

**ANP-DEPENDENT ANTIBODIES****CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of International Application Serial No. PCT/CN2023/137087, filed December 7, 2023, the content of which is hereby incorporated by reference in its entirety.

**REFERENCE TO AN ELECTRONIC SEQUENCE LISTING**

[0002] The content of the electronic sequence listing (379428.xml; Size: 76,648 bytes; and Date of Creation: December 5, 2024) is herein incorporated by reference in its entirety.

**BACKGROUND**

[0003] Cancer immunotherapy is the stimulation of the immune system to treat cancer, improving on the immune system's natural ability to fight the disease. Cancer immunotherapy exploits the fact that cancer cells often have tumor associated antigens (TAA), molecules on their surface that can bind to antibody proteins or T-cell receptors, triggering an immune system response. The tumor associated antigens are often proteins or other macromolecules.

[0004] Immune checkpoints are regulators of the immune system. These pathways are crucial for self-tolerance, which prevents the immune system from attacking cells indiscriminately. However, some cancers can protect themselves from attack by stimulating immune checkpoint targets. Inhibitory checkpoint molecules thus can be suitable targets for cancer immunotherapy due to their potential for use in multiple types of cancers. Currently approved checkpoint inhibitors include those that inhibit CTLA4, PD-1 or PD-L1.

[0005] The expression of TAA and checkpoint molecules, however, is typically not restricted to tumor tissues. Their expression in non-tumor tissues can lead to adverse effects. It is therefore desirable to develop therapeutics that target TAA and checkpoint molecules but whose activation is restricted to tumor tissues.

**SUMMARY**

[0006] Disclosed herein are antibodies capable of binding a TAA or immune checkpoint molecule in an adenine nucleotide-dependent manner. Examples of adenine nucleotides include ATP (adenosine triphosphate) ADP (adenosine diphosphate), and AMP (adenosine

monophosphate), collectively referred to as ANP. Tumor cells produce and release large amount of adenine nucleotides into the tumor microenvironment. Therefore, the tumor microenvironment includes considerably higher concentrations of adenine nucleotides than normal tissues. These new antibodies' dependency on adenine nucleotides, therefore, further limits their activity in normal tissue, resulting in improved safety of the therapeutics.

**[0007]** In accordance with one embodiment of the present disclosure, provided is an antibody or antigen-binding fragment thereof capable of binding to ATP (adenosine triphosphate) ADP (adenosine diphosphate), or AMP (adenosine monophosphate), wherein the antibody or the fragment thereof comprises a heavy chain variable region (VH) and a light chain variable region (VL), wherein the VH has R at position 50; R at position 52b; R, E, K, T, S, Q, G, P, V, M or W at position 97; R, K, H, T, S, N, Q, G, A, P, V, L, M or W at position 100c; and R, T, L or M at position 100d, and the VL has Q at position 90; G, T, A, S, L, Y or H at position 91; F at position 94; P at position 95; and S, G, T or A at position 96, all positions according to Kabat numbering.

**[0008]** In some embodiments, the antibody or antigen-binding fragment thereof has binding specificity to a tumor associated antigen or immune checkpoint molecule, and binds to the tumor associated antigen or immune checkpoint molecule at higher affinity in the presence of the ATP, ADP or AMP than in the absence of the ATP, ADP or AMP.

**[0009]** In some embodiments, the VH has R at position 50; R at position 52b; R at position 97; R at position 100c; and R at position 100d, and the VL has Q at position 90; G at position 91; F at position 94; P at position 95; and S at position 96.

**[0010]** In some embodiments, the VH comprises heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and wherein the VH CDR1 comprises the amino acid sequence of SEQ ID NO:9 or SEQ ID NO:9 with 1, 2, 3, 4 or 5 amino acid substitutions; and the VH CDR2 comprises the amino acid sequence of SEQ ID NO:10 or SEQ ID NO:10 with 1, 2, 3, 4 or 5 amino acid substitutions.

**[0011]** In some embodiments, the VH CDR1 comprises the amino acid sequence of SEQ ID NO:9 or SEQ ID NO:9 with 1, 2, or 3 amino acid substitutions; and the VH CDR2 comprises the amino acid sequence of SEQ ID NO:10 or SEQ ID NO:10 with 1, 2, or 3 amino acid substitutions.

**[0012]** In some embodiments, the VL comprises light chain complementarity determining regions (CDR) VL CDR1, VL CDR2, and VL CDR3, and wherein the VL CDR1 comprises the amino acid sequence of SEQ ID NO:12 or SEQ ID NO:12 with 1, 2, 3, 4 or 5 amino acid substitutions; and the VL CDR2 comprises the amino acid sequence of SEQ ID NO:13 or SEQ ID NO:14 with 1, 2, 3, 4 or 5 amino acid substitutions.

**[0013]** In some embodiments, the VL CDR1 comprises the amino acid sequence of SEQ ID NO:12 or SEQ ID NO:12 with 1, 2, or 3 amino acid substitutions; and the VL CDR2 comprises the amino acid sequence of SEQ ID NO:13 or SEQ ID NO:13 with 1, 2, or 3 amino acid substitutions.

**[0014]** In some embodiments, the VH comprises a first framework region, a second framework region, a third framework region, and a fourth framework region which, collectively have at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:33, 34, 35 and 36, and the VL comprises a first framework region, a second framework region, a third framework region, and a fourth framework region which, collectively have at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:37, 38, 39 and 40.

**[0015]** In some embodiments, the first, second, third and fourth framework regions of the VH, respectively, has at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:33, 34, 35 and 36. In some embodiments, the first, second, third and fourth framework regions of the VL, respectively, has at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO: 37, 38, 39 and 40.

**[0016]** In some embodiments, the antibody or antigen-binding fragment thereof has binding specificity to CTLA4. To this end, in some embodiments, the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3, and wherein the VH CDR1 comprises the amino acid sequence of SEQ ID NO:15, or SEQ ID NO:15 in which position 32 is an amino acid selected from L, E, F, A, M, W, Q, Y, H, K or R; the VH CDR2 comprises the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having at least 60%, 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:16; the VH CDR3 comprises the amino acid sequence of SEQ ID NO:17, or SEQ ID NO:17 with an amino acid substitution at any one of the positions 95, 99, 100, 100a, 100b, 100e, 100f, 101, or 102; the

VL CDR1 comprises the amino acid sequence of SEQ ID NO:18, or SEQ ID NO:18 with an amino acid substitution; the VL CDR2 comprises the amino acid sequence of SEQ ID NO:19, or SEQ ID NO:19 in which position 53 is G, or position 55 is M, F, Y or L; and the VL CDR3 comprises the amino acid sequence of SEQ ID NO:20, or SEQ ID NO:20 an amino acid substitution at any one of the positions 88, 89, 92, 93 or 97, all positions according to Kabat numbering.

**[0017]** In some embodiments, the VH CDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:15 and 58-68; the VH CDR2 comprises the amino acid sequence of SEQ ID NO:16; the VH CDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:17 and 69-75; the VL CDR1 comprises the amino acid sequence of SEQ ID NO:18; the VL CDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:19 and 76-84; or the VL CDR3 comprises the amino acid sequence of SEQ ID NO:20.

