1008), CDR2-L8 (SEQ ID NO:2008) and CDR3-L6 (SEQ ID NO:3008)	H8 (SEQ ID NO:108) H8 comprises CDR1-H8 (SEQ ID NO:4008), CDR2-H8 (SEQ ID NO:5008) and CDR3-H8 (SEQ ID NO:6008)
A9	L9 (SEQ ID NO:9)	H9 (SEQ ID NO:109)

	L9 comprises CDR1-L9 (SEQ ID NO:1009), CDR2-L9 (SEQ ID NO:2009) and CDR3-L9 (SEQ ID NO:3009)	H9 comprises CDR1-H9 (SEQ ID NO:4009), CDR2-H9 (SEQ ID NO:5009) and CDR3-H9 (SEQ ID NO:6009)
A10	L10 (SEQ ID NO:10) L10 comprises CDR1-L10 (SEQ ID NO:1010), CDR2-L10 (SEQ ID NO:2010) and CDR3-L10 (SEQ ID NO:3010)	H10 (SEQ ID NO:110) H10 comprises CDR1-H10 (SEQ ID NO:4010), CDR2-H10 (SEQ ID NO:5010) and CDR3-H10 (SEQ ID NO:6010)
A11	L11 (SEQ ID NO:11) L11 comprises CDR1-L11 (SEQ ID NO:1011), CDR2-L11 (SEQ ID NO:2011) and CDR3-L11 (SEQ ID NO:3011)	H11 (SEQ ID NO:111) H11 comprises CDR1-H11 (SEQ ID NO:4011), CDR2-H11 (SEQ ID NO:5011) and CDR3-H11 (SEQ ID NO:6011)
A12	L12 (SEQ ID NO:12) L12 comprises CDR1-L12 (SEQ ID NO:1012), CDR2-L12 (SEQ ID NO:2012) and CDR3-L12 (SEQ ID NO:3012)	H12 (SEQ ID NO:112) H12 comprises CDR1-H12 (SEQ ID NO:4012), CDR2-H12 (SEQ ID NO:5012) and CDR3-H12 (SEQ ID NO:6012)
A13	L13 (SEQ ID NO:13) L13 comprises CDR1-L13 (SEQ ID NO:1013), CDR2-L13 (SEQ ID NO:2013) and CDR3-L13 (SEQ ID NO:3013)	H13 (SEQ ID NO:113) H13 comprises CDR1-H13 (SEQ ID NO:4013), CDR2-H13 (SEQ ID NO:5013) and CDR3-H13 (SEQ ID NO:6013)
A14	L14 (SEQ ID NO:14) L14 comprises CDR1-L14 (SEQ ID NO:1014), CDR2-L14 (SEQ ID NO:2014) and CDR3-L14 (SEQ ID NO:3014)	H14 (SEQ ID NO:114) H14 comprises CDR1-H14 (SEQ ID NO:4014), CDR2-H14 (SEQ ID NO:5014) and CDR3-H14 (SEQ ID NO:6014)
A15	L15 (SEQ ID NO:15) L15 comprises CDR1-L15 (SEQ ID NO:1015), CDR2-L15 (SEQ ID NO:2015) and CDR3-L15 (SEQ ID NO:3015)	H15 (SEQ ID NO:115) H15 comprises CDR1-H15 (SEQ ID NO:4015), CDR2-H15 (SEQ ID NO:5015) and CDR3-H15 (SEQ ID NO:6015)
A16	L16 (SEQ ID NO:16) L16 comprises CDR1-L16 (SEQ ID NO:1016), CDR2-L16 (SEQ ID NO:2016) and CDR3-L16 (SEQ ID NO:3016)	H16 (SEQ ID NO:116) H16 comprises CDR1-H16 (SEQ ID NO:4016), CDR2-H16 (SEQ ID NO:5016) and CDR3-H16 (SEQ ID NO:6016)
A17	L17 (SEQ ID NO:17) L17 comprises CDR1-L17 (SEQ ID NO:1017), CDR2-L17 (SEQ ID NO:2017) and CDR3-L17 (SEQ ID NO:3017)	H17 (SEQ ID NO:117) H17 comprises CDR1-H17 (SEQ ID NO:4017), CDR2-H17 (SEQ ID NO:5017) and CDR3-H17 (SEQ ID NO:6017)
A18	L18 (SEQ ID NO:18) L18 comprises CDR1-L18 (SEQ ID NO:1018), CDR2-L18 (SEQ ID NO:2018) and CDR3-L18 (SEQ ID NO:3018)	H18 (SEQ ID NO:118) H18 comprises CDR1-H18 (SEQ ID NO:4018), CDR2-H18 (SEQ ID NO:5018) and CDR3-H18 (SEQ ID NO:6018)
A19	L19 (SEQ ID NO:19) L19 comprises CDR1-L19 (SEQ ID NO:1019), CDR2-L19 (SEQ ID NO:2019) and CDR3-L19 (SEQ ID NO:3019)	H19 (SEQ ID NO:119) H19 comprises CDR1-H19 (SEQ ID NO:4019), CDR2-H19 (SEQ ID NO:5019) and CDR3-H19 (SEQ ID NO:6019)
A20	L20 (SEQ ID NO:20) L20 comprises CDR1-L20 (SEQ ID NO:1020), CDR2-L20 (SEQ ID NO:2020) and CDR3-L20 (SEQ ID NO:3020)	H20 (SEQ ID NO:120) H20 comprises CDR1-H20 (SEQ ID NO:4020), CDR2-H20 (SEQ ID NO:5020) and CDR3-H20 (SEQ ID NO:6020)
A21	L21 (SEQ ID NO:21)	H21 (SEQ ID NO:121) H21 comprises CDR1-H21 (SEQ ID NO:4021), CDR2-H21 (SEQ ID

	L21 comprises CDR1-L21 (SEQ ID NO:1021), CDR2-L21 (SEQ ID NO:2021) and CDR3-L21 (SEQ ID NO:3021)	NO:5021) and CDR3-H21 (SEQ ID NO:6021)
A22	L22 (SEQ ID NO:22) L22 comprises CDR1-L22 (SEQ ID NO:1022), CDR2-L22 (SEQ ID NO:2022) and CDR3-L22 (SEQ ID NO:3022)	H22 (SEQ ID NO:122) H22 comprises CDR1-H22 (SEQ ID NO:4022), CDR2-H22 (SEQ ID NO:5022) and CDR3-H22 (SEQ ID NO:6022)
A23	L23 (SEQ ID NO:23) L23 comprises CDR1-L23 (SEQ ID NO:1023), CDR2-L23 (SEQ ID NO:2023) and CDR3-L23 (SEQ ID NO:3023)	H23 (SEQ ID NO:123) H23 comprises CDR1-H23 (SEQ ID NO:4023), CDR2-H23 (SEQ ID NO:5023) and CDR3-H23 (SEQ ID NO:6023)
A24	L24 (SEQ ID NO:24) L24 comprises CDR1-L24 (SEQ ID NO:1024), CDR2-L24 (SEQ ID NO:2024) and CDR3-L24 (SEQ ID NO:3024)	H24 (SEQ ID NO:124) H24 comprises CDR1-H24 (SEQ ID NO:4024), CDR2-H24 (SEQ ID NO:5024) and CDR3-H24 (SEQ ID NO:6024)
A25	L25 (SEQ ID NO:25) L25 comprises CDR1-L25 (SEQ ID NO:1025), CDR2-L25 (SEQ ID NO:2025) and CDR3-L25 (SEQ ID NO:3025)	H25 (SEQ ID NO:125) H25 comprises CDR1-H25 (SEQ ID NO:4025), CDR2-H25 (SEQ ID NO:5025) and CDR3-H25 (SEQ ID NO:6025)
A26	L26 (SEQ ID NO:26) L26 comprises CDR1-L26 (SEQ ID NO:1026), CDR2-L26 (SEQ ID NO:2026) and CDR3-L26 (SEQ ID NO:3026)	H26 (SEQ ID NO:126) H26 comprises CDR1-H26 (SEQ ID NO:4026), CDR2-H26 (SEQ ID NO:5026) and CDR3-H26 (SEQ ID NO:6026)
A27	L27 (SEQ ID NO:27) L27 comprises CDR1-L27 (SEQ ID NO:1027), CDR2-L27 (SEQ ID NO:2027) and CDR3-L27 (SEQ ID NO:3027)	H27 (SEQ ID NO:127) H27 comprises CDR1-H27 (SEQ ID NO:4027), CDR2-H27 (SEQ ID NO:5027) and CDR3-H27 (SEQ ID NO:6027)
A28	L28 (SEQ ID NO:28) L28 comprises CDR1-L28 (SEQ ID NO:1028), CDR2-L28 (SEQ ID NO:2028) and CDR3-L28 (SEQ ID NO:3028)	H28 (SEQ ID NO:128) H28 comprises CDR1-H28 (SEQ ID NO:4028), CDR2-H28 (SEQ ID NO:5028) and CDR3-H28 (SEQ ID NO:6028)

7.8. Pharmaceutical compositions

[00387] Pharmaceutical compositions containing the proteins and polypeptides of the present disclosure are also provided. Specifically, the present disclosure provides a pharmaceutical composition comprising anti-CTLA-4 ABP. In some embodiments, the pharmaceutical composition comprises GIGA-564. In some embodiments, the pharmaceutical composition comprises GIGA-2328. Such compositions comprise a therapeutically or prophylactically effective amount of the polypeptide or protein in a mixture with pharmaceutically acceptable materials, and physiologically acceptable formulation materials.

[00388] The pharmaceutical composition may contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition.

[00389] Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium hydrogen-sulfite); buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, other organic acids); bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin); fillers; monosaccharides; disaccharides and other carbohydrates (such as glucose, mannose, or dextrins); proteins (such as serum albumin, gelatin or immunoglobulins); coloring; flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides (preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. Neutral buffered saline or saline mixed with conspecific serum albumin are examples of appropriate diluents. In accordance with appropriate industry standards, preservatives such as benzyl alcohol may also be added. The composition may be formulated as a lyophilizate using appropriate excipient solutions (*e.g.*, sucrose) as diluents. Suitable components are nontoxic to recipients at the dosages and concentrations employed. Further examples of components that may be employed in pharmaceutical formulations are presented in Remington's Pharmaceutical Sciences, 16th Ed. (1980) and 20th Ed. (2000), Mack Publishing Company, Easton, PA.

[00390] In some embodiments, less than 50% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, less than 40% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, less than 30% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, less than 20% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, less than 10% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, less than 5% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, less than 3% of the ABP in the pharmaceutical composition is fucosylated.

[00391] In some embodiments, more than 99% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 95% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 90% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 85% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 80% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 75% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 70% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 65% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 60% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 50% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 40% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 30% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 20% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 10% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 5% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 3% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, more than 1% of the ABP in the pharmaceutical composition is fucosylated.

[00392] In some embodiments, 30-70% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, 20-50% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, 10-40% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, 10-30% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, 5-20% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, 1-10% of the ABP in the pharmaceutical composition is fucosylated. In some embodiments, 5-20% of the ABP in the pharmaceutical composition is fucosylated.

[00393] In some embodiments, the pharmaceutical formulation materials include one or more of: histidine buffer, citrate buffer, sucrose, sodium chloride, succinate, polysorbate 20, and polysorbate-80. In certain embodiments, the pharmaceutical formulation comprises 1-20 mM of histidine or citrate buffer. In certain embodiments, the pharmaceutical formulation comprises 100-350 mM of sucrose. In certain embodiments, the pharmaceutical formulation comprises 0-75 mM of sucrose. In certain embodiments, the pharmaceutical formulation comprises 0.002 to 0.1% by weight of polysorbate-20. In certain embodiments, the pharmaceutical formulation

comprises 0.002 to 0.1% by weight of polysorbate-80. In certain embodiments, the pharmaceutical formulation material includes 20 mM of citrate or histidine, 170 to 270 mM of sucrose, 0 to 50 mM of sodium chloride, and 0.02% by weight of polysorbate-20. In certain embodiments, the pharmaceutical formulation material includes 20 mM of citrate or histidine, 170 to 270 mM of sucrose, 0 to 50 mM of sodium chloride, and 0.02% by weight of polysorbate-80.

[00394] In some embodiments, the pharmaceutical composition has a pH from 5.0 to 6.5. In some embodiments, the pharmaceutical composition has a pH from 5.5 to 6.5. In some embodiments, the pharmaceutical composition has a pH from 6.0 to 6.5. In some embodiments, the pharmaceutical composition has pH 6.1, 6.2, 6.3, 6.4, or 6.5. In some embodiments, the pharmaceutical composition has pH 6.2.

[00395] In some embodiments, the pharmaceutical composition comprises 20mM of histidine or citrate buffer. In some embodiments, the pharmaceutical composition comprises 20mM of histidine buffer. In some embodiments, the pharmaceutical composition comprises 20mM of citrate buffer.

[00396] In some embodiments, the pharmaceutical composition comprises 50mM of NaCl. In some embodiments, the pharmaceutical composition is devoid of NaCl.

[00397] In some embodiments, the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration of 170mM. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration of 200mM. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration of 250mM. In some embodiments, the pharmaceutical composition comprises sucrose at a concentration of 270mM.

[00398] In some embodiments, the pharmaceutical composition comprises 170mM or 270mM of sucrose.

[00399] In some embodiments, the pharmaceutical composition comprises Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 0.05-0.5 mg/ml Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 0.1-1 mg/ml Polysorbate 20. In some embodiments, the pharmaceutical composition comprises 0.2 mg/ml Polysorbate 20.

[00400] In some embodiments, the pharmaceutical composition comprises 20mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 5mg/mL of the ABP. In some embodiments, the pharmaceutical composition comprises 5mg/mL to 20 mg/mL of the ABP.

[00401] In some embodiments, the pharmaceutical composition comprises trehalose.

[00402] In some embodiments, the pharmaceutical composition comprises about 0.04 to 30 mg/ml of the ABP, 0.9% Sodium Chloride Injection, USP or 5% Dextrose Injection, USP. In some embodiments, the pharmaceutical composition comprises 20mM histidine, 270mM sucrose, and 0.2 mg/ml Polysorbate 20, and has pH 6.2.

[00403] In some embodiments, the ABP is formulated at a concentration of 1 mg/ml to 80 mg/ml. In certain embodiments, the ABP is formulated at a concentration of 5 mg/ml to 20 mg/ml. In certain embodiments, the ABP is formulated at a concentration of 20mg/ml. In certain embodiments, the ABP is formulated at a concentration of 10mg/ml. In certain embodiments, the ABP is formulated at a concentration of 5mg/ml.

[00404] In some embodiments, the pharmaceutical composition comprises 20mg/ml of the ABP, 20mM histidine, 270mM sucrose, and 0.2 mg/ml Polysorbate 20, and has pH 6.2. In some embodiments, the pharmaceutical composition comprises 10mg/ml of the ABP, 20mM histidine, 270mM sucrose, and 0.2 mg/ml Polysorbate 20, and has pH 6.2. In some embodiments, the pharmaceutical composition comprises 5mg/ml of the ABP, 20mM histidine, 270mM sucrose, and 0.2 mg/ml Polysorbate 20, and has pH 6.2.

[00405] In some embodiments, the pharmaceutical composition comprises a pH of about 5 to 7. In some embodiments, the pharmaceutical formulation comprises a pH of about 5.0 to 6.5. In some embodiments, the pharmaceutical formulation comprises a pH of about 5.0, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, or 7.

[00406] Optionally, the composition additionally comprises one or more physiologically active agents, for example, an anti-angiogenic substance, a chemotherapeutic substance (such as capecitabine, 5-fluorouracil, or doxorubicin), an analgesic substance, *etc.*, non-exclusive examples of which are provided herein. In various embodiments, the composition comprises one, two, three, four, five, or six physiologically active agents in addition to a CTLA-4-binding protein.

[00407] In another embodiment of the present disclosure, the compositions disclosed herein may be formulated in a neutral or salt form. Illustrative pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine,

trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

[00408] The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[00409] The optimal pharmaceutical composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format, and desired dosage. *See* for example, Remington's Pharmaceutical Sciences, *supra.* Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the polypeptide. For example, suitable compositions may be water for injection, physiological saline solution for parenteral administration.

7.8.1. Content of pharmaceutically active ingredient

[00410] In typical embodiments, the active ingredient (*i.e.,* the proteins and polypeptides, ABP, of the present disclosure) is present in the pharmaceutical composition at a concentration of at least 0.01mg/ml, at least 0.005 mg/ml, at least 0.004 mg/ml, at least 0.05 mg/ml, 0.04 mg/ml, 0.1mg/ml, at least 0.5mg/ml, or at least 1mg/ml. In some embodiments, the active ingredient is present in the pharmaceutical composition at a concentration of 0.1-20mg/ml, 0.1-10mg/ml, or 0.2-10mg/ml. In some embodiments, the active ingredient is present in the pharmaceutical composition at a concentration of 0.24-9.54 mg/ml.

[00411] In certain embodiments, the active ingredient is present in the pharmaceutical composition at a concentration of at least 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 25 mg/ml, or 30 mg/ml. In certain embodiments, the active ingredient is present in the pharmaceutical composition at a concentration of 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 25 mg/ml, or 30 mg/ml. In certain embodiments, the active ingredient is present in the pharmaceutical composition at a concentration of at least 20 mg/ml, 25 mg/ml, 30 mg/ml, 35 mg/ml, 40 mg/ml, 45 mg/ml, 50 mg/ml, 55 mg/ml, 60 mg/ml, 65 mg/ml, 70 mg/ml, 75 mg/ml, 80 mg/ml, 85 mg/ml, 90 mg/ml, 95 mg/ml, or 100 mg/ml. In certain embodiments, the active ingredient is present in the

pharmaceutical composition at a concentration of 20 mg/ml, 25 mg/ml, 30 mg/ml, 35 mg/ml, 40 mg/ml, 45 mg/ml, 50 mg/ml, 55 mg/ml, 60 mg/ml, 65 mg/ml, 70 mg/ml, 75 mg/ml, 80 mg/ml, 85 mg/ml, 90 mg/ml, 95 mg/ml, or 100 mg/ml. In certain embodiments, the active ingredient is present in the pharmaceutical composition at a concentration ranging from 1 mg/ml to 80 mg/ml. In certain embodiments, the active ingredient is present in the pharmaceutical composition at a concentration ranging from 5 mg/ml to 20 mg/ml. In certain embodiments, the active ingredient is present in the pharmaceutical composition at a concentration ranging from 0.04 mg/ml to 30 mg/ml.

[00412] In some embodiments, the pharmaceutical composition comprises one or more additional active ingredients in addition to the proteins or polypeptides of the present disclosure. The one or more additional active ingredients can be a drug targeting a different check-point receptor, such as PD-1 inhibitor (*e.g.*, anti-PD-1 antibody), PD-L1 inhibitor (*e.g.*, anti-PD-L1 antibody), LAG-3 inhibitor, CD47 inhibitor, or TIGIT inhibitor (*e.g.*, anti-TIGIT antibody).

7.8.2. Formulation Generally

[00413] The pharmaceutical composition can be in any form appropriate for human or veterinary medicine, including a liquid, an oil, an emulsion, a gel, a colloid, an aerosol or a solid.

[00414] The pharmaceutical composition can be formulated for administration by any route of administration appropriate for human or veterinary medicine, including enteral and parenteral routes of administration.

[00415] In various embodiments, the pharmaceutical composition is formulated for administration by inhalation. In certain of these embodiments, the pharmaceutical composition is formulated for administration by a vaporizer. In certain of these embodiments, the pharmaceutical composition is formulated for administration by a nebulizer. In certain of these embodiments, the pharmaceutical composition is formulated for administration by an aerosolizer.

[00416] In various embodiments, the pharmaceutical composition is formulated for oral administration, for buccal administration, or for sublingual administration.

[00417] In some embodiments, the pharmaceutical composition is formulated for intravenous, intramuscular, or subcutaneous administration.

[00418] In some embodiments, the pharmaceutical composition is formulated for intrathecal or intracerebroventricular administration.

[00419] In some embodiments, the pharmaceutical composition is formulated for an infusion. In some embodiments, the pharmaceutical composition is formulated for i.v. infusion.

7.8.3. Pharmacological compositions adapted for injection

[00420] For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution

which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives can be included, as required.

[00421] In various embodiments, the unit dosage form is a vial, ampule, bottle, or pre-filled syringe. In some embodiments, the unit dosage form contains 0.005 mg, 0.05 mg, 0.01 mg, 0.1 mg, 0.5 mg, 1 mg, 2.5 mg, 5 mg, 10 mg, 12.5 mg, 25 mg, 50 mg, 75 mg, or 100 mg of the pharmaceutical composition. In some embodiments, the unit dosage form contains 125 mg, 150 mg, 175 mg, or 200 mg of the pharmaceutical composition. In some embodiments, the unit dosage form contains 250 mg of the pharmaceutical composition. In some embodiments, a vial contains 50mg, 60mg, 70mg, 80mg, 90mg, or 100mg of the pharmaceutical composition.

[00422] In some embodiments, the unit dosage form contains from 50mg to 2500mg of the anti-CTLA-4 ABP. In some embodiments, the unit dosage form contains from 50mg to 1500mg of the anti-CTLA-4 ABP. In some embodiments, the unit dosage form contains from 50mg to 1000mg of the anti-CTLA-4 ABP. In some embodiments, the unit dosage form contains from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg of the anti-CTLA-4 ABP. In some embodiments, the unit dosage form contains 80mg, 240mg, 720mg, 800mg, 1440mg or 2160mg of the anti-CTLA-4 ABP. In some embodiments, the unit dose form contains 50mg, 100mg, 150mg, 250mg, 700mg, 800mg, 900mg, 1000mg, 1500mg, 2000mg, or 2500mg of the anti-CTLA-4 ABP. In some embodiments, a vial contains 50mg, 60mg, 70mg, 80mg, 90mg, or 100mg of the anti-CTLA-4 ABP.

[00423] In typical embodiments, the pharmaceutical composition in the unit dosage form is in liquid form. In various embodiments, the unit dosage form contains between 0.1 mL and 50 ml of the pharmaceutical composition. In some embodiments, the unit dosage form contains 1 ml, 2.5 ml, 5 ml, 7.5 ml, 10 ml, 25 ml, or 50 ml of pharmaceutical composition.

[00424] In particular embodiments, the unit dosage form is a vial containing 1 ml of the pharmaceutical composition at a concentration of 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, or 1mg/ml. In some embodiments, the unit dosage form is a vial containing 2 ml of the pharmaceutical composition at a concentration of 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, or 1mg/ml. In some embodiments, the unit dosage form is a vial containing a 1 to 150 ml of the pharmaceutical composition at a concentration of 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 25 mg/ml, 30 mg/ml, 35 mg/ml, 40 mg/ml, 45 mg/ml, 50 mg/ml, 55 mg/ml, 60 mg/ml, 65 mg/ml, 70 mg/ml, 75 mg/ml, 80 mg/ml, 85

mg/ml, 90 mg/ml, 95 mg/ml, or 100 mg/ml. In some embodiments, the unit dosage form is a vial containing a 1 to 150 ml of the pharmaceutical composition at a concentration of 0.01-0.1mg/ml, 0.1-0.5 mg/ml, 0.5-1 mg/ml, 1-10 mg/ml, 1-50 mg/ml, 10-50 mg/ml, 10-100 mg/ml, 50-100 mg/ml, or 50-200 mg/ml.

[00425] In some embodiments, the pharmaceutical composition in the unit dosage form is in solid form, such as a lyophilate, suitable for solubilization.

[00426] Unit dosage form embodiments suitable for subcutaneous, intradermal, or intramuscular administration include preloaded syringes, auto-injectors, and autoinject pens, each containing a predetermined amount of the pharmaceutical composition described hereinabove.

[00427] In various embodiments, the unit dosage form is a preloaded syringe, comprising a syringe and a predetermined amount of the pharmaceutical composition. In certain preloaded syringe embodiments, the syringe is adapted for subcutaneous administration. In certain embodiments, the syringe is suitable for self-administration. In particular embodiments, the preloaded syringe is a single use syringe.

[00428] In various embodiments, the preloaded syringe contains about 0.1 mL to about 0.5 mL of the pharmaceutical composition. In certain embodiments, the syringe contains about 0.5 mL of the pharmaceutical composition. In specific embodiments, the syringe contains about 1.0 mL of the pharmaceutical composition. In particular embodiments, the syringe contains about 2.0 mL of the pharmaceutical composition.

[00429] In certain embodiments, the unit dosage form is an autoinject pen. The autoinject pen comprises an autoinject pen containing a pharmaceutical composition as described herein. In some embodiments, the autoinject pen delivers a predetermined volume of pharmaceutical composition. In other embodiments, the autoinject pen is configured to deliver a volume of pharmaceutical composition set by the user.

[00430] In various embodiments, the autoinject pen contains about 0.1 mL to about 5.0 mL of the pharmaceutical composition. In specific embodiments, the autoinject pen contains about 0.5 mL of the pharmaceutical composition. In particular embodiments, the autoinject pen contains about 1.0 mL of the pharmaceutical composition. In other embodiments, the autoinject pen contains about 5.0 mL of the pharmaceutical composition.

7.9. Dosage Regimens

[00431] In some embodiments, the ABP is administered at a dose sufficient to produce a therapeutic effect.

[00432] In various embodiments, the ABP is administered using a weight based dose. In some embodiments, the ABP is administered in an amount of at least 0.05 mg/kg. In some embodiments, the ABP is administered in an amount of at least 0.01 mg/kg. In some

embodiments, the ABP is administered in an amount of at least 0.1 mg/kg. In some embodiments, the ABP is administered in an amount of at least 0.5 mg/kg. In certain embodiments, the ABP is administered in an amount of at least 1 mg/kg. In certain embodiments, the dose is at least 2 mg/kg, at least 3 mg/kg, at least 4 mg/kg, at least 5 mg/kg, at least 6 mg/kg, at least 7 mg/kg, at least 8 mg/kg, at least 9 mg/kg, or at least 10 mg/kg.

[00433] In some embodiments, the ABP is administered in an amount of less than 30mg/kg. In some embodiments, the ABP is administered in an amount of at least 0.01mg/kg, 0.03mg/kg, 0.1mg/kg, 0.3mg/kg, 1mg/kg, 9 mg/kg or 27 mg/kg. In some embodiments, the ABP is administered in an amount from 0.5mg/kg to 30mg/kg. In some embodiments, the ABP is administered in an amount from 1mg/kg to 18mg/kg. In some embodiments, the ABP is administered in an amount from 1mg/kg to 10mg/kg. In some embodiments, the ABP is administered in an amount of 1mg/kg, 3mg/kg, 9 mg/kg, 27 mg/kg or 30mg/kg.

[00434] In various embodiments, the dose of the ABP is at least 10 mg/kg. In certain embodiments, the dose is at least 15 mg/kg, at least 20 mg/kg, at least 25 mg/kg, 30 mg/kg, at least 35 mg/kg, at least 40 mg/kg, at least 45 mg/kg, at least 50 mg/kg, at least 55 mg/kg, at least 60 mg/kg, at least 65 mg/kg, at least 70 mg/kg, at least 75 mg/kg, at least 80 mg/kg, at least 85 mg/kg, at least 90 mg/kg, at least 95 mg/kg, at least 100 mg/kg, at least 150 mg/kg, at least 175 mg/kg, or at least 200 mg/kg. In certain embodiments, the dose is 250 mg/kg, 300 mg/kg, 350 mg/kg, 400 mg/kg, 450 mg/kg, 500 mg/kg, 600 mg/kg, 650 mg/kg, 700 mg/kg, 750 mg/kg, 800 mg/kg, 850 mg/kg, 900 mg/kg, 950 mg/kg, or 1000 mg/kg. In certain embodiments, the dose is 0.5 mg/kg to 100 mg/kg per day. In certain embodiments, the dose is 2 mg/kg to 100 mg/kg per day. In certain embodiments, the dose is 25 mg/kg to 1000 mg/kg per day.

[00435] In some embodiments, the dose of the ABP is from 50mg to 2500mg. In some embodiments, the dose of the ABP is from 50mg to 2000mg. In some embodiments, the dose of the ABP is from 50mg to 1500mg. In some embodiments, the dose of the ABP is from 50mg to 1000mg. In some embodiments, the dose of the ABP is from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg. In some embodiments, the dose of the ABP is 80mg, 240mg, 720mg, 800mg, 1440mg or 2160mg. In some embodiments, the dose of the ABP is 100mg, 150mg, 200mg, 250mg, 500mg, 700mg, 750mg, 800mg, 1400mg, 1500mg, 2000mg, 2100mg, 2200mg, 2300mg, 2400mg, or 2500mg.

7.10. Unit dosage forms

[00436] The pharmaceutical compositions may conveniently be presented in unit dosage form.

[00437] The unit dosage form will typically be adapted to one or more specific routes of administration of the pharmaceutical composition.

[00438] In various embodiments, the unit dosage form is adapted for administration by inhalation. In certain of these embodiments, the unit dosage form is adapted for administration by a vaporizer. In certain of these embodiments, the unit dosage form is adapted for administration by a nebulizer. In certain of these embodiments, the unit dosage form is adapted for administration by an aerosolizer.

[00439] In various embodiments, the unit dosage form is adapted for oral administration, for buccal administration, or for sublingual administration.

[00440] In some embodiments, the unit dosage form is adapted for intravenous, intramuscular, intratumoral, peritumoral or subcutaneous administration. In some embodiments, the unit dosage form is adapted for intravenous infusion.

[00441] In some embodiments, the unit dosage form is adapted for intrathecal or intracerebroventricular administration.

[00442] In some embodiments, the pharmaceutical composition is formulated for topical administration.

[00443] The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

[00444] In some embodiments, the unit dosage form comprises 50mg to 5000mg of the ABP. In some embodiments, the unit dosage form comprises 50mg to 2500mg of the ABP. In some embodiments, the unit dosage form comprises 50mg to 2000mg of the ABP. In some embodiments, the unit dosage form comprises 10mg to 2000mg of the ABP. In some embodiments, the unit dosage form comprises 50mg to 1000mg of the ABP.

[00445] In some embodiments, the unit dosage form comprises the ABP at an amount from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg.

[00446] In some embodiments, the unit dosage form comprises the ABP at an amount of 80mg, 100mg, 160mg, 200mg, 240mg, 300mg, 400mg, 500mg, 720mg, 800mg, 900mg, 1000mg, 1440mg or 2160mg.

[00447] In some embodiments, the unit dose is in a vial and comprises 20mg/mL of the ABP. In some embodiments, the unit dose is in a vial and comprises 5mg/mL of the ABP. In some embodiments, the unit dose is in a vial and comprises 5mg/mL to 20mg/mL of the ABP.

[00448] In some embodiments, the unit dose is in a diluted form and comprises 0.1-20mg/mL of ABP. In some embodiments, the unit dose is in a diluted form and comprises 0.1-10mg/mL, 0.2-10mg/mL, 0.24-9.54mg/mL or 0.2-9mg/mL of ABP. In some embodiments, the unit dose is diluted in 0.9% sodium chloride. In some embodiments, the unit dose is diluted in dextrose.

7.11. Methods of use

[00449] In one aspect, therapeutic antibodies may be used that specifically bind to intact CTLA-4.

[00450] *In vivo* and/or *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each subject's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

[00451] An oligopeptide or polypeptide is within the scope of the present disclosure if it has an amino acid sequence that is at least 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to least one of the CDRs provided herein; and/or to a CDR of a CTLA-4 binding agent that cross-blocks the binding of at least one of antibodies A1-A28 to CTLA-4, and/or is cross-blocked from binding to CTLA-4 by at least one of antibodies A1-A28; and/or to a CDR of a CTLA-4 binding agent wherein the binding agent can block the binding of CTLA-4 to its ligands.

[00452] CTLA-4 binding agent polypeptides and antibodies are within the scope of the present disclosure if they have amino acid sequences that are at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to a variable region of at least one of antibodies A1-A28, and cross-block the binding of at least one of antibodies A1-A28 to CTLA-4, and/or are cross-blocked from binding to CTLA-4 by at least one of antibodies A1-A28; and/or can block the inhibitory effect of CTLA-4 on its ligands.

[00453] Antibodies according to the disclosure may have a binding affinity for human CTLA-4 of less than or equal to 5×10^{-7}M, less than or equal to 1×10^{-7}M, less than or equal to 0.5×10^{-7}M, less than or equal to 1×10^{-8}M, less than or equal to 1×10^{-9}M, less than or equal to 1×10^{-10}M, less than or equal to 1×10^{-11}M, or less than or equal to 1×10^{-12} M.