**[0018]** In some embodiments, the VH CDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:15 and 58-66; the VH CDR2 comprises the amino acid sequence of SEQ ID NO:16; the VH CDR3 comprises the amino acid sequence of SEQ ID NO:17; the VL CDR1 comprises the amino acid sequence of SEQ ID NO:18; the VL CDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:19 and 76-83; or the VL CDR3 comprises the amino acid sequence of SEQ ID NO:20.

**[0019]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:15, 16, 17, 18, 19 and 20; SEQ ID NO:58, 16, 17, 18, 76 and 20; SEQ ID NO:59, 16, 17, 18, 19 and 20; SEQ ID NO:66, 16, 17, 18, 19 and 20; SEQ ID NO:58, 16, 17, 18, 19 and 20; SEQ ID NO:15, 16, 17, 18, 77 and 20; SEQ ID NO:15, 16, 17, 18, 79 and 20; SEQ ID NO:66, 16, 17, 18, 77 and 20; SEQ ID NO:66, 16, 17, 18, 79 and 20; SEQ ID NO:66, 16, 17, 18, 76 and 20; SEQ ID NO:58, 16, 17, 18, 77 and 20; SEQ ID NO:58, 16, 17, 18, 79 and 20; SEQ ID NO:58, 16, 17, 18, 80 and 20; SEQ ID NO:58, 16, 17, 18, 78 and 20; SEQ ID NO:58, 16, 17, 18, 83 and 20; SEQ ID NO:58, 16, 17, 18, 82 and 20; SEQ ID NO:59, 16, 17, 18, 80 and 20; SEQ ID NO:59, 16, 17, 18, 79 and 20; SEQ ID NO:59, 16, 17, 18, 78 and 20; SEQ ID NO:59, 16, 17, 18, 76 and 20; SEQ ID NO:59, 16, 17, 18, 77 and 20; SEQ ID NO:60, 16, 17, 18, 80 and 20; SEQ ID NO:60, 16, 17, 18, 79 and 20; SEQ ID NO:60, 16, 17, 18, 76 and 20; SEQ ID NO:60, 16, 17, 18, 77 and 20; SEQ ID NO:61, 16, 17, 18, 77 and 20; SEQ ID NO:61, 16, 17, 18, 78 and 20;

SEQ ID NO:61, 16, 17, 18, 80 and 20; SEQ ID NO:61, 16, 17, 18, 79 and 20; SEQ ID NO:61, 16, 17, 18, 76 and 20; SEQ ID NO:62, 16, 17, 18, 78 and 20; SEQ ID NO:62, 16, 17, 18, 79 and 20; SEQ ID NO:62, 16, 17, 18, 76 and 20; SEQ ID NO:63, 16, 17, 18, 77 and 20; SEQ ID NO:63, 16, 17, 18, 78 and 20; SEQ ID NO:63, 16, 17, 18, 79 and 20; SEQ ID NO:63, 16, 17, 18, 76 and 20; SEQ ID NO:63, 16, 17, 18, 81 and 20; SEQ ID NO:64, 16, 17, 18, 78 and 20; SEQ ID NO:65, 16, 17, 18, 78 and 20; or SEQ ID NO:65, 16, 17, 18, 79 and 20.

**[0020]** In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:3 and 4, SEQ ID NO:41 and 42, SEQ ID NO:43 and 4, SEQ ID NO:44 and 4, SEQ ID NO:41 and 4, SEQ ID NO:3 and 45, SEQ ID NO:3 and 46, SEQ ID NO:44 and 45, SEQ ID NO:44 and 46, SEQ ID NO:44 and 42, SEQ ID NO:41 and 45, SEQ ID NO:41 and 46, SEQ ID NO:41 and 47, SEQ ID NO:41 and 48, SEQ ID NO:41 and 49, SEQ ID NO:41 and 50, SEQ ID NO:43 and 47, SEQ ID NO:43 and 46, SEQ ID NO:43 and 48, SEQ ID NO:43 and 42, SEQ ID NO:43 and 45, SEQ ID NO:51 and 47, SEQ ID NO:51 and 46, SEQ ID NO:51 and 42, SEQ ID NO:51 and 45, SEQ ID NO:52 and 45, SEQ ID NO:52 and 48, SEQ ID NO:52 and 47, SEQ ID NO:52 and 46, SEQ ID NO:52 and 42, SEQ ID NO:53 and 48, SEQ ID NO:53 and 46, SEQ ID NO:53 and 42, SEQ ID NO:54 and 45, SEQ ID NO:54 and 48, SEQ ID NO:54 and 46, SEQ ID NO:54 and 42, SEQ ID NO:54 and 55, SEQ ID NO:56 and 48, SEQ ID NO:57 and 48, or SEQ ID NO:57 and 46.

**[0021]** In some embodiments, the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3, and wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:21, 22, 23, 24, 25 and 26. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:5 and 6.

**[0022]** In some embodiments, the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3, and wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:27, 28, 29, 30, 31 and 32. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:7 and 8.

[0023] In some embodiments, antibody or antigen-binding fragment thereof comprises an IgG Fc fragment that is ADCC competent.

[0024] Also provided are methods and uses for treating cancer with the antibody or antigen-binding fragment thereof, polynucleotide(s) encoding them, or cells containing such polynucleotide(s).

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0025] FIG. 1A-B show the ATP binding, and competition binding of antibody R4P4F7 to ATP, ADP and AMP, without binding to ADO.

[0026] FIG. 2 presents structures obtained by X-ray crystallography showing hydrogen bond interactions between ATP and the heavy chain and light chain of antibody R4P4F7. Residues that formed hydrogen bond are shown as sticks; key hydrogen bonds are highlighted as dash lines.

[0027] FIG. 3 presents structures obtained by X-ray crystallography showing water-bridged hydrogen bond interactions between ATP and the heavy chain and light chain. Residues that formed hydrogen bond with waters are shown as sticks, and the water molecules are shown as spheres. Key hydrogen bonds are highlighted as dash lines.

[0028] FIG. 4 presents structures obtained by X-ray crystallography showing pi/amide stacking interactions between ATP and the heavy chain and light chain. Residues that formed pi-pi interactions and amide-pi interactions are shown as sticks.

[0029] FIG. 5 shows the binding of test antibodies to hCTLA4-expressing CHOK1 or regular CHOK1 cells.

[0030] FIG. 6 shows the binding of test antibodies to hCTLA4-expressing CHOK1 or regular CHOK1 cells.

[0031] FIG. 7 shows the binding of test antibodies to hCTLA4-expressing CHOK1 cells.

[0032] FIG. 8 shows the binding of P106A2 to hCTLA4-expressing CHOK1 cells at different concentrations of ATP, ADP or AMP.

[0033] FIG. 9 shows the binding of P106A7 to hCTLA4-expressing CHOK1 cells at different concentrations of ATP, ADP or AMP.

[0034] FIG. 10 shows the binding, measured by Octet BLI of P106A2 and P106A7 to human CTLA4 at different concentrations of ATP, ADP or AMP.

[0035] FIG. 11A-E show the binding of P106A2 with mutations in VH N32 and/or VL Q55 to hCTLA4-expressing CHOK1 cells in the presence or absence of ANP (ATP + ADP + AMP).