[00454] The affinity of an antibody or binding partner, as well as the extent to which an antibody inhibits binding, can be determined by one of ordinary skill in the art using conventional techniques, for example those described by Scatchard *et al.* (*Ann. N.Y. Acad. Sci.* 51:660-672 (1949)) or by surface plasmon resonance (SPR; BIAcore, Biosensor, Piscataway, NJ). For surface plasmon resonance, target molecules are immobilized on a solid phase and exposed to ligands in a mobile phase running along a flow cell. If ligand binding to the immobilized target occurs, the local refractive index changes, leading to a change in SPR angle, which can be monitored in real time by detecting changes in the intensity of the reflected light. The rates of change of the SPR signal can be analyzed to yield apparent rate constants for the association and dissociation phases of the binding reaction. The ratio of these values gives the apparent equilibrium constant (affinity) (*see, e.g.,* Wolff *et al.*, *Cancer Res.* 53:2560-65 (1993)).

[00455] An antibody according to the present disclosure may belong to any immunoglobin class, for example IgG, IgE, IgM, IgD, or IgA.

7.11.1. Methods of treating a disease responsive to a CTLA-4 inhibitor or activator

[00456] In another aspect, methods are presented for treating a subject having a disease responsive to a CTLA-4 inhibitor or activator. The disease can be cancer, autoimmune disease, or viral or bacterial infection. In some embodiments, the disease is autoimmune disease, autoinflammatory disease or inflammation.

[00457] The terms "treatment," "treating," and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic, in terms of completely or partially preventing a disease, condition, or symptoms thereof, and/or may be therapeutic in terms of a partial or complete cure for a disease or condition and/or adverse effect, such as a symptom, attributable to the disease or condition. "Treatment" as used herein covers any treatment of a disease or condition of a mammal, particularly a human, and includes: (a) preventing the disease or condition from occurring in a subject which may be predisposed to the disease or condition but has not yet been diagnosed as having it; (b) inhibiting the disease or condition (*e.g.*, arresting its development); or (c) relieving the disease or condition (*e.g.*, causing regression of the disease or condition, providing improvement in one or more symptoms). Improvements in any conditions can be readily assessed according to standard methods and techniques known in the art. The population of subjects treated by the method of the disease includes subjects suffering from the undesirable condition or disease, as well as subjects at risk for development of the condition or disease.

[00458] In some embodiments, the pharmaceutical composition is administered by inhalation, orally, by buccal administration, by sublingual administration, by injection or by topical application. In some embodiments, the pharmaceutical composition is administered by i.v. infusion.

[00459] In some embodiments, the major cannabinoid is administered in an amount less than 1g, less than 500 mg, less than 100 mg, less than 10 mg per dose.

[00460] In some embodiments, the pharmaceutical composition is administered once a day, 2-4 times a day, 2-4 times a week, once a week, or once every two weeks.

[00461] A composition can be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. For example, the pharmaceutical composition can be administered in combination with one or more drugs targeting a different check-point receptor, such as PD-1 inhibitor (*e.g.*, anti-PD-1 antibody), PD-L1 inhibitor (*e.g.*, anti-PD-L1 antibody), LAG-3 inhibitor, CD47 inhibitor, or TIGIT inhibitor (*e.g.*, anti-TIGIT antibody).

[00462] In some embodiments, the disease is selected from the group consisting of cancer, AIDS, Alzheimer's disease and viral or bacterial infection. In certain embodiments, the disease is cancer. In some embodiments, the disease is selected from the group consisting of autoimmune disease, autoinflammatory disease, and inflammation.

[00463] In certain embodiments, the disease is cancer. In certain embodiments, the subject has a tumor. Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. Types of cancers to be treated with the pharmaceutical composition described herein include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included. In some embodiments, the cancer is RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, MSI colorectal cancer, ovarian cancer, or cervical cancer. In some embodiments, the subject has a tumor with high levels of Tregs, high levels of CTLA-4, high levels of NK cells, or high levels of activating FcRs.

[00464] Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma,

Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases). In some embodiments, the subject has a tumor with high levels of Tregs, high levels of CTLA-4, high levels of NK cells, or high levels of activating FcRs.

[00465] In some embodiments, the subject is suffering from a cancer selected from the group consisting of colon carcinoma, breast cancer, pancreatic cancer, ovarian cancer, prostate cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, merkel cell carcinoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acute lymphocytic leukemia, acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and combinations thereof.

[00466] In some embodiments, the subject has a tumor with high levels of Tregs, high levels of CTLA-4, high levels of NK cells, or high levels of activating FcRs.

[00467] In additional embodiments, the cancer is a solid tumor selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma,

colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases.

[00468] In some embodiments, the cancer patient has melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, hepatocellular carcinoma, esophageal cancer, breast cancer, sarcoma, MSI-Hi/dMMR colorectal cancer, ovarian cancer, or cervical cancer, bladder, prostate, TMB-HI tumors of any origin, a tumor which is MSI, a tumor that is dMMR, a T cell leukemia/lymphoma, NHL, a tumor expressing CTLA-4 by the cancer cell.

[00469] In certain embodiments, the cancer is a solid tumor selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma/colorectal cancer, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases.

[00470] In some embodiments, the cancer is resistant to anti-PD-1 or anti-PD-L1 treatment. In some embodiments, the cancer is resistant to treatment of anti-PD-1 antibody or

anti-PD-L1 antibody. In some embodiments, the anti-CTLA-4 ABP is used to treat a patient who has been previously treated with anti-PD-1 antibody or anti-PD-L1 antibody. In some embodiments, the cancer patient has progressed or relapsed after anti-PD-1 or anti-PD-L1 treatment.

[00471] In some embodiments, the method of treatment using anti-CTLA-4 ABP further comprises the step of screening patients appropriate for the treatment. In some embodiments, the method of treatment further comprises the step of deciding whether the cancer is resistant to anti-PD-1 treatment or anti-PD-L1 treatment.

[00472] In some embodiments, the method of treatment using anti-CTLA-4 ABP further comprises the step of administering an antigen binding protein (anti-PD-1 ABP or anti-PD-L1 ABP) that specifically binds a human PD-1 or anti-PD-L1. In some embodiments, the patient is treated with anti-CTLA-4 ABP and followed by treatment with anti-PD-1 ABP or anti-PD-L1 ABP. In some embodiments, the patient is treated with anti-PD-1 ABP or anti-PD-L1 ABP, and followed by treatment with anti-CTLA-4 ABP. In some embodiments, the patient is treated with both anti-CTLA-4 ABP and anti-PD-1 ABP or anti-PD-L1 ABP in the same treatment cycle.

[00473] In some embodiments, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on the same day. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on different days. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on different days in the same cycle. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on different days in different cycles.

[00474] In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on the same day. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on different days. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on different days in the same cycle. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on different days in different cycles.

[00475] In some embodiments, the anti-CTLA-4 ABP is administered over multiple cycles. In some embodiments, the anti-CTLA-4 ABP is repeatedly administered over two, three, four, five, six, seven or more cycles. In some embodiments, each cycle is two weeks apart, three weeks apart, four weeks apart, five weeks apart, six weeks apart, seven weeks apart, eight weeks apart, or nine weeks apart from each other. In some embodiments, each cycle is one month apart, two months apart, three months apart, four months apart, five months apart, six months apart, or seven months apart from each other.

[00476] In some embodiments, the anti-CTLA-4 ABP is administered once. In some embodiments, the anti-CTLA-4 ABP is administered twice, three times, four times, five times, six times, or more.

[00477] In some embodiments, the anti-CTLA-4 ABP is repeatedly administered every 1-2 weeks, every 2-3 weeks, every 3-4 weeks, every 4-5 weeks, every 5-6 weeks, every 6-7 weeks, every 7-8 weeks, every 8-9 weeks, every 9-10 weeks, every 10-11 weeks, every 11-12 weeks, every 12-13 weeks, every 13-14 weeks, or every 14-15 weeks. In some embodiments, the anti-CTLA-4 ABP is administered every month, every two months, every three months, every four months, every five months, or less frequent. In some embodiments, the anti-CTLA-4 ABP is administered every 1-2 months, every 2-3 months, every 3-4 months, every 4-5 months, or every 5-6 months.

[00478] In some embodiments, the anti-PD-1 ABP or anti-PD-L1 ABP is administered in combination with the anti-CTLA-4 ABP in each of the repeated administrations. In some embodiments, the anti-PD-1 ABP or anti-PD-L1 ABP is administered in combination with the anti-CTLA-4 ABP in some but not all of the repeated administrations. In some embodiments, the anti-PD-1 ABP or anti-PD-L1 ABP is administered in combination with the anti-CTLA-4 ABP in some of the repeated administrations, and the anti-PD-1 ABP or anti-PD-L1 ABP is administered without the anti-CTLA-4 ABP in other repeated administrations.

[00479] In some embodiments, the anti-PD-1 ABP is pembrolizumab. In some embodiments, the anti-PD-1 ABP is nivolumab, cemiplimab, atezolizumab, dostarlimab, or durvalumab. In some embodiments, the anti-PD-L1 ABP is atezolizumab, or avelumab.

[00480] In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio selected from 3:1, 3:10, 1:3, 1:10, 10:1, 10:3, 9:1, and 1:1. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio from 2:1 to 10:1. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio from 1:1 to 1:10. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio of 3:1.

[00481] In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered at a weight ratio selected from 3:1, 3:10, 1:3, 1:10, 10:1, 10:3, 9:1, and 1:1. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered at a weight ratio from 2:1 to 10:1. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered at a weight ratio from 1:1 to 1:10. In some embodiments, the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered at a weight ratio of 3:1.

[00482] In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 100mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 70mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 60mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 50mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 30mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 20mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 10mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose less than 5mg/kg.

[00483] In some embodiments, the anti-CTLA-4 ABP is administered at a dose greater than 0.01mg/kg, 0.03mg/kg, 0.1mg/kg, 0.3mg/kg, or 1mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose greater than 2 mg/kg, 3mg/kg, 4 mg/kg, 5 mg/kg, or 10 mg/kg.

[00484] In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 0.5mg/kg to 30mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 1mg/kg to 25mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 1mg/kg to 20mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 1mg/kg to 18mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 1mg/kg to 10mg/kg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose of 1mg/kg, 3mg/kg, 9 mg/kg, 27 mg/kg, or 30mg/kg.

[00485] In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 50mg to 2500mg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 50mg to 2000mg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 50mg to 1500mg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 50mg to 1000mg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg. In some embodiments, the anti-CTLA-4 ABP is administered at a dose of 40mg, 80mg, 120mg, 240mg, 720mg, 800mg, 1440mg or 2160mg in each administration. In some embodiments, the anti-CTLA-4 ABP is administered at a dose of 50mg, 100mg, 150mg, 250mg, 700mg, 800mg, 900mg, 1000mg, 1500mg, 2000mg, or 2500mg in each administration.

[00486] In another aspect, the present disclosure provides a method of reducing CTLA-4^{HI} Tregs in a subject with limited proliferation of remaining Tregs comprising administering to the subject an effective dose of an antigen binding protein.

[00487] In some embodiments, the subject is a human subject, optionally, a human subject with cancer. In some embodiments, the subject is a human subject, optionally, a human subject with melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, MSI colorectal cancer, ovarian cancer, or cervical cancer.

[00488] In some embodiments, the method further comprising the step of administering one or more additional therapeutic agents to the subject.

[00489] In some embodiments, the subject has a tumor with high levels of Tregs, high levels of CTLA-4, high levels of NK cells, or high levels of activating FcRs.

8. EXAMPLES

[00490] Below are examples of specific embodiments for carrying out the present disclosure. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present disclosure in any way. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, *etc.*), but some experimental error and deviation should, of course, be allowed for.

[00491] The practice of the present disclosure will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992). Furthermore, methods of generating and selecting antibodies explained in Adler *et al.*, A natively paired antibody library yields drug leads with higher sensitivity and specificity than a randomly paired antibody library, MAbs (2018), and Adler *et al.*, Rare, high-affinity mouse anti-CTLA-4 antibodies that function in checkpoint blockade, discovered using microfluidics and molecular genomics, MAbs (2017), which are incorporated by reference in its entirety herein, can be employed.

8.1. Example 1: Generation of antigen binding protein

Mouse Immunization and Sample Preparation:

[00492] First, transgenic mice carrying inserted human immunoglobulin genes were immunized with soluble CTLA-4 immunogen of SEQ ID NO: 7001 (*i.e.,* His-tagged CTLA-4 protein (R&D Systems)) using TiterMax as an adjuvant. One μg of immunogen was injected into each hock and 3 μg of immunogen was administered intraperitoneally, every third day for 15 days. Titer was assessed by enzyme-linked immunosorbent assay (ELISA) on a 1:2 dilution series of each animal's serum, starting at a 1:200 dilution. A final intravenous boost of 2.5 μg/hock without adjuvant was given to each animal before harvest. Lymph nodes (popliteal, inguinal, axillary, and mesenteric) were surgically removed after sacrifice. Single cell suspensions for each animal were made by manual disruption followed by passage through a 70 μm filter. Next, the EasySep™ Mouse Pan-B Cell Isolation Kit (Stemcell Technologies) negative selection kit was used to isolate B cells from each sample. The lymph node B cell populations were quantified by counting on a C-Chip hemocytometer (Incyto) and assessed for viability using Trypan blue. The cells were then diluted to 5,000–6,000 cells/mL in phosphate-buffered saline (PBS) with 12% OptiPrep™ Density Gradient Medium (Sigma). This cell mixture was used for microfluidic encapsulation. Approximately one million B cells were run from each of the six animals through an emulsion droplet microfluidics platform.

Generating paired heavy and light chain libraries:

[00493] A DNA library encoding scFv from RNA of single cells, with native heavy-light Ig pairing intact, was generated using the emulsion droplet microfluidics platform or vortex emulsions. The method for generating the DNA library was divided into 1) poly(A) + mRNA capture, 2) multiplexed overlap extension reverse transcriptase polymerase chain reaction (OE-RT-PCR), and 3) nested PCR to remove artifacts and add adapters for deep sequencing or yeast display libraries. The scFv libraries were generated from approximately one million B cells from each animal that achieved a positive ELISA titer.

[00494] For poly(A) + mRNA capture, a custom designed co-flow emulsion droplet microfluidic chip fabricated from glass (Dolomite) was used. The microfluidic chip has two input channels for fluorocarbon oil (Dolomite), one input channel for the cell suspension mix described above, and one input channel for oligo-dT beads (NEB) at 1.25 mg/ml in cell lysis buffer (20 mM Tris pH 7.5, 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Tween-20, and 20 mM dithiothreitol). The input channels were etched to 50 μm by 150 μm for most of the chip's length, narrow to 55 μm at the droplet junction, and were coated with hydrophobic Pico-Glide (Dolomite). Three Mitos P-Pump pressure pumps (Dolomite) were used to pump the liquids through the chip. Droplet size depends on pressure, but typically droplets of ~45 mm diameter are optimally stable. Emulsions were collected into chilled 2 ml microcentrifuge tubes

and incubated at 40 °C for 15 minutes for mRNA capture. The beads were extracted from the droplets using Pico-Break (Dolomite). In some embodiments, similar single cell partitioning emulsions were made using a vortex.

[00495] For multiplex OE-RT-PCR, glass Telos droplet emulsion microfluidic chips were used (Dolomite). mRNA-bound beads were re-suspended into OE-RT-PCR mix and injected into the microfluidic chips with a mineral oil-based surfactant mix (available commercially from GigaGen) at pressures that generate 27 μm droplets. The OE-RT-PCR mix contains 2x one-step RT-PCR buffer, 2.0 mM $MgSO_4$, SuperScript III reverse transcriptase, and Platinum Taq (Thermo Fisher Scientific), plus a mixture of primers directed against the IgK C region, the IgG C region, and all V regions (FIG. 2). The overlap region was a DNA sequence that encodes a Gly-Ser rich scFv linker sequence. The DNA fragments were recovered from the droplets using a droplet breaking solution (available commercially from GigaGen) and then purified using QIAquick PCR Purification Kit (Qiagen). In some embodiments, similar OE-RT-PCR emulsions were made using a vortex.

[00496] For nested PCR (FIG. 2), the purified OE-RT-PCR product was first run on a 1.7% agarose gel for 80 minutes at 150 V. A band at 1200–1500 base pair (bp) corresponding to the linked product was excised and purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey Nagel). PCR was then performed to add adapters for Illumina sequencing or yeast display; for sequencing, a randomer of seven nucleotides is added to increase base calling accuracy in subsequent next generation sequencing steps. Nested PCR was performed with 2x NEBNext High-Fidelity amplification mix (NEB) with either Illumina adapter containing primers or primers for cloning into the yeast expression vector. The nested PCR product was run on a 1.2% agarose gel for 50 minutes at 150V. A band at 800–1100 bp was excised and purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey Nagel).

[00497] In some embodiments, scFv libraries were not natively paired, for example, randomly paired by amplifying scFv directly from RNA isolated from B cells.

8.2. Example 2: Isolation of CTLA-4 binders by yeast display

Library Screening:

[00498] Human IgG1-Fc (Thermo Fisher Scientific) and CTLA-4 (R&D Systems) proteins were biotinylated using the EZ-Link Micro Sulfo-NHS-LC-Biotinylation kit (Thermo Fisher Scientific). The biotinylation reagent was resuspended to 9 mM and added to the protein at a 50-fold molar excess. The reaction was incubated on ice for 2 hours and then the biotinylation reagent was removed using Zeba desalting columns (Thermo Fisher Scientific). The final protein concentration was calculated with a Bradford assay.

[00499] Next, the six DNA libraries were expressed as surface scFv in yeast. A yeast surface display vector (pYD) that contains a GAL1/10 promoter, an Aga2 cell wall tether, and a C-terminal c-Myc tag was built. The GAL1/10 promoter induces expression of the scFv protein in medium that contains galactose. The Aga2 cell wall tether was required to shuttle the scFv to the yeast cell surface and tether the scFv to the extracellular space. The c-Myc tag was used during the flow sort to stain for yeast cells that express in-frame scFv protein. *Saccharomyces cerevisiae* cells (ATCC) were electroporated (Bio-Rad Gene Pulser II; 0.54 kV, 25 uF, resistance set to infinity) with gel-purified nested PCR product and linearized pYD vector for homologous recombination *in vivo*. Transformed cells were expanded and induced with galactose to generate yeast scFv display libraries.

[00500] Two million yeast cells from the expanded scFv libraries were stained with anti-c-Myc (Thermo Fisher Scientific A21281) and an AF488-conjugated secondary antibody (Thermo Fisher Scientific A11039). To select scFv-expressing cells that bind to CTLA-4, biotinylated CTLA-4 antigen was added to the yeast culture (7 nM final) during primary antibody incubation and then stained with PE-streptavidin (Thermo Fisher Scientific). Yeast cells were flow sorted on a BD Influx (Stanford Shared FACS Facility) for double- positive cells (AF488C/PEC), and recovered clones were then plated on SD-CAA plates with kanamycin, streptomycin, and penicillin (Teknova) for expansion. The expanded first round FACS clones were then subjected to a second round of FACS with the same antigen at the same molarity (7 nM final). Plasmid minipreps (Zymo Research) were prepared from yeast recovered from the final FACS sort. Tailed-end PCR was used to add Illumina adapters to the plasmid libraries for deep sequencing.

[00501] In a typical FACS dot plot, the upper right quadrant contains yeast that stain for both antigen binding and scFv expression (identified by a C-terminal c-Myc tag). The lower left quadrant contains yeast that do not stain for either the antigen or scFv expression. The lower right quadrant contains yeast that express the scFv but do not bind the antigen. The frequency of binders in each repertoire was estimated by dividing the count of yeast that double stain for antigen and scFv expression by the count of yeast that express an scFv. Libraries generated from immunized mice yielded low percentages of scFv binders (ranging from 0.08%–1.28%) when sorted at 7 nM final antigen concentration. There was no clear association between serum titer and the frequency of binders in a repertoire. Following expansion of these sorted cells, a second round of FACS at 7 nM final antigen concentration was used to increase the specificity of the screen. The frequency of binders in the second FACS was always substantially higher than the first FACS, ranging from 8.39%–84.4%. Generally, lower frequency of binders in the first sort

yielded lower frequency of binders in the second sort. Presumably, this is due to lower gating specificity for samples that have fewer bona fide binders in the original repertoire.

Deep repertoire sequencing:

[00502] CTLA-4-binding clones were recovered as a library ("a library of CTLA-4 binding clones"), and subjected to deep repertoire sequencing. Deep repertoire sequencing determines the sequences of all paired variable (V(D)J) regions of both heavy and light chain sequences. The library of CTLA-4 binding clones was deposited under ATCC Accession No. PTA-125512 under the Budapest Treaty on November 20, 2018, under ATCC Account No. 197361 (American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110 USA). Each clone in the library contains an scFv comprising a paired variable (V(D)J) regions of both heavy and light chain sequences originating from a single cell. Deep repertoire sequencing determines the sequences of all paired variable (V(D)J) regions of both heavy and light chain sequences. Some of the heavy and light chain sequences obtained from sequencing the yeast scFv library are provided in SEQ ID NOS: 1-28 and SEQ ID NOS: 101-128. Additional sequences obtained from sequencing the yeast scFv library are provided in SEQ ID NOS 8000-8991. Specifically, their variable light chain (V_L) sequences include SEQ ID NOS: 8000-8495. Their heavy chain (V_H) sequences include SEQ ID NOS: 8496-8991.

[00503] Deep antibody sequencing libraries were quantified using a quantitative PCR Illumina Library Quantification Kit (KAPA) and diluted to 17.5 pM. Libraries were sequenced on a MiSeq (Illumina) using a 500 cycle MiSeq Reagent Kit v2, according to the manufacturer's instructions. To obtain high quality sequence reads with maintained heavy and light chain linkage, sequencing was performed in two separate runs. In the first run ("linked run"), the scFv libraries were directly sequenced to obtain forward read of 340 cycles for the light chain V-gene and CDR3, and reverse read of 162 cycles that cover the heavy chain CDR3 and part of the heavy chain V-gene. In the second run ("unlinked run"), the scFv library was first used as a template for PCR to separately amplify heavy and light chain V-genes. Then, forward reads of 340 cycles and reverse reads of 162 cycles for the heavy and light chain Ig were obtained separately. This produces forward and reverse reads that overlap at the CDR3 and part of the V-gene, which increases confidence in nucleotide calls.

[00504] To remove base call errors, the expected number of errors (E) for a read were calculated from its Phred scores. By default, reads with E >1 were discarded, leaving reads for which the most probable number of base call errors is zero. As an additional quality filter, singleton nucleotide reads were discarded because sequences found two or more times have a high probability of being correct. Finally, high-quality, linked antibody sequences by merging

filtered sequences were generated from the linked and unlinked runs. Briefly, a series of scripts that first merged forward and reverse reads from the unlinked run were written in Python. Any pairs of forward and reverse sequences that contained mismatches were discarded. Next, the nucleotide sequences from the linked run were used to query merged sequences in the unlinked run. The final output from the scripts is a series of full-length, high-quality variable (V(D)J) sequences, with native heavy and light chain Ig pairing.

[00505] To identify reading frame and FR/CDR junctions, a database of well-curated immunoglobulin sequences was first processed to generate position-specific sequence matrices (PSSMs) for each FR/CDR junction. These PSSMs were used to identify FR/CDR junctions for each of the merged nucleotide sequences generated using the processes described above. This identified the protein reading frame for each of the nucleotide sequences. CDR sequences that have a low identify score to the PSSMs are indicated by an exclamation point. Python scripts were then used to translate the sequences. Reads were required to have a valid predicted CDR3 sequence, so, for example, reads with a frame-shift between the V and J segments were discarded. Next, UBLAST was run using the scFv nucleotide sequences as queries and V and J gene sequences from the IMGT database as the reference sequences. The UBLAST alignment with the lowest E-value was used to assign V and J gene families and compute %ID to germline.

[00506] Each animal yielded 38–50 unique scFv sequences present at 0.1% frequency or greater after the second FACS selection, including a total of 28 unique scFv candidate binders (SEQ ID Nos: 1-28 for light chains; SEQ ID Nos: 101-128 for heavy chains). The light chain having a sequence of SEQ ID NO: [n] and the heavy chain having a sequence of SEQ ID NO: [100+n] are a cognate pair from a single cell, and forming a single scFv. For example, the light chain of SEQ ID NO:1 and the heavy chain of SEQ ID NO:101 are a cognate pair, the light chain of SEQ ID NO:28 and the heavy chain of SEQ ID NO:128 are a cognate pair, *etc.*

[00507] In this method, the two rounds of FACS resulted in enrichment of the CTLA-4-binding scFvs. In addition, many scFv were not detected in the sequencing data from the initial population of B cells from the immunized mice and most of the scFv present in the pre-sort mouse repertoires were eliminated following FACS. Therefore, this work suggests that most of the antibodies present in the repertoires of immunized mice are not strong binders to the immunogen and that this method can enrich for rare nM-affinity binders from the initial population of B cells from immunized mice.

8.3. Example 3: Biological characteristics of antigen binding protein

[00508] scFv sequences that were present at low frequency in pre-sort libraries and became high frequency in post-sort libraries were then synthesized as full-length mAbs in

Chinese hamster ovary (CHO) cells. These mAbs comprise the 2–3 most abundant sequences in the second round of FACS for each animal.

CTLA-4 Target Binding Profiles

[00509] The binding specificity and affinity of each full-length antibody towards CTLA-4 were determined using biolayer interferometry (BLI) and/or surface plasmon resonance (SPR). Anti-cyno CTLA-4 and anti-mouse CTLA-4 affinities were tested using ForteBio (BLI). Anti-human CTLA-4 affinities were measured using Carterra (SPR).

[00510] For BLI, antibodies were loaded onto an Anti-Human IgG Fc (AHC) biosensor using the Octet Red96 system (ForteBio). Loaded biosensors were dipped into antigen dilutions beginning at 300 nM, with 6 serial dilutions at 1:3. Kinetic analysis was performed using a 1:1 binding model and global fitting.

[00511] For SPR, a moderate density (»1,000 Response Units) of an antihuman IgG-Fc reagent (Southern Biotech 2047-01) was amine-coupled to a Xantec CMD-50M chip (50nm carboxymethyldextran medium density of functional groups) activated with 133 mM EDC (Sigma) and 33.3 mM S-NHS (ThermoFisher) in 100 mM MES pH 5.5. Then, goat anti-Human IgG Fc (Southern Biotech 2047-01) was coupled for 10 minutes at 25 mg/m L in 10 mM Sodim Acetate pH 4.5 (Carterra Inc.). The surface was then deactivated with 1 M ethanolamine pH 8.5 (Carterra Inc.). Running buffer used for the lawn immobilization was HBS-EPC (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20, pH 7.4; Teknova).

[00512] The sensor chip was then transferred to a continuous flow microspotter (CFM; Carterra Inc.) for array capturing. The mAb supernatants were diluted 50-fold (3–10 mg/mL final concentration) into HBS-EPC with 1 mg/mL BSA. The samples were each captured twice with 15-minute and 4-minute capture steps on the first and second prints, respectively, to create multiple densities, using a 65 mL/min flow rate. The running buffer in the CFM was also HBS-EPC.

[00513] Next, the sensor chip was loaded onto an SPR reader (MX- 96 system; Ibis Technologies) for the kinetic analysis. CTLA-4 was injected at five increasing concentrations in a four-fold dilution series with concentrations of 1.95, 7.8, 31.25, 125, and 500 nM in running buffer (HBS-EPC with 1.0 mg/mL BSA). CTLA-4 injections were 5 minutes with a 15-minute dissociation at 8 mL/second in a non-regenerative kinetic series. An injection of the goat anti-Human IgG Fc capture antibody at 75 mg/mL was injected at the end of the series to verify the capture level of each mAb. Binding data was double referenced by subtracting an interspot surface and a blank injection and analyzed for ka (on-rate), kd (off-rate), and KD (affinity) using the Kinetic Interaction Tool software (Carterra Inc.).

[00514] For cell surface binding studies, stable CTLA-4 expressing Flp-In CHO (Thermo Fisher Scientific) cells were generated and mixed at a 50:50 ratio. One million cells were stained with 1 μg of the disclosed anti-CTLA-4 recombinant antibodies in 200 μl of MACS Buffer (DPBS with 0.5% bovine serum albumin and 2 mM EDTA) for 30 minutes at 4°C. Cells were then co-stained with anti-human irrelevant target APC and anti-human IgG Fc-PE [M1310G05] (BioLegend 41070) antibodies for 30 minutes at 4°C. An anti-human CTLA-4-FITC antibody was used as a control for these mixing experiments and cell viability was assessed with DAPI. Flow cytometry analysis was conducted on a BD Influx at the Stanford Shared FACS Facility and data was analyzed using FlowJo.

[00515] Antibodies that specifically bind to CTLA-4 were identified. Affinity to CTLA-4 (K_D) of each antibody is provided in TABLE 6. The Promega assay % inhibition was calculated relative to the strongest inhibitor, which is antibody A5. The affinity of each antibody against human CTLA-4, the on rate, off rate, and KD are shown in TABLE 7.

TABLE 6

Ab#	Binds by FACS?	Promega assay antagonist (EC50, ug/mL)	Promega assay % inhibition	Affinity to Human CTLA-4(nM)	Affinity to Cyno CTLA-4 (nM)	Affinity to Mouse CTLA-4 (nM)
Ipilimumab	Yes	0.51	50.2%	4.9	1.3	no binding
A1	Yes	0.13	43.2%	0.77	0.96	51
A2	Yes	0.12	75.4%	5.1	3.1	no binding
A3	Yes	0.18	49.8%	1.5	1.2	no binding
A4	Yes	0.15	81%	3	1.2	no binding
A5	Yes	0.18	100%	4.6	2.2	39.4
A6	Yes	0.18	61.7%	5.7	1.6	87.8
A7	Yes	0.17	11.5%	5.7	0.75	no binding
A8	Yes	0.15	54.7%	4.6	1	no binding
A9	Yes	0.19	60.8%	6.8	0.92	no binding
A10	Yes	0.26	24.5%	22	3.1	no binding
A11	Yes	0.42	26.6%	7.5	1.6	no binding
A12	Yes	no blocking	0%	5.3	4.5	not tested
A13	Yes	0.09	13.3%	3.2	9.6	not tested
A14	Yes	0.09	6%	9.8	23.6	not tested

A15	Yes	no blocking	0%	10	2.6	not tested
A16	Yes	no blocking	0%	23	2.8	not tested
A17	Yes	no blocking	0%	51	6.3	not tested
A18	Yes	0.89	8.6%	48	no binding	not tested
A19	No	not tested	not tested	3.3	not tested	not tested
A20	No	not tested	not tested	20	not tested	not tested
A21	No	not tested	not tested	31	not tested	not tested
A22	No	not tested	not tested	32	not tested	not tested
A23	No	no blocking	0%	35	not tested	not tested
A24	No	not tested	not tested	55	not tested	not tested
A25	No	not tested	not tested	58	not tested	not tested
A26	No	not tested	not tested	74	not tested	not tested
A27	No	not tested	not tested	120	not tested	not tested
A28	No	no blocking	0%	46	not tested	not tested

TABLE 7

	kon (M-1 s-1)	**koff (s-1)**	**KD (M)**
Ipilimumab	6.50E+04	3.20E-04	4.90E-09
A1	2.10E+05	1.60E-04	7.70E-10
A2	1.00E+05	5.20E-04	5.10E-09
A3	1.90E+05	2.80E-04	1.50E-09
A4	7.60E+04	2.30E-04	3.00E-09
A5	9.00E+04	4.20E-04	4.60E-09
A6	6.10E+04	3.50E-04	5.70E-09
A7	6.40E+04	3.60E-04	5.70E-09
A8	8.50E+04	3.90E-04	4.60E-09
A9	6.30E+04	4.30E-04	6.80E-09
A10	2.30E+04	5.10E-04	2.20E-08
A11	4.70E+04	3.50E-04	7.50E-09
A12	5.10E+04	2.70E-04	5.30E-09
A13	1.30E+05	4.20E-04	3.20E-09
A14	5.40E+04	5.20E-04	9.80E-09
A15	7.00E+04	7.30E-04	1.00E-08
A16	3.10E+04	7.20E-04	2.30E-08
A17	6.60E+04	3.40E-03	5.10E-08
A18	4.20E+03	2.00E-04	4.80E-08
A19	1.20E+05	3.80E-04	3.30E-09
A20	3.20E+04	6.40E-04	2.00E-08
A21	1.80E+04	5.50E-04	3.10E-08
A22	1.10E+04	3.60E-04	3.20E-08
A23	3.40E+04	1.20E-03	3.50E-08
A24	2.50E+04	1.40E-03	5.50E-08

A25	3.00E+04	1.80E-03	5.80E-08
A26	3.50E+03	2.60E-04	7.40E-08
A27	3.10E+04	3.60E-03	1.20E-07
A28	5.20E+04	2.40E-03	4.60E-08

CTLA-4 ligand blocking assay:

[00516] For analysis of the antibodies' ability to block the CTLA-4/ligand interaction, the CTLA-4 Blockade Bioassay (Promega) was used according to the manufacturer's instructions. On the day prior to the assay, aAPC/Raji cells that express CTLA-4 ligands CD80 and CD86 were thawed into 90% Ham's F-12/10% fetal bovine serum (FBS) and plated into the inner 60 wells of two 96-well plates. The cells were incubated overnight at 37 °C, 5% CO_2. On the day of assay, antibodies were diluted in 99% RPMI/1% FBS. The antibody dilutions were added to the wells containing the CTLA-4 ligand expressing aAPC/Raji cells, followed by addition of CTLA-4 effector cells (thawed into 99% RPMI/1% FBS). The cell/antibody mixtures were incubated at 37 °C, 5% CO_2 for 6 hours, after which Bio-Glo Reagent was added and luminescence was read using a Spectramax i3x plate reader (Molecular Devices). Fold-induction was plotted by calculating the ratio of [signal with antibody]/[signal with no antibody], and the plots were used to calculate the EC50 using SoftMax Pro (Molecular Devices). In-house produced ipilimumab was used as a positive control, and an antibody binding to an irrelevant antigen was used as a negative control.

[00517] Binding of CTLA-4 to its ligand leads to inhibition of T cell signaling. Antibodies that bind CTLA-4 and antagonize CTLA-4/ligand interactions can therefore remove this inhibition, allowing T cells to be activated. CTLA-4/ligand checkpoint blockade was tested through an *in vitro* cellular Nuclear Factor of Activated T cells (NFAT) luciferase reporter assay. In this assay, antibodies whose anti-CTLA-4 epitopes fall inside the ligand binding domain antagonize CTLA-4/ligand interactions, resulting in an increase of the NFAT-luciferase reporter. The full-length mAb candidates that can bind CTLA-4 expressed in CHO cells were assayed. To generate an EC50 value for each mAb, measurements were made across several concentrations. It was found that some full-length mAbs are functional in checkpoint blockade in a dose dependent manner as summarized in TABLE 6, 8.

[00518] The ability of the CTLA-4 antibodies (indicated in TABLE 8) to prevent the binding of CD80 or CD86 to plate-bound CTLA4 was evaluated using ELISA. The EC50 and the percent inhibition of each interaction is shown in the TABLE 8. Plates were coated with rhCTLA-4-Fc and then blocked with 1x PBST with 5% w/v nonfat dry milk. After blocking a dilution series of the indicated antibody was added to the plate. Then, to determine how much CD80 or CD86 was still able to bind plate bound CTLA-4, after the plates were washed,

rhCD80-His or rhCD86-His, respectively, was added to the plate. Unbound CD80-His/CD86-His was washed away and mouse anti-His-HRP was added. TMB was used to determine how much CD80-His/CD86-His bound to the plate bound CTLA-4 in the presence of each antibody.

[00519] In some embodiments of the present disclosure, the anti-CTLA-4 antibodies function pharmacologically by antibody-dependent cell-mediated cytotoxicity (ADCC). In some embodiments of the present disclosure, immune-related toxicities related to anti-CTLA-4 antibody therapy are abrogated with an antibody that functions in ADCC but which does not function in checkpoint blockade.

TABLE 8

Antibody	**CD80 EC50 (ug/mL)**	**CD80 % inhibition**	**CD86 EC50 (ug/ml)**	**CD86 % inhibition**
Ipilimumab	0.08211	96.5%	0.1136	90.6%
CTLA4.A7	0.9955	92.1%	1.98	78.8%
CTLA4.A2	0.1006	95.4%	0.1452	91.5%
CTLA4.A12	0.2427	89.2%	0.3704	77.4%
CTLA4.A14	0.2262	83.7%	0.2442	54.5%
CTLA4.A5	0.07049	96.3%	0.1218	90.6%

Epitope binning:

[00520] Epitope binning was performed using high-throughput Array SPR in a modified classical sandwich approach. A sensor chip was functionalized using the Carterra CFM and methods similar to the SPR affinity studies, except a CMD-200M chip type was used (200nm carboxymethyl dextran, Xantec) and mAbs were coupled at 50 mg/mL to create a surface with higher binding capacity (~3,000 reactive units immobilized). The mAb supernatants were diluted at 1:1 or 1:10 in running buffer, depending on the concentration of the mAb in the supernatant.

[00521] The sensor chip was placed in the MX-96 instrument, and the captured mAbs ("ligands") were crosslinked to the surface using the bivalent amine reactive linker bis(sulfosuccinimidyl) suberate (BS3, ThermoFisher), which was injected for 10 minutes at 0.87 mM in water. Excess activated BS3 was neutralized with 1 M ethanolamine pH 8.5. For each binning cycle, a 7-minute injection of 250 mg/mL human IgG (Jackson ImmunoResearch 009-000-003) was used to block reference surfaces and any remaining capacity of the target spots.

[00522] Next, 250 nM CTLA-4 protein was injected onto the sensor chip, followed by injections of the diluted mAb supernatants ("analytes") or buffer blanks as negative controls. Thus, the analyte mAb only bound to the antigen if it was not competitive with the ligand mAb. At the end of each cycle, a one minute regeneration injection was performed using a solution of 4 parts Pierce IgG Elution Buffer (ThermoFisher #21004), one part 5 M NaCl (0.83 M final), and 1.25 parts 0.85% H3PO4 (0.17% final).

[00523] A network community plot algorithm was then used in an SPR epitope data analysis software package (Carterra Inc.) to determine epitope bins. Note that the clustering algorithm groups mAbs for which only analyte data are available separately from the mAbs for which both ligand and analyte data are available. This phenomenon is an artifact of the incomplete competitive matrix. mAbs with both ligand and analyte data had more mAb-mAb measurements, resulting in more mAb-mAb connections, which led to a closer relationship in the community plot.

[00524] The epitope binning showed that all the mAbs were in distinct bins from ipilimumab (FIG. 3).

8.4. Example 4: Influence of CTLA-4 ABPs on tumor growth

[00525] Transgenic mice expressing human CTLA-4 (hCTLA-4 KI mice) were implanted subcutaneously with MC38 tumor cells on the right flank. The hCTLA-4 KI mice were treated with one mg/kg of the indicated CTLA-4 antibody on Days 8, 11, and 14 post-implantation. Specifically, the mice were treated with a control antibody (n=8), ipilumumab (n=8), CTLA4.A2 antibody (n=8), CTLA4.A14 antibody (n=9), CTLA4.A14.2a antibody (n=8), CTLA4.A7 antibody (n=9), CTLA4.A7 antibody (n=9), and CTLA4.A12 antibody (n=8). CTLA-4.A14.2a antibody is the A14 antibody cloned onto a mouse IgG2a background, which enhances antibody-dependent cellular cytotoxicity (ADCC) activity. Tumor volume was measured and tumor growth inhibition was calculated using the formula below:

$$\textit{Mean \% Inhibition} = \textit{(mean(C)-mean(T))/mean(C) * 100\%}$$

$$\textit{T - current group value}$$

$$\textit{C - control group value}$$

[00526] Tumors were implanted subcutaneously in the right flank region with MC38 tumor cells ($1x10^6$) in 0.1 ml of PBS for tumor development. The cells in exponential growth phase were harvested and quantitated by cell counter before tumor implantation. Tumor volumes were measured twice per week in two dimensions using a caliper, and the volume will be expressed in mm^3 using the formula: "V = (L x W x W)/2, where V is tumor volume, L is tumor length (the longest tumor dimension) and W is tumor width (the longest tumor dimension perpendicular to L). Dosing as well as tumor and body weight measurements were conducted in a Laminar Flow Cabinet. The body weights and tumor volumes were measured by using StudyDirector™ software (version 3.1.399.19). Animals were dosed i.p. (intraperitoneally) with the indicated protein in a sterile saline solution including 0.1 mg/ml of the indicated protein. Each mouse received 10 microliters of the indicated solution per a gram of body weight, which leads to a dosing of 1 mg/kg. Animals were dosed days 0, 3, and 6 post randomization.

[00527] TABLE 9 shows the percentage of mice in which the tumor had a complete response (CR) to the treatment. At least 2 consecutive tumor measurements of 0 mm^3 following treatment initiation qualifies as a CR.

TABLE 9	
Antibody	**% of tumors with CR**
Control	12.5% (1 of 8)
Ipilimumab	75% (6 of 8)
CTLA4.A2	100% (8 of 8)
CTLA4.A14	66.67% (6 of 9)
CTLA4.A14.2a	75% (6 of 8)
CTLA4.A7	55.6% (5 of 9)
CTLA4.A12	50% (4 of 8)

[00528] TABLE 10 shows the percentage of mice with tumors that had a CR but then later relapsed by day 56. The group treated with CTLA4.A14.2a that had previously shown a CR had 0% relapse by day 56, indicating that enhancing ADCC can prolong the anti-tumor immunity.

TABLE 10	
Antibody	**% of relapse by Day 56 in tumors that had previously shown CR**
Ipilimumab	16.67% (1 of 6)
CTLA4.A2	25% (2 of 8)
CTLA4.A14	33.3% (2 of 6)
CTLA4.A14.2a	0% (0 of 6)

[00529] TABLE 11 shows the mean inhibition of tumor volume over time when the hCTLA-4 KI mice implanted with MC38 tumor cells were treated with one mg/kg of the control or one mg/kg of the indicated CTLA-4 antibodies.

TABLE 11: Mean Inhibition											
Study day	**8**	**11**	**14**	**17**	**21**	**24**	**27**	**29**	**31**	**35**	**38**
Control	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Ipilimumab	-3.81%	0.44%	31.41%	69.65%	84.04%	90.73%	96.00%	96.92%	96.61%	87.45%	88.27%
CTLA4.A7	-7.49%	10.91%	34 54%	60.11%	70.73%	81.04%	78.25%	78.75%	70.60%	50.50%	43.41%
CTLA4.A2	-6.79%	-6.10%	26.70%	61.14%	85.22%	97.98%	99.58%	100.00%	100.00%	99.55%	99.36%
CTLA4.A12	-5.89%	12.35%	34.21%	49.59%	70.56%	86.82%	90.22%	91.82%	91.13%	68.63%	70.44%
CTLA4.A14	-7.37%	-6.91%	26.70%	58.54%	80.64%	93.71%	95.73%	97.23%	97.26%	92.22%	90.17%

CTLA4.A14.2a	-4.74 %	-20.04 %	9.74%	40.80 %	65.98 %	87.85 %	93.48 %	95.01%	93.66%	81.40 %	82.75 %

This data is also shown in **FIG. 17A**.

8.5. Example 5: Influence of CTLA-4 ABPs on Systemic Anti-Tumor Immunity

[00530] The hCTLA-4 KI mice bearing MC38 tumors were treated with the indicated anti-CTLA-4 on days 8, 11, and 14 post tumor cell implantation, as explained *supra*. The mice in which tumors displayed a CR were re-challenged with implantation of MC38 cells on the opposite flank. TABLE 12 shows the individual mouse tumor volumes (mm^3) of the original or re-challenge tumors on the final day of the study (73 days after the original tumor cell implantation and 30 days after the re-challenge implantation). There was no growth of re-challenge tumors in mice in which the original tumor remained a CR. The 3 instances of growth seen in the re-challenge tumors were in mice in which the original tumor had started to re-grow (*see* TABLE 12). The results also indicated that CTLA4.A2 may induce protective systemic anti-tumor immunity even when the primary tumor (original tumor) relapses (*see* TABLE 13).

TABLE 12

	Ipilimumab (original tumor)	Ipilimumab (re-challenge tumor)	CTLA4.A2 (original tumor)	CTLA4.A2 (re-challenge tumor)	CTLA4.A14 (original tumor)	CTLA4.A14 (re-challenge tumor)
Tumor Volume (mm^3)	0	0	0	0	105.7	85.3
	1822.3	149.6	0	0	0	0
	0	0	0	0	913.3	98.1
	0	0	0	0	0	0
	0	0	1415	0	0	0
	0	0	0	0	0	0
			678.9	0		
			0	0		

TABLE 13

Antibody	% of secondary tumors that grew if primary tumor relapsed
Ipilimumab	100% (1 of 1)
CTLA4.A2	0% (0 of 2)
CTLA4.A14	100% (2 of 2)

Data from this experiment is also shown in **FIG. 17B**.

8.6. Example 6: Influence of Increased Dosage of CLTA-4 ABPs *MC38 tumors treated with anti-CTLA-4*

[00531] Transgenic mice expressing human CTLA-4 (hCTLA-4 KI mice) were implanted with MC38 tumor cells on the right flank. Randomization started when the mean tumor size reached $98.5mm^2$. The hCTLA-4 KI mice were treated with 5 mg/kg of the indicated anti-CTLA-4 bi-weekly for 5 doses starting on day 0 post-randomization. The administered antibodies are shown in TABLE 14. CTLA4.A14.2a is antibody A14 cloned onto a mouse IgG2a backbone,

which enhances ADCC activity. The 297 suffix denotes that the hIgG1 Fc was mutated at the N297 amino acid to eliminate glycosylation and thus Fc effector function including ADCC.

A) Tumor growth inhibition

[00532] Over the course of the study, tumor growth inhibition was determined using the formula below:

*Mean % Inhibition = (mean(C)-mean(T))/mean(C) * 100%*

T - current group value

C - control group value

[00533] The results showed that antibodies lacking Fc activity had reduced efficacy overall. These antibodies were still able to induce tumor regression in some animals, indicating that anti-CTLA-4 works by both Fc-dependent and Fc-independent mechanisms of action, and indicating that anti-CTLA-4s lacking Fc activity, including ADCC and ADCP, can induce anti-tumor responses (TABLEs 14 and 15).

TABLE 14

	Mean Inhibition						
Study Day	**0**	**3**	**6**	**10**	**13**	**17**	**20**
1x PBS (negative control)	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Ipilimumab	0.31%	-21.76%	12.56%	75.43%	91.55%	95.54%	98.71%
CTLA4.A5	0.74%	-28.09%	-6.22%	64.17%	87.32%	98.32%	100.00%
CTLA4.A14	-0.56%	-13.30%	5.33%	68.17%	86.21%	92.79%	97.52%
CTLA4.A12	0.40%	-32.59%	-14.13%	57.48%	83.00%	94.99%	98.04%
CTLA4.A8	0.26%	-18.48%	-14.18%	52.53%	78.37%	96.15%	97.96%
CTLA4.A7	-0.61%	-7.67%	10.04%	76.74%	94.36%	98.88%	100.00%
CTLA4.A2	0.27%	-23.97%	-9.95%	60.95%	89.33%	98.35%	100.00%
CTLA4.A13	-1.15%	-7.61%	6.23%	49.18%	64.82%	93.82%	95.23%
CTLA4.A5.2a	0.88%	11.78%	27.51%	66.64%	89.02%	96.85%	100.00%
CTLA4.A14.2a	1.04%	-7.89%	21.12%	45.60%	84.83%	97.30%	100.00%
Ipilimumab.297	0.68%	8.27%	21.37%	22.75%	16.02%	25.70%	26.39%
CTLA4.A5.297	-0.33%	17.56%	18.58%	5.70%	-15.40%	4.65%	13.61%

TABLE 15	
% of each group in which tumor had started to regress by day 13 post treatment initiation	
1x PBS (negative control)	0%
Ipilimumab	100%
CTLA4.A5	100%
CTLA4.A14	100%
CTLA4.A12	88%
CTLA4.A8	100%
CTLA4.A7	100%
CTLA4.A2	100%
CTLA4.A13	86%
CTLA4.A5.2a	100%
CTLA4.A14.2a	100%
Ipilimumab.297	25%
CTLA4.A5.297	14%

Data from this experiment is also shown in **FIGs. 12B, 14A and 27.**

B) Histopathological Analysis:

[00534] The hCTLA-4 mice were euthanized and their right kidneys were harvested for histopathological analysis. Tissue was formalin-fixed and paraffin-embedded, and cut in 5μm sections that were placed on glass slides for standard hematoxylin and eosin (H&E) staining as well as anti-IgG and anti-C3 immunohistochemistry (IHC) staining. Stained slides were prepared as digital images. A board-certified veterinary pathologist with experience in laboratory animals and toxicologic pathology evaluated the H&E images for any findings and evaluated the anti-IgG and C3 slides for location, intensity, and percent of positive staining. Findings in H&E images were scored on a scale from 0 to 5 (0=within normal limits, 1= minimal findings or the least change discernible, 2= mild findings, 3= moderate, 4=marked, and 5=severe or to the greatest extent possible). Findings in IHC images were scored on a scale of 1 to 4 for intensity (0=negative, 1=minimal or slightly positive and 4=very dark), and as a percent of the positive cells in the glomeruli (after reviewing at least 5 glomeruli).

[00535] The H&E, immunoglobulin, or C3 stain images were scored by a blinded pathologist and the results are shown in **FIG. 4A-4C**. The main H&E finding was that leukocytes in the renal interstitium are not usually involved in glomeruli. Ig and C3 deposition in the glomeruli scoring are also shown in **FIG. 4A-4C**.

C) Alkaline Phosphatase:

[00536] The hCTLA-4 mice were also analyzed for changes in alkaline phosphatase level. The level of alkaline phosphatase in the serum was determined using comprehensive diagnostic rotors on ABAXIS VetScan VS2.

[00537] The study found that ipilimumab (IPI) elevates alkaline phosphatase levels, which may be an indication of immune-mediated hepatitis. The CTLA-4 antibodies (*e.g.*, CTLA4.A14.2A) showed a decrease in elevation of alkaline phosphatase levels (**FIG. 5**). This decreased elevation in alkaline phosphatase induced by the presently disclosed CTLA-4 antibodies may indicate they are less likely to induce immune-mediated hepatitis than treatments such as ipilimumab.

8.7. Example 7: Influence of CTLA-4 ABPs on a second tumor model *RM1 tumors treated with anti-CTLA-4*

[00538] Transgenic mice expressing human CTLA-4 (hCTLA-4 KI mice) were implanted with RM1 tumor cells on the right flank. (Human IgG1 isotype negative control n=7, atezolimumab n=8, n=11 for all other groups). The hCTLA-4 KI mice were treated with the antibodies indicated in TABLE 16. The CTLA-4 antibodies were dosed at 5 mg/kg on days 0, 3, and 6 post randomization and atezolizumab was dosed at 5 mg/kg biweekly for 3 weeks starting at day 0 post randomization. Human IgG1 isotype negative control was dosed at 5mg/kg on Days 0, 3, and 6 post randomization. The mean inhibition of tumor growth was determined at Days 0, 4, 7, 11, 14, and 18 using the following formula:

$$\textit{Mean \% Inhibition} = \textit{(mean(C)-mean(T))/mean(C) * 100\%}$$

T - current group value

C - control group value

[00539] **TABLE 16** shows that mean inhibition values for the control, the CTLA-4 antibodies, and the atezolizumab treatments over the course of the study.

TABLE 16

	Mean Inhibition					
Study day	**0**	**4**	**7**	**11**	**14**	**18**
Human IgG1 isotype (negative control)	n/a	n/a	n/a	n/a	n/a	n/a
Ipilimumab	2.14%	11.33%	30.07%	41.96%	52.16%	57.56%
CTLA4.A2	0.09%	-12.28%	25.30%	43.83%	50.20%	59.41%
CTLA4.A14	-0.17%	-7.72%	22.10%	36.93%	48.84%	55.18%
Atezolizumab	1.83%	17.66%	0.41%	-3.02%	-7.89%	-2.89%
Atezolizumab + Ipilimumab	-0.47%	-3.31%	18.00%	29.81%	30.39%	35.21%
Atezolizumab + CTLA4.A2	1.50%	-6.72%	23.00%	41.64%	40.93%	44.30%

Atezolizumab + CTLA4.A14	0.87%	2.55%	31.58%	45.65%	47.43%	55.18%

[00540] Data from this experiment is also shown in FIG. 14B.

8.8. Example 8: Combination treatment (pembrolizumab and anti-CTLA-4s)

[00541] Transgenic mice expressing human CTLA-4 and PD-1 (hCTLA4-hPD1 knock in (KI) mice, n=8 per treatment group) were implanted subcutaneously with 1x 10^6 MC38 tumor cells in the right flank. The hCTLA4-hPD1 KI mice were treated with a control (1x phosphate buffered saline, or PBS); 2 mg/kg pembrolizumab (pembro) or 2 mg/kg pembro + 5 mg/kg anti-CTLA-4, administered i.p. with a dose volume of 10ml/kg per animal as indicated in TABLE 17 twice weekly for three weeks starting on day 1 post-randomization. The mean (%) delta inhibition of tumor growth induced by each treatment in comparison to control treatment was calculated using the formula below and the results are shown in TABLE 17.

$$Mean\ \%\ \Delta Inhibition = ((mean(C)-mean(C_0))-(mean(T)-mean(T_0)))/(mean(C)-mean(C_0))*100\%$$

T - current group value

T_0 - current group initial value

C - control group value

C_0 - control group initial value

TABLE 17

	Mean Delta Inhibition						
Study day	**4**	**7**	**10**	**14**	**17**	**21**	**24**
Control	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Pembrolizumab	44.33%	44.20%	30.78%	20.92%	10.08%	8.62%	-11.94%
Pembrolizumab + CTLA4.A5	29.57%	79.32%	85.83%	87.28%	86.27%	78.55%	97.36%
Pembrolizumab + CTLA4.A1	-82.05%	-5.30%	41.81%	50.97%	55.73%	52.23%	66.74%

[00542] The study showed that mice treated with pembro alone did not exhibit tumor growth inhibition at day 24, however the addition of the indicated CTLA-4 antibodies increased the tumor growth inhibition over the course of the study.

[00543] At the end of the experiment, select tumors were harvested and flow cytometry was conducted to investigate intratumoral immune cell populations. The data indicate that anti-CTLA-4s decrease intratumoral Treg populations while increasing intratumoral NK cell populations (FIG. 6).

8.9. Example 9: Immune Related Adverse Events

[00544] Transgenic mice expressing human CTLA-4 (hCTLA-4 KI mice) were implanted with MC38 tumor cells on the right flank. The hCTLA-4 KI mice were treated with one mg/kg of

the indicated CTLA-4 antibody on days 8, 11, and 14 post-implantation. The mice were weighed on days 8, 11, 14, and 17 post-implantation. Animal counts were: n=8 for ipilimumab, *n*=9 for A7, *n*=8 for A2, *n*=9 for A14, and *n*=8 for A14.2. The percent changes in body weight of the mice receiving the indicated anti-CTLA-4 treatments are shown in FIG. 7.

[00545] The mice treated with CTLA4.A7, CTLA4.A14, and CTLA4.A14.2a did not appear to exhibit weight loss after the final dose of anti-CTLA-4 (FIG. 7). This finding was unexpected because immune-related Adverse Events (irAE) have been reported to be greater when anti-CTLA-4s with enhanced ADCC (*e.g.*, CTLA.A14.2a) are administered. This data suggests that anti-CTLA-4s with reduced blocking activity may limit induction of irAEs even when ADCC is enhanced.

8.10. Example 10: Peripheral Flow Cytometry

[00546] Transgenic mice expressing human CTLA-4 (hCTLA-4 KI mice) were implanted with MC38 tumor cells in the right flank. The hCTLA-4 KI mice were treated with one mg/kg of the indicated CTLA-4 antibody on days 8, 11, and 14 post-implantation. Peripheral flow cytometry was performed on day 27. 100μL of blood was used for staining. The findings from the peripheral blood flow cytometry are shown in FIGs. 8A-8F, 9A-9D and 10.

[00547] The results provided in FIG. 8A indicated that CTLA4.A2 and CTLA4.A14 decrease the elevation of peripheral T cells (CD3+). Enhancing ADCC with CTLA4.A14.2a increased newly activated T cells (CD69+). CTLA4.A2 and CTLA4.A14 resulted in fewer non-conventional regulatory cells (CD4+PD1+, CD4+ICOS+). (*See* FIG. 8D and 8E).

[00548] The results also indicated that CTLA4.A2 and CTLA4.A14 better enhance CD8+ T cells (FIG. 9A). CTLA4.A2 better enhanced newly activated T cells (CD8+CD69+) and led to decreased T cell exhaustion (CD8+PD1+) relative to Ipilimumab. ICOS has been described as a pharmacodynamic marker for anti-CTLA-4. Enhancing ADCC with CTLA4.A14.2a appeared to further elevate CD8+ICOS+ cells (FIG. 9A-9D). The results also indicated that CTLA4.A2 and CTLA4.A14.2a lead to decreased peripheral immune activation relative to Ipilimumab, as judged by the frequency of dendritic cells (DCs) and activated DCs (CD86+). (*see* FIG. 10).

8.11. Example 11: Treatment with Low Dose CTLA-4 Study

[00549] Transgenic mice expressing human CTLA-4 (hCTLA-4 KI mice) were implanted subcutaneously in the right flank region with MC38 tumor cells (1E6) in 0.1 ml of PBS for tumor development. The cells in exponential growth phase were harvested and quantitated by cell counter before tumor implantation. The hCTLA-4 KI mice were randomized when the mean tumor volume was 96.15 mm^3 and treated with 0.3 mg/kg of the indicated anti-CTLA-4, ipilimumab, or human IgG1 isotype control (Isotype) on days 0, 3 and 6 post-randomization. Tumor volume and mean % of inhibition was determined as described in Example 4. CTLA4.A2

and CTLA4.A14 resulted in significantly higher tumor inhibition over the 18 days of the study. The results of the study are shown in TABLE 18 and FIG. 11.

TABLE 18						
	Dates/Study Days					
Group	12/6/2019	12/10/2019	12/13/2019	12/17/2019	12/20/2019	12/24/2019
	0	4	7	11	14	18
Isotype						
Ipilimumab	-0.17%	9.33%	18.68%	22.37%	27.23%	11.84%
CTLA4.A2	0.22%	13.00%	23.03%	49.09%	60.03%	59.54%
CTLA4.A14	0.01%	23.39%	35.06%	53.05%	57.50%	45.41%

Data from this experiment is also shown in FIGs. 11, 14C and 30.

8.12. Example 12: Epitope mapping of CTLA-4

[00550] Epitope mapping of commercially available anti CTLA-4 monoclonal antibody ipilimumab and anti CTLA-4 monoclonal antibody GIGA-564 (also described as clone A14 and CTLA4.A14 herein) was performed. The CDR sequences are described below in TABLE 21:

TABLE 21: CDR sequences of Ipilimumab and GIGA-564

	Ipilimumab		GIGA-564	
	Heavy	Light	Heavy	Light
CDR1	GFTFSSY (SEQ ID NO: 12069)	QSVGSSYLA (SEQ ID NO: 12072)	GFTFSSY (SEQ ID NO: 12075)	QSVSSSYLA (SEQ ID NO: 12078)
CDR2	SYDGNN(SEQ ID NO: 12070)	GAFSRAT (SEQ ID NO: 12073)	WYEGRN (SEQ ID NO: 12076)	GASSRAT (SEQ ID NO: 12079)
CDR3	TGWLGPFDY (SEQ ID NO: 12071)	QQYGSSPWT (SEQ ID NO: 12074)	AGDLGAFDI (SEQ ID NO: 12077)	QQYGSSPWT (SEQ ID NO: 12080)

[00551] Briefly, comprehensive alanine scanning mutagenesis was performed across the CTLA-4 protein. The mutant proteins were expressed in human cells, and binding by the two antibodies was determined. Protein expression and folding of the mutant proteins was validated and binding to wild-type CTLA-4 was used to normalize the data. Stringency was increased using, e.g., pH or salt modifications, to determine only the most important residues. The results are presented in TABLE 22.

TABLE 22: Critical residues for binding of Ipilimumab and GIGA-564 to CTLA-4

	Ipilimumab	GIGA-564
CTLA-4 residues	R70, K130, Y139, L141, I143	K130, Y139, L141, I143

This data is also described in **FIGs. 13C-D.**

[00552] The results show that the antibodies share a common epitope. However, R70 on CTLA-4 is a critical residue for Ipilimumab but not for GIGA-564 binding. It is known that R70 is also involved in CD80 and CD86 binding to CTLA-4 (see, e.g., Li, Dong et al. "A functional antibody cross-reactive to both human and murine cytotoxic T-lymphocyte-associated protein 4 via binding to an N-glycosylation epitope." *mAbs* vol. 12,1 (2020): 1725365 and Udupi A. et al. "Structural basis for cancer immunotherapy by the first-in-class checkpoint inhibitor ipilimumab." PNAS May 2017, 114 (21) E4223-E4232). It is also known that R70 is contacted by the heavy chain CDR2 in the crystal structure of Ipilimumab bound to CTLA-4; the heavy chain CDR2 sequences of the two antibodies are dissimilar.

[00553] In addition, the data show that L74A is a secondary residue for both, and impacts GIGA 564 binding more than Ipilimumab.

[00554] It is known that the shared epitope residues are contacted by the heavy chain CDR3 and light chain CDR3 in the crystal structure. The light chain CDR3 sequences of the two antibodies are identical.

[00555] The experiments were also performed with a Fab version of each antibody. The data show that E68 is also an important residue for GIGA-564 binding.

[00556] In conclusion, Ipilimumab and GIGA-564 have overlapping but distinct epitopes. R70 in critical for the binding of Ipilimumab but not GIGA-564. It is likely that R70 is available to associate with CD80/CD86 when GIGA-564 is bound. E68 and L74 are possible differentiators between monoclonal antibody Ipilimumab and monoclonal antibody GIGA-564.

8.13. Example 13: Mechanism of Action of GIGA-564 compared to ipilimumab in a murine model

[00557] The inventors of the present disclosure developed a novel CTLA-4 monoclonal antibody with minimal ability to block CTLA-4 binding to its CD80/CD86 ligands that has superior anti-tumor activity, and reduced toxicity compared to ipilimumab in murine models expressing human CTLA-4.

[00558] The study described herein assesses and characterizes the mechanism of action of GIGA-564 CTLA-4 antibody compared to ipilimumab in a murine model. Additionally, the anti-tumor effect of conventional anti-CTLA-4 mAbs, including ipilimumab, was investigated in the presence and absence of Fc effector functions.

MATERIALS AND METHODS

[00559] Some of the methods described previously are repeated below. .

Antibody sequences

[00560] Amino acid sequences for the IgK and IgG variable regions for the 14 antibodies described here that were expressed as full-length antibodies and tested for *in vitro* blocking activity are listed in **TABLE 26**.

TABLE 26: CTLA-4 antibody sequences

CTLA-4 antibody sequences. Amino acid sequences are provided of the IgK and IgG variable regions for the 14 characterized full-length CTLA-4 antibodies.