[0036] FIG. 12A-B show the binding of P106A2 with mutations in VH N32 and/or VL S53/Q55 to hCTLA4-expressing CHOK1 cells in the presence or absence of ANP.

[0037] FIG. 13 shows the binding of P106A2 with mutations in VH N32 and/or VL S53/Q55 (with or without ADCC-enhancing Fc mutations) to hCTLA4-expressing CHOK1 cells in the presence or absence of ANP.

[0038] FIG. 14 shows cross-reactivity of the mutant antibodies to human, cyno or mouse CTLA4 on CHOK1 cells.

[0039] FIG. 15 shows cross-reactivity of the mutant antibodies to human, cyno or mouse CTLA4 by ELISA.

[0040] FIG. 16 shows the ADCC activities of the test antibodies measured by the ADCC reporter assay.

[0041] FIG. 17 shows the activities of the test antibodies in blocking the binding between CTLA4 and its ligands CD80 and CD86.

[0042] FIG. 18 shows the impact of Fc variants on the antibody binding activity.

[0043] FIG. 19 shows the activities of the test antibodies in inhibiting tumor growth in MC38 model.

[0044] FIG. 20 shows the anti-FolR antibody FP010-F01 bound to FolR-positive cells in an ANP-dependent manner.

[0045] FIG. 21 shows that the anti-FolR antibody FP010-F01 bound to FolR in an ATP dose-dependent manner.

[0046] FIG. 22 shows that the anti-FolR antibody FP010-F01 found the FolR proteins from both human and cyno cells.

## DETAILED DESCRIPTION

### *Definitions*

[0047] It is to be noted that the term “a” or “an” entity refers to one or more of that entity; for example, “an antibody,” is understood to represent one or more antibodies. As such, the terms “a” (or “an”), “one or more,” and “at least one” can be used interchangeably herein.

[0048] As used herein, the term “polypeptide” is intended to encompass a singular “polypeptide” as well as plural “polypeptides,” and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term “polypeptide” refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, “protein,” “amino acid chain,” or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of “polypeptide,” and the term “polypeptide” may be used instead of, or interchangeably with any of these terms. The term “polypeptide” is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non- naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis.

[0049] The term “isolated” as used herein with respect to cells, nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the macromolecule. The term “isolated” as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an “isolated nucleic acid” is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term “isolated” is also used herein to refer to cells or

polypeptides which are isolated from other cellular proteins or tissues. Isolated polypeptides is meant to encompass both purified and recombinant polypeptides.

**[0050]** As used herein, the term “recombinant” as it pertains to polypeptides or polynucleotides intends a form of the polypeptide or polynucleotide that does not exist naturally, a non-limiting example of which can be created by combining polynucleotides or polypeptides that would not normally occur together.

**[0051]** “Homology” or “identity” or “similarity” refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An “unrelated” or “non-homologous” sequence shares less than 40% identity, though preferably less than 25% identity, with one of the sequences of the present disclosure.

**[0052]** A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 98 % or 99 %) of “sequence identity” to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Ausubel *et al.* eds. (2007) Current Protocols in Molecular Biology. Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Biologically equivalent polynucleotides are those having the above-noted specified percent homology and encoding a polypeptide having the same or similar biological activity.

**[0053]** The term “an equivalent nucleic acid or polynucleotide” refers to a nucleic acid having a nucleotide sequence having a certain degree of homology, or sequence identity, with the nucleotide sequence of the nucleic acid or complement thereof. A homolog of a double

stranded nucleic acid is intended to include nucleic acids having a nucleotide sequence which has a certain degree of homology with or with the complement thereof. In one aspect, homologs of nucleic acids are capable of hybridizing to the nucleic acid or complement thereof. Likewise, “an equivalent polypeptide” refers to a polypeptide having a certain degree of homology, or sequence identity, with the amino acid sequence of a reference polypeptide. In some aspects, the sequence identity is at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99%. In some aspects, the equivalent polypeptide or polynucleotide has one, two, three, four or five addition, deletion, substitution and their combinations thereof as compared to the reference polypeptide or polynucleotide. In some aspects, the equivalent sequence retains the activity (*e.g.*, epitope-binding) or structure (*e.g.*, salt-bridge) of the reference sequence.

**[0054]** Hybridization reactions can be performed under conditions of different “stringency”. In general, a low stringency hybridization reaction is carried out at about 40°C in about 10 x SSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50°C in about 6 x SSC, and a high stringency hybridization reaction is generally performed at about 60°C in about 1 x SSC. Hybridization reactions can also be performed under “physiological conditions” which is well known to one of skill in the art. A non-limiting example of a physiological condition is the temperature, ionic strength, pH and concentration of  $Mg^{2+}$  normally found in a cell.

**[0055]** A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term “polynucleotide sequence” is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching. The term “polymorphism” refers to the coexistence of more than one form of a gene or portion thereof. A portion of a gene of which there are at least two different forms, *i.e.*, two different nucleotide sequences, is referred to as a “polymorphic region of a gene”. A polymorphic region can be a single nucleotide, the identity of which differs in different alleles.

**[0056]** The terms “polynucleotide” and “oligonucleotide” are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples

of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, dsRNA, siRNA, miRNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this disclosure that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

**[0057]** The term “encode” as it is applied to polynucleotides refers to a polynucleotide which is said to “encode” a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

**[0058]** As used herein, an “antibody” or “antigen-binding polypeptide” refers to a polypeptide or a polypeptide complex that specifically recognizes and binds to an antigen. An antibody can be a whole antibody and any antigen binding fragment or a single chain thereof. Thus the term “antibody” includes any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule having biological activity of binding to the antigen. Examples of such include, but are not limited to a complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework (FR) region, or any portion thereof, or at least one portion of a binding protein.

**[0059]** The terms “antibody fragment” or “antigen-binding fragment”, as used herein, is a portion of an antibody such as F(ab')<sub>2</sub>, F(ab)<sub>2</sub>, Fab', Fab, Fv, scFv and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. The term “antibody fragment” includes aptamers, spiegelmers, and diabodies. The term “antibody fragment” also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex.

[0060] A “single-chain variable fragment” or “scFv” refers to a fusion protein of the variable regions of the heavy ( $V_H$ ) and light chains ( $V_L$ ) of immunoglobulins. In some aspects, the regions are connected with a short linker peptide of ten to about 25 amino acids. The linker can be rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the  $V_H$  with the C-terminus of the  $V_L$ , or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. ScFv molecules are known in the art and are described, *e.g.*, in US patent 5,892,019.

[0061] The term antibody encompasses various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon ( $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\delta$ ,  $\epsilon$ ) with some subclasses among them (*e.g.*,  $\gamma$  1-  $\gamma$ 4). It is the nature of this chain that determines the “class” of the antibody as IgG, IgM, IgA, IgE, or IgG, respectively. The immunoglobulin subclasses (isotypes) *e.g.*, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgG<sub>5</sub>, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant disclosure. All immunoglobulin classes are clearly within the scope of the present disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000. The four chains are typically joined by disulfide bonds in a “Y” configuration wherein the light chains bracket the heavy chains starting at the mouth of the “Y” and continuing through the variable region.