Antibody	IgK variable sequence	IgG variable region sequence
aCTLA-4.1	EIVLTQSPGTLSLSPGERATLSCRASQSVSYLAW YQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGT DFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGT KVEIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSRYG MHWVRQAPGKGLEWVAVIWYDGRNKYYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTALYSC ARAGELGPFDYWGQGTLVTVSSA
aCTLA-4.2	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGSSPFTFGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCIASGFTFSSYG MHWVRQAPGKGLEWVAVNWYDGSNKHYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARGGVWGPYFDYWGQGTLVTVSSA
aCTLA-4.3	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGSSPFTFGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTLSSFG MHWVRQAPGKGLEWVAVIWYDGSNKYYADS VKGRFIISRDNSKTALYLQMNSLRAEDTAVYYC ARAHYFGAFDIWGQGTMVTVSSA
aCTLA-4.4	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGRSPFTFGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSNYG MNWVRQAPGKGLEWVAVIWYDGRNKHYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARGGDWGPYFDYWGQGTLVTVSSA
aCTLA-4.9	EIVLTQSPGTLSLSPGEGATLSCRASQSFSSNYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGTSPFTFGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSFG MHWVRQAPGKGLEWVAVIWYDGRNKYYVDS VKGRFTISRDNSNNTLYLQMNSLRAEDTAVYY CARGEFFGEFFDYWGQGTLVTVSSA
aCTLA-4.11	EIVLTQSPGTLSLSPGERATLSCRASQSVSYLAW YQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGT DFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGT KVEIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG MHWVRQAPGKGLEWVAVIWYDGSNKFYADSV KGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCA RGGHLGSFDYWGQGTLVTVSSA
aCTLA-4.12	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGTSPWTFGQ GTKVEIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG MHWVRQAPGKGLEWVAVIWYDGSNKYYADS VKGRFTFSRDNSKNTLYLQMNSLRAEDTAVYY CARGGLMGAFDYWGQGTLVTVSSA

aCTLA-4.14	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGSSPFTFGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG MHWVRQAPGKGLEWVAVIWYDGRNKDYADS VKGRITISRDNSKNTLYLQMNSLRAEDTAVYYC ARGGLLGPYFDYWGQGTLVTVSSA
aCTLA-4.15 (GIGA-564)	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGSSPWTSGQ GTKVEIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG MHWVRQAPGTGLEWVAVIWYEGRNKYYADP VKGRFTISRDNSKNTLYLQMNSLRDDDTAVYY CARAGDLGAFDIWGQGTMVTVSSA
aCTLA-4.22	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGSSPFTFGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCVASGFTLSSYG MHWVRQAPGKGLEWVAVIWYDGSNKHYADS VKGRFTISRDNSKNTLSLQMNSLRAEDTAVYYC ARGGQLGPFDYWGQGTLVTVSSA
aCTLA-4.24	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLN WYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGS GTDFTLTISSLQPEDFATYYCQQSYSTPFTFGPGT KVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSHG MHWVRQAPGKGLEWVAVIWYDGSNKHYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY CARGDILTGYYGYWGQGTLVTVSSA
aCTLA-4.26	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLN WYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGS GTDFTLTISSLQPEDFATYYCQQSYSTPFTFGPGT KVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG MHWVRQAPGKGLEWVAVIWYDGSYKYYADS VKGRFTISRDDSKNTLYLQMSSLRAEDTAVYYC ARAPHYAILTGYYEDYWGQGTLVTVSSA
aCTLA-4.28	EIVLTQSPGTLSLSPGDRATLSCRASQSGSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGTSPFTSGPG TKVDIK	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYG MHWVRQAPGKGLEWVAVIWYDGNNKYYADS VKGRFTISRDNSKNTLYLQMYSLRAEDTAVYY CARGGILAAGIFDYWGQGTLVTVSSA
aCTLA-4.29	EIVLTQSPGTLSLSPGDRATLSCRASQSGSSSYLA WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGS GTDFTLTISRLEPEDFAVYYCQQYGTSPFTSGPG TKVDIK	QVQLVESGGGVVQPGRSLRISCAASGFTFSSYGI HWVRQAPGKGLQWVAVIWYDGRNKYYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCS RSGSFGAFDIWGQGTMVTVSSA

Generation of full-length antibodies and expression in CHO cells

[00561] Five Trianni Mouse® mice expressing antibodies with fully-human variable regions were immunized at Antibody Solutions (Sunnyvale, CA, USA), as described elsewhere (41). Briefly, mice were immunized with soluble His-tagged CTLA-4 (CT4-H5229; Acro Biosystems, Newark, DE, USA) and alendronate (ALD) / muramyl dipeptide (MDP) adjuvant twice per week for four weeks. Mice were euthanized and inguinal and popliteal lymph nodes and spleen were harvested and processed into a single cell suspension. Cells from all mice were pooled by tissue type and B cells were selected from lymph node and spleen using a mouse Pan-B negative selection kit (Stemcell Technologies, Vancouver, BC, Canada). Generation of natively paired heavy and light chain libraries, yeast surface display of scFvs and FACS sorting, and antibody repertoire analysis was described elsewhere.

[00562] From this analysis, scFv sequences were selected for full-length antibody expression based on enrichment after sorting. Expression constructs were Gibson assembled using GeneBlocks (Integrated DNA Technologies, Coralvile, IA, USA) and NEBuilder HiFi DNA Assembly Master Mix (NEB, Ipswich, MA, USA) to integrate sequences into a vector appropriate for transient expression in CHO cells. The vector used was a variant of the pCDNA5/FRT mammalian expression vector (Thermo Fisher Scientific, Waltham, MA, USA). The vector has an elongation factor 1 alpha (EF1α) promoter to express light chain followed by a bovine growth hormone (BGH) polyA sequence and a cytomegalovirus (CMV) promoter to express heavy chain followed by a second BGH polyA sequence. All constructs were synthesized as human IgG1 isotype, regardless of a given antibody's IgG isotype in the original repertoire. Constructs were transformed into NEB 10-beta *E. coli* for amplification and purified with the ZymoPURE Plasmid Maxiprep Kit (Zymo Research, Irvine, CA, USA). The purified plasmid was then used for transient transfection in the ExpiCHO system (Thermo Fisher Scientific, Waltham, MA, USA). Transfected cells were cultured for 7–9 days in ExpiCHO medium, and then antibodies were purified from filtered supernatant using Protein A columns (MilliporeSigma, St. Louis, MO, USA). Antibody purity and proper size was verified by Coomassie stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Thermo Fisher Scientific, Waltham, MA, USA).

Transient transfection and mAb protein production

[00563] For larger-scale expression, the full-length kappa chain of GIGA-564 (A14; "aCTLA-4.15") was cloned into a separate pSF expression vector (MilliporeSigma, St. Louis, MO, USA) with a CMV promoter. The immunoglobulin kappa-only and dual-gene

(immunoglobulin kappa and immunoglobulin gamma) plasmids were transfected at a 2:1 molar ratio into ExpiCHO cells using ExpiFectamine (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, for every 100 mL of culture 100 μg of total plasmid was mixed with 320 μl of ExpiFectamine in 4 mL of OptiPro serum free media (Thermo Fisher Scientific, Waltham, MA, USA), incubated at room temperature for 5 minutes, then added to cells freshly passaged to 6×10^6 cells/mL. Cells were fed on day 1 and day 5 after transfection with 16 mL of ExpiCHO feed, then harvested on Day 8 or when the viability dropped below 75%.

[00564] Harvested cell-culture fluid (HCCF) was loaded onto a HiTrap MabSelect Prism A column (Cytiva, Marlborough, MA, USA), equilibrated with PBS pH 7.0-7.4 using a fast protein liquid chromatography (FPLC) instrument (AKTA pure 25, Cytiva, Marlborough, MA, USA), and eluted with 100 mM citrate pH 3. Fractions were pooled to collect >90% of eluted material and neutralized to pH 6.2 using 1 M tris pH 9. Neutralized eluate was dialyzed into 40 mM histidine + 240 mM sucrose pH 6.2 and formulated with 0.2% tween-20. Concentration was determined by absorbance (NanoDrop 8000, Thermo Fisher Scientific, Waltham, MA, USA) and endotoxin quantified by limulus amebocyte lysate assay (NexGen PTS, Charles River, Wilmington, MA, USA). Routine biophysical characterization included size exclusion chromatography high performance liquid chromatography (SEC-HPLC; 7.8 × 300 mm, 2.7 μM, 300A column, Agilent, Santa Clara, CA, USA), SDS-PAGE (12% tris-glycine, Thermo Fisher Scientific, Waltham, MA, USA), and capillary electrophoresis sodium dodecyl sulphate (CE-SDS; protein 230, BioAnalyzer 2100, Agilent, Santa Clara, CA, USA).

Clonal cluster analysis and visualization

[00565] USEARCH was used to compute the total amino acid differences between each pairwise alignment of FACS-sorted scFv sequences. The R package igraph (version 1.2.6) was then used to generate a clustering plot for the pairwise alignments. The sequences were represented as "nodes", while "edges" were the links between nodes. Edges indicate pairwise alignments with $\leq$9 amino acid differences. The layout_with_graphopt (charge = 0.03, niter = 1000) option was used to format the output.

Affinity measurements

[00566] Kinetic analysis of CTLA-4 mAb HCCF from CHO expression was performed on an MX-96 instrument (IBIS Technologies, Enschede, Netherlands) by Carterra (Dublin, CA, USA). A medium density capture chip was created with anti-Human IgG Fc (SouthernBiotech, Birmingham, AL, USA) with a surface density of 925-1200 RU. A CFM printer (IBIS Technologies, Enschede, Netherlands) printed one 10 minute print of mAb HCCF for each

sample. His-tagged human CTLA-4 antigen (CT4-H5229; Acro Biosystems, Newark, DE, USA) injections for kinetic analysis were 5 minutes and dissociations were 10 minutes. Kinetic analysis was performed with five 5-fold serial titrations starting at 500 nM antigen and fitted to a 1:1 monovalent model.

[00567] Binding/dissociation experiments to determine the affinity of mAb HCCF from CHO expression to His-tagged cynomolgus CTLA-4 (CT4-C5227; Acro Biosystems, Newark, DE, USA) were performed at 30°C on an Octet Red96 instrument (ForteBio, Fremont, CA) by a CRO (Bionova Scientific, Fremont, CA, USA). Antibodies were loaded at 5 μg/mL onto Protein A biosensors that were dipped into 80 nM antigen, and kinetic constants were calculated using a monovalent model.

Flow cytometry for in vitro characterization of mAbs

[00568] To determine if the CTLA-4 mAbs bound the appropriate antigen expressed on the surface of mammalian cells, CHO cell lines stably expressing human CTLA-4 or an irrelevant antigen (CD27) were generated. Cells (0.5×10^6 each cell line) were combined and washed with MACS buffer (Dulbecco's Phosphate Buffered Saline [DPBS], with 0.5% bovine serum albumin [BSA], and 2mM ethylenediaminetetraacetic acid [EDTA]). The cells were incubated with 10 μg/mL anti-CTLA-4 for 30 min at 4°C, then excess, unbound mAbs were removed by washing twice with MACS buffer. The cells were then stained with PE-conjugated anti-Human IgG Fc antibody (clone M1310G05, BioLegend 410720, San Diego, CA, USA) to detect bound anti-CTLA-4 and with fluorescein isothiocyanate (FITC)-conjugated anti-CD27 (clone O323, BioLegend 302806, San Diego, CA, USA) to distinguish the cells expressing the irrelevant antigen. Cells were washed twice with MACS buffer, fixed with 4% paraformaldehyde fixation buffer (BioLegend 420801, San Diego, CA, USA) for 20 minutes at room temperature and washed twice more with MACS buffer. For validation of the N297Q variants of the ipilimumab analog and aCTLA-4.28, a similar procedure was followed except CHO cells lacking expression of human CTLA-4 were used in place of the CD27-expressing cells, the two cell lines were stained separately, FITC-conjugated anti-Human IgG Fc antibody (clone M1310G05, BioLegend 410719, San Diego, CA, USA) was used to detect bound anti-CTLA-4, cells were not treated with fixation buffer, and cells were instead stained with 4′,6-diamidino-2-phenylindole (DAPI; BioLegend, San Diego, CA, USA) and gated for live ($DAPI^-$) cells on the cytometer. Binding of mAbs to CTLA-4 on the cell surface was determined using LSR II (BD Biosciences, San Jose, CA, USA) or CytoFLEX LX (Beckman Coulter, Brea, CA, USA) flow cytometers and analyzed using FlowJo (v10.6.1, BD Biosciences, San Jose, CA, USA).

[00569] To determine accumulation of anti-CTLA-4s on the cell surface, suspension CHO cells expressing wildtype human CTLA-4 were plated at 1×10^6 cells/well in DPBS (Lonza, Basel, Switzerland) containing 0.5% BSA (MilliporeSigma, St. Louis, MO, USA) and 0.5 M EDTA (MilliporeSigma, St. Louis, MO, USA). Titrations of ipilimumab or GIGA-564 from 0-50 μg/mL were added to the cells and the plate was incubated at 37°C for 30 minutes to allow for internalization to occur. Anti-human IgG Fc allophycocyanin (APC) (BioLegend, San Diego, CA, USA) was added as a secondary antibody at 10 μg/mL and the plate was incubated at 4°C for 30 minutes. Following viability staining with DAPI (BioLegend, San Diego, CA, USA), data was acquired on the CytoFLEX LX (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo (v10.6.1, BD Biosciences, San Jose, CA, USA).

Cell-based CTLA-4 blocking assay

[00570] CTLA-4 Blockade Bioassay was purchased from Promega (JA3001; Madison, WI, USA) and performed according to the manufacturer's recommendations. Briefly, a serial dilution of each antibody was generated in assay buffer (90% RPMI 1640/10% FBS, supplied in the kit) in a sterile 96-well plate. CTLA-4 effector cells (provided with kit) were thawed and diluted into 3.2 mL of assay buffer and 25 μL of the cell suspension was added to each of the inner 60 wells of a 96-well, white flat-bottom plates. 25 μL of the appropriate antibody dilutions were added to the wells containing CTLA-4 effector cells. Artificial antigen presenting (aAPC)/Raji cells (provided with kit) were thawed and diluted into 7.2 mL of assay buffer, and 25 μL of the cell suspension was added to wells containing the diluted antibodies and CTLA-4 effector cells. Plates were incubated for 6 hours at 37°C in a tissue culture incubator with 5% CO_2 before 75 μL of Bio-Glo Reagent was added to wells containing cell and antibody mixtures. Plates were incubated for 5-15 minutes at room temperature and luminescence was measured on a Spectramax i3x plate reader (Molecular Devices, San Jose, CA, USA). Data analysis was performed using the Softmax Pro (Molecular Devices, San Jose, CA, USA) or Prism (GraphPad, San Diego, CA, USA) software packages.

CD80/CD86 blocking ELISAs

[00571] ELISA plates (Nunc, MaxiSorp ELISA plates, flat bottom, uncoated, BioLegend, San Diego, CA, USA) were coated with recombinant human CTLA-4-Fc (7268-CT, R&D Systems, Minneapolis, MN, USA) at 1 μg/mL overnight at 4°C. Plates were then blocked with 5% milk in phosphate buffered saline with tween 20 (PBST) for 1 hour at room temperature. A titration series of the indicated mAbs was then added to the plates and then the plates were incubated for 1 hour at room temperature to allow mAb binding. Excess, unbound mAb was

removed by washing with PBST. Recombinant human His-tagged CD80 (R&D Systems 9050-B1, Minneapolis, MN, USA) or CD86 (R&D Systems 9090-B2, Minneapolis, MN, USA) was then added to the plates at 1 μg/mL. After incubation for 1 hour at room temperature, the plates were washed to remove unbound ligand. Bound ligand was detected with a horseradish peroxidase (HRP)-conjugated anti-His antibody (652504, BioLegend, San Diego, CA, USA). After incubation for 1 hour at room temperature and washing, the plates were developed with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (34028, Pierce, Waltham, MA, USA). After sufficient signal was achieved, development was stopped by the addition of 1 N hydrochloric acid. Absorbance at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices, San Jose, CA, USA). Half maximal inhibitory concentration (IC50) values were calculated by plotting absorbance versus the log of concentration using Prism (GraphPad, San Diego, CA, USA).

Murine models

[00572] Murine experiments were done in compliance with all relevant ethical regulations and approved by the Institutional Animal Care and Use Committee of Crown Bioscience. Experiments using full-length hCTLA-4 knock-in HuGEMM™ mice (Shanghai Model Organisms Center, Inc.) were performed at Crown Bioscience (Taicang Jiangsu Province, China). Full-length hCTLA-4 knock-in HuGEMM™ mice were generated by knocking in human *CTLA4* cDNA with a polyA sequence at exon 1 of the murine *Ctla4* locus, replacing murine *Ctla4* expression with human *CTLA4* expression. Crown Bioscience acquired MC38 cells from FDCC (The Institutes of Biomedical Sciences (IBS), Fudan University, China) and RM-1 cells from SIBS (Shanghai Institutes for Biological Sciences, China) and authenticated cell line identity of research cell banks by single nucleotide polymorphism (SNP) analysis. Cell lines were mycoplasma negative. 8-12-week-old, female, full-length hCTLA-4 knock-in HuGEMM™ mice were injected subcutaneously at the right lower flank with MC38 or RM-1 cells (10^6 cells suspended in 100 μL of PBS) as indicated for tumor development. Tumors were allowed to establish until tumor volume reached the indicated size. Mice were then randomized and dosing, as described for each experiment, was initiated on the same day as randomization. Tumor volumes and body weight were measured in a blinded fashion at least twice a week. Tumor volumes were calculated using the formula: $V = (L \times W \times W)/2$, where V is tumor volume, L is tumor length (the longest tumor dimension), and W is tumor width (the longest tumor dimension perpendicular to L). Individual animals were removed from the study as their tumor volumes measured greater than 3000 mm^3. Where indicated mice with a complete response on day 35 post randomization were re-challenged on day 43. For the re-challenge experiment, MC38 tumor cells

(10^6 in 100 µL PBS) were implanted subcutaneously in the lower left (opposite) flank region. Tumor cells were also implanted in 6-8 week old naïve, WT C57BL/6 mice (Shanghai Lingchang Biotechnology Co., Ltd. (Shanghai, China)) as a positive control for tumor growth at the time of the re-challenge. Mice and any tumor progression were observed for another 30 days. Several of these experiments are previously described in **Examples 4, 5, 6, 7, and 11**.

[00573] Single cell suspensions from tumors were prepared using the murine tumor dissociation kit (Miltenyi, Bergisch Gladbach, Germany) and GentleMACS™ Octo Dissociator with Heaters set to the dissociation program (37_c_m_TDK_1). Single cell suspensions from lymph nodes were pushed through a 70 µm cell strainer using the plunger from a 5 mL syringe. Single cell suspensions were then incubated at room temperature with 1× RBC lysis buffer for 90 seconds. RBC lysis was then quenched, washed, strained through a 70 µm cell strainer, and counted. For cell staining cells were first blocked with 1 µg/ml Fc-Block (mouse Fc Block, BD Biosciences, San Jose, CA, USA) for 15 minutes at 4°C. Cells were then stained with the indicated surface antibodies for 30 minutes on ice in the dark (**Table 27**). Cells were then washed twice and fixed with Fixation/Permeabilization working solution (eBioscience, San Diego, CA, USA) and then washed twice with 1× Permeabilization buffer (eBioscience, San Diego, CA, USA), after which intracellular staining was performed in 1× Permeabilization buffer. Following intracellular staining, cells were washed twice with 1× Permeabilization buffer and data was collected using a flow cytometer (LSRFortessa X-20, BD Biosciences, San Jose, CA, USA). Data were analyzed using Kaluza or FlowJo 10.

TABLE 27: Reagent antibodies for immunophenotyping.

Reagent antibodies for immunophenotyping. Antibody clone details used for immunophenotyping are listed.

Marker	Fluorochrome	Clone	Catalog	Vender
CD107a	FITC	1D4B	121606	BioLegend
CD11c	FITC	N418	117306	BioLegend
CD335 (NKp46)	BV605	29A1.4	137619	BioLegend
CD4	BUV395	GK1.5	563790	BD
CD4	BV421	GK1.5	100438	BioLegend
CD44	BV421	IM7	563970	BD
CD45	BV785	30-F11	103149	BioLegend
CD62L	BV785	MEL-14	104440	BioLegend
CD69	PE-Cy7	H1.2F3	104512	BioLegend
CD8	PE-eFluor 610	53-6.7	61-0081-82	eBioscience
CD80	APC	16-10A1	560016	BD

CD86	BV605	GL-1	105037	BioLegend
FoxP3*	PE	FJK-16s	12-5773-82	eBioscience
hCD152 (CTLA-4)	Percp-cy5.5	L3D10	349928	BioLegend
hCD152 (CTLA-4)*	Alexa Fluor® 647	L3D10	349920	BioLegend
Ki67*	Alexa Fluor® 700	SolA15	56-5698-82	eBioscience
Live/Dead	Ef780	n/a	65-0865-18	eBioscience
Live/Dead	APC-eFluor 780	n/a	65-0865-14	eBioscience
TCR-beta	BUV395	H57-597	742485	BD
Counting beads	-	-	01-1234-42	Invitrogen
*Used for internal staining				

[00574] Experiments using hCTLA-4/hPD-1 double knock-in HuGEMM™ mice on the BALB/c background (GemPharmatech Co., Ltd) were generated by crossing mice in which exon 2 of murine *Ctla4* was replaced with exon 2 from human *CTLA4* with mice in which exon 2 and 3 of murine *Pdcd1* were replaced with exon 2 and 3 of human *PDCD1*. The murine toxicity study using these mice was performed at Crown Bioscience (Taicang Jiangsu Province, China). Female mice were 4-5 weeks old at start of dosing and were treated with PBS, pembrolizumab (15 mg/kg), ipilimumab (10 mg/kg) plus pembrolizumab, or GIGA-564 (10 mg/kg) plus pembrolizumab every 3 days for 9 doses, and mice were euthanized 10 days after completion of dosing and tissues collected for histopathology analysis.

Epitope mapping

[00575] Identification of the key energetic residues involved in binding was performed by Integral Molecular (Philadelphia, PA, USA). Full-length human CTLA-4 with a mutation to reduce internalization (Y201G) was cloned into a transient expression vector, with each extracellular position (36-161) individually mutated to alanine (or alanine mutated to serine). The mutant library was arrayed in 384-well microplates and transiently transfected into HEK-293T cells. The optimal staining concentrations of ipilimumab, GIGA-564, and L3D10 control (BioLegend, San Diego, CA, USA) were determined using wild-type CTLA-4, then applied to the CTLA-4 mutant library. Antibody binding was detected using goat-anti-human IgG-Alexa fluor-488 or anti-human IgG F(ab')2-Alexa fluor-488 (Jackson ImmunoResearch, West Grove, PA, USA), and mean cellular fluorescence was determined using flow cytometry (Intellicyt iQue, Ann Arbor, MI, USA). Mutated residues were considered critical if mutation resulted in significant loss of binding to the test antibody compared to the control antibody. This epitope mapping experiment is also described in Example 12.

In vitro Treg activation assay

[00576] Anti-CD3 (OKT3, Ultra-LEAF purified, BioLegend, San Diego, CA, USA) with and without CD80 (R&D Systems rhCD80-Fc, 10107-B1-100, Minneapolis, MN, USA) were covalently linked to the surface of M-450 Tosyl beads (Dynabead M-450, 140130, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's guidelines. To confirm that the surfaces of each bead type were properly coated, fluorescent antibodies were used to detect either the mouse Fc (anti-CD3) or the CD80 on the bead surface via flow cytometry.

[00577] Donor-matched human Treg and Tconv cells (Stemcell Technologies, 70096, Vancouver, BC, Canada) were thawed and stained with CellTrace Violet (C34571, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. 10^5 cells were then cultured with 10^5 beads as indicated in Complete Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific, Waltham, MA, USA) with non-essential amino acids, sodium pyruvate, Glutamax, and 5% human antibody serum at 37°C, 5% CO_2 in a 96-well U-bottom plate (Falcon-Corning U-bottom tissue culture plates, VWR, Radnor, PA, USA). In conditions where rhCD80-Fc (Abatacept, BMS, Princeton, NJ, USA) or aCTLA-4.28 were used, these were mixed with beads first prior to being added to the cells. Test samples with both rhCD80-Fc and aCTLA-4.28 were mixed and incubated together prior to mixing with beads and then cells. After a 4 day incubation, cells were washed, stained, and analyzed using the CytoFLEX LX (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed in FlowJo v10.7.1 (BD Biosciences, San Jose, CA, USA).

FcR effector activity bioassays

[00578] Fc effector activity bioassays for mFcγRIIIa (CS1779B08), mFcγRIV (M1151), hFcγRIIb (CS1781E02), hFcγRIIa-H variant (G9981), hFcγRIIa-R variant (CS1781B08), hFcγRIIIa-V variant (G7011), and hFcγRIIIa-F variant (G9791) were purchased from Promega Corporation (Madison, WI, USA). The assays were carried out following the manufacturer's instructions. Briefly, CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were suspended in RPMI 1640 + 4% FBS media with GIGA-564, ipilimumab, or GIGA-564 with the LALA-PG mutation to eliminate Fc function, and incubated at 37°C for 30 minutes. The following starting concentrations and dilution factors were used for each assay: 1 μg/mL, 2.5-fold dilution series (mFcγRIIIa, hFcγRIIIa-V variant), 500 μg/mL, 2.5-fold (mFcγRIV), 3 μg/mL, 3-fold (hFcγRIIb, hFcγRIIa-H and R variant), or 10 μg/mL, 2.5-fold (hFcγRIIIa-F variant). Jurkat/NFAT-Luc effector cells solely expressing one type of FcγR were added to each well (effector:target ratio was 5:1) and incubated at 37°C for 6 hours. Luciferase activity was measured by using the included Bio-Glo

Luciferase Assay Reagent with the SpectraMax i3x or iD3 plate readers (Molecular Devices, San Jose, CA, USA). Luciferase activity measured in relative luminescence units (RLU) were plotted against the concentration of CTLA-4 mAbs. The IC50 value of each mAb was calculated by logistic regression using Prism (GraphPad, San Diego, CA, USA).

Determination of pH-sensitivity of antibodies

[00579] 96-well plates (Nunc, MaxiSorp, flat bottom, uncoated, BioLegend, San Diego, CA, USA, 423501) were coated with 0.5 μg/mL rhCTLA-4-Fc chimera (7268-CT, R&D Systems, Minneapolis, MN, USA) diluted in 1× PBS pH 7.0 and incubated at 2-8°C overnight. Plates were washed with 1× PBST and blocked with PBST + 1% BSA (PBSTB). Purified CTLA-4 mAbs were diluted in 10 mM sodium phosphate + 150 mM NaCl adjusted to pH 4.0, 5.0, 6.0, or 7.0 then added to the plate for 1 hour. Unbound antibodies were washed away with PBST and bound antibodies detected with 0.5 μg/mL HRP-conjugated goat anti-human constant kappa (2060-05, Southern Biotech, Birmingham, AL, USA) diluted in PBSTB. Plate was washed with PBST once more, then TMB substrate (1-Step™ Ultra TMB-ELISA Substrate Solution, Thermo Fisher Scientific, Waltham, MA, USA) was added and developed for approximately 1 minute before stopping the reaction with 1 N HCl. Absorbance at 450 nm was measured using a spectrophotometer (i3x, Molecular Devices, San Jose, CA, USA) and plotted using Prism (GraphPad, San Diego, CA, USA).

Generation of cell pools stably expressing GIGA-564

[00580] GIGA-564 was expressed from a variant of the pCDNA5/FRT mammalian expression vector (Thermo Fisher Scientific; Waltham, MA, USA). The vector has a promoter for glutamine synthetase selection and uses an EF1α promoter to drive light chain expression, followed by a BGH polyA sequence and a CMV promoter to drive expression of the heavy chain, followed by a second BGH polyA sequence. The antibody expression construct was built using GeneBlocks (Integrated DNA Technologies, Coralville, IA, USA) and NEBuilder® HiFi DNA Assembly Master Mix (New England BioLabs, Ipswich, MA, USA). The construct was amplified in NEB 10-beta *E. coli* and purified with the ZymoPURE™ Plasmid Maxiprep Kit (Zymo Research, Irvine, CA, USA). It was then linearized for transfection by digesting 150 μg of DNA with 3,000 units PvuI-HF restriction enzyme (New England Biolabs, Ipswich, MA, USA), precipitated, and washed.

[00581] Sigma Aldrich's CHOZN cells were cultured in EX-CELL CD CHO Fusion medium (MilliporeSigma, Burlington, MA, USA) supplemented with 6 mM GlutaMAX (Gibco, Waltham, MA, USA), shaking at 37°C, 5% CO_2, 125 RPM (25 mm throw) for 7 days prior to

electroporation. The day before electroporation, suspension cells were seeded at 0.5×10^6 viable cells/mL (vc/mL). Linearized plasmid was transfected and pulsed using CM-150 on the Amaxa 4D Nucleofector (Lonza, Basel, Switzerland) and SE Cell Line 4D Nucleofector X kit (Lonza, Basel, Switzerland). Each cuvette had 4 μg of DNA and 10^7 CHOZN cells concentrated in SE cell solution. Post-electroporation, the transfected cells were transferred to T-75 flasks at a 2×10^6 vc/mL density for a 1-day recovery in EX-CELL CD CHO Fusion medium (MilliporeSigma, Burlington, MA, USA) with 6mM GlutaMAX (Gibco; Waltham, MA, USA). After 1-day recovery, transfected cells were pelleted and resuspended into 80% EX-CELL CD CHO Cloning medium (MilliporeSigma, Burlington, MA, USA) and 20% EX-CELL CD CHO Fusion medium (MilliporeSigma, Burlington, MA, USA) and plated into 96-well plates (non-TC treated, flat bottom, Greiner One-Bio, Kremsmünster, Austria) at 5,000 cell per well and incubated 37°C, 5% CO_2 with humidity.

[00582] After 4-5 weeks, confluent transfected wells (minipools) were screened for antibody concentration using the Gator bio-layer interferometry system and Protein-A biosensors (GatorBio, Palo Alto, CA, USA). Minipools with the highest antibody titer were scaled-up using EX-CELL CD CHO Fusion medium in static 24-well TC-treated plates incubated at 37°C, 5% CO_2 with humidity. Minipools continued to expand and shaker adapted to shaking 6-well plates (non-TC treated, flat bottom, Greiner One-Bio, Kremsmünster, Austria) on a 19 mm shaking platform set at 145 RPM, 37°C, 5% CO_2, with humidity. Material from an 8-day terminal batch in 24-well plates (TC treated, flat bottom, Corning, Corning, NY, USA) was screened using the Gator system as a second method to screen minipools, in order to identify the high producers. Cultures were expanded into shaking 50 mL conical tubes (Midsci, St. Louis, MO, USA) incubated at 37°C, 5% CO_2, 80% humidity, shaking at 225 RPM with 25 mm throw. Top minipools were combined to generate enriched pools (EPs).

[00583] EPs were cultured in shake flasks at 125 RPM, 25 mm throw, seeded at $0.2\text{-}0.4 \times 10^6$ viable cell density (VCD)/mL for 3-4 days. EPs were then inoculated in EX-CELL Advanced Fed Batch medium at 0.4×10^6 VCD/mL in 1 L shake flasks (Corning, Corning, NY, USA) with a vent cap and working volume of 200 mL. Incubator conditions were held constant throughout fed-batch at 37°C, 5% CO_2, 80% humidity, and shaking at 125 RPM (25 mm throw). Beginning day 3 of fed-batch to harvest, growth, viability, and metabolite concentrations were measured offline. Glucose was supplemented when concentration dropped below 4 g/L, up to 6 g/L using 45% glucose stock (Corning, Corning, NY, USA). Cultures were fed days 3, 5, 7, 9, and 11 with EX-CELL Advanced CHO Feed 1 (MilliporeSigma, Burlington, MA, USA) (4% of working

culture volume) and CellVento 4Feed COMP (MilliporeSigma, Burlington, MA, USA) (2% of working culture volume). Cultures were harvested when viability dropped below 80% and cell culture supernatant was clarified by centrifugation and filtered. Protein was purified using Protein A eluted with 100 mM acetate pH 3. One EP was used for subsequent analysis (EP-1).

Glycan analysis

[00584] This analysis was performed by the CRO Bionova Scientific (Fremont, CA, USA). 150 μg of antibody was digested with PNGase F (Prozyme, Hayward, CA, USA) to liberate glycans. Glycans were isolated and labeled with a fluorescent molecule using a GlykoPrep InstantAB labeling kit (Prozyme, Hayward, CA, USA) following the manufacturer's instructions. Labeled glycans were injected over a 3.5 μm, 2.1 × 150 mm XBridge Amide column (Waters, Milford, MA, USA) on a UPLC (Dionex Ultimate 3000, Waters, Milford, MA, USA) with a fluorescent detector. Peaks were identified based on known glycans from the Glyko InstantAB biantennary and high-mannose partitioned library standards (Prozyme, Hayward, CA, USA), with the total percent of each glycoform reported as the integrated area under the identified peak divided by the sum of the AUC (area under the curve) for all peaks.

Skin inflammation, colonic epithelial and heart toxicity scoring

[00585] Hematoxylin and eosin (H&E)-stained sections of skin from near the whisker region was scored as follows 1: 1-3 small foci of lymphocyte aggregates per section, 2: 4-10 small foci or 1–3 intermediate foci, 3: 4 or more intermediate or the presence of large foci, 4: marked interstitial fibrosis in parenchyma and large foci of lymphocyte aggregates.

[00586] The colonic epithelial score was determined by assessment of H&E-stained colon rolls. To determine this score, after reviewing the whole slide the four regions in which damage was most severe were selected for scoring, and the score from all four areas was added together to determine the cumulative score. To determine the score for individual areas, both the epithelial structures affected and the consistency of the damage were taken into account. For this the damage to epithelial structures was scored as 0: no lesions, 1: mucosa damage, 2: submucosa damage, 3: muscularis/serosa damage. The consistency of damage in each area was scored as 1: focal, 2: patchy, 3: diffuse. Both scores were then multiplied together to determine the area score.

[00587] For the heart pathology score, lymphocyte infiltration on H&E-stained heart sections in pericardium, right or left atrium, base of aorta, and left or right ventricle each count for 1 point each. The number of $CD45^+$ cells were counted on heart formalin-fixed paraffin-embedded (FFPE) sections stained with CD45. The visual fields for inflammatory cell infiltration

analysis were randomly selected, and the average score of three fields for each sample was determined.