[0062] Antibodies, antigen-binding polypeptides, variants, or derivatives thereof of the disclosure include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, *e.g.*, Fab, Fab' and F(ab')<sub>2</sub>, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VK or VH domain, fragments produced by a Fab expression library, and anti- idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to LIGHT antibodies disclosed herein). Immunoglobulin or antibody

molecules of the disclosure can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

**[0063]** Light chains are classified as either kappa or lambda ( $K$ ,  $\lambda$ ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the “tail” portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

**[0064]** Both the light and heavy chains are divided into regions of structural and functional homology. The terms “constant” and “variable” are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VK) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CK) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen-binding site or amino- terminus of the antibody. The N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 and CK domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

**[0065]** As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VK domain and VH domain, or subset of the complementarity determining regions (CDRs), of an antibody combine to form the variable region that defines a three dimensional antigen-binding site. This quaternary antibody structure forms the antigen-binding site present at the end of each arm of the Y. More specifically, the antigen-binding site is defined by three CDRs on each of the VH and VK chains (*i.e.* CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2 and CDR-L3). In some instances, *e.g.*, certain immunoglobulin molecules derived from camelid species or engineered based on camelid immunoglobulins, a complete immunoglobulin molecule may consist of heavy chains only, with no light chains. *See, e.g.*, Hamers-Casterman *et al.*, *Nature* 363:446-448 (1993).

**[0066]** In naturally occurring antibodies, the six “complementarity determining regions” or “CDRs” present in each antigen-binding domain are short, non-contiguous sequences of amino

acids that are specifically positioned to form the antigen-binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen-binding domains, referred to as “framework” regions, show less inter-molecular variability. The framework regions largely adopt a  $\beta$ -sheet conformation and the CDRs form loops which connect, and in some cases form part of, the  $\beta$ -sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been precisely defined (*see* “Sequences of Proteins of Immunological Interest,” Kabat, E., *et al.*, U.S. Department of Health and Human Services, (1983); and Chothia and Lesk, *J. Mol. Biol.*, 196:901-917 (1987)).

[0067] In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term “complementarity determining region” (“CDR”) to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat *et al.*, U.S. Dept. of Health and Human Services, “Sequences of Proteins of Immunological Interest” (1983) and by Chothia *et al.*, *J. Mol. Biol.* 196:901-917 (1987), which are incorporated herein by reference in their entireties. The CDR definitions according to Kabat and Chothia include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth in the table below as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

|        | Kabat  | Chothia |
|--------|--------|---------|
| CDR-H1 | 31-35  | 26-32   |
| CDR-H2 | 50-65  | 52-58   |
| CDR-H3 | 95-102 | 95-102  |
| CDR-L1 | 24-34  | 26-32   |
| CDR-L2 | 50-56  | 50-52   |
| CDR-L3 | 89-97  | 91-96   |

**[0068]** Kabat *et al.* also defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of “Kabat numbering” to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, “Kabat numbering” refers to the numbering system set forth by Kabat *et al.*, U.S. Dept. of Health and Human Services, “Sequence of Proteins of Immunological Interest” (1983).

**[0069]** In addition to table above, the Kabat number system describes the CDR regions as follows: CDR-H1 begins at approximately amino acid 31 (*i.e.*, approximately 9 residues after the first cysteine residue), includes approximately 5-7 amino acids, and ends at the next tryptophan residue. CDR-H2 begins at the fifteenth residue after the end of CDR-H1, includes approximately 16-19 amino acids, and ends at the next arginine or lysine residue. CDR-H3 begins at approximately the thirty third amino acid residue after the end of CDR-H2; includes 3-25 amino acids; and ends at the sequence W-G-X-G, where X is any amino acid. CDR-L1 begins at approximately residue 24 (*i.e.*, following a cysteine residue); includes approximately 10-17 residues; and ends at the next tryptophan residue. CDR-L2 begins at approximately the sixteenth residue after the end of CDR-L1 and includes approximately 7 residues. CDR-L3 begins at approximately the thirty third residue after the end of CDR-L2 (*i.e.*, following a cysteine residue); includes approximately 7-11 residues and ends at the sequence F or W-G-X-G, where X is any amino acid.

**[0070]** Antibodies disclosed herein may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In another embodiment, the variable region may be chondrichthoid in origin (*e.g.*, from sharks).

**[0071]** As used herein, the term “heavy chain constant region” includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain constant region comprises at least one of: a CH1 domain, a hinge (*e.g.*, upper, middle, and/or

lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. For example, an antigen-binding polypeptide for use in the disclosure may comprise a polypeptide chain comprising a CH1 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain; a polypeptide chain comprising a CH1 domain and a CH3 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain, or a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, a CH2 domain, and a CH3 domain. In another embodiment, a polypeptide of the disclosure comprises a polypeptide chain comprising a CH3 domain. Further, an antibody for use in the disclosure may lack at least a portion of a CH2 domain (*e.g.*, all or part of a CH2 domain). As set forth above, it will be understood by one of ordinary skill in the art that the heavy chain constant region may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

**[0072]** The heavy chain constant region of an antibody disclosed herein may be derived from different immunoglobulin molecules. For example, a heavy chain constant region of a polypeptide may comprise a CH1 domain derived from an IgG<sub>1</sub> molecule and a hinge region derived from an IgG<sub>3</sub> molecule. In another example, a heavy chain constant region can comprise a hinge region derived, in part, from an IgG<sub>1</sub> molecule and, in part, from an IgG<sub>3</sub> molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG<sub>1</sub> molecule and, in part, from an IgG<sub>4</sub> molecule.

**[0073]** As used herein, the term “light chain constant region” includes amino acid sequences derived from antibody light chain. Preferably, the light chain constant region comprises at least one of a constant kappa domain or constant lambda domain.

**[0074]** A “light chain-heavy chain pair” refers to the collection of a light chain and heavy chain that can form a dimer through a disulfide bond between the CL domain of the light chain and the CH1 domain of the heavy chain.

**[0075]** As previously indicated, the subunit structures and three dimensional configuration of the constant regions of the various immunoglobulin classes are well known. As used herein, the term “VH domain” includes the amino terminal variable domain of an immunoglobulin heavy chain and the term “CH1 domain” includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

[0076] As used herein the term “CH2 domain” includes the portion of a heavy chain molecule that extends, *e.g.*, from about residue 244 to residue 360 of an antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering system; and residues 231-340, EU numbering system; *see* Kabat *et al.*, U.S. Dept. of Health and Human Services, “Sequences of Proteins of Immunological Interest” (1983). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It is also well documented that the CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 residues.

[0077] As used herein, the term “hinge region” includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen-binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux *et al.*, *J. Immunol* 161:4083 (1998)).

[0078] As used herein the term “disulfide bond” includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the CH1 and CK regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system).

[0079] As used herein, the term “chimeric antibody” will be held to mean any antibody wherein the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant disclosure) is obtained from a second species. In certain embodiments the target binding region or site will be from a non-human source (*e.g.* mouse or primate) and the constant region is human.

[0080] As used herein, “percent humanization” is calculated by determining the number of framework amino acid differences (*i.e.*, non-CDR difference) between the humanized domain and the germline domain, subtracting that number from the total number of amino acids, and then dividing that by the total number of amino acids and multiplying by 100.

[0081] By “specifically binds” or “has specificity to,” it is generally meant that an antibody binds to an epitope via its antigen-binding domain, and that the binding entails some

complementarity between the antigen-binding domain and the epitope. According to this definition, an antibody is said to “specifically bind” to an epitope when it binds to that epitope, via its antigen-binding domain more readily than it would bind to a random, unrelated epitope. The term “specificity” is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody “A” may be deemed to have a higher specificity for a given epitope than antibody “B,” or antibody “A” may be said to bind to epitope “C” with a higher specificity than it has for related epitope “D.”

**[0082]** As used herein, the terms “treat” or “treatment” refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the progression of cancer. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

**[0083]** By “subject” or “individual” or “animal” or “patient” or “mammal,” is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sport, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, cows, and so on.

**[0084]** As used herein, phrases such as “to a patient in need of treatment” or “a subject in need of treatment” includes subjects, such as mammalian subjects, that would benefit from administration of an antibody or composition of the present disclosure used, *e.g.*, for detection, for a diagnostic procedure and/or for treatment.

#### ***ANP-Dependent Antibodies and Fragments***

**[0085]** The instant inventors identified a new antibody derived from human germline sequences, R4P4F7, which is capable of binding to adenine nucleotides including ATP (adenosine triphosphate) ADP (adenosine diphosphate), and AMP (adenosine monophosphate) (collectively referred to as ANP). Mutation analysis revealed that certain residues in some

“extended” CDR regions are involved in such binding. For instance, R50, R52b, R97, R100c, and R100d of the VH and G91 and S96 of the VL are involved in forming hydrogen bonds (or water-bridged hydrogen bonds) with the ANP; Q90, F94 and P95 of the VL, on the other hand, are important for the structure of the VL CDR3 (all positions according to Kabat numbering).

**[0086]** Within these important residues, some do not allow any mutation (*e.g.*, R50 and R52b of the VH, and Q90, F94 and P95 of the VL) while some can be substituted with certain analogous amino acids. For instance, R97 can be substituted with E, K, T, S, Q, G, P, V, M, or W; R110c can be substituted with K, H, T, S, N, Q, G, A, P, V, L, M, or W; R110d can be substituted with T, L, or M; G91 can be substituted with T, A, S, L, Y, or H; and S96 can be substituted with G, T or A.

**[0087]** Understanding of the key residues in R4P4F7 in ANP-binding allowed the inventors to screen for antibody libraries to identify candidates that retained the ANP-binding activity but also had desired affinity to a tumor associated antigen (TAA) or immune checkpoint molecule, such as CTLA4. Subsequently, the inventors identified a large number of anti-CTLA4 antibodies, three of which were further studied extensively, including P106A2, P106A7, and P119H7.

**[0088]** As demonstrated in the experimental examples, these new antibodies bound to CTLA4 in an ANP-dependent manner. For instance, as shown in **FIG. 7**, in the absence of ANP, P106A2 and P119H7 almost had no observable binding to the human CTLA4 protein expressed on the surface of CHOK1 cells; in the presence of 300  $\mu$ M ANP, both of these antibodies bound to CTLA4 potently. By contrast, the benchmark antibody (BMK001-I) bound to CTLA4 at high affinity under both conditions.

**[0089]** Mutation analysis was also conducted to understand the involvement of amino acid residues in the CDR regions in term of CTLA4 binding, in particular at the N32 of the VH and S53 and Q55 of the VL (Kabat numbering). Many of the mutations further improved the binding to CTLA4. The results are summarized in **Table 17**.

**[0090]** In addition to targeting CTLA4, as shown in Example 10, the same screening process was also successfully used to identify anti-FolR antibodies that were ANP-dependent in binding to the target protein. The identified anti-FolR antibodies harbored the key residues for ANP binding and further validated the new screening technology and these residues.

[0091] In accordance with one embodiment of the present disclosure, provided is an antibody platform as the basis for identifying ANP-dependent antibodies having specificity to a target antigen, such as a tumor associated antigen or immune checkpoint molecule.

[0092] In one embodiment, an antibody capable of binding to ANP is provided. In some embodiments, the antibody (or antigen-binding fragment thereof) has a heavy chain variable region (VH) and a light chain variable region (VL), each including a CDR1, CDR2 and CDR3. In some embodiments, the VH and VL include the amino acids as shown in **Table A**.

**Table A. Preferred amino acids at positions**

| Position (Kabat) | Most preferred amino acids | Preferred amino acids                   |
|------------------|----------------------------|-----------------------------------------|
| VH 50            | R                          |                                         |
| VH 52b           | R                          |                                         |
| VH 97            | R                          | E, K, T, S, Q, G, P, V, M or W          |
| VH 100c          | R                          | K, H, T, S, N, Q, G, A, P, V, L, M or W |
| VH 100d          | R                          | T, L or M                               |
| VL 90            | Q                          |                                         |
| VL 91            | G                          | T, A, S, L, Y or H                      |
| VL 94            | F                          |                                         |
| VL 95            | P                          |                                         |
| VL 96            | S                          | G, T or A                               |

[0093] In some embodiments, the VH has an arginine at position 50 (Kabat numbering, same below). In some embodiments, the VH has an arginine at position 52b. In some embodiments, the VH has an arginine at position 97. In some embodiments, the VH has an amino acid selected from E, K, T, S, Q, G, P, V, M or W at position 97. In some embodiments, the VH has an arginine at position 100c. In some embodiments, the VH has an amino acid selected from K, H, T, S, N, Q, G, A, P, V, L, M or W at position 100c. In some embodiments, the VH has an arginine at position 100d. In some embodiments, the VH has an amino acid selected from T, L or M at position 100d.

[0094] In some embodiments, the VL has a glutamine at position 90. In some embodiments, the VL has a glycine at position 91. In some embodiments, the VL has an amino acid selected from T, A, S, L, Y or H at position 91. In some embodiments, the VL has a phenylalanine at position 94. In some embodiments, the VL has a proline at position 95. In some embodiments, the VL has a serine at position 96. In some embodiments, the VL has an amino acid selected from G, T or A at position 91.

[0095] In some embodiments, the VH includes a VH CDR1 having the amino acid sequence of SEQ ID NO:9, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative amino acid substitutions. In some embodiments, the VH includes a VH CDR1 having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:9.

[0096] In some embodiments, the VH includes a VH CDR2 having the amino acid sequence of SEQ ID NO:10, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative amino acid substitutions. In some embodiments, the VH includes a VH CDR2 having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:10. In some embodiments, the VH CDR2 at least has an arginine at position 50. In some embodiments, the VH CDR2 at least has an arginine at position 52b. In some embodiments, the VH CDR2 at least has arginine at positions 50 and 52b.