Kidney pathology staining and scoring

[00588] Tissues were collected and were placed in 10% buffered neutral formalin and then paraffin embedded. Sectioning, staining, and scoring was performed by Allele (San Diego, CA, USA). Briefly, blocks were cut in 5 μm sections that were placed on glass slides for anti-IgG (UltraPolymer Goat anti-Mouse heavy and light chain IgG-HRP, Cell IDx, San Diego, CA, USA) or anti-C3 (EPR19394, Abcam, Cambridge, UK) immunohistochemistry (IHC) staining. Stained slides were prepared as digital images.

[00589] A board-certified veterinary pathologist with experience in laboratory animals and toxicologic pathology, who was blinded to the study, evaluated the anti-IgG slides for location, intensity, and percent of positive staining. Findings were scored on a scale of 1 to 4 for intensity (0: negative, 1: minimal or slightly positive, 4: very dark), and as a percent of the positive cells in the glomeruli (after reviewing at least 5 glomeruli). This experiment is also described in Example 6.

Statistical analysis

[00590] Comparison of changes in tumor volume measured longitudinally in multiple groups was determined using a linear mixed effects model with treatment group and day as fixed effects and animal identifier as a random effect, to account for the dependence of repeated measures. All tests were two-sided without adjustments to type I error rates. These analyses were conducted using R version 3.6.2.

[00591] For the re-challenge study, tumor volume (in mm^3) was analyzed using a linear mixed effects model including treatment group and day as fixed effects and animal identifier as a random effect, to account for repeated measures. Statistical comparisons were made using the Wald test against the isotype control group for the initial challenge and against the naïve control group for the re-challenge portion of the study. All tests were two-sided with an alpha level of 0.05.

[00592] Adjusted p-values for comparison of skin inflammation, colonic epithelial damage, $CD45^+$ cell infiltration into the heart, heart pathology score, colon length, spleen weight, and kidney Ig or C3 deposition were calculated using the Benjamini-Hochberg step-down procedure to account for multiple comparisons. Analyses were performed using R version 3.6.2. To determine if there was any statistical difference in the percent body weight induced by

pembrolizumab or pembrolizumab plus a CTLA-4 mAb in comparison to control, a mixed effect model on change in body weight including day and treatment group as fixed effects and animal identifier as a random effect, to account for repeated measurements within animal, was used.

[00593] For flow cytometry data, the Wilcoxon rank sum test was used to determine statistical significance, with an alpha level of 0.05.

RESULTS

[00594] This study presented evidence that checkpoint inhibition was not a primary mechanism of action for efficacy of anti-CTLA-4 antibodies. Instead, the primary mechanism for efficacy is FcR-mediated Treg depletion in the tumor microenvironment. The study identified a monoclonal antibody (mAb) (GIGA-564) that binds to CTLA-4 at an epitope that differs from ipilimumab's by only a few amino acids, yet has limited checkpoint inhibitor activity. Surprisingly, the weak checkpoint inhibitor had superior anti-tumor activity compared to ipilimumab in a murine model. The weak checkpoint inhibitor also induced less Treg proliferation and has increased ability to induce *in vitro* FcR signaling and *in vivo* depletion of intratumoral Tregs. Further experiments showed that the enhanced FcR activity of the weak checkpoint inhibitor likely contributes to its enhanced anti-tumor activity. The results of the present study showed that weak checkpoint inhibition was associated with lower toxicity in murine models.

[00595] The results herein showed that compared to ipilimumab, the weak B7 ligand blocking anti-CTLA-4 antibody GIGA-564 induced less peripheral Treg proliferation, more efficient intratumoral Treg depletion, superior anti-tumor efficacy, and less toxicity in human *CTLA4* knock-in mouse models. This work suggests a translational path forward to improve outcomes for cancer patients.

[00596] The results of the study showed that the efficacy of anti-CTLA-4 drugs is due to depletion of Tregs in the tumor microenvironment. Checkpoint inhibition by anti-CTLA-4 is not necessary and may cause toxicities.

[00597] The inventors surprisingly found that GIGA-564 is:

(1) an anti-CTLA-4 with limited checkpoint inhibitor activity, GIGA-564 has superior efficacy to ipilimumab in mouse models;

(2) induced less Treg proliferation than ipilimumab in tumor bearing mice;

(3) had superior Treg depletion in the tumor microenvironment and more efficiently depleted intratumoral CTLA-4^{Hi};

(4) improved cell signaling via interactions between CTLA-4-bound GIGA-564 and FcR; and

(5) induced less toxicity than ipilimumab in murine models expressing human CTLA-4.

In vitro characterization of a diverse panel of anti-CTLA-4 antibodies

[00598] A library of natively paired single chain variable fragments (scFvs) specific to CTLA-4 were generated by immunizing Trianni Mouse® animals, which produce antibodies with fully-human variable domains and mouse constant regions; then used a proprietary microfluidics platform and yeast scFv display to select for CTLA-4 binders. Fourteen of the antibodies were cloned as full-length human IgG1 antibodies, produced in Chinese hamster ovary (CHO) cells, and were shown to bind to recombinant human CTLA-4 by surface plasmon resonance (SPR) with low nanomolar affinity (average equilibrium dissociation constant, KD: 8.7 nM), similar to that of ipilimumab (**FIG. 20A-20B** and **TABLE 23**).

TABLE 23: *In vitro* characterization of scFvs reformatted as full-length antibodies.

In vitro characterization of scFvs reformatted as full-length antibodies. kon, koff, and KD of the indicated CTLA-4 mAbs for soluble human CTLA-4 was determined by SPR (Carterra). Geometric MFI of the indicated CTLA-4 mAbs bound to CTLA-4$^+$ or CD27$^+$ CHO cells as detected by a secondary human IgG antibody and flow cytometry. Affinity (KD) of the indicated CTLA-4 mAbs for soluble cynomolgus CTLA-4 was determined by a single antigen concentration BLI measurement (Octet). The ability of each aCTLA-4 mAb to block CTLA-4 binding to CD80/CD86 was determined by a cell-based assay (Promega). Due to constraints on sample size in each Promega bioassay kit, this set of aCTLA-4 mAbs was analyzed using multiple plates (plate letters correspond to Plate A-D in **Fig. 20D**). To control for plate-to-plate variability in maximum signal, the ipilimumab analog was run on each plate; a representative sample was used for this table.

Antibody	kon human CTLA-4 (M-1 s-1)	koff human CTLA-4 (s-1)	KD human CTLA-4 (nM)	MFI of CTLA-4+ CHO cells	MFI of CD27+ CHO cells	KD cyno CTLA-4 (nM)	Cell-based blocking activity EC50 (µg/mL)	Cell-based blocking activity max signal (RLU)	Cell-based assay plate
Ipilimumab analog	6.50E+04	3.20E-04	4.9	860	26.6	1.3	0.34	307	A
aCTLA-4.1	8.50E+04	3.90E-04	4.6	1008	18.6	1.0	0.10	349	A
aCTLA-4.2	1.90E+05	2.80E-04	1.5	1050	18.2	1.2	0.18	273	C
aCTLA-4.3	6.40E+04	3.60E-04	5.7	854	15.3	0.8	0.17	63	C
aCTLA-4.4	1.00E+05	5.20E-04	5.1	1123	11.7	3.1	0.12	413	A
aCTLA-4.9	2.10E+05	1.60E-04	0.77	1328	18.5	1.0	0.13	237	C
aCTLA-4.11	4.20E+03	2.00E-04	48	219	14.8	no binding	0.89	47	D
aCTLA-4.12	5.10E+04	2.70E-04	5.3	1013	17.1	4.5	2.17	13	D
aCTLA-4.14	1.30E+05	4.20E-04	3.2	1024	17	9.6	0.09	73	A
aCTLA-4.15 (GIGA-564)	5.40E+04	5.20E-04	9.8	717	15.9	23.6	0.09	33	A

aCTLA-4.22	2.30E+04	5.10E-04	22	641	15.2	3.1	0.26	134	A
aCTLA-4.24	6.30E+04	4.30E-04	6.8	934	26	0.9	0.19	333	B
aCTLA-4.26	6.10E+04	3.50E-04	5.7	939	17.8	1.6	0.18	338	B
aCTLA-4.28	9.00E+04	4.20E-04	4.6	1033	17.2	2.2	0.18	548	B
aCTLA-4.29	7.60E+04	2.30E-04	3	999	16.3	1.2	0.15	444	B

[00599] All 14 mAbs bound CHO cells stably expressing human CTLA-4, with no off-target binding to CHO cells expressing the irrelevant target CD27 (**FIG. 20C** and **TABLE 23**). Next, the ability of these mAbs to block interaction of CTLA-4 with its endogenous ligands (the B7 proteins CD80 and CD86) was assessed by a cell-based assay. The mAbs exhibited a variety of half maximal effective concentration (EC50, μg/mL; average: 0.34; range: 0.1-2.17), and a broad range of maximum signals (average: 240; range: 13-548), where lower values indicated less blocking (**FIG. 20D** and **TABLE 23**). The correlations between affinity KD and blocking EC50 or between affinity KD and blocking maximum signal were not significant (linear regression; R^2=0.06 and R^2=0.16, respectively; $p>0.05$, **FIG. 20E**). For this set of 14 antibodies, it was speculated that B7 ligand blocking is a function of anti-CTLA-4 binding epitope and not CTLA-4 binding affinity. Of interest, aCTLA-4.15, subsequently re-named GIGA-564, showed a combination of high affinity and low blocking maximum signal.

FcR effector function is required for the anti-tumor efficacy of ipilimumab in a murine model

[00600] The role of Treg depletion in the efficacy of CTLA-4 mAbs has remained controversial. Additionally, the role of the Fc effector function in the anti-tumor efficacy of ipilimumab has not been reported. Thus, the anti-tumor effect of conventional anti-CTLA-4 mAbs, including ipilimumab, was investigated in the presence and absence of Fc effector functions.

[00601] First, wild-type human IgG1 and human IgG1 N297Q mutant Fc variants of ipilimumab and another strong blocker, aCTLA-4.28 (**FIG. 20** and **TABLE 23**) were generated. The N297Q mutation eliminates Fc glycosylation and the ability to bind FcRs, thus abrogating Fc effector functions such as ADCC. The CTLA-4 mAbs with the N297Q mutation were still able to bind CTLA-4$^+$ CHO cells (**FIG. 21**). As ipilimumab does not bind murine CTLA-4, a human *CTLA4* knock-in (hCTLA-4 KI) mouse model, where the human *CTLA4* coding region is knocked-in to the murine *Ctla4* locus, was used for *in vivo* efficacy studies. The majority (89.9% and 91.5%, respectively) of CD4 conventional T cells (CD4$^+$FOXP3$^-$) in the lymph node of hCTLA-4 KI and wildtype mice were naïve (CD44^{Lo}CD62L$^+$) (**FIG. 12A**), suggesting that CTLA-4 is functional in hCTLA-4 KI mice. Both the ipilimumab analog and aCTLA-4.28 with a wildtype human IgG1 Fc led to strong regression of MC38 tumors (colon cancer model) in hCTLA-4 KI mice (**FIG. 12B**). However, in mice treated with ipilimumab_N297Q or aCTLA-4.28_N297, tumor growth was not significantly different from isotype control treated mice (**FIG. 12B**). These data indicated that anti-CTLA-4 mAbs, including ipilimumab, require FcR binding

for anti-tumor activity and that blocking the binding of B7 ligands to CTLA-4 is not sufficient for the anti-tumor activity of anti-CTLA-4 mAbs.

GIGA-564 has weak ability to block CTLA-4 from binding to CD80/CD86

[00602] To confirm the weaker blocking ability of GIGA-564, ELISAs were performed to separately determine the ability of CD80 or CD86 to bind CTLA-4 in the presence of CTLA-4 mAbs (**FIG. 13A**). If binding of the mAb to CTLA-4 blocked CTLA-4 from binding to the ligand, the amount of ligand detected would decrease with increasing mAb concentration. GIGA-564 had much weaker blocking efficiency for both CD80 and CD86 than ipilimumab or aCTLA-4.28, as the curves were strongly shifted to the right (**FIG. 13B** and **Table 24**). Additionally, GIGA-564 was unable to fully block either ligand, even at the highest concentrations (**FIG. 13B**). This is consistent with its inability to induce full CD28 signaling in the cell-based signaling assay (**FIG. 20D**), which required the mAbs to strongly block binding of CTLA-4 to CD80 and CD86.

Table 24: GIGA-564 has minimal ability to block CD80 or CD86 from binding CTLA-4

GIGA-564 has minimal ability to block CD80 or CD86 from binding CTLA-4. Summary of blocking ELISA data from **FIG. 13B**. Absorbance values from the detected CD80 or CD86 were normalized to signal from ELISA plates incubated with pembrolizumab. The data were then analyzed and fit by nonlinear regression. The normalized maximum and minimum signals and the IC50 for blocking are provided.

	CD80				CD86		
Antibody	**Max**	**Min**	**IC50 (μg/mL)**		**Max**	**Min**	**IC50 (μg/mL)**
Ipilimumab	0.96	0.04	0.07		1.04	0.09	0.07
GIGA-564	0.97	0.18	2.23		1.07	0.36	0.30
aCTLA-4.28	1.09	0.04	0.12		1.05	0.12	0.12

Max: maximum absorbance at 450 nm, normalized to prembrolizumab

Min: minimum absorbance at 450 nm, normalized to pembrolizumab

[00603] The key amino acids in the CTLA-4 epitope for GIGA-564 and ipilimumab were determined using the shotgun mutagenesis epitope mapping method. Epitope mapping revealed that GIGA-564 and ipilimumab have overlapping but distinct epitopes. In particular K130, Y139, L141, and I143 are key amino acids that are part of the epitope for both GIGA-564 and ipilimumab (**FIG. 13C-13D**). However, R70 is a key amino acid for the epitope of ipilimumab but not GIGA-564 (**FIG. 13C-13D**). Importantly, R70 is also important for CD80 and CD86 binding to CTLA-4 (**FIG. 13D**). It was speculated that R70 on CTLA-4 may be more available to

bind CD80 and CD86 when CTLA-4 is bound by GIGA-564 compared to ipilimumab, explaining why GIGA-564 only minimally blocks CD80/CD86 binding to CTLA-4.

GIGA-564 treatment induces anti-tumor responses in murine models

[00604] The anti-tumor efficacy of GIGA-564 was tested. Both GIGA-564 and commercial ipilimumab led to nearly complete control of MC38 tumors in hCTLA-4 KI mice when they were dosed at 5 mg/kg twice a week (**FIG. 14A**). Similarly, in hCTLA-4 KI mice bearing RM-1 tumors (prostate cancer model), which are more resistant to anti-CTLA-4, GIGA-564 led to similar tumor growth inhibition to commercial ipilimumab when dosed at 5 mg/kg on days 0, 3, and 6 (**FIG. 14B**). Thus, it was found that anti-CTLA-4 anti-tumor activity is independent of checkpoint inhibition via CTLA-4 blocking in multiple tumor models.

[00605] Highly efficacious dosing of anti-CTLA-4 mAbs at 5 mg/kg made it difficult to identify any potential difference in the anti-tumor efficacy of ipilimumab and GIGA-564. Therefore, the ability of 0.3 mg/kg of ipilimumab or GIGA-564 to control progression of MC38 tumors in hCTLA-4 KI mice was assessed. GIGA-564 was more effective in limiting tumor progression than commercial ipilimumab at this low dose (**FIG. 14C**).

GIGA-564 induces less peripheral Treg proliferation and efficiently depletes intratumoral Tregs

[00606] Tregs stimulated via CD3/CD80 proliferate more than Tregs stimulated via CD3 alone, and CTLA-4 blockade overcomes inhibition by exogenous CTLA-4-Fc (**FIG. 22**). In agreement with this, blocking or acute loss of CTLA-4 enhances Treg proliferation *in vivo*. Thus, it was hypothesized that GIGA-564 would induce less Treg proliferation than ipilimumab. To test this possibility, hCTLA-4 KI mice bearing established MC38 tumors were treated with mAbs at 5 mg/kg on days 0, 3, and 6 and analyzed the proliferation status of peripheral T cell subsets from a non-draining lymph node on day 7 (**FIG. 15A**). This analysis revealed that ipilimumab increased the proliferation of Tregs more than it increased the proliferation of conventional T cells (Tconv) or CD8 T cells (**FIG. 15A**). Furthermore, GIGA-564 induced significantly less peripheral Treg proliferation than ipilimumab (**FIG. 15A**; Wilcoxon rank sum test, $p = <0.05$). These data are consistent with the *in vitro* data that showed that GIGA-564 only weakly blocks the interaction of CTLA-4 with its B7 ligands (**FIG. 13B**, **FIG. 20D**, and **TABLE 23**).

[00607] Intratumoral Tregs express more surface CTLA-4 than effector T cells or even peripheral Tregs. Thus, as FcR effector function activity of antibodies is dependent on the

amount of antibody that binds to the cell surface, CTLA-4 mAbs selectively deplete intratumoral Tregs. Furthermore, antibody-mediated depletion of CTLA-4^{Hi} Tregs in the tumor microenvironment (TME) has been shown to begin within the first 24 hours after antibody administration. Thus, to determine the ability of GIGA-564 to deplete intratumoral Tregs hCTLA-4 KI mice bearing established MC38 tumors were treated with 5 mg/kg of isotype, ipilimumab, or GIGA-564 and then analyzed Treg populations in the periphery and tumor by flow cytometry 1 day later. It was found that both anti-CTLA-4 antibodies depleted intratumoral Tregs (Wilcoxon rank sum test, $p<0.05$) but not peripheral Tregs (**FIG. 15B**; Wilcoxon rank sum test, $p>0.05$). It was also found that for animals administered GIGA-564, the population of remaining intratumoral Tregs had a lower CTLA-4 mean fluorescence intensity (MFI) than analogous populations from animals administered ipilimumab (**FIG. 15C-15D**; Wilcoxon rank sum test, $p<0.05$). Additionally, GIGA-564 weakly enhanced the intratumoral CD8/Treg ratio compared to ipilimumab (**FIG. 15E**). GIGA-564 was more effective at depleting CTLA-4^{Hi} intratumoral Tregs, which in addition to the reduced Treg proliferation induced by GIGA-564, may explain why GIGA-564 is superior at controlling tumor growth at low doses than ipilimumab. The results show that GIGA-564 eliminates intratumoral Tregs. Human CTLA-4 knock-in mice bearing MC38 tumors were treated with 5 mpk of ipilimumab or GIGA-564 and sacrified 1 day later. Flow cytometry showed that GIGA-564 is more efficient than ipilumab at depleting CTLA-4+ Tregs in the tumor microenvironment.

[00608] Additional flow analysis of the TME or peripheral lymph node of hCTLA-4 KI mice with established MC38 tumors treated with 2 or 3 doses of GIGA-564 or ipilimumab was performed (harvesting on day 4 or 7, respectively). Even with additional treatments, both GIGA-564 and ipilimumab were able to specifically deplete intratumoral Tregs but not peripheral Tregs (**FIG. 23**).

GIGA-564 induces more FcR signaling than ipilimumab

[00609] The increased efficacy of low dose GIGA-564 compared to ipilimumab (**FIG. 14C**) may be in part due to the enhanced ability by which GIGA-564 depletes CTLA-4^{Hi} intratumoral Tregs (**FIG. 15C-15E**). To investigate this, the ability of GIGA-564 and ipilimumab to induce mouse FcR signal transduction were compared. It was found that GIGA-564 resulted in more FcR signaling than ipilimumab in Jurkat effector cells expressing murine FcγRIV, but not FcγRIII, when co-cultured with CHO cells stably expressing CTLA-4 **(FIG. 16A** and **TABLE 25).** This increased FcγRIV signaling induced by GIGA-564 may explain why GIGA-564 more efficiently depletes intratumoral Tregs than ipilimumab.

TABLE 25: Fc receptor signaling of GIGA-564 and ipilimumab

	Maximum luminescence (RLU)			EC50 (μg/mL)	
FcR assay	Ipilimumab	GIGA-564	GIGA-564_LALAPG	Ipilimumab	GIGA-564
mFcγRIV	990	2,557	297	2.21	not determined*
mFcγRIII	17,496	14,311	1,163	0.22	0.15
hFcγRIIIa-V	59,205	87,184	4,255	0.04	0.02
hFcγRIIIa-F	6,880	15,818	2,434	0.09	0.04
hFcγRIIa-H	973	802	210	0.05	0.04
hFcγRIIa-R	2,641	2,560	1,572	0.21	0.08
hFcγRIIb	5,801	5,173	978	0.17	0.11

*GIGA-564 EC50 not determined due to poor curve fit

Fc receptor signaling of GIGA-564 and ipilimumab. Summary of FcR Bioassay results from **Fig. 16**. Luminescence (RLU) data from the Bioassay were fit by nonlinear regression. The maximum signal and EC50 for FcR signaling are provided.

As the increased murine FcγRIV signaling induced by GIGA-564 may contribute to the enhanced depletion of intratumoral Tregs and efficacy induced by GIGA-564 in hCTLA-4 KI MC38 tumor-bearing mice, it was important to determine if GIGA-564 also induces more human FcR signal transduction. It was found that GIGA-564 induced more human FcγRIIIa signaling than ipilimumab (**FIG. 16B** and **TABLE 25**). However, the increased FcR signaling induced by GIGA-564 was FcR-specific (**FIG. 16C-16D** and **TABLE 25**). From a translational perspective, GIGA-564 may have increased ability to induce FcR signaling and thus FcR effector functions in patients, especially if expression of FcγRIIIa is used as a biomarker.

[00610] It has previously been suggested that CTLA-4 mAbs with reduced binding at lower pH have enhanced ability to accumulate on the surface of Tregs because they dissociate from CTLA-4 in the early endosome. The hypothesis is that reduced binding at low pH allows for more CTLA-4 recycling, and thus more total CTLA-4 surface expression. A CTLA-4 mAb with reduced binding at low pH would selectively bind CTLA-4 in the periphery over CTLA-4 in the TME. Since the purpose of this study was to generate a CTLA-4 mAb that would target intratumoral Tregs, it was determined the pH sensitivity of several CTLA-4 mAbs and found that GIGA-564 similarly bound to CTLA-4 at all tested pHs, including the at lower pHs often present in the TME (**FIG. 24A**).

[00611] FcR signaling can also be affected by several other factors including the amount of antibody that accumulates on the cell surface and the affinity of the Fc portion of mAb to FcRs. No evidence was found that more GIGA-564 accumulates on the surface of CTLA-4$^+$ cells than ipilimumab (**FIG. 24B**). The affinity of an Fc for FcγRIIIa is in part controlled by Fc glycosylation, specifically Fc fucosylation, because afucosylation enhances the affinity of the Fc for FcγRIIIa and thus ADCC. It was found that GIGA-564 transiently expressed in ExpiCHO

cells was less fucosylated than ipilimumab, while a CHO cell pool stably expressing GIGA-564 produced material that was 95% fucosylated (**FIG. 24C**). Human FcγRIIIa assays revealed that the amount of GIGA-564 afucosylation in each preparation correlated with FcγRIIIa signaling (**FIG. 24D**). Accordingly, an afucosylated form of GIGA-564 (GIGA-2328) has been generated as described in Example 17.

[00612] Further investigation revealed that GIGA-564 still demonstrates higher FcR activity than ipilimumab, even in the context of decreased afucosylation (**FIG. 24E**). The increased FcR activity of GIGA-564 likely contributes to its enhanced anti-tumor efficacy, and this may translate well to patients as GIGA-564 also induces enhanced human FcγRIIIa signaling.

GIGA-564 induces durable anti-tumor immune responses

[00613] While first generation anti-CTLA-4s led to durable anti-tumor immunity, little is known about the ability of an anti-CTLA-4 with minimal blocking activity to induce a durable anti-tumor immune response. To investigate this translationally important question, hCTLA-4 KI mice bearing established MC38 tumors were treated on days 0, 3, and 6 with control (isotype), ipilimumab, or GIGA-564 at 1 mg/kg, an FDA-approved dose of ipilimumab that induced a durable complete response in approximately 50% of animals (5/8 ipilimumab treated animals and 5/9 GIGA-564 treated animals) (**FIG. 6A**). Animals with a complete response on day 43 were then re-challenged with MC38 tumor cells on the opposite flank from the original tumor site. All durable complete responders in the group previously treated with commercial ipilimumab and 4/5 of the durable complete responders in the group previously treated with GIGA-564 did not develop tumors at the re-challenge site (**FIG. 17B**). Thus, GIGA-564, an anti-CTLA-4 with minimal blocking activity, leads to durable anti-tumor immunity by preventing tumor development at a distant site.

GIGA-564 induces less toxicity than ipilimumab in murine models

[00614] In the clinic, CTLA-4 antibodies are most frequently used in combination with PD-1 antibodies, but this combination can induce relatively high frequency of severe adverse events. Thus, a CTLA-4 mAb with enhanced efficacy and reduced toxicity would be beneficial to patients. Several lines of evidence reveal that a significant portion of the toxicities induced by conventional CTLA-4 mAbs likely arises through blocking CTLA-4 from binding CD80 and CD86. This suggests that a CTLA-4 mAb with minimal blocking activity would not only have enhanced anti-tumor efficacy but may also result in less toxicity. Accordingly, the toxicity induced by ipilimumab and GIGA-564 was compared in the presence of anti-PD-1.

[00615] hCTLA-4 KI mice treated with a CTLA-4 mAb have been shown to recapitulate some of the toxicity induced by CTLA-4 mAbs in patients, and repeated treatment of BALB/c mice with anti-mouse CTLA-4 antibodies was shown to induce intestinal inflammation.

[00616] Thus, in the present study, hCTLA-4/hPD-1 double knock-in mice on the BALB/c background were treated with vehicle, pembrolizumab, pembrolizumab plus ipilimumab, or pembrolizumab plus GIGA-564 every 3 days for 9 doses (**FIG. 18A**). One week after the last dose, mice were euthanized and tissues were collected for pathology analysis (**FIG. 18B-18C** and **FIG. 25A-25B**). Histopathological analysis revealed that pembrolizumab plus ipilimumab, but not pembrolizumab plus GIGA-564, induced more colonic epithelial damage and skin inflammation than vehicle treated mice or mice treated with pembrolizumab alone (**FIG. 18B-18C**), while both combination treatments led to a minor increase in immune cell infiltration to the heart (**FIG. 25C-25D**). As CTLA-4 mAbs have been shown to induce kidney deposition in hCTLA-4 KI mice on the C57BL/6 background, kidneys were collected on day 20 post initiation of treatment from hCTLA-4 KI mice bearing MC38 tumors treated with vehicle, 5 mg/kg ipilimumab, or 5 mg/kg GIGA-564 twice a week for 5 doses (**FIG. 14A**). Kidneys were processed into formalin fixed paraffin embedded (FFPE) blocks. FFPE sections were stained for anti-mouse immunoglobin and scored by a board-certified veterinary pathologist blinded from the study. Ipilimumab, but not GIGA-564, significantly increased the percent of glomeruli with murine Ig deposition (**FIG. 25E**), suggesting that ipilimumab induces more kidney damage than GIGA-564 in hCTLA-4 KI mice. Thus, GIGA-564 induces less toxicity than ipilimumab in these murine models.

[00617] In conclusion, GIGA-564 is a novel CTLA-4 mAb with minimal B7 ligand blocking activity resulting in enhanced efficacy but reduced toxicity in multiple murine models (**FIG. 19**), suggesting that reducing CTLA-4 checkpoint inhibition yields translationally relevant benefits for both efficacy and safety.

[00618] It was shown herein that GIGA-564, an anti-CTLA-4 with minimal ability to block CTLA-4 binding to its CD80/CD86 ligands and enhanced FcR activity, had superior anti-tumor activity and reduced toxicity compared to ipilimumab in murine models expressing human CTLA-4. It was found that in hCTLA-4 KI mice, ipilimumab enhanced Treg proliferation more than proliferation of $CD4^{+}FOXP3^{-}$ or CD8 T cells. GIGA-564 leads to less Treg proliferation and increased anti-tumor activity in a murine model when compared to the first generation anti-CTLA-4 mAb ipilimumab.

[00619] It has recently been suggested that anti-CTLA-4-mediated depletion of intratumoral Tregs may be transient, explaining why intratumoral Treg depletion is not obvious at all timepoints following anti-CTLA-4 therapy. Thus, one possibility is that anti-CTLA-4 mediated expansion of peripheral Tregs provides a reservoir that reseeds the intratumoral Treg niche. Additionally, while anti-CTLA-4 mAbs efficiently induce depletion of intratumoral Tregs in the MC38 model primarily used here, the ability of anti-CTLA-4s to deplete intratumoral Tregs in patients is expected to be variable. This is because the expression of CTLA-4 by intratumoral Tregs varies among and within patients as can the affinity of FcRs, which are able to mediate antibody-dependent killing of CTLA-4^{Hi} Tregs. Furthermore, GIGA-564 may synergize well with anti-PD-1s, because it would be expected to deplete the intratumoral Tregs an anti-PD-1 could induce.

[00620] Several lines of evidence suggest that blocking the ability of CTLA-4 to bind CD80/CD86 may strongly contribute to the severe adverse events induced by first generation CTLA-4 mAbs. First, *CTLA4* mutations in humans that decrease binding of CTLA-4 to its B7 ligands are associated with immune infiltration into the brain, gastrointestinal tract, and lung, and can result in diarrhea, colitis, lymphocytic interstitial lung disease, and occasionally autoimmune thyroiditis. Clinically it has also been reported that the gastrointestinal biopsies of CHAI patients (CTLA-4 haploinsufficiency with autoimmune infiltration) are reminiscent of patients treated with anti-CTLA-4 blocking antibodies. Other relevant clinical data has been reported after treatment with abatacept (CTLA-4-Ig), which strongly binds the B7 proteins CD80 and CD86. This study found that pembrolizumab plus GIGA-564 induced less colonic epithelial damage and skin pathology than pembrolizumab plus ipilimumab in human CTLA-4/PD-1 double knock-in mice on the BALB/c background. Gastrointestinal and dermatological adverse events in patients are among the most common severe adverse events associated with ipilimumab or ipilimumab plus anti-PD-1 therapy.

[00621] Thus, the data shown herein suggested that clinical translation of GIGA-564 may result in reduced rates of these adverse events commonly associated with conventional CTLA-4 mAbs and thus benefit patients.

[00622] Surprisingly, it was found that GIGA-564 has enhanced FcR activity *in vitro* and *in vivo*. As next-generation anti-CTLA-4s with enhanced FcR activity have improved anti-tumor efficacy, it is likely that the enhanced FcR activity of GIGA-564 contributes to the improved anti-tumor efficacy of this mAb. The data presented herein indicate that the enhanced FcR function of transiently produced GIGA-564 can be due to the partially afucosylated status of this

material; however, further investigation revealed that GIGA-564 still demonstrated higher human FcγRIIIa activity even in the context of decreased afucosylation, suggesting that our results will translate to patients. Of note, the GIGA-564 material used in the murine toxicity study had relatively high levels of afucosylation (80.9%) and Fc effector function, further emphasizing the role of checkpoint inhibition in the induction of adverse events induced by conventional anti-CTLA-4 mAbs.

[00623] Another strategy that has been reported to enhance FcR function of CTLA-4 mAbs is selection of mAbs that dissociate from CTLA-4 at acidic pHs, leading to enhanced recycling of CTLA-4 and thus accumulation of the anti-CTLA-4 mAb on the cell surface. However, the tumor microenvironment is often acidic, and thus anti-CTLA-4s that preferentially bind at neutral pH will be less able to bind CTLA-4 in the tumor microenvironment, precisely where it was aimed to selectively deplete Tregs. Importantly, GIGA-564 was able to bind to CTLA-4 efficiently at low pH and can thus target intratumoral Tregs.

[00624] It has been argued that the FcR-dependent activity of anti-CTLA-4s may result from FcRs holding CTLA-4 away from the immune synapse and thus serves to further functionally inhibit the CTLA-4/B7 immune checkpoint. As CTLA-4 is most highly expressed by Tregs and co-stimulation is also important for Treg proliferation, anti-CTLA-4s that block the ability of CTLA-4 to bind its B7 ligands induce significant Treg proliferation. Thus, the fact that GIGA-564 only weakly blocks the ability of CTLA-4 to bind its B7 ligands and has enhanced FcR activity but induces less Treg proliferation than ipilimumab, suggests that the Fc activity of anti-CTLA-4s play little role in the inhibiting the ability of anti-CTLA-4s to bind its B7 ligands.

[00625] In conclusion, the inventors of the present application showed that the weak blocking CTLA-4 mAb GIGA-564 reduced proliferation of peripheral Tregs, more efficiently depleted intratumoral CTLA-4^{Hi}, and had superior anti-tumor activity while inducing less toxicity than ipilimumab in murine models expressing human CTLA-4.