[0097] In some embodiments, the VH includes a VH CDR3 having the amino acid sequence of SEQ ID NO:11, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative amino acid substitutions. In some embodiments, the VH includes a VH CDR3 having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:11. In some embodiments, the VH CDR3 at least has an arginine at position 97. In some embodiments, the amino acid at position 97 is selected from E, K, T, S, Q, G, P, V, M or W. In some embodiments, the VH CDR3 at least has an arginine at position 100c. In some embodiments, the amino acid at position 100c is selected from K, H, T, S, N, Q, G, A, P, V, L, M or W. In some embodiments, the VH CDR3 at least has an arginine at position 100d. In some embodiments, the amino acid at position 100c is selected from T, L or M. In some embodiments, the VH CDR3 at least has arginine at positions 97, 100c and 100d. In some embodiments, the VH CDR3 at least has arginine or E, K, T, S, Q, G, P, V, M, or W at position 97, arginine or K, H, T, S, N, Q, G, A, P, V, L, M, or W at position 100c, and arginine or T, L, or M at position 100d.

[0098] In some embodiments, the VL includes a VL CDR1 having the amino acid sequence of SEQ ID NO:12, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative amino acid substitutions. In some embodiments, the VL includes a VL CDR1 having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:12.

[0099] In some embodiments, the VL includes a VL CDR2 having the amino acid sequence of SEQ ID NO:13, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative

amino acid substitutions. In some embodiments, the VL includes a VL CDR2 having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:13.

**[0100]** In some embodiments, the VL includes a VL CDR3 having the amino acid sequence of SEQ ID NO:14, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative amino acid substitutions. In some embodiments, the VL includes a VL CDR3 having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:14. In some embodiments, the VL CDR3 has at least has a glutamine at position 90. In some embodiments, the VL CDR3 has at least has a glycine at position 91. In some embodiments, the amino acid at position 91 is selected from T, A, S, L, Y or H. In some embodiments, the VL CDR3 has at least has a phenylalanine at position 94. In some embodiments, the VL CDR3 has at least has a proline at position 95. In some embodiments, the VL CDR3 has at least has a serine at position 96. In some embodiments, the amino acid at position 96 is selected from G, T or A. In some embodiments, the VL CDR3 has glutamine at position 90, glycine at position 91, phenylalanine at position 94, proline at position 95, and serine at position 96. In some embodiments, the VL CDR3 has glutamine at position 90, glycine or T, A, S, L, Y or H at position 91, phenylalanine at position 94, proline at position 95, and serine or G, T or A at position 96.

**[0101]** In some embodiments, the antibody includes the same framework regions as R4P4F7. In some embodiments, the antibody's framework regions, collectively, have at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to the framework regions of R4P4F7.

**[0102]** In some embodiments, the antibody includes a VH framework 1 (FM1, positions 1-25 Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:33. In some embodiments, the VH FM1 has the amino acid sequence of SEQ ID NO:33, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0103]** In some embodiments, the antibody includes a VH framework 2 (FM2, positions 36-49 Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:34. In some embodiments, the VH FM2 has the amino acid sequence of SEQ ID NO:34, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0104]** In some embodiments, the antibody includes a VH framework 3 (FM3, positions 66-94, Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to

SEQ ID NO:35. In some embodiments, the VH FM3 has the amino acid sequence of SEQ ID NO:35, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0105]** In some embodiments, the antibody includes a VH framework 4 (FM4, positions 103-113, Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:36. In some embodiments, the VH FM4 has the amino acid sequence of SEQ ID NO:36, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0106]** In some embodiments, the antibody includes a VL framework 1 (FM1, positions 1-22, Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:37. In some embodiments, the VL FM1 has the amino acid sequence of SEQ ID NO:37, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0107]** In some embodiments, the antibody includes a VL framework 2 (FM2, positions 36-48, Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:38. In some embodiments, the VL FM2 has the amino acid sequence of SEQ ID NO:38, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0108]** In some embodiments, the antibody includes a VL framework 3 (FM3, positions 58-87, Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:39. In some embodiments, the VL FM3 has the amino acid sequence of SEQ ID NO:39, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0109]** In some embodiments, the antibody includes a VL framework 4 (FM4, positions 98-107, Kabat numbering) having at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:40. In some embodiments, the VL FM3 has the amino acid sequence of SEQ ID NO:40, optionally with 1, 2, 3, 4 or 5 amino acid substitutions, preferably conservative substitutions.

**[0110]** In some embodiments, the VH includes the amino acid sequence of SEQ ID NO:1. In some embodiments, the VH has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity

to SEQ ID NO:1. In some embodiments, the VH has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:1, and includes arginine at positions 50, 52b, 97, 100c and 100d. In some embodiments, the VH has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:1, and includes arginine at position 50, arginine at position 52b, arginine or any one of E, K, T, S, Q, G, P, V, M or W at position 97, arginine or any one of K, H, T, S, N, Q, G, A, P, V, L, M or W at position 100c and arginine or any one of T, L or M at position 100d.

[0111] In some embodiments, the VL includes the amino acid sequence of SEQ ID NO:2. In some embodiments, the VL has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:2. In some embodiments, the VL has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:2, and includes glutamine at position 90, glycine at position 91, phenylalanine at position 94, proline at position 95, and serine at position 96. In some embodiments, the VL has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:2, and includes glutamine at position 90, glycine or any one of T, A, S, L, Y or H at position 91, phenylalanine at position 94, proline at position 95, and serine or any one of G, T or A at position 96.

[0112] In some embodiments, the antibody (or antigen-binding fragment thereof), in addition to binding to ANP, has specificity to an antigen that is a tumor associated antigen or immune checkpoint molecule. In some embodiments, the antigen is, without limitation, CD3, CD47, PD1, PD-L1, LAG3, TIM3, CTLA4, VISTA, CSFR1, A2AR, CD73, CD39, CD40, CEA, HER2, CMET, 4-1BB, OX40, SIRPA CD16, CD28, ICOS, CTLA4, BTLA, TIGIT, HVEM, CD27, VEGFR, or VEGF.

#### ***ANP-Dependent Antibody against CTLA4***

[0113] Based on the platform as described above, the instant inventors were able to identify antibodies having specificity to the CTLA4 protein. These anti-CTLA4 antibodies were considerably more active in the presence of ANP and thus have greatly improved therapeutic index as compared to conventional anti-CTLA4 antibodies such as ipilimumab.

[0114] Accordingly, one embodiment of the present disclosure provides an anti-CTLA4 antibody, which includes a heavy chain variable region (VH) and a light chain variable region (VL), each including a CDR1, CDR2 and CDR3.

[0115] In some embodiments, the VH CDR1 includes the amino acid sequence of SEQ ID NO:15, the VH CDR2 includes the amino acid sequence of SEQ ID NO:16, and the VH CDR3 includes the amino acid sequence of SEQ ID NO:17. In some embodiments, the VL CDR1 includes the amino acid sequence of SEQ ID NO:18, the VL CDR2 includes the amino acid sequence of SEQ ID NO:19, and the VL CDR3 includes the amino acid sequence of SEQ ID NO:20.

[0116] Mutation analysis showed that mutations at certain positions of the CDRs can further enhance the activity of the antibody. For instance, the N32 of VH CDR1 (Kabat numbering) can be substituted with L, E, F, A, M, W, Q, Y, H, K, or R; the S53 of VL CDR2 can be substituted with G; and the Q55 can be substituted with M, F, Y or L. Certain other positions of the CDRs, it is to be appreciated, can also be substituted, preferably with conservative mutations, to preserve or improve the activity.

[0117] One embodiment of the present disclosure, therefore, provides an anti-CTLA4 antibody, in which the VH CDR1 includes the amino acid sequence of SEQ ID NO:15, or SEQ ID NO:15 in which position 32 is an amino acid selected from L, E, F, A, M, W, Q, Y, H, K or R; the VH CDR2 includes the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having at least 60%, 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:16; the VH CDR3 includes the amino acid sequence of SEQ ID NO:17, or SEQ ID NO:17 with an amino acid substitution at any one of the positions 95, 99, 100, 100a, 100b, 100e, 100f, 101, or 102; the VL CDR1 includes the amino acid sequence of SEQ ID NO:18, or SEQ ID NO:18 with an amino acid substitution; the VL CDR2 includes the amino acid sequence of SEQ ID NO:19, or SEQ ID NO:19 in which position 53 is G, or position 55 is M, F, Y or L; and the VL CDR3 includes the amino acid sequence of SEQ ID NO:20, or SEQ ID NO:20 an amino acid substitution at any one of the positions 88, 89, 92, 93 or 97, all positions according to Kabat numbering.

[0118] One embodiment of the present disclosure, therefore, provides an anti-CTLA4 antibody, in which the VH CDR1 includes the amino acid sequence of SEQ ID NO:15, or SEQ ID NO:15 in which position 32 is an amino acid selected from L, E, F, A, M, W, Q, Y, H, K or R; the VH CDR2 includes the amino acid sequence of SEQ ID NO:16; the VH CDR3 includes the amino acid sequence of SEQ ID NO:17; the VL CDR1 includes the amino acid sequence of SEQ ID NO:18; the VL CDR2 includes the amino acid sequence of SEQ ID NO:19, or SEQ ID NO:19

in which position 53 is G, or position 55 is M, F, Y or L; and the VL CDR3 includes the amino acid sequence of SEQ ID NO:20, all positions according to Kabat numbering.

**[0119]** In some embodiments, the VH CDR1 includes an amino acid sequence selected from the group consisting of SEQ ID NO:15 and 58-68. In some embodiments, the VH CDR2 includes the amino acid sequence of SEQ ID NO:16. In some embodiments, the VH CDR3 includes an amino acid sequence selected from the group consisting of SEQ ID NO:17 and 69-75. In some embodiments, the VL CDR1 includes the amino acid sequence of SEQ ID NO:18. In some embodiments, the VL CDR2 includes an amino acid sequence selected from the group consisting of SEQ ID NO:19 and 76-84. In some embodiments, the VL CDR3 includes the amino acid sequence of SEQ ID NO:20.

**[0120]** In some embodiments, the VH CDR1 includes an amino acid sequence selected from the group consisting of SEQ ID NO:15 and 58-66. In some embodiments, the VH CDR2 includes the amino acid sequence of SEQ ID NO:16. In some embodiments, the VH CDR3 includes the amino acid sequence of SEQ ID NO:17. In some embodiments, the VL CDR1 includes the amino acid sequence of SEQ ID NO:18. In some embodiments, the VL CDR2 includes an amino acid sequence selected from the group consisting of SEQ ID NO:19 and 76-83. In some embodiments, the VL CDR3 includes the amino acid sequence of SEQ ID NO:20.

**[0121]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:15, 16, 17, 18, 19 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 76 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:59, 16, 17, 18, 19 and 20.

**[0122]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:66, 16, 17, 18, 19 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 19 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:15, 16, 17, 18, 77 and 20.

[0123] In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:15, 16, 17, 18, 79 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:66, 16, 17, 18, 77 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:66, 16, 17, 18, 79 and 20.

[0124] In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:66, 16, 17, 18, 76 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 77 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 79 and 20.

[0125] In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 80 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 78 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 83 and 20.

[0126] In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 82 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:59, 16, 17, 18, 80 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:59, 16, 17, 18, 79 and 20.

[0127] In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:59, 16, 17, 18, 78 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2

and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:59, 16, 17, 18, 76 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:59, 16, 17, 18, 77 and 20.

**[0128]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:60, 16, 17, 18, 80 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:60, 16, 17, 18, 79 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:60, 16, 17, 18, 76 and 20.

**[0129]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:60, 16, 17, 18, 77 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:61, 16, 17, 18, 77 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:61, 16, 17, 18, 78 and 20.

**[0130]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:61, 16, 17, 18, 80 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:61, 16, 17, 18, 79 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:61, 16, 17, 18, 76 and 20.

**[0131]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:62, 16, 17, 18, 78 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:62, 16, 17, 18, 79 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2

and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:62, 16, 17, 18, 76 and 20.

**[0132]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:63, 16, 17, 18, 77 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:63, 16, 17, 18, 78 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:63, 16, 17, 18, 79 and 20.

**[0133]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:63, 16, 17, 18, 76 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:63, 16, 17, 18, 81 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:64, 16, 17, 18, 78 and 20.

**[0134]** In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:65, 16, 17, 18, 78 and 20. In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 include, respectively, the amino acid sequences of SEQ ID NO:65, 16, 17, 18, 79 and 20.

**[0135]** In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:3 and 4. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 42. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:43 and 4.

**[0136]** In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:44 and 4. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 4. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:3 and 45.

[0137] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:3 and 46. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:44 and 45. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:44 and 46.

[0138] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:44 and 42. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 45. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 46.

[0139] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 47. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 48. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 49.

[0140] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 50. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:43 and 47. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:43 and 46.

[0141] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:43 and 48. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:43 and 42. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:43 and 45.

[0142] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:51 and 47. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:51 and 46. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:51 and 42.

[0143] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:51 and 45. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:52 and 45. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:52 and 48.

[0144] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:52 and 47. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:52 and 46. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:52 and 42.

[0145] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:53 and 48. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:53 and 46. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:53 and 42.

[0146] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:54 and 45. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:54 and 48. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:54 and 46.

[0147] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:54 and 42. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:54 and 55. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:56 and 48.

[0148] In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:57 and 48. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:57 and 46.

[0149] In some embodiments, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 76 and 20. In some embodiments, the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 42.

[0150] In some embodiments, the VH CDR1 includes the amino acid sequence of SEQ ID NO:21, the VH CDR2 includes the amino acid sequence of SEQ ID NO:22, and the VH CDR3 includes the amino acid sequence of SEQ ID NO:23. In some embodiments, the VL CDR1 includes the amino acid sequence of SEQ ID NO:24, the VL CDR2 includes the amino acid sequence of SEQ ID NO:24, and the VL CDR3 includes the amino acid sequence of SEQ ID NO:26.

[0151] In some embodiment, the VH includes the amino acid sequence of SEQ ID NO:5 or has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:5 while having the CDR sequences as recited above. In some embodiment, the VL includes the amino acid sequence of SEQ ID NO:6 or has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:6 while having the CDR sequences as recited above.