8.14. Example 14: Development and validation of CHO cells expressing cyno CTLA-4 on the cell surface

[00626] To determine the ability of GIGA-564 vs ipilimumab (Ipi) to bind cynomolgus CTLA-4 on the cell surface, CHO cells stably expressing cynomolgus CTLA-4 (cyno CTLA-4) or human CTLA-4 (hCTLA-4) were generated.

[00627] The ability of PE-labeled anti-CTLA-4 antibodies, clones L3D10 or BNI3, to bind to cyno CTLA-4 or hCTLA-4+ CHO cells was tested to validate the expression of cyno CTLA-4

or hCTLA-4 on the surface of these cells. In this experiment, parental CHO cells and cyno CTLA-4 CHO cells or hCTLA-4 CHO cells (1E6 cells each cell line) were first washed with MACS buffer (DPBS + 0.5% BSA + 2mM EDTA) and then incubated with either PE-labeled anti-CTLA-4 clones L3D10 or BNI3. Afterwards, the cells were washed twice with MACS buffer to remove excess, unbound antibody. Then, binding of mAbs to cyno or human CTLA-4 on the cell surface was analyzed by flow cytometry (**FIG. 34**). Flow histograms of **FIG. 34** show that the cyno CTLA-4 and hCTLA-4 CHO cell lines express antigen on the cell surface bound by anti-CTLA-4 clone L3D10 or BNI3, this validates expression of cyno or human CTLA-4 on the surface of these cell lines, respectively.

8.15. Example 15: GIGA-564 has reduced ability to bind cyno CTLA-4 compared to Ipilimumab

[00628] To determine the ability of GIGA-564 to bind to cynomolgus (cyno) and human CTLA-4 expressed on the surface of mammalian cells, parental CHO cells and cyno CTLA-4 CHO cells or hCTLA-4 CHO cells (1E6 cells each cell line) were first washed with MACS buffer (DPBS + 0.5% BSA + 2mM EDTA) and then incubated with 10 ug/mL of Ipilimumab, Atezolizumab (negative control), or GIGA-564 at 4°C.

[00629] Afterwards, the cells were washed twice with MACS buffer to remove excess, unbound antibodies. The cells were then stained with PE-labeled rat anti-human IgG Fc antibody to detect bound human antibodies. After staining, the cells were washed twice with MACS buffer to remove excess, unbound PE-conjugated anti-human antibodies. Then, binding of mAbs to cyno or human CTLA-4+ CHO cells was analyzed by flow cytometry. **FIG. 35** shows that while GIGA-564 and ipilimumab (Ipi) have relatively similar ability to bind hCTLA-4; compared to Ipi, GIGA-564 has a greatly reduced ability to bind to cyno CTLA-4 expressed on the cell surface. The results suggest that Ipi and GIGA-564 bind to different CTLA-4 epitopes.

[00630] Next, the ability of GIGA-564 to bind recombinant cynomolgus monkey CTLA-4 (rcmCTLA-4) was tested by ELISA.

[00631] In these ELISAs binding of GIGA-564 to various CTLA-4 proteins sourced from multiple manufacturers, including Fc chimera and his-tagged formats, was tested. To test the binding of GIGA-564 to Fc chimera CTLA-4 proteins, rcmCTLA-4-Fc from R&D systems (9336-CT-200, **FIG. 36A**), Sino Biological (90213-C02H, **FIG. 36B**), or ACROBiosystems (CT4-C5256, **FIG. 36C**) were coated at 1 ug/mL on one half of separate ELISA plates. Recombinant human CTLA-4-Fc (rhCTLA-4) from R&D Systems (7268-CT-100, **FIGs. 36A-36C**) was coated at 1 ug/mL, on the other half of each of the plates. Similarly, to test the binding

of GIGA-564 to his-tagged CTLA-4, rcmCTLA-4-His from Sino Biological (90213-C08H, **FIGs. 36D**) or ACROBiosystems (CT4-C5227, **FIGs. 36E**) were coated at 1ug/mL on one half of separate ELISA plates. RhCTLA-4-Fc (ACROBiosystems, CT4-H5229, **FIGs. 36D-E**) was coated at 1 ug/mL, on the other half of each of these plates. After coating, the plates were incubated overnight at 4°C.

[00632] The following day, the plates were blocked with 5% milk in PBST for 1 hour on a plate shaker at room temperature. Titration series of Ipilimumab, Atezolizumab (negative control), and GIGA-564 (starting at 5 ug/mL) were added to the plates and incubated on a plate shaker for 1 hour at room temperature to allow mAb binding. Excess, unbound mAbs were removed by washing with PBST. Bound mAbs were then detected with an HRP-conjugated anti-kappa light chain antibody (0.5 ug/ml, Southern Biotech 2060-50). After incubation on a plate shaker for 1 hour at room temperature and washing, the plates were developed with TMB substrate. After sufficient signal was achieved, 1 N hydrochloric acid was added to stop development. Absorbance at 450 nm was read using a Spectramax i3x plate reader (Molecular Devices). EC50 values were calculated by plotting absorbance vs. the log of concentration using Prism (GraphPad). As Ipi has similar binding to human and cyno CTLA-4, in most cases, GIGA-564 had reduced ability to bind cyno CTLA-4 compared to human CTLA-4 (TABLE 28). These results suggest that the Ipi and GIGA-564 epitopes are different in practice.

TABLE 28: GIGA-564 has reduced binding to cynomologous CTLA-4 in some formats. TABLE 28 summarizes ELISA data from FIG. 36A-E with the EC50 values and fold change for each antibody tested in the ELISA assays.

mAb	Antigen	EC50	Fold Change*
GIGA-564	R&D rcmCTLA-4 Fc	1.71**	126
GIGA-564	R&D rhCTLA-4 Fc	0.0136	
Ipilimumab	R&D rcmCTLA-4 Fc	0.0111	1.2
Ipilimumab	R&D rhCTLA-4 Fc	0.0092	
GIGA-564	Sino Biological rcmCTLA-4 Fc	1.7290**	170
GIGA-564	R&D rhCTLA-4 Fc	0.0102	
Ipilimumab	Sino Biological rcmCTLA-4 Fc	0.0112	1.6
Ipilimumab	R&D rhCTLA-4 Fc	0.0070	
GIGA-564	ACROBiosystems rcmCTLA-4 Fc	2.43**	254
GIGA-564	R&D rhCTLA-4 Fc	0.0096	
Ipilimumab	ACROBiosystems cCTLA-4 Fc	0.0077	1.3
Ipilimumab	R&D rhCTLA-4 Fc	0.0060	

GIGA-564	Sino Biological rcmCTLA-4 His	0.5117	10
GIGA-564	ACROBiosystems rhCTLA-4 His	0.0496	
Ipilimumab	Sino Biological rcmCTLA-4 His	0.0118	0.6
Ipilimumab	ACROBiosystems rhCTLA-4 His	0.0203	
GIGA-564	ACROBiosystems rcmCTLA-4 His	2.65**	57
GIGA-564	ACROBiosystems rhCTLA-4 His	0.0468	
Ipilimumab	ACROBiosystems rcmCTLA-4 His	0.0226	1.3
Ipilimumab	ACROBiosystems rhCTLA-4 His	0.0176	

*fold change: EC50 of cyno divided by EC50 of human

**did not reach signal saturation

8.16. Example 16: Fucosylation analysis of GIGA-564, GIGA-2328, and Ipilimumab

[00633] **TABLE 30** shows a fucosylation analysis of GIGA-564 and Ipiliumumab produced with unmanipulated fucosylation, and **TABLE 31** shows a fucosylation analysis of GIGA-564 and Ipiliumumab produced with conditions to promote afucosylation. Each antibody was digested with PNGase F to liberate glycans. Glycans were isolated and labeled with a fluorescent molecule using a GlykoPrep InstantAB labeling kit (Prozyme) following the manufacturer's instructions. Labeled glycans were injected over a XBridge Amide column on an Ultra Performance Liquid Chromatography (UPLC) system with a fluorescent detector. Peaks were identified based on known glycans from the Glyko InstantAB biantennary and high-mannose partitioned library standards (Prozyme), with the total percent of each glycoform reported as the integrated area under the identified peak divided by the sum of the area under the curve for all peaks. Each value represents and independent measurement of the indicated lot. If more than one measurement was made, the minimum, maximum, and mean values are also provided.

TABLE 30: Fucosylation analysis of GIGA-564 and Ipiliumumab produced with unmanipulated fucosylation

GIGA-564 (transient expression)		GIGA-564 (stable expression)		Ipilimumab (transient expression)		Ipilimumab (stable expression)		Ipilimumab (Yervoy)	
	% fucosylated		% fucosylated		% fucosylated		% fucosylated		% fucosylated
min	77.4	min	81.6	min	82.7	min	88.0	min	88.4
max	80.9	max	95.0	max	83.5	max	88.3	max	89.7
average	79.4	average	90.3	average	83.1	average	88.2	average	89.1
Lot	% fucosylated	Lot	% fucosylated	Lot	% fucosylated	Lot	% fucosylated	Lot	% fucosylated
PN-2758.01	80.9	PN-4261.01	95.0	PN-4265.01	82.7	PN-4489	88.3	PN-4094	89.1
PN-4088.01	77.4	PN-4261.02	94.3	PN-4265.01	83.5	PN-4532.01	88.0	PN-2167	89.4
PN-2758.01	79.8	PN-4262.01	89.9					PN-2753	88.8
		PN-4324.01	92.0					PN-4094	88.4
		PN-4324.01	91.6					PN-4094	89.7
		PN-4554.01	85.5						
		PN-4555.01	81.6						
		PN-4324.01	91.2						
		PN-4324.02	91.4						

Some of the data shown in **TABLE 30** is also provided in **Example 13.**

TABLE 31: Fucosylation analysis of GIGA-564 and Ipiliumumab produced with conditions to promote afucosylation

GIGA-564 (transient expression)		GIGA-564 (stable expression)		Ipilimumab (transient expression)		Ipilimumab (stable expression)	
Lot	% fucosylated	Lot	% fucosylated		% fucosylated		% fucosylated
PN-2328.01	5.2	PN-4534.01	25.1	min	5.2	min	3.3
				max	6.3	max	3.9
				average	5.9	average	3.6
				Lot	% fucosylated	Lot	% fucosylated
				PN-2326.01	5.2	PN-4491.01	3.3
				PN-4266.01	6.3	PN-4533.01	3.9
				PN-4266.01	6.2		

[00634] GIGA-2328 was designed to have low fucosylation for the purpose of enhancing FcγRIIIa signaling. As shown in **FIG. 41**, an ADCC reporter bioassay for human FcγRIIIa, V variant (G7011) from Promega Corporation was carried out following the manufacturer's instructions. Briefly, CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were suspended in RPMI 1640 + 4% FBS media with the indicated antibody and incubated at 37°C for 30 minutes. Jurkat/NFAT-Luc effector cells expressing human FcγRIIIa, V variant were added to each well at an effector:target ratio of 5:1 and incubated at 37°C for 6 hours. Luciferase activity was measured by using the included Bio-Glo Luciferase Assay Reagent with the SpectraMax i3x reader. Luciferase activity measured in relative luminescence units (RLU) were plotted against the concentration of antibody.

TABLE 34. GIGA-2328 has lower overall fucosylation than GIGA-564.

Glycoform	**GIGA-564**	**GIGA-2328[1]**	**GIGA-2328[2]**
fucosylated	91.6	5.2	25.1
afucosylated	7.2	91.6	74.0
G0-GlcNac	0.4	7.5	1.4
G0F-GlcNac	0.6	0.6	0.3
G0	3.9	55.5	60.2
G0F	64.1	2.9	19.4
Man5/G1-1*	2.8	11.7	8.3
G1-2			3.5
G1F-1	17.7	1.0	3.5
G1F-2	7.0	0.7	1.5
G2			0.6
Man6	0.1	6.3	
G2F	2.2		0.4
Man7		4.2	
Man8		4.3	
Man9		2.0	
Unknown	1.1	3.2	0.9

1, antibody was expressed by transient transfection in ExpiCHO cells (Thermo Fisher)
2, antibody was stably expressed in the CHOZN cell line (Sigma Aldrich)
*, in some cases separate values for Man5/G1-1 were summed to calculate this value.
Some data in **TABLE 34** includes repeated data shown in **TABLE 30** and **TABLE 31.**

[00635] Each antibody was digested with PNGase F to liberate glycans. Glycans were isolated and labeled with a fluorescent molecule using a GlykoPrep InstantAB labeling kit (Prozyme) following the manufacturer's instructions. Labeled glycans were injected over a XBridge Amide column on an Ultra Performance Liquid Chromatography (UPLC) system UPLC with a fluorescent detector. Peaks were identified based on known glycans from the Glyko

InstantAB biantennary and high-mannose partitioned library standards (Prozyme), with the total percent of each glycoform reported as the integrated area under the identified peak divided by the sum of the area under the curve for all peaks.

[00636] **FIGs. 42A-42B**. GIGA-564 induces antibody-dependent cellular cytotoxicity (ADCC) and/or antibody-dependent cellular phagocytosis (ADCP) by human PBMCs against a target cell line expressing CTLA-4. Cryopreserved human PBMCs from two donors were thawed and recovered overnight in RMPI media containing 100 U/mL IL-2 and 10% FBS. CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were stained with CellTrace Violet (Thermo Fisher), then incubated with either GIGA-564 or a human IgG1 isotype control at the indicated concentrations at 37°C for 30 minutes. PMBC effector cells were then added at a 20:1 effector to target ratio and the samples were incubated at 37°C for 4 hours to allow for ADCC. The samples were then washed with MACS buffer, stained with the viability dye 7-Aminoactinomycin D (7-AAD) to label dead cells, and analyzed by flow cytometry. (**FIG. 42A**) Representative gating strategy to determine ADCC. Samples were first gated for target cells (CellTrace Violet+), then single cells, then dead (7-AAD+) cells. (FIG. 42B) Plots of percent dead cells at each concentration of GIGA-564 and human IgG1 isotype. GIGA-564 leads to higher percent dead CTLA-4+ target cells than the isotype control.

8.17. Example 17: Effect of different IgG1 Allotypes on FcγRIIIa signaling

[00637] IgG1 allotype may impact signaling via the FcγRIIIa receptor. **FIG. 43A** shows sequence information of IgG1 allotype IGHG1*08 and IGHG1*01, which differ by one amino acid in the CH1 domain. The differing residue between these two allotypes is highlighted, and surrounding residues are provided for reference. Position given in both IMGT and EU numbering.

[00638] **FIG. 43B** shows clinical ipilimumab (Yervoy) uses the IGHG1*08 allotype, and results in lower FcγRIIIa signaling than Ipilimumab and GIGA-564 produced by GigaGen, which use the IGHG1*01. These results suggest that allotype may impact FcγRIIIa signaling. ADCC reporter bioassays for human FcγRIIIa, V variant (G7011) were purchased from Promega Corporation. The assays were carried out following the manufacturer's instructions. Briefly, CHO target cells stably expressing human CTLA-4 with the Y201G mutation to enhance cell surface expression were suspended in RPMI 1640 + 4% FBS media with the indicated antibody and incubated at 37°C for 30 minutes. Jurkat/NFAT-Luc effector cells expressing human FcγRIIIa, V variant were added to each well at an effector:target ratio of 5:1 and incubated at 37°C for 6 hours. Luciferase activity was measured by using the included Bio-Glo Luciferase

Assay Reagent with the SpectraMax i3x reader. Luciferase activity measured in relative luminescence units (RLU) were plotted against the concentration of antibody.

8.18. Example 16: Formulation of ABPs for clinical use

[00639] The study was performed to determine a formulation with optimal protein stability and ease of administration and to have a tonicity (near isotonic, 290 mOsm/kg) for simpler administration.

[00640] GIGA-564 was formulated at 20mg/mL in a variety of buffers, then measured using differential scanning fluorimetry to look for conformation and unfolding.

- *Range of formulations tested:* from pH 5 to pH 7.6
- *Buffer:* The buffer was either citrate or phosphate (of varying concentrations) as was appropriate for the pH
- *Salt Concentration:* The salt concentration ranged from 0 – 200 mM NaCl
- *Sucrose:* Sucrose was tested at a range from 0 – 10 % (weight to volume), 10% is about 290 nM

[00641] Samples run on differential scanning fluorimetry over 25-95°C and scattering was reported as mAU. Measurement output was the ratio of fluorescence at 350nm to 330 nm. The 350 signal doesn't change much, however as the protein unfolds the 330 signal decreases significantly (driven by tryptophan becoming more accessible).

[00642] **Tonset** is the temperature at which the signal starts to increase.

[00643] **Tm-1-2,-3** etc. are unfolding temperatures of different domains, defined by an inflection point in the 350/330 data and local maximum in the first derivative.

[00644] For an antibody, there are not always 3 distinct Tm, but generally: Tm1 = CH_2; TM2 = fab; Tm3 = CH_3.

[00645] **Tagg** = the temperature at which the scattering increases, indicating an increase in size of the particles.

[00646] The **350/330 ratio at 25°C** indicates the conformation – lower is more folded. **FIG. 37** provides response plots that show the impact of pH and sucrose concentration when NaCl and buffer concentration are fixed at 100mM and 30mM, respectively. The results show that (i) higher Tms and Tagg are better; (ii) higher [sucrose] improves results; (iii) best pH in the middle (~6.3); (iv) pH has little effect on Tagg.

[00647] Next, optimal conditions for the formulation were calculated. Theoretical response plots as shown in **FIG. 38** were generated to determine the buffer concentration and NaCl

amount expected to result in the highest Tm as shown. The results show that (i) best buffer concentration varies from 10-50mM; and (ii) the calculated ideal [NaCl] for all parameters is high, from 80-100mM. Next, a pareto analysis was performed to determine which variables had the most impact on the formulation. Data in **FIG. 39** suggested (i) for all, sucrose concentration was the most important variable; and (ii) pH was moderately important for Tm-onset, TM-3. Additionally, **FIG. 40** shows (i) high NaCl was the most important for conformation; (ii) pH had some effect, with lower pH being better; and (iii) sucrose had some effect, with higher being better.

[00648] Based on these findings, the below formulations were generated and tested.

form.	mg/mL	pH	[buffer]	type	[NaCl]	[sucrose]	PS-20	Osmo
1	20	6.5	20mM	histidine	---	270mM	0.02%	290 mOsm/kg
2	20	6.5	20mM	histidine	50mM	270mM	0.02%	390 mOsm/kg
3	20	6.5	20mM	histidine	50mM	170mM	0.02%	290 mOsm/kg
4	20	6	20mM	histidine	---	270mM	0.02%	290 mOsm/kg
5	20	6	20mM	histidine	50mM	270mM	0.02%	390 mOsm/kg
6	20	6	20mM	histidine	50mM	170mM	0.02%	290 mOsm/kg
7	20	5.5	20mM	citrate	---	270mM	0.02%	290 mOsm/kg
8	20	5.5	20mM	citrate	50mM	270mM	0.02%	390 mOsm/kg
9	20	5.5	20mM	citrate	50mM	170mM	0.02%	290 mOsm/kg

Each of the formulations was prepared with GIGA-564 at 20mg/ml and tested under four different conditions – (A) 5°C storage, (B) 5x F/T (5 cycles of freeze and thaw), (C) agitation, and (D) 40°C storage for 2 weeks. After the treatment, the samples were analyzed by SE-HPLC, differential scanning fluorimetry (DSF), dynamic light scattering (DLS), BioAnalyzer, UV spec, ELISA to assess ability to bind to antigen (CTLA-4), cell-based assay to assess FcR signaling strength, or visual inspection.

[00649] FIG. 44A and FIG. 44B show DSF analysis results. Buffers 1 (G1) and 4 (G4) provided the highest Tm1, whereas Buffers 3 (G3), 6 (G6) and 9 (G9) performed worse. Samples in Buffers 1 (G1) and 4 (G4) did not aggregate even at high temperatures.

[00650] FIG. 45 provides DLS analysis results with cumulant radius. In the analysis, the cumulant radius represents the protein structure where smaller radius indicates that the proteins are more tightly folded. The data show that all the tested formulations provided cumulant radius within acceptable ranges, but samples in Buffers 1 (G1) and 4 (G4) had significantly smaller radius compared to other buffers.

[00651] FIG. 46A and FIG. 46B provide SE-HPLC analysis results. FIG. 46A shows % monomers in each sample following the four different treatment conditions, (A) 5°C storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks. FIG. 46B shows total AUC calculated by SE-HPLC for each sample. The results show that most samples in citrate/pH5.5 had reduced %monomer and product loss. There was no clear evidence that additional NaCl made much impact on the product stability. The product is stable in histidine 6.5 or 6.0 and 270mM sucrose (G1 and G4), the formulations that led to the best results for DSF (FIG. 44) and DLS (FIG. 45).

[00652] FIG. 47A and FIG. 47B provide results from analysis using BioAnalyzer. FIG. 47A provides % area of monomer peak. FIG. 47B provides the raw data. The data show that formulation 5 (G5) had the most stable values across the treatment conditions.

[00653] FIG. 48A and FIG. 48B show CTLA-4 binding affinities of GIGA-564 in formulations 1 (G1) or 4 (G4) tested by ELISA. The results show that there was no significant difference in the CTLA-4 binding in the two buffers under the four different conditions, (A) 5°C storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks.

[00654] FIG. 49A and FIG. 49B show human FcRIIIa (V variant) signaling induced by GIGA-564 in formulations 1 (G1) or 4 (G4) as determined by cell-based assay. The results show that there was no significant difference in the FcR binding in the two buffers under the four different conditions, (A) 5°C storage, (B) 5x F/T, (C) agitation, and (D) 40°C storage for 2 weeks.

[00655] Alternative or additional components of the formulation can include Trehalose instead of sucrose, and at appropriate pHs or succinate could be used instead of citrate/phosphate.

8.19. Example 17: Manufacturing of GIGA-564 and GIGA-2328

[00656] GIGA-564 can be made in CHO cells. In some variations it can be expressed transiently, for example using ExpiCHO cells, Expi293 cells, or other cell lines commonly used for transient transfection. In some variations it can be expressed from a cell line modified to stably express the heavy and light chain sequences of GIGA-564 using a cell line commonly used for stable expression, such as DG44, CHOZN GS, or others. The stable expression construct may be integrated using methods such as targeted integration, random integration, transposase-mediated integration, lentiviral transduction, or others. In some variations an enhancer element of some kind may be used to enhance transcription or translation and thus titers, for example a 2G UNic element, UCOE element, or others.

[00657] GIGA-2328 (aCTLA-4.15 with low fucosylation) is produced in cells selected due to their ability to produce proteins with reduced core fucosylation. In some variations GIGA-

2328 will be produced in CHO cells expressing the bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase), which prevents the production of UDP-Fucose from UDP-Mannose. In some variations, fucosylated GIGA-564 can also be produced using cells expressing the bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) if an external source of fucose is given to the cells. In some variations, GIGA-2328 may be produced in CHO cells lacking or having reduced expression of Fut8. In some variations, GIGA-2328 may be produced by adding the fucosylation inhibitor 2-Fluorfucose (2FF) to the media the cells are grown in. In some variations, GIGA-2328 may be produced in cells overexpressing the glycosyltransferase (GnTIII). Additionally, GIGA-2328 can be produced by other CHO cells selected or modified to have reduced fucosylation levels or by another cell line selected for its ability to produce proteins with reduced fucosylation.

[00658] To manufacture GIGA-564, cells are thawed from storage in liquid nitrogen and expanded to reach a sufficient density for inoculation of a vessel for bioproduction (including but not limited batch, fed batch or perfusion culture in a shake flask, TPP or bioreactor). In one variation, the fed batch culture is grown for approximately 14 days with addition of glucose and feeds, as appropriate. After the fed batch culture is complete the culture is harvested by methods that may include cell settling, centrifugation, and/or filtration.

[00659] GIGA-564 is purified from the harvested cell culture fluid using one or more of several chromatographic steps. The first step is Protein A or similar affinity chromatography, using a resin such as MabSelect Sure, MabSelect PrismA, CaptivA, or others. The affinity chromatography step may be followed by low pH viral inactivation, followed subsequently by cation exchange chromatography in bind and elute mode, using a resin such as POROS XS, CaptoS ImpaAct or others. Anion exchange filtration in flow-through mode is performed using a filter such as Natrix Q, SartobindQ, Mustang Anionic Exchange (Q) or others, and nanofiltration for virus removal is performed using filters such as Planova BioEX, Viresolve, Pegasus or others. Finally, GIGA-564 is subjected to ultrafiltration/diafiltration to concentrate and buffer exchange to the final formulation conditions. A similar approach for manufacturing and purifying can be used for manufacturing and purification of GIGA-2328.

8.20. Example 18: Combination of pembrolizumab and GIGA-564

[00660] Transgenic mice expressing human CTLA-4 and PD-1 (hCTLA4-hPD1 knock in (KI) mice, n=8 for the control arm [PBS] and n=12 for each treatment arm) were implanted subcutaneously with 1x 10^6 MC38 tumor cells expressing human PD-L1 in the flank. The hCTLA4-hPD1 KI mice were treated with different doses of pembrolizumab (pembro) and/or

anti-CTLA-4 (GIGA-564) as indicated in TABLE 36. The antibodies were administered i.p. on days 0, 3, and 6 post randomization.

TABLE 36

Group no	Treatment	No that reached tumor max within 28 days	% reached max (3000 mm^3)	No. of CRs	% CRs	# OR	% OR
1	PBS	7 of 8	87.5	0	0	0	0
2	pembro 1 mg/kg	11 of 12	91.67	0	0	1 of 12	8.33
3	pembro 0.3 mg/kg	10 of 12	83.33	0	0	0	0
4	GIGA-564 3 mg/kg	0 of 12	0	0	0	6 of 12	50
5	GIGA-564 0.3 mg/kg	9 of 12	75	0	0	2 of 12	16.67
6	pembro 1 mg/kg, GIGA-564 3 mg/kg	0 of 12	0	9 of 12	75	11 of 12	91.67
7	pembro 1 mg/kg, GIGA-564 0.3 mg/kg	8 of 12	66.67	2 of 12	16.67	2 of 12	16.67
8	pembro 0.3 mg/kg, GIGA-564 3 mg/kg	4 of 12	33.33	4 of 12	33.33	6 of 12	50%
9	pembro 0.3 mg/kg, GIGA-564 0.3 mg/kg	11 of 12	91.67	0	0	0	0

[00661] The data show that in this experiment at these doses neither pembro (Keytruda) nor GIGA-564 leads to complete responses (CRs) within 28 days individually, nonetheless combination therapy with Keytruda (1 mg/kg) + GIGA-564 (3 mg/kg) led to a 75% complete response rate and a 91.67% overall response (OR, which indicates that at anytime post initiation of dosing tumors had decreased in size by at least 20% compared to their size at initiation of doisng) rate.

[00662] Even low dose (0.3 mg/kg) GIGA-564 did overcome resistance to 1 mg/kg pembro with this combination leading to a complete response rate of 16.67%. This data suggests that GIGA-564 can overcome resistance to anti-PD-1 therapy, better than ipilimumab. Without wishing to be bound by a theory, GIGA-564 can work better at overcoming resistance to immunotherapy compared to ipilimumab because it is more efficient at depleting intratumoral Tregs and induces less proliferation of the remaining Tregs.

8.21. Example 19: Toxicology study of GIGA-564

[00663] The adverse effects of GIGA-564 were tested in comparison to ipilimumab (Yervoy). Transgenic mice expressing human CTLA-4 (hCTLA-4 KI) BALB/c mice were

treated with saline (group 1), 30 mg/kg GIGA-564 (group 2), or 30 mg/kg Ipilimumab (group 3) every 3 days for 9 doses.

[00664] Blood concentrations of various physiological molecules in the mice were measured on Day 26 and summarized in the below TABLE 37. The results show that Group 3 showed elevated AST and ALT levels, whereas Group 2 did not, suggesting that GIGA-564 leads to less toxicity than ipilimumab.

TABLE 37

Day: 26 Relative to Start Date

Sex: Female		ALT (U/L) [a]	AST (U/L) [a]	TP (g/L) [a]	ALB (g/L) [a]	BIL-T (µmol/L) [a]	ALP (U/L) [a]	GGT (U/L)	sGLU (mmol/L) [a]	UREA (mmol/L) [a]	CRE (µmol/L) [a]	Ca (mmol/L) [a]	P (mmol/L) [a]
Group 1,	Mean	39	88	49.5	31.5	1.70	193	.	7.58	7.24	8 n	2.18	2.46
0	SD	3	15	1.0	0.6	0.29	25	.	1.85	1.24	1	0.04	0.70
mg/kg/dose	N	4	4	4	4	4	4	0	4	4	2	4	4
Group 2,	Mean	45	106	53.5	31.6	2.07	204	.	6.70	6.42	13 n	2.19	2.58
30	SD	5	14	2.7	2.0	0.42	42	.	1.15	1.10	2	0.10	0.53
mg/kg/dose	N	6	6	6	6	6	6	0	6	6	2	6	6
	%Diff	14	20	8.1	0.3	22.12	6	.	-11.51	-11.32	56	0.54	4.57
Group 3,	Mean	52 **	127 *	53.6	30.8	2.08	174	.	7.01	6.24	9 n	2.21	2.79
30	SD	6	23	3.3	1.8	0.35	18	.	2.09	1.01	.	0.05	0.58
mg/kg/dose	N	6	6	6	6	6	6	0	6	6	1	6	6
	%Diff	32	44	8.4	-2.3	22.81	-10	.	-7.46	-13.83	13	1.38	13.43

[a]-Anova & Dunnett: *=p≤0.05; **=p≤0.01; Group 1 vs Group 2-3;n- Inappropriate for statistics

[00665] Their food consumptions over the period were also measured and provided in the TABLE 38. It shows that ipilimumab (Group 3) but not GIGA-564 (Group 2) leads to reduced food consumption in the mice.

TABLE 38

Food Total Consumption (g)

Sex: Female		Day(s) Relative to Start Date						
		-4 ~ -3 [a]	-3 ~ -2 [a]	-2 ~ -1 [a]	-1 ~ 4 [a1]	4 ~ 7 [a]	7 ~ 10 [a]	10 ~ 13 [a1]
Group 1,	Mean	2.7	2.7	3.0	12.6	9.2	9.9	9.7
0	SD	0.4	0.0	0.2	0.3	1.3	0.8	0.4
mg/kg/dose	N	7	7	7	7	7	7	7
Group 2,	Mean	2.4	2.9	3.0	12.4	9.8*	10.0*	9.3
30	SD	0.0	0.0	0.2	1.0	1.4	1.0	1.7
mg/kg/dose	N	13	11	11	12	12	13	13
	%Diff	-13.2	7.2	-0.7	-1.5	6.4	1.0	-4.5
Group 3,	Mean	2.5	2.8	3.0	11.7**t	8.7 tt	9.5 ###	7.7***tt
30	SD	0.3	0.2	0.1	0.8	0.8	0.9	0.2
mg/kg/dose	N	12	12	12	12	12	12	12
	%Diff	-5.9	2.5	2.1	-7.1	-5.9	-3.8	-21.2

[a] – Kruskal-Wallis & Dunnett on Ranks: *=p≤0.05; Group 1 vs Group 2-3
[a1] – Anova & Dunnett; **= p≤0.01; ***= p≤0.001; Group 1 vs Group 2-3 Wilcoxon (Rank): ###= p≤0.001; Group 2 vs Group 3
T-test: t= p≤0.05; tt= p≤0.01; Group 2 vs Group 3

Food Total Consumption (g)

Sex: Female		Day(s) Relative to Start Date			
		13 → 16	16 → 19	19 → 22	22 → 25
Group 1,	Mean	9.7	10.0	9.7	9.6
0	SD	0.7	0.8	0.8	0.3
mg/kg/dose	N	7	7	7	7
Group 2,	Mean	9.8	10.2	9.6	10.1
30	SD	1.5	2.1	1.5	2.0
mg/kg/dose	N	11	11	11	11
	%Diff	0.7	2.3	-0.9	5.0
Group 3,	Mean	8.5*t	8.6 t	8.7	8.5 tt
30	SD	0.6	0.2	0.2	0.2
mg/kg/dose	N	12	12	12	12
	%Diff	-12.9	-13.8	-9.9	-12.1

Anova & Dunnett: *= p≤0.05; Group 1 vs Group 2-3
T-test; t= p≤0.05; tt= p≤0.01; Group 2 vs Group 3

[00666] As another indication to measure adverse effects, hematological analysis results are provided in TABLE 39. In the study, hCTLA-4 KI mice treated with ipilimumab but not GIGA-564 showed decreased platelet levels.

TABLE 39

Day: 26 Relative to Start Date

Sex: Female		#LYMP (10^3/μL) [a]	%LYMP (%) [a]	#MONO (10^3/μL) [a1]	%MONO (%) [a1]	#EOS (10^3/μL) [a1]	%EOS (%) [a1]	#BASO (10^3/μL) [a1]	%BASO (%) [a1]	PLT (10^3/μL) [a1]	MPV (fL) [a1]
Group 1,	Mean	1.23	76.4	0.01	0.5	0.05	3.1	0.01	0.9	1153	10.4
0	SD	0.14	4.9	0.01	0.2	0.03	1.2	0.01	0.7	74	0.1
mg/kg/dose	N	3	3	3	3	3	3	3	3	3	3
Group 2,	Mean	0.96	80.8	0.00	0.2	0.02	1.7	0.00*	0.5	955	10.5
30	SD	0.53	4.9	0.00	0.1	0.02	0.8	0.00	0.2	75	0.3
mg/kg/dose	N	5	5	5	5	5	5	5	5	5	5
	%Diff	-22.49	5.7	-70.00	-52.0	-58.75	-44.5	-85.00	-44.6	-12	1.3
Group 3,	Mean	1.29	83.4	0.00	0.2	0.03	1.9	0.01 tt	0.9	860 *	10.8
30	SD	0.41	9.4	0.00	0.2	0.03	1.9	0.00	0.6	190	0.4
mg/kg/dose	N	5	5	5	5	5	5	5	5	6	6
	%Diff	4.43	9.2	-70.00	-56.0	-47.50	-40.0	-10.00	3.8	-25	3.8

[a]- Kruskal-Wallis & Dunnett on Ranks
[a1] – Anova & Dunnett: *= p≤0.05; Group 1 vs Group 2-3
T-Test: tt= p≤0.01; Group 2 vs Group 3

8.22. Example 20: Response of non-human primates to GIGA-564

[00667] Six naïve male cynomolgus monkeys (n=3/group) were dosed with a single IV bolus of 3 mg/kg or 30 mg/kg of GIGA-564 administered at 5ml/kg per animal for assessing the pharmacokinetic (PK) properties of 3 mg/kg or 30 mg/kg of GIGA-564 over the 28-day period. No adverse effects were observed during or in the 28 days following IV bolus dosing. Blood or urine samples were also collected pre- and on- study for hematology, clinical chemistry, coagulation, cytokine levels and urinalysis. No significant weight gains or losses or significant abnormal clinical pathology were documented over the course of the study.

[00668] The analysis timelines were as followed:

- Body weights: Pre-Dose: (Day -7 and Day -1), Post-Dose: twice weekly and once at the end of study (Day 29).
- Detailed Clinical Observations: Pre-Dose: (Day -1), twice weekly, and once at the end of the study (Day 29)
- Clinical Pathology (Chemistry/Hematology/Coagulation): Pre-Dose, Day -7, Day -1, and Day 29 Post-Dose.
- Cytokine: Pre-dose (Day 1), 3hrs and 24 hrs Post-Dose.
- PK Timepoints: Day 1 (Pre- Dose), 0.5hr, 1hr, 6hrs, 24hrs, 48hrs, 168hrs, 240hrs, 336hrs, 504hrs, and 672hrs Post-Dose
- Urinalysis: Pre-Dose and Day 29 Post-Dose
- Food Consumption: Daily, qualitative

[00669] ***Clinical Chemistry*:** Lithium Heparin tube 0.5 ml was used for Clinical Chemistry; Samples were run through a standard clinical chemistry panel using Comprehensive Profile rotor using whole blood.

[00670] ***Hematology:*** K2 EDTA tube 0.5 ml was used for hematology; samples were run for a Part 5 Hematology using IDEXX Procyte, which included absolute Reticulocyte count and Reticulocyte percentage.

[00671] ***Coagulation:*** Sodium Citrate tube 0.5ml was used for coagulation analysis, where the sample was analyzed using the VetScan Pro. Coagulation assessment included measuring Fibrinogen, aPTT, and PT. For this analysis, 100 µl of whole blood was aliquoted to run aPTT and PT parameters. The remaining 0.4 mL of whole blood was centrifuged to plasma, yielding 0.2 ml of plasma. 100 µl of the plasma was used to run the Fibrinogen analysis.

[00672] ***Cytokine Analysis:*** The blood samples for Cytokine analysis (0.5ml) were collected from the monkey's femoral, saphenous, or other available vein via direct venipuncture, (vein used was recorded in study records) and placed into a K2 EDTA tube. Blood samples were placed on ice. The samples were centrifuged at a temperature of 4°C, at 3,000g for 10 minutes. After centrifugation of the blood, plasma was aliquoted into a polypropylene tube. Plasma samples were store frozen at -80 C until used for analysis. Cytokine analysis was done using Accuri Flow Cytometer C6 using a Cytometric Bead Array Kit.

[00673] ***Urinalysis:*** Urine samples were collected using steel pans placed under the cages for at least 8 hours but no more than 12 hours. The cage pans were inclined so that the urine runs

through a tubing mechanism that was attached to the cage pan. The tube was connected to a container that was placed in a cooler with cold gel packs or wet ice to minimize contamination and maintain integrity of the samples. Thus, samples were collected on wet ice (or cold gel packs or an alternative cooling method) and maintained on wet ice or stored refrigerated (2 to 8 °C) until analysis which was no more than 12 hours after finishing the collection. The samples were allowed to acclimate to ambient temperature for 20 to 30 minutes prior to analysis.

[00674] ***PK:*** Blood samples processed for PK analysis (1.5ml) as indicated were collected into a serum separator polypropylene tube and processed (30 min at room temperature). Samples were centrifuged at a temperature of 4°C, at 3,000xg, for 15 minutes. After centrifugation of the blood, the samples were aliquoted into 6 polypropylene tubes. Samples were transferred to at -80°C within 1 hour and stored frozen at -80°C. Concentration of GIGA-564 in the serum samples was determined by ELISA.

[00675] The PK study data show that CL (clearance, the volume of sera from which GIGA-564 is completes cleared per day) in NHPs of GIGA-564 for all groups were in the expected range for an IgG1 (about 3-8 mL/day/kg) with linear PK (FIG. 52A and FIG. 52B). C_{max} and AUC were approximately dose proportional. The difference in C_{max}/D and AUC/D across two dose groups was less than 2-fold. The terminal half-life did not change much with dose (mean ranging from 10.2 to 12 days). FIG. 53 provides PK profiles of individual animals. The individual PK profile shows no evidence indicating formation of anti-drug antibody (ADA).

Dose	ID	HL	Cmax	Cmax_D	AUClast	AUClast_D	AUCINF_obs	AUC_%Extrap_obs	Cl_obs	Vss_obs
mg/kg		(day)	(µg/mL)	(kg*µg/mL/mg)	(day*µg/mL)	(day*kg*µg/mL/mg)	(day*µg/mL)	(%)	(mL/day/kg)	(mL/kg)
3	1	10.3	63.4	21.1	366	122	431	15.1	6.95	99.6
	2	11.7	62.4	20.8	302	101	365	17.2	8.21	125
	3	8.56	65.8	21.9	333	111	373	10.7	8.05	94.2
	N	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
	Mean	**10.2**	**63.9**	**21.3**	**334**	**111**	**390**	**14.3**	**7.74**	**106**
	SD	**1.57**	**1.71**	**0.569**	**32**	**10.7**	**36.2**	**3.34**	**0.684**	**16.3**
	CV%	**15.5**	**2.67**	**2.67**	**9.59**	**9.59**	**9.29**	**23.3**	**8.84**	**15.4**
30	4	10.1	648	21.6	3900	130	4470	12.8	6.71	87.6
	5	11.9	681	22.7	3990	133	4760	16.2	6.3	93.2
	6	14	755	25.2	4760	159	6160	22.7	4.87	89.3
	N	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
	Mean	**12**	**695**	**23.2**	**4220**	**141**	**5130**	**17.2**	**5.96**	**90**
	SD	**1.94**	**54.9**	**1.83**	**472**	**15.7**	**904**	**5.07**	**0.967**	**2.89**
	CV%	**16.1**	**7.9**	**7.9**	**11.2**	**11.2**	**17.6**	**29.4**	**16.2**	**3.21**

[00676] PK data of GIGA-564 in the NHPs was used to model human PK, which showed that GIGA-564 will have PK in the range expected for a human IgG1. Species-invariant time method was used to project GIGA-564 PK from cyno to humans:

$$Time_{human} = Time_{cyno} * \left(\frac{\text{Body weight}_{human}}{\text{Body weight}_{cyno}}\right)^{Exponent_{volume} - Exponent_{clearance}} \quad (5)$$

$$Concentration_{human} = Concentration_{cyno} * \left(\frac{Dose_{human}}{Dose_{cyno}}\right) * \left(\frac{\text{Body weight}_{cyno}}{\text{Body weight}_{human}}\right)^{Exponent_{volume}} \quad (6)$$

A volume exponent of 1 ($V_{exponent}$) and clearance exponent of 0.85 ($CL_{exponent}$) were chosen based on recent publications of interspecies scaling for therapeutic antibodies (Deng 2011). Average cynomolgus monkey and human body weights were assumed to be 3 kg and 70 kg, respectively.

[00677] As shown in FIGs. 54A and 54B, human CL is projected to be 4.27 mL/day/kg (12.5 mL/h). Human half-life is projected to be 17.8 days. GIGA-564 projected human PK is consistent with the expectation for a IgG1 with linear PK in humans (CL 3-5 mL/day/kg and half-life of about 21 days). Projected PK of individual human subjects are provided in FIG. 55A and time profiles after monthly dosing are provided in FIG. 55B. As further specified in the below table, mathematical modeling shows that GIGA-564 is expected to have no or only mild accumulation after monthly (every 28 day) dosing based on the projected human PK.

	accumulation ratio		
	based on AUC	based on Cmax	Based on Ctrough
Dose 2	1.28	1.13	1.31
Dose 3	1.36	1.17	1.40
Dose 4	1.38	1.18	1.43

8.23. Example 21: Cytokine release assay

[00678] GIGA-564 and ipilimumab were tested for their effects on cytokine/chemokine release from human peripheral blood mononuclear cells (PBMCs) under wet bound and soluble conditions. Further, potential cytotoxic effects in PBMCs were tested using alamarBlue™ cytotoxicity assay. Tested antibody compositions including GIGA-564 or ipilimumab are summarized in the below table.

Compound ID	Batch	Amount received (mL)	Test concentration (µg/mL)
GIGA-564	PN-4654.02	1.5	750, 250, 83, 27.8, 9.3, 3.1, 1.0
Ipilimumab	PN-4094	1.5	250, 83, 27.8, 9.3, 3.1, 1.0

[00679] For wet-bound presentation, test articles and controls were diluted in PBS, added to wells for coating, incubated overnight at 4°C, and washed before addition of PBMCs. For soluble presentation, test articles were diluted in an appropriate formulation buffer such that the amount of the formulation buffer was the same for each antibody concentration tested.

[00680] **Positive controls:** anti-CD3 (clone OKT3, BioLegend, Cat # 317315) and super agonist anti-CD28 (clone ANC28.1/5D10, Ancell, catalogue No. 177-820), were used at 3 µg/well (15 µg/mL). LPS R595 (100 ng/mL) and PHA (10 µg/mL) were used as positive controls in soluble condition.

[00681] **Isotype controls:** Mouse IgG1k (BioLegend, catalogue No. 400153) and Mouse IgG2a (BioLegend, catalogue No. 400224), were used at 3 µg/well (15 µg/mL).

[00682] **Analytes:** human IFNγ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-17A, MIP-1α, and TNFα.

[00683] FIG. 56 provides cytokine/chemokine release in response to GIGA-564 and FIG. 57 provides cytokine/chemokine release in response to ipilimumab under wet bound conditions. Under the wet bound conditions, both GIGA-564 and Ipilimumab failed to elicit release of cytokines including IFNγ, IL-2, IL-10, and IL-17A, from human PBMCs unlike assay positive controls. Stimulation of IL-1β, MIP-1α, and TNFα are comparable to isotype control levels or lesser than assay positive controls. IL-6 was observed in a dose-dependent manner but to lesser level than that induced by the anti-CD28 Induction of IL-8 was observed in a dose-dependent manner and comparable to that observed with the vehicle buffers or isotype controls. Immobilization of antibodies stimulates non-physiological activation of Fc receptors in the immune cells, which release specific cytokines/chemokines. Induction of IL-8 was observed at moderate levels due to non-physiological activation of immune cells. The positive stimulant controls, anti-CD3 (clone OKT3) and super agonistic anti-CD28, induced characteristically higher and specific cytokine secretion compared to both the test compound (GIGA-564 and ipilimumab) and isotype controls.

[00684] FIG. 58 provides cytokine/chemokine release in response to soluble GIGA-564 and FIG. 59 provides cytokine/chemokine release in response to soluble ipilimumab. Both soluble GIGA-564 and ipilimumab failed to elicit release of cytokines including IFNγ, IL-1β, IL-

2, IL-6, IL-10, IL-17A, MIP-1α, and TNFα from human PBMCs unlike assay positive controls. The positive stimulant controls, anti-CD3 (clone OKT3) and super agonistic anti-CD28, LPS and PHA induced characteristically higher and specific cytokine secretion compared to both the test compound and isotype controls

[00685] FIG. 60 shows cytotoxicity effects of GIGA-564 (top) and ipilimumab (bottom) in PBMCs under wet bound or soluble conditions. GIGA-564 and ipilimumab did not exhibit cytotoxic effects in human PBMCs when tested alone. The cell viability control, staurosporine (1 μM), exhibited cytotoxicity in PBMCs (90 % toxicity @ 48 hours) in all PBMC donors confirming assay performance.

8.24. Example 22: Efficacy of GIGA-2328

[00686] The hCTLA-4 KI mice bearing established MC38 tumors were treated with PBS, the indicated amount of ipilimumab (Yervoy), GIGA-564, afucosylated ipilimumab (next-generation), or GIGA-2328 (GIGA-564 with more afucosylation) on days 0, 3, and 6 after randomization.

[00687] Data for mice euthanized due to large tumor size was carried forward. Tumor sizes of the animals measured over time are provided in FIGs. 50A and 50B. The data show that GIGA-2328 is more efficacious than ipilimumab or even afucosylated ipilimumab (next-generation).

8.25. Example 23: Efficacy of GIGA-564

[00688] In a separate set of experiments, the hCTLA-4 KI mice bearing established MC38 tumors were treated with the indicated amount of GIGA-564 (1 mg/kg, 0.3 mg/kg, 0.1 mg/kg, 0.03 mg/kg, 0.01 mg/kg) on days 0, 3, and 6 and tumor growth was followed. Individual animals were euthanized as tumors reached 3000 mm^3 or at day 28. Data from animals that tumors reached 3000 mm^3 were carried forward until there were no remaining animals in that group. Data from the experiments are provided in FIG. 61 as median +/- CI. They show anti-tumoral effects of GIGA-564 in a dose dependent manner. Data suggests that GIGA-564 is more effective in inducing anti-tumor efficacy than ipilimumab that did not significantly induce tumor growth inhibition in this model when dosed at 0.3 mg/kg on days 0, 3, and 6 (FIG 30).

8.26. Example 24: FcR effector activity bioassays of fucosylated vs afucosylated anti-CTLA-4s

[00689] Fc effector activity bioassays for hFcγRIIIa were performed using the kit purchased from Promega Corporation (Madison, WI, USA). The assays were carried out following the manufacturer's instructions. Briefly, CHO target cells stably expressing human

CTLA-4 with the Y201G mutation to enhance cell surface expression were suspended media + sera with GIGA-564, GIGA-564_XF (afucosylated GIGA-564, also known as GIGA-2328, produced from a stable cell line), GIGA-564_AEX (GIGA-564 purified by protein A and then also purified with anion exchange), GIGA-564 with the LALA-PG mutation to eliminate Fc function, ipilimumab, ipilimumab_XF (afucosylated ipilimumab, also known as next-generation ipilimumab), or GIGA-2328 produced transiently, and incubated at 37°C for 30 minutes.

[00690] Jurkat/NFAT-Luc effector cells expressing human FcgammaRIIIa (V variant) were added to each well (effector:target ratio was 5:1) and incubated at 37°C for 6 hours. Luciferase activity was measured by using the included Bio-Glo Luciferase Assay Reagent with the SpectraMax i3x or iD3 plate readers (Molecular Devices, San Jose, CA, USA). Luciferase activity measured in relative luminescence units (RLU) were plotted against the concentration of CTLA-4 mAbs. (FIG. 51A and FIG. 51B) The EC50 value of each mAb was calculated by logistic regression using Prism (GraphPad, San Diego, CA, USA) and provided in TABLE 40. In TABLE 40, the %Fuco and %Afuco correspond to the percentages of those assigned by UPLC analysis. Briefly, PNGase digestion is used to liberate glycans, which are labeled with fluorescent molecules using GlykoPrep InstantAB kit and injected on a UPLC with fluorescent detector and compared to known glycan standards from kit.

TABLE 40

				EC50 (ug/mL)	
PN	**Ab**	**%Fuco**	**%Afuco**	**Plate 1**	**Plate 2**
4532.01	Ipi	88.0	10.4	0.0057	0.0063
4533.01	Ipi_XF	3.9	93.3	0.0011	0.0013
4534.01	GIGA-564_XF	25.1	74.0	0.00041	0.0019
4324.01	GIGA-564	91.2	7.7	0.0036	0.0102
4324.02	GIGA-564_AEX	91.4	7.5	0.0048	-
2328.01	GIGA-2328	5.2	91.6	-	0.0011

8.27. Example 25: Combination of pembrolizumab and GIGA-2328

[00691] Transgenic mice expressing human CTLA-4 and PD-1 (hCTLA4-hPD1 knock in (KI) mice, n=6 for the control arm and n=12 for the experimental groups) were treated with pembrolizumab (15 mg/kg) alone or in combination with GIGA-564 (10mg/kg) or GIGA-2328 (10mg/kg) every 3 days for 9 doses. Animals were euthanized on day 35 and tissues were collected for histology.

[00692] Histology of various organs of the animals was analyzed and the results are provided in TABLE 41. GIGA-2328 in combination with pembrolizumab (Keytruda) led to little to no increase in pathology in key organs compared to GIGA-564 in combination with pembrolizumab (Keytruda). This suggested that GIGA-2328 (afucosylation) can enhance efficacy with while having little increase on toxicity.

TABLE 41

		Heart		Colon		Liver		Skin	Spleen
Group	Treatment	Histopathology score - Inflammatory cells infiltration	Mean value of CD45+ cell density (/mm2)	histopath score - lymphocyte infiltration	Mean value of CD45+ cell density (/mm2)	histopath score - inflammatory cell infiltration	Mean value of CD45+ cell density (/mm2)	histopath score - inflammatory cell infiltration	histopath score - cellularity of lymphocytes in white pulp increased
1	PBS	0.0 ± 0.0	75.4 ± 6.0	0.0 ± 0.0	997.2 ± 87.2	1.2 ± 0.2	1099.1 +/- 34.4	0.2 +/- 0.2	1.0 ± 4.0
2	Keytruda (15 mg/kg)	0.4 ± 0.1	215.2 ± 61.2	0.0 ± 0.0	1107.7 ± 116.9	1.1 ± 0.1	1187.6 +/- 47.2	0.4 +/- 0.1	1.3 ± 0.2
3	Keytruda (15 mg/kg) + GIGA-564 (10 mg/kg)	1.2 ± 0.3	1437.9 ± 630.8	0.5 ± 0.2	883.1 ± 59.5	1.7 ± 0.1	1546.3 +/- 99.5	0.5 +/- 0.2	3.0 ± 0.2
4	Keytruda (15 mg/kg) + GIGA-2328 (10 mg/kg)	0.8 ± 0.1	192.4 ± 35.6	0.3 ± 0.1	1162.2 ± 97.1	2.0 ± 0.2	1261.1 +/- 35.0	0.7 +/- 0.1	3.1 ± 0.2

9. INCORPORATION BY REFERENCE

[00693] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same

extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes.

10. EQUIVALENTS

[00694] While various specific embodiments have been illustrated and described, the above specification is not restrictive. It will be appreciated that various changes can be made without departing from the spirit and scope of the present disclosure(s). Many variations will become apparent to those skilled in the art upon review of this specification.

TABLE 19 provides sequences for antibody light chains, heavy chains, CDRs, and human CTLA4.

TABLE 19	
SEQ ID NO	**Chain (Antibody)**
1	L1 (A1) "aCTLA-4.9"
2	L2 (A2) "aCTLA-4.4"
3	L3 (A3) "aCTLA-4.2"
4	L4 (A4) "aCTLA-4.29"
5	L5 (A5) "aCTLA-4.28"
6	L6 (A6) "aCTLA-4.26"
7	L7 (A7) "aCTLA-4.3"
8	L8 (A8) "aCTLA-4.1"
9	L9 (A9) "aCTLA-4.24"
10	L10 (A10) "aCTLA-4.22"
11	L11 (A11) "aCTLA-4.31"
12	L12 (A12) "aCTLA-4.12"
13	L13 (A13) "aCTLA-4.14"
14	L14 (A14) "aCTLA-4.15"
15	L15 (A15) "aCTLA-4.18"
16	L16 (A16) "aCTLA-4.5"
17	L17 (A17) "aCTLA-4.17"
18	L18 (A18) "aCTLA-4.11"
19	L19 (A19) "aCTLA-4.7"
20	L20 (A20) "aCTLA-4.25"
21	L21 (A21) "aCTLA-4.10"
22	L22 (A22) "aCTLA-4.21"
23	L23 (A23) "aCTLA-4.23"
24	L24 (A24) "aCTLA-4.27"
25	L25 (A25) "aCTLA-4.32"
26	L26 (A26) "aCTLA-4.20"
27	L27 (A27) "aCTLA-4.8"
28	L28 (A28) "aCTLA-4.19"
101	H1 (A1) "aCTLA-4.9"
102	H2 (A2) "aCTLA-4.4"
103	H3 (A3) "aCTLA-4.2"
104	H4 (A4) "aCTLA-4.29"
105	H5 (A5) "aCTLA-4.28"
106	H6 (A6) "aCTLA-4.26"

107	H7 (A7) “aCTLA-4.3”
108	H8 (A8) “aCTLA-4.1”
109	H9 (A9) “aCTLA-4.24”
110	H10 (A10) “aCTLA-4.22”
111	H11 (A11) “aCTLA-4.31”
112	H12 (A12) “aCTLA-4.12”
113	H13 (A13) “aCTLA-4.14”
114	H14 (A14) “aCTLA-4.15”
115	H15 (A15)“aCTLA-4.18”
116	H16 (A16)“aCTLA-4.5”
117	H17 (A17)“aCTLA-4.17”
118	H18 (A18) “aCTLA-4.11”
119	H19 (A19) “aCTLA-4.7”
120	H20 (A20) “aCTLA-4.25”
121	H21 (A21) “aCTLA-4.10”
122	H22 (A22) “aCTLA-4.21”
123	H23 (A23) “aCTLA-4.23”
124	H24 (A24) “aCTLA-4.27”
125	H25 (A25) “aCTLA-4.32”
126	H26 (A26) “aCTLA-4.20”
127	H27 (A27) “aCTLA-4.8”
128	H28 (A28) “aCTLA-4.19”
1001	CDR1-L1 (A1)
1002	CDR1-L2 (A2)
1003	CDR1-L3 (A3)
1004	CDR1-L4 (A4)
1005	CDR1-L5 (A5)
1006	CDR1-L6 (A6)
1007	CDR1-L7 (A7)
1008	CDR1-L8 (A8)
1009	CDR1-L9 (A9)
1010	CDR1-L10 (A10)
1011	CDR1-L11 (A11)
1012	CDR1-L12 (A12)
1013	CDR1-L13 (A13)
1014	CDR1-L14 (A14)
1015	CDR1-L15 (A15)
1016	CDR1-L16 (A16)
1017	CDR1-L17 (A17)
1018	CDR1-L18 (A18)
1019	CDR1-L19 (A19)
1020	CDR1-L20 (A20)
1021	CDR1-L21 (A21)
1022	CDR1-L22 (A22)
1023	CDR1-L23 (A23)
1024	CDR1-L24 (A24)
1025	CDR1-L25 (A25)
1026	CDR1-L26 (A26)

1027	CDR1-L27 (A27)
1028	CDR1-L28 (A28)
2001	CDR2-L1 (A1)
2002	CDR2-L2 (A2)
2003	CDR2-L3 (A3)
2004	CDR2-L4 (A4)
2005	CDR2-L5 (A5)
2006	CDR2-L6 (A6)
2007	CDR2-L7 (A7)
2008	CDR2-L8 (A8)
2009	CDR2-L9 (A9)
2010	CDR2-L10 (A10)
2011	CDR2-L11 (A11)
2012	CDR2-L12 (A12)
2013	CDR2-L13 (A13)
2014	CDR2-L14 (A14)
2015	CDR2-L15 (A15)
2016	CDR2-L16 (A16)
2017	CDR2-L17 (A17)
2018	CDR2-L18 (A18)
2019	CDR2-L19 (A19)
2020	CDR2-L20 (A20)
2021	CDR2-L21 (A21)
2022	CDR2-L22 (A22)
2023	CDR2-L23 (A23)
2024	CDR2-L24 (A24)
2025	CDR2-L25 (A25)
2026	CDR2-L26 (A26)
2027	CDR2-L27 (A27)
2028	CDR2-L28 (A28)
3001	CDR3-L1 (A1)
3002	CDR3-L2 (A2)
3003	CDR3-L3 (A3)
3004	CDR3-L4 (A4)
3005	CDR3-L5 (A5)
3006	CDR3-L6 (A6)
3007	CDR3-L7 (A7)
3008	CDR3-L8 (A8)
3009	CDR3-L9 (A9)
3010	CDR3-L10 (A10)
3011	CDR3-L11 (A11)
3012	CDR3-L12 (A12)
3013	CDR3-L13 (A13)
3014	CDR3-L14 (A14)
3015	CDR3-L15 (A15)
3016	CDR3-L16 (A16)
3017	CDR3-L17 (A17)
3018	CDR3-L18 (A18)

3019	CDR3-L19 (A19)
3020	CDR3-L20 (A20)
3021	CDR3-L21 (A21)
3022	CDR3-L22 (A22)
3023	CDR3-L23 (A23)
3024	CDR3-L24 (A24)
3025	CDR3-L25 (A25)
3026	CDR3-L26 (A26)
3027	CDR3-L27 (A27)
3028	CDR3-L28 (A28)
4001	CDR1-H1 (A1)
4002	CDR1-H2 (A2)
4003	CDR1-H3 (A3)
4004	CDR1-H4 (A4)
4005	CDR1-H5 (A5)
4006	CDR1-H6 (A6)
4007	CDR1-H7 (A7)
4008	CDR1-H8 (A8)
4009	CDR1-H9 (A9)
4010	CDR1-H10 (A10)
4011	CDR1-H11 (A11)
4012	CDR1-H12 (A12)
4013	CDR1-H13 (A13)
4014	CDR1-H14 (A14)
4015	CDR1-H15 (A15)
4016	CDR1-H16 (A16)
4017	CDR1-H17 (A17)
4018	CDR1-H18 (A18)
4019	CDR1-H19 (A19)
4020	CDR1-H20 (A20)
4021	CDR1-H21 (A21)
4022	CDR1-H22 (A22)
4023	CDR1-H23 (A23)
4024	CDR1-H24 (A24)
4025	CDR1-H25 (A25)
4026	CDR1-H26 (A26)
4027	CDR1-H27 (A27)
4028	CDR1-H28 (A28)
5001	CDR2-H1 (A1)
5002	CDR2-H2 (A2)
5003	CDR2-H3 (A3)
5004	CDR2-H4 (A4)
5005	CDR2-H5 (A5)
5006	CDR2-H6 (A6)
5007	CDR2-H7 (A7)
5008	CDR2-H8 (A8)
5009	CDR2-H9 (A9)
5010	CDR2-H10 (A10)

5011	CDR2-H11 (A11)
5012	CDR2-H12 (A12)
5013	CDR2-H13 (A13)
5014	CDR2-H14 (A14)
5015	CDR2-H15 (A15)
5016	CDR2-H16 (A16)
5017	CDR2-H17 (A17)
5018	CDR2-H18 (A18)
5019	CDR2-H19 (A19)
5020	CDR2-H20 (A20)
5021	CDR2-H21 (A21)
5022	CDR2-H22 (A22)
5023	CDR2-H23 (A23)
5024	CDR2-H24 (A24)
5025	CDR2-H25 (A25)
5026	CDR2-H26 (A26)
5027	CDR2-H27 (A27)
5028	CDR2-H28 (A28)
6001	CDR3-H1 (A1)
6002	CDR3-H2 (A2)
6003	CDR3-H3 (A3)
6004	CDR3-H4 (A4)
6005	CDR3-H5 (A5)
6006	CDR3-H6 (A6)
6007	CDR3-H7 (A7)
6008	CDR3-H8 (A8)
6009	CDR3-H9 (A9)
6010	CDR3-H10 (A10)
6011	CDR3-H11 (A11)
6012	CDR3-H12 (A12)
6013	CDR3-H13 (A13)
6014	CDR3-H14 (A14)
6015	CDR3-H15 (A15)
6016	CDR3-H16 (A16)
6017	CDR3-H17 (A17)
6018	CDR3-H18 (A18)
6019	CDR3-H19 (A19)
6020	CDR3-H20 (A20)
6021	CDR3-H21 (A21)
6022	CDR3-H22 (A22)
6023	CDR3-H23 (A23)
6024	CDR3-H24 (A24)
6025	CDR3-H25 (A25)
6026	CDR3-H26 (A26)
6027	CDR3-H27 (A27)
6028	CDR3-H28 (A28)
7001	Human CTLA4

Table 20 provides the sequence identifiers for the light chain, heavy chain, and CDRs of the indicated clones

TABLE 20								
	SEQ ID NO							
Antibody Clone Number	**Light Chain**	**Heavy Chain**	**CDR1 (Light)**	**CDR2 (Light)**	**CDR3 (Light)**	**CDR1 (Heavy)**	**CDR2 (Heavy)**	**CDR3 (Heavy)**
1	8000	8496	8992	9488	9984	10480	10976	11472
2	8001	8497	8993	9489	9985	10481	10977	11473
3	8002	8498	8994	9490	9986	10482	10978	11474
4	8003	8499	8995	9491	9987	10483	10979	11475
5	8004	8500	8996	9492	9988	10484	10980	11476
6	8005	8501	8997	9493	9989	10485	10981	11477
7	8006	8502	8998	9494	9990	10486	10982	11478
8	8007	8503	8999	9495	9991	10487	10983	11479
9	8008	8504	9000	9496	9992	10488	10984	11480
10	8009	8505	9001	9497	9993	10489	10985	11481
11	8010	8506	9002	9498	9994	10490	10986	11482
12	8011	8507	9003	9499	9995	10491	10987	11483
13	8012	8508	9004	9500	9996	10492	10988	11484
14	8013	8509	9005	9501	9997	10493	10989	11485
15	8014	8510	9006	9502	9998	10494	10990	11486
16	8015	8511	9007	9503	9999	10495	10991	11487
17	8016	8512	9008	9504	10000	10496	10992	11488
18	8017	8513	9009	9505	10001	10497	10993	11489
19	8018	8514	9010	9506	10002	10498	10994	11490
20	8019	8515	9011	9507	10003	10499	10995	11491
21	8020	8516	9012	9508	10004	10500	10996	11492
22	8021	8517	9013	9509	10005	10501	10997	11493
23	8022	8518	9014	9510	10006	10502	10998	11494
24	8023	8519	9015	9511	10007	10503	10999	11495
25	8024	8520	9016	9512	10008	10504	11000	11496
26	8025	8521	9017	9513	10009	10505	11001	11497
27	8026	8522	9018	9514	10010	10506	11002	11498
28	8027	8523	9019	9515	10011	10507	11003	11499
29	8028	8524	9020	9516	10012	10508	11004	11500
30	8029	8525	9021	9517	10013	10509	11005	11501
31	8030	8526	9022	9518	10014	10510	11006	11502
32	8031	8527	9023	9519	10015	10511	11007	11503
33	8032	8528	9024	9520	10016	10512	11008	11504
34	8033	8529	9025	9521	10017	10513	11009	11505
35	8034	8530	9026	9522	10018	10514	11010	11506
36	8035	8531	9027	9523	10019	10515	11011	11507
37	8036	8532	9028	9524	10020	10516	11012	11508
38	8037	8533	9029	9525	10021	10517	11013	11509
39	8038	8534	9030	9526	10022	10518	11014	11510
40	8039	8535	9031	9527	10023	10519	11015	11511
41	8040	8536	9032	9528	10024	10520	11016	11512
42	8041	8537	9033	9529	10025	10521	11017	11513

43	8042	8538	9034	9530	10026	10522	11018	11514
44	8043	8539	9035	9531	10027	10523	11019	11515
45	8044	8540	9036	9532	10028	10524	11020	11516
46	8045	8541	9037	9533	10029	10525	11021	11517
47	8046	8542	9038	9534	10030	10526	11022	11518
48	8047	8543	9039	9535	10031	10527	11023	11519
49	8048	8544	9040	9536	10032	10528	11024	11520
50	8049	8545	9041	9537	10033	10529	11025	11521
51	8050	8546	9042	9538	10034	10530	11026	11522
52	8051	8547	9043	9539	10035	10531	11027	11523
53	8052	8548	9044	9540	10036	10532	11028	11524
54	8053	8549	9045	9541	10037	10533	11029	11525
55	8054	8550	9046	9542	10038	10534	11030	11526
56	8055	8551	9047	9543	10039	10535	11031	11527
57	8056	8552	9048	9544	10040	10536	11032	11528
58	8057	8553	9049	9545	10041	10537	11033	11529
59	8058	8554	9050	9546	10042	10538	11034	11530
60	8059	8555	9051	9547	10043	10539	11035	11531
61	8060	8556	9052	9548	10044	10540	11036	11532
62	8061	8557	9053	9549	10045	10541	11037	11533
63	8062	8558	9054	9550	10046	10542	11038	11534
64	8063	8559	9055	9551	10047	10543	11039	11535
65	8064	8560	9056	9552	10048	10544	11040	11536
66	8065	8561	9057	9553	10049	10545	11041	11537
67	8066	8562	9058	9554	10050	10546	11042	11538
68	8067	8563	9059	9555	10051	10547	11043	11539
69	8068	8564	9060	9556	10052	10548	11044	11540
70	8069	8565	9061	9557	10053	10549	11045	11541
71	8070	8566	9062	9558	10054	10550	11046	11542
72	8071	8567	9063	9559	10055	10551	11047	11543
73	8072	8568	9064	9560	10056	10552	11048	11544
74	8073	8569	9065	9561	10057	10553	11049	11545
75	8074	8570	9066	9562	10058	10554	11050	11546
76	8075	8571	9067	9563	10059	10555	11051	11547
77	8076	8572	9068	9564	10060	10556	11052	11548
78	8077	8573	9069	9565	10061	10557	11053	11549
79	8078	8574	9070	9566	10062	10558	11054	11550
80	8079	8575	9071	9567	10063	10559	11055	11551
81	8080	8576	9072	9568	10064	10560	11056	11552
82	8081	8577	9073	9569	10065	10561	11057	11553
83	8082	8578	9074	9570	10066	10562	11058	11554
84	8083	8579	9075	9571	10067	10563	11059	11555
85	8084	8580	9076	9572	10068	10564	11060	11556
86	8085	8581	9077	9573	10069	10565	11061	11557
87	8086	8582	9078	9574	10070	10566	11062	11558
88	8087	8583	9079	9575	10071	10567	11063	11559
89	8088	8584	9080	9576	10072	10568	11064	11560
90	8089	8585	9081	9577	10073	10569	11065	11561
91	8090	8586	9082	9578	10074	10570	11066	11562

92	8091	8587	9083	9579	10075	10571	11067	11563
93	8092	8588	9084	9580	10076	10572	11068	11564
94	8093	8589	9085	9581	10077	10573	11069	11565
95	8094	8590	9086	9582	10078	10574	11070	11566
96	8095	8591	9087	9583	10079	10575	11071	11567
97	8096	8592	9088	9584	10080	10576	11072	11568
98	8097	8593	9089	9585	10081	10577	11073	11569
99	8098	8594	9090	9586	10082	10578	11074	11570
100	8099	8595	9091	9587	10083	10579	11075	11571
101	8100	8596	9092	9588	10084	10580	11076	11572
102	8101	8597	9093	9589	10085	10581	11077	11573
103	8102	8598	9094	9590	10086	10582	11078	11574
104	8103	8599	9095	9591	10087	10583	11079	11575
105	8104	8600	9096	9592	10088	10584	11080	11576
106	8105	8601	9097	9593	10089	10585	11081	11577
107	8106	8602	9098	9594	10090	10586	11082	11578
108	8107	8603	9099	9595	10091	10587	11083	11579
109	8108	8604	9100	9596	10092	10588	11084	11580
110	8109	8605	9101	9597	10093	10589	11085	11581
111	8110	8606	9102	9598	10094	10590	11086	11582
112	8111	8607	9103	9599	10095	10591	11087	11583
113	8112	8608	9104	9600	10096	10592	11088	11584
114	8113	8609	9105	9601	10097	10593	11089	11585
115	8114	8610	9106	9602	10098	10594	11090	11586
116	8115	8611	9107	9603	10099	10595	11091	11587
117	8116	8612	9108	9604	10100	10596	11092	11588
118	8117	8613	9109	9605	10101	10597	11093	11589
119	8118	8614	9110	9606	10102	10598	11094	11590
120	8119	8615	9111	9607	10103	10599	11095	11591
121	8120	8616	9112	9608	10104	10600	11096	11592
122	8121	8617	9113	9609	10105	10601	11097	11593
123	8122	8618	9114	9610	10106	10602	11098	11594
124	8123	8619	9115	9611	10107	10603	11099	11595
125	8124	8620	9116	9612	10108	10604	11100	11596
126	8125	8621	9117	9613	10109	10605	11101	11597
127	8126	8622	9118	9614	10110	10606	11102	11598
128	8127	8623	9119	9615	10111	10607	11103	11599
129	8128	8624	9120	9616	10112	10608	11104	11600
130	8129	8625	9121	9617	10113	10609	11105	11601
131	8130	8626	9122	9618	10114	10610	11106	11602
132	8131	8627	9123	9619	10115	10611	11107	11603
133	8132	8628	9124	9620	10116	10612	11108	11604
134	8133	8629	9125	9621	10117	10613	11109	11605
135	8134	8630	9126	9622	10118	10614	11110	11606
136	8135	8631	9127	9623	10119	10615	11111	11607
137	8136	8632	9128	9624	10120	10616	11112	11608
138	8137	8633	9129	9625	10121	10617	11113	11609
139	8138	8634	9130	9626	10122	10618	11114	11610
140	8139	8635	9131	9627	10123	10619	11115	11611

141	8140	8636	9132	9628	10124	10620	11116	11612
142	8141	8637	9133	9629	10125	10621	11117	11613
143	8142	8638	9134	9630	10126	10622	11118	11614
144	8143	8639	9135	9631	10127	10623	11119	11615
145	8144	8640	9136	9632	10128	10624	11120	11616
146	8145	8641	9137	9633	10129	10625	11121	11617
147	8146	8642	9138	9634	10130	10626	11122	11618
148	8147	8643	9139	9635	10131	10627	11123	11619
149	8148	8644	9140	9636	10132	10628	11124	11620
150	8149	8645	9141	9637	10133	10629	11125	11621
151	8150	8646	9142	9638	10134	10630	11126	11622
152	8151	8647	9143	9639	10135	10631	11127	11623
153	8152	8648	9144	9640	10136	10632	11128	11624
154	8153	8649	9145	9641	10137	10633	11129	11625
155	8154	8650	9146	9642	10138	10634	11130	11626
156	8155	8651	9147	9643	10139	10635	11131	11627
157	8156	8652	9148	9644	10140	10636	11132	11628
158	8157	8653	9149	9645	10141	10637	11133	11629
159	8158	8654	9150	9646	10142	10638	11134	11630
160	8159	8655	9151	9647	10143	10639	11135	11631
161	8160	8656	9152	9648	10144	10640	11136	11632
162	8161	8657	9153	9649	10145	10641	11137	11633
163	8162	8658	9154	9650	10146	10642	11138	11634
164	8163	8659	9155	9651	10147	10643	11139	11635
165	8164	8660	9156	9652	10148	10644	11140	11636
166	8165	8661	9157	9653	10149	10645	11141	11637
167	8166	8662	9158	9654	10150	10646	11142	11638
168	8167	8663	9159	9655	10151	10647	11143	11639
169	8168	8664	9160	9656	10152	10648	11144	11640
170	8169	8665	9161	9657	10153	10649	11145	11641
171	8170	8666	9162	9658	10154	10650	11146	11642
172	8171	8667	9163	9659	10155	10651	11147	11643
173	8172	8668	9164	9660	10156	10652	11148	11644
174	8173	8669	9165	9661	10157	10653	11149	11645
175	8174	8670	9166	9662	10158	10654	11150	11646
176	8175	8671	9167	9663	10159	10655	11151	11647
177	8176	8672	9168	9664	10160	10656	11152	11648
178	8177	8673	9169	9665	10161	10657	11153	11649
179	8178	8674	9170	9666	10162	10658	11154	11650
180	8179	8675	9171	9667	10163	10659	11155	11651
181	8180	8676	9172	9668	10164	10660	11156	11652
182	8181	8677	9173	9669	10165	10661	11157	11653
183	8182	8678	9174	9670	10166	10662	11158	11654
184	8183	8679	9175	9671	10167	10663	11159	11655
185	8184	8680	9176	9672	10168	10664	11160	11656
186	8185	8681	9177	9673	10169	10665	11161	11657
187	8186	8682	9178	9674	10170	10666	11162	11658
188	8187	8683	9179	9675	10171	10667	11163	11659
189	8188	8684	9180	9676	10172	10668	11164	11660

190	8189	8685	9181	9677	10173	10669	11165	11661
191	8190	8686	9182	9678	10174	10670	11166	11662
192	8191	8687	9183	9679	10175	10671	11167	11663
193	8192	8688	9184	9680	10176	10672	11168	11664
194	8193	8689	9185	9681	10177	10673	11169	11665
195	8194	8690	9186	9682	10178	10674	11170	11666
196	8195	8691	9187	9683	10179	10675	11171	11667
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215	8214	8710	9206	9702	10198	10694	11190	11686
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218	8217	8713	9209	9705	10201	10697	11193	11689
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494	8493	8989	9485	9981	10477	10973	11469	11965
495	8494	8990	9486	9982	10478	10974	11470	11966
496	8495	8991	9487	9983	10479	10975	11471	11967

TABLE 32-33. A14 Light Chain CDR sequences and A14 Heavy Chain CDR sequences for GIGA-564 identified by different algorithms (Antibody A14).

TABLE 32: A14 Light Chain CDR sequences for GIGA-564 identified by different algorithms (Antibody A14):

Region	Definition	Sequence Fragment	Residues	Length
LFR1	Chothia	EIVLTQSPGTLSLSPGERATLSC------ (SEQ ID NO: 11999)	1 - 23	23
	AbM	EIVLTQSPGTLSLSPGERATLSC------ (SEQ ID NO: 12000)	1 - 23	23
	Kabat	EIVLTQSPGTLSLSPGERATLSC------ (SEQ ID NO: 12001)	1 - 23	23
	Contact	EIVLTQSPGTLSLSPGERATLSCRASQSV (SEQ ID NO: 12002)	1 - 29	29
	IMGT	EIVLTQSPGTLSLSPGERATLSCRAS--- (SEQ ID NO: 12003)	1 - 26	26
CDR-L1	Chothia	RASQSVSSSYLA- (SEQ ID NO: 12004)	24 - 35	12
	AbM	RASQSVSSSYLA- (SEQ ID NO: 12005)	24 - 35	12
	Kabat	RASQSVSSSYLA- (SEQ ID NO: 12006)	24 - 35	12
	Contact	------SSSYLAWY (SEQ ID NO: 12007)	30 - 37	8
	IMGT	---QSVSSSY---- (SEQ ID NO: 12008)	27 - 33	7
LFR2	Chothia	--WYQQKPGQAPRLLIY (SEQ ID NO: 12009)	36 - 50	15
	AbM	--WYQQKPGQAPRLLIY (SEQ ID NO: 12010)	36 - 50	15
	Kabat	--WYQQKPGQAPRLLIY (SEQ ID NO: 12011)	36 - 50	15
	Contact	----QQKPGQAPR---- (SEQ ID NO: 12012)	38 - 46	9
	IMGT	LAWYQQKPGQAPRLLIY (SEQ ID NO: 12013)	34 - 50	17
CDR-L2	Chothia	----GASSRAT (SEQ ID NO: 12014)	51 - 57	7
	AbM	----GASSRAT (SEQ ID NO: 12015)	51 - 57	7
	Kabat	----GASSRAT (SEQ ID NO: 12016)	51 - 57	7
	Contact	LLIYGASSRA- (SEQ ID NO: 12017)	47 - 56	10
	IMGT	----GA----- (SEQ ID NO: 12018)	51 - 52	2
LFR3	Chothia	-----GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC (SEQ ID NO: 12019)	58 - 89	32
	AbM	-----GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC (SEQ ID NO: 12020)	58 - 89	32
	Kabat	-----GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	58 - 89	32

		(SEQ ID NO: 12021)		
	Contact	----TGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC (SEQ ID NO: 12022)	57 - 89	33
	IMGT	SSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC (SEQ ID NO: 12023)	53 - 89	37
CDR-L3	Chothia	QQYGSSPWT (SEQ ID NO: 12024)	90 - 98	9
	AbM	QQYGSSPWT (SEQ ID NO: 12025)	90 - 98	9
	Kabat	QQYGSSPWT (SEQ ID NO: 12026)	90 - 98	9
	Contact	QQYGSSPW- (SEQ ID NO: 12027)	90 - 97	8
	IMGT	QQYGSSPWT (SEQ ID NO: 12028)	90 - 98	9
LFR4	Chothia	-SGQGTKVEIK (SEQ ID NO: 12029)	99 - 108	10
	AbM	-SGQGTKVEIK (SEQ ID NO: 12030)	99 - 108	10
	Kabat	-SGQGTKVEIK (SEQ ID NO: 12031)	99 – 108	10
	Contact	TSGQGTKVEIK (SEQ ID NO: 12032)	98 - 108	11
	IMGT	-SGQGTKVEIK (SEQ ID NO: 12033)	99 - 108	10

TABLE 33: A14 Heavy Chain CDR sequences for GIGA-564 identified by different algorithms (Antibody A14):

Region	Definition	Sequence Fragment	Residues	Length
HFR1	Chothia	QVQLVESGGGVVQPGRSLRLSCAAS----- (SEQ ID NO: 12034)	1 - 25	25
	AbM	QVQLVESGGGVVQPGRSLRLSCAAS----- (SEQ ID NO: 12035)	1 - 25	25
	Kabat	QVQLVESGGGVVQPGRSLRLSCAASGFTFS (SEQ ID NO: 12036)	1 - 30	30
	Contact	QVQLVESGGGVVQPGRSLRLSCAASGFTF- (SEQ ID NO: 12037)	1 – 29	29
	IMGT	QVQLVESGGGVVQPGRSLRLSCAAS----- (SEQ ID NO: 12038)	1 - 25	25
CDR-H1	Chothia	GFTFSSY--- (SEQ ID NO: 12039)	26 - 32	7
	AbM	GFTFSSYGMH (SEQ ID NO: 12040)	26 - 35	10
	Kabat	-----SYGMH (SEQ ID NO: 12041)	31 - 35	5
	Contact	----SSYGMH(SEQ ID NO: 12042)	30 - 35	6
	IMGT	GFTFSSYG--(SEQ ID NO: 12043)	26 - 33	8
HFR2	Chothia	GMHWVRQAPGTGLEWVAVI(SEQ ID NO: 12044)	33 - 51	19
	AbM	---WVRQAPGTGLEWVA--(SEQ ID NO: 12045)	36 - 49	14
	Kabat	---WVRQAPGTGLEWVA--(SEQ ID NO: 12046)	36 - 49	14
	Contact	---WVRQAPGTGLE-----(SEQ ID NO: 12047)	36 - 46	11
	IMGT	-MHWVRQAPGTGLEWVAV-(SEQ ID NO: 12048)	34 - 50	17
CDR-H2	Chothia	-----WYEGRN---------(SEQ ID NO: 12049)	52 - 57	6
	AbM	---VIWYEGRNKY-------(SEQ ID NO: 12050)	50 - 59	10
	Kabat	---VIWYEGRNKYYADPVKG (SEQ ID NO: 12051)	50 - 66	17
	Contact	WVAVIWYEGRNKY-------(SEQ ID NO: 12052)	47 - 59	13
	IMGT	----IWYEGRNK--------(SEQ ID NO: 12053)	51 - 58	8
HFR3	Chothia	KYYADPVKGRFTISRDNSKNTLYLQMNSLRDDDTAVYYCAR (SEQ ID NO: 12054)	58 - 98	41
	AbM	--YADPVKGRFTISRDNSKNTLYLQMNSLRDDDTAVYYCAR (SEQ ID NO: 12055)	60 - 98	39
	Kabat	---------RFTISRDNSKNTLYLQMNSLRDDDTAVYYCAR (SEQ ID NO: 12056)	67 - 98	32
	Contact	--YADPVKGRFTISRDNSKNTLYLQMNSLRDDDTAVYYC– (SEQ ID NO: 12057)	60 - 96	37
	IMGT	-YYADPVKGRFTISRDNSKNTLYLQMNSLRDDDTAVYYC– (SEQ ID NO: 12058)	59 - 96	38
CDR-H3	Chothia	--AGDLGAFDI (SEQ ID NO: 12059)	99 - 107	9
	AbM	--AGDLGAFDI (SEQ ID NO: 12060)	99 - 107	9
	Kabat	--AGDLGAFDI (SEQ ID NO: 12061)	99 - 107	9
	Contact	ARAGDLGAFD- (SEQ ID NO: 12062)	97 - 106	10
	IMGT	ARAGDLGAFDI (SEQ ID NO: 12063)	97 - 107	11
HFR4	Chothia	-WGQGTMVTVSS (SEQ ID NO: 12064)	108 - 118	11

AbM	-WGQGTMVTVSS(SEQ ID NO: 12065)	108 - 118	11
Kabat	-WGQGTMVTVSS (SEQ ID NO: 12066)	108 - 118	11
Contact	IWGQGTMVTVSS (SEQ ID NO: 12067)	107 - 118	12
IMGT	-WGQGTMVTVSS (SEQ ID NO: 12068)	108 - 118	11

TABLE 35: Light Chain sequences, light chain CDR sequences, heavy chain sequences, and heavy chain CDR sequences of Ipilimumab.

Ipilimumab	**Amino Acid Sequence**	**SEQ ID NO:**
Variable Heavy Chain (HC)	QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGL EWVTFISYDGNNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDT AIYYCARTGWLGPFDYWGQGTLVTVSSA	12087
Variable Light Chain (LC)	EIVLTQSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRL LIYGAFSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSP WTFGQGTKVEIK	12088
LC CDR1	QSVGSSY	12081
LC CDR2	GAF	12082
LC CDR3	QQYGSSPWT	12083
HC CDR1	GFTFSSYT	12084
HC CDR2	ISYDGNNK	12085
HC CDR3	ARTGWLGPFDY	12086

WHAT IS CLAIMED IS:

1. A method of treating cancer comprising the step of:

administering a cancer patient an effective amount of an antigen binding protein (anti-CTLA-4 ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4,

wherein the anti-CTLA-4 ABP comprises a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID NO:12080, a CDR1-H consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077.

2. The method of claim 1, wherein the cancer is resistant to anti-PD-1 or anti-PD-L1 treatment.
3. The method of claim 1 or 2, wherein the cancer is resistant to treatment of anti-PD-1 antibody or anti-PD-L1 antibody.
4. The method of any one of claims 1-3, wherein the cancer patient has progressed or relapsed after anti-PD-1 or anti-PD-L1 treatment.
5. The method of any one of claims 1-4, further comprising the step of deciding whether the cancer is resistant to anti-PD-1 treatment or anti-PD-L1 treatment.
6. The method of any one of claims 1-5, wherein the cancer patient has melanoma, RCC (renal cell cancer), NSCLC (non-small cell lung cancer), Merkel cell carcinoma, cSCC, mesothelioma, hepatocellular carcinoma, esophageal cancer, breast cancer, sarcoma, MSI-Hi/dMMR colorectal cancer, ovarian cancer, or cervical cancer, bladder, prostate, TMB-HI tumors of any origin, a tumor which is MSI, a tumor that is dMMR, a T cell leukemia/lymphoma, NHL, a tumor expressing CTLA-4 by the cancer cell.
7. The method of any one of claims 1-6, further comprising the step of administering an antigen binding protein (anti-PD-1 ABP or anti-PD-L1 ABP) that specifically binds a human PD-1 or anti-PD-L1.
8. The method of claim 7, wherein the anti-PD-1 ABP is pembrolizumab.
9. The method of claim 7 or 8, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio selected from 3:1, 3:10, 1:3, 1:10, 10:1, 10:3, 9:1, and 1:1.
10. The method of claim 7 or 8, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio from 2:1 to 10:1.
11. The method of claim 7 or 8, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio from 1:1 to 1:10.
12. The method of claim 9 or 10, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered at a weight ratio of 3:1.

13. The method of any one of claims 7-12, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on the same day.
14. The method of any one of claims 7-12, wherein the anti-CTLA-4 ABP and the anti-PD-1 ABP are administered on different days.
15. The method of claim 7, wherein the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on the same day.
16. The method of claim 7, wherein the anti-CTLA-4 ABP and the anti-PD-L1 ABP are administered on different days.
17. The method of any one of claims 1-16, wherein the effective amount of the anti-CTLA-4 ABP is less than 30mg/kg.
18. The method of any one of claims 1-17, wherein the effective amount of the anti-CTLA-4 ABP is at least 0.01mg/kg, 0.03mg/kg, 0.1mg/kg, 0.3mg/kg, 1mg/kg, 9 mg/kg or 27 mg/kg.
19. The method of claim 17 or 18, wherein the effective amount of the anti-CTLA-4 ABP is from 0.5mg/kg to 30mg/kg.
20. The method of claim 19, wherein the effective amount of the anti-CTLA-4 ABP is from 1mg/kg to 18mg/kg.
21. The method of claim 20, wherein the effective amount of the anti-CTLA-4 ABP is from 1mg/kg to 10mg/kg.
22. The method of claim 21, wherein the effective amount of the anti-CTLA-4 ABP is 1mg/kg, 3mg/kg, or 30mg/kg.
23. The method of any one of claims 1-22, wherein the effective amount of the anti-CTLA-4 ABP is from 50mg to 2500mg.
24. The method of claim 23, wherein the effective amount of the anti-CTLA-4 ABP is from 50mg to 1000mg.
25. The method of claim 23, wherein the effective amount of the anti-CTLA-4 ABP is from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg.
26. The method of claim 23, wherein the effective amount of the anti-CTLA-4 ABP is 80mg, 240mg, 720mg, 800mg, 1440mg, 2160mg, 50mg, 100mg, 150mg, 250mg, 700mg, 800mg, 900mg, 1000mg, 1500mg, 2000mg, or 2500mg.
27. The method of any one of claims 1-26, wherein the anti-CTLA-4 ABP comprises

a variable light chain (V_L) comprising a sequence at least 97% identical to SEQ ID NO:14 and a variable heavy chain (V_H) comprising a sequence at least 97% identical to SEQ ID NO:114.

28. The method of claim 27, wherein the anti-CTLA-4 ABP comprises

a variable light chain (V_L) comprising the sequence of SEQ ID NO:14 and a variable heavy chain (V_H) comprising the sequence of SEQ ID NO:114.

29. The method of any one of claims 1-28, wherein the anti-CTLA-4 ABP comprises an scFv or a full length monoclonal antibody.

30. The method of any one of claims 1-29, wherein the anti-CTLA-4 ABP comprises an immunoglobulin constant region.

31. The method of any one of claims 1-30, wherein the anti-CTLA-4 ABP is a IgG1 ABP.

32. The method of any one of claims 1-31, wherein the anti-CTLA-4 ABP comprises an IGHG1*01 human heavy chain constant region gene segment.

33. The method of any one of claims 1-32, wherein the anti-CTLA-4 ABP comprises a lysine at amino acid position 97 (R97) according to IMGT exon numbering.

34. The method of any one of claims 1-32, wherein the anti-CTLA-4 ABP comprises a lysine at amino acid position 97 (R214) according to EU numbering.

35. The method of any one of claims 1-34, wherein the anti-CTLA-4 ABP comprises an afucosylated Fc region.

36. The method of any one of claims 1-35, wherein the anti-CTLA-4 ABP is produced from a cell comprising a bacterial protein RMD (GDP-6-deoxy-D-lyxo-4-hexulose reductase) or a modification thereof.

37. The method of claim 36, wherein the cell is cultured in the absence of fucose.

38. The method of any one of claims 1-37, wherein the anti-CTLA-4 ABP is produced from a cell lacking or with reduced expression of Fut8.

39. The method of any one of claims 1-37, wherein the anti-CTLA-4 ABP is produced from a cell cultured in the presence of a fucosylation inhibitor, 2-Fluorfucose (2FF).

40. The method of any one of claims 1-37, wherein the anti-CTLA-4 ABP is produced from a cell overexpressing glycosyltransferase (GnTIII).

41. The method of any one of claims 1-40, wherein the anti-CTLA-4 ABP has been isolated based on its fucosylation status.

42. The method of any one of claims 1-41, wherein the anti-CTLA-4 ABP comprises an Fc region lacking core fucosylation of the N-glycan of the Fc portion.

43. The method of any one of claims 1-42, wherein the ABP is an afucosylated monoclonal antibody.

44. The method of any one of claims 1-43, wherein the anti-CTLA-4 ABP is administered in a pharmaceutical composition.

45. The method of claim 44, wherein the pharmaceutical composition has pH from 5.0 to 6.5.
46. The method of claim 45, wherein the pharmaceutical composition has pH from 6.0 to 6.5.
47. The method of any one of claims 44-46, wherein the pharmaceutical composition comprises 20mM of histidine or citrate buffer.
48. The method of claim 47, wherein the pharmaceutical composition comprises 20mM of histidine.
49. The method of any one of claims 44-48, wherein the pharmaceutical composition comprises 50mM of NaCl.
50. The method of any one of claims 44-49, wherein the pharmaceutical composition comprises sucrose at a concentration from 170mM to 270mM.
51. The method of any one of claims 44-50, wherein the pharmaceutical composition comprises 0.1-1 mg/ml Polysorbate 20.
52. The method of claim 51, wherein the pharmaceutical composition comprises 0.2 mg/ml Polysorbate 20.
53. The method of any one of claims 44-52, wherein the pharmaceutical composition comprises 20mM histidine, 270mM sucrose, and 0.2 mg/ml Polysorbate 20, and has pH 6.2.
54. The method of any one of claims 44-53, wherein the pharmaceutical composition comprises from 5 mg/mL to 20 mg/mL of the anti-CTLA-4 ABP.
55. The method of claim 54, wherein the pharmaceutical composition comprises 20 mg/mL of the anti-CTLA-4 ABP.
56. The method of claim 54, wherein the pharmaceutical composition comprises 10 mg/mL of the anti-CTLA-4 ABP.
57. The method of claim 54, wherein the pharmaceutical composition comprises 5 mg/mL of the anti-CTLA-4 ABP.
58. The method of any one of claims 1-57, wherein the step of administering the anti-CTLA-4 ABP is repeated.
59. The method of claim 58, wherein the step of administering the anti-CTLA-4 ABP is repeated at least twice, three times, four times, or more.
60. The method of claim 58 or 59, wherein the step of administering the anti-CTLA-4 ABP is repeated every week, every two weeks, every three weeks, every four weeks , every five weeks, every six weeks or every seven weeks.
61. The method of claim 58 or 59, wherein the step of administering the anti-CTLA-4 ABP is repeated every 1-2 weeks, every 2-3 weeks, every 3-4 weeks, every 4-5 weeks, every 5-6

weeks, every 6-7 weeks, every 7-8 weeks, every 8-9 weeks, every 9-10 weeks, every 10-11 weeks, every 11-12 weeks, every 12-13 weeks, every 13-14 weeks, or every 14-15 weeks.

62. The method of claim 58 or 59, wherein the step of administering the anti-CTLA-4 ABP is repeated every month, every two months, every three months, every four months, every five months, or less frequent.

63. The method of claim 58 or 59, wherein the step of administering the anti-CTLA-4 ABP is repeated every 1-2 months, every 2-3 months, every 3-4 months, every 4-5 months, or every 5-6 months.

64. The method of any one of claims 58-63, wherein the anti-PD-1 antibody or anti-PD-L1 antibody is administered in combination with the anti-CTLA-4 ABP in each of the repeated administrations.

65. A pharmaceutical composition comprising an anti-CTLA-4 ABP and a pharmaceutically acceptable excipient,

wherein the anti-CTLA-4 ABP is an isolated antigen binding protein (ABP) that specifically binds a human cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and comprises a CDR1-L consisting of SEQ ID NO:12078, a CDR2-L consisting of SEQ ID NO:12079, a CDR3-L consisting of SEQ ID NO:12080, a CDR1-H consisting of SEQ ID NO:12075, a CDR2-H consisting of SEQ ID NO:12076 and a CDR3-H consisting of SEQ ID NO:12077.

66. The pharmaceutical composition of claim 65, wherein the anti-CTLA-4 ABP comprises a variable light chain (V_L) comprising the sequence of SEQ ID NO:14 and a variable heavy chain (V_H) comprising the sequence of SEQ ID NO:114.

67. The pharmaceutical composition of any one of claims 65-66, having pH from 5.0 to 6.5.

68. The pharmaceutical composition of claim 67, having a pH from 6.0 to 6.5.

69. The pharmaceutical composition of claim 68, having pH 6.2.

70. The pharmaceutical composition of any one of claims 65-69, comprising 20mM of histidine or citrate buffer.

71. The pharmaceutical composition of claim 70, wherein the pharmaceutical composition comprises 20mM of histidine.

72. The pharmaceutical composition of any one of claims 65-71, comprising 50mM of NaCl.

73. The pharmaceutical composition of any one of claims 65-72, comprising sucrose at a concentration from 170mM to 270mM.

74. The pharmaceutical composition of any one of claims 65-73, comprising 0.1-1 mg/ml Polysorbate 20.

75. The pharmaceutical composition of claim 74, comprising 0.2 mg/ml Polysorbate 20.
76. The pharmaceutical composition of any one of claims 65-75, comprising 20mM histidine, 270mM sucrose, and 0.02% PS-20, and has pH 6.2.
77. The pharmaceutical composition of any one of claims 65-76, comprising 5 mg/mL to 20 mg/mL of the anti-CTLA-4 ABP.
78. The pharmaceutical composition of claim 77, comprising 20 mg/mL of the anti-CTLA-4 ABP.
79. The pharmaceutical composition of claim 77, comprising 10 mg/mL of the anti-CTLA-4 ABP.
80. The pharmaceutical composition of claim 77, comprising 5 mg/mL of the anti-CTLA-4 ABP.
81. The pharmaceutical composition of any one of claims 65-80, wherein less than 50% of the anti-CTLA-4 ABP is fucosylated.
82. The pharmaceutical composition of claim 81, wherein less than 40% of the anti-CTLA-4 ABP is fucosylated.
83. The pharmaceutical composition of claim 82, wherein less than 30% of the anti-CTLA-4 ABP is fucosylated.
84. The pharmaceutical composition of claim 83, wherein less than 20% of the anti-CTLA-4 ABP is fucosylated.
85. The pharmaceutical composition of claim 84, wherein less than 10% of the anti-CTLA-4 ABP is fucosylated.
86. The pharmaceutical composition of any one of claims 65-80, wherein 3% to 30% of the anti-CTLA-4 ABP is fucosylated.
87. The pharmaceutical composition of claim 86, wherein 10% to 30% of the anti-CTLA-4 ABP is fucosylated.
88. The pharmaceutical composition of claim 87, wherein 15% to 25% of the anti-CTLA-4 ABP is fucosylated.
89. The pharmaceutical composition of any one of claims 65-88, formulated for injection.
90. The pharmaceutical composition of any one of claims 65-88, formulated for iv infusion.
91. A unit dose form of the pharmaceutical composition of any one of claims 65-90.
92. The unit dose form of claim 91, comprising 50mg to 5000mg of the anti-CTLA-4 ABP.
93. The unit dose form of claim 92, comprising 50mg to 2500mg of the anti-CTLA-4 ABP.
94. The unit dose form of claim 93, comprising 50mg to 2000mg of the anti-CTLA-4 ABP.

95. The unit dose form of claim 92, comprising the anti-CTLA-4 ABP at an amount from 70mg to 150mg, from 150mg to 500mg, from 500mg to 800mg, from 700mg to 900mg, from 800mg to 1200mg, from 1200mg to 1500mg, or from 1500mg to 2500mg.

96. The unit dose form of claim 95, comprising the anti-CTLA-4 ABP at an amount of 80mg, 240mg, 720mg, 800mg, 1440mg or 2160mg.