[0152] In some embodiments, the VH CDR1 includes the amino acid sequence of SEQ ID NO:27, the VH CDR2 includes the amino acid sequence of SEQ ID NO:28, and the VH CDR3 includes the amino acid sequence of SEQ ID NO:29. In some embodiments, the VL CDR1 includes the amino acid sequence of SEQ ID NO:30, the VL CDR2 includes the amino acid sequence of SEQ ID NO:31, and the VL CDR3 includes the amino acid sequence of SEQ ID NO:32.

[0153] In some embodiment, the VH includes the amino acid sequence of SEQ ID NO:7 or has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:7 while having the CDR sequences as recited above. In some embodiment, the VL includes the amino acid sequence of SEQ ID NO:8 or has at least 60%, 70%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:8 while having the CDR sequences as recited above.

[0154] Also provided, in some embodiments, are antibodies and antigen-binding fragments that include CDR sequences derived from the presently disclosed CDR sequences, with one, two or three amino acid substitutions, deletions, and/or additions.

[0155] A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in an immunoglobulin polypeptide is preferably replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[0156] In some embodiments, the antibody is ADCC competent. ADCC activities of an antibody can be enhanced with methods known in the art, such as mutations to the human IgG Fc fragment, including those demonstrated in the experimental examples.

#### *Antibody-Drug Conjugates*

[0157] In some embodiments, the antibodies or fragments may be conjugated to therapeutic agents, prodrugs, peptides, proteins, enzymes, viruses, lipids, biological response modifiers, pharmaceutical agents, or PEG.

[0158] In one embodiment, the antibodies or fragments of the disclosure are covalently attached to a drug moiety. The drug moiety may be, or be modified to include, a group reactive with a conjugation point on the antibody. For example, a drug moiety can be attached by alkylation (*e.g.*, at the epsilon-amino group lysines or the N-terminus of antibodies), reductive amination of oxidized carbohydrate, transesterification between hydroxyl and carboxyl groups, amidation at amino groups or carboxyl groups, and conjugation to thiols.

[0159] In some embodiments, the number of drug moieties, *p*, conjugated per antibody molecule ranges from an average of 1 to 8; 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2. In some embodiments, *p* ranges from an average of 2 to 8, 2 to 7, 2 to 6, 2 to 5, 2 to 4 or 2 to 3. In other embodiments, *p* is an average of 1, 2, 3, 4, 5, 6, 7 or 8. In some embodiments, *p* ranges from an average of about 1 to about 20, about 1 to about 10, about 2 to about 10, about 2 to about 9, about 1 to about 8, about 1 to about 7, about 1 to about 6, about 1 to about 5, about 1 to about 4, about 1 to about 3, or about 1 to about 2. In some embodiments, *p* ranges from about 2 to about 8, about 2 to about 7, about 2 to about 6, about 2 to about 5, about 2 to about 4 or about 2 to about 3.

[0160] For example, when chemical activation of the protein results in formation of free thiol groups, the protein may be conjugated with a sulfhydryl reactive agent. In one aspect, the agent is one which is substantially specific for free thiol groups. Such agents include, for example, maleimide, haloacetamides (*e.g.*, iodo, bromo or chloro), haloesters (*e.g.*, iodo, bromo or chloro), halomethyl ketones (*e.g.*, iodo, bromo or chloro), benzylic halides (*e.g.*, iodide, bromide or chloride), vinyl sulfone and pyridylthio.

[0161] The drug can be linked to the antibody or fragment by a linker. Suitable linkers include, for example, cleavable and non-cleavable linkers. A cleavable linker is typically susceptible to

cleavage under intracellular conditions. Suitable cleavable linkers include, for example, a peptide linker cleavable by an intracellular protease, such as lysosomal protease or an endosomal protease. In exemplary embodiments, the linker can be a dipeptide linker, such as a valine-citrulline (val-cit), a phenylalanine-lysine (phe-lys) linker, or maleimidocapronic-valine-citrulline-p-aminobenzyloxycarbonyl (mc-Val-Cit-PABA) linker. Another linker is Sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (smcc). Sulfo-smcc conjugation occurs via a maleimide group which reacts with sulfhydryls (thiols, —SH), while its Sulfo-NHS ester is reactive toward primary amines (as found in Lysine and the protein or peptide N-terminus). Yet another linker is maleimidocaproyl (mc). Other suitable linkers include linkers hydrolyzable at a specific pH or a pH range, such as a hydrazone linker. Additional suitable cleavable linkers include disulfide linkers. The linker may be covalently bound to the antibody to such an extent that the antibody must be degraded intracellularly in order for the drug to be released *e.g.*, the mc linker and the like.

**[0162]** A linker can include a group for linkage to the antibody. For example, linker can include an amino, hydroxyl, carboxyl or sulfhydryl reactive groups (*e.g.*, maleimide, haloacetamides (*e.g.*, iodo, bromo or chloro), haloesters (*e.g.*, iodo, bromo or chloro), halomethyl ketones (*e.g.*, iodo, bromo or chloro), benzylic halides (*e.g.*, iodide, bromide or chloride), vinyl sulfone and pyridylthio).

**[0163]** In some embodiments, the drug moiety is a cytotoxic or cytostatic agent, an immunosuppressive agent, a radioisotope, a toxin, or the like. The conjugate can be used for inhibiting the multiplication of a tumor cell or cancer cell, causing apoptosis in a tumor or cancer cell, or for treating cancer in a patient. The conjugate can be used accordingly in a variety of settings for the treatment of animal cancers. The conjugate can be used to deliver a drug to a tumor cell or cancer cell. Without being bound by theory, in some embodiments, the conjugate binds to or associates with a cancer cell expressing CTLA4, and the conjugate and/or drug can be taken up inside a tumor cell or cancer cell through receptor-mediated endocytosis.

**[0164]** In some embodiments, the drug moiety is a maytansinoid or an auristatin. In some embodiments, the drug moiety is a macrocyclic ketone analogue such as eribulin. In some embodiments, the drug moiety is a topoisomerase inhibitor, such as exatecan and exatecan derivatives.

[0165] Once inside the cell, one or more specific peptide sequences within the conjugate (*e.g.*, in a linker) are hydrolytically cleaved by one or more tumor-cell or cancer-cell-associated proteases, resulting in release of the drug. The released drug is then free to migrate within the cell and induce cytotoxic or cytostatic or other activities. In some embodiments, the drug is cleaved from the antibody outside the tumor cell or cancer cell, and the drug subsequently penetrates the cell, or acts at the cell surface.

[0166] Examples of drug moieties or payloads are selected from the group consisting of eribulin (2-(3-Amino-2-hydroxypropyl)hexacosahydro-3-methoxy-26-methyl-20,27-bis(methylene)11,15-18,21-24,28-triepoxy-7,9-ethano-12,15-methano-9H,15H-furo(3,2-i)furo(2',3'-5,6)pyrano(4,3-b)(1,4)dioxacyclopentacosin-5-(4H)-one), DM1 (maytansine, N2'-deacetyl-N2'-(3-mercapto-1-oxopropyl)- or N2'-deacetyl-N2'-(3-mercapto-1-oxopropyl)-maytansine), mc-MMAD (6-maleimidocaproyl-monomethylauristatin-D or N-methyl-L-valyl-N-[(1S,2R)-2-methoxy-4-[(2S)-2-[(1R,2R)-1-methoxy-2-methyl-3-oxo-3-[(1S)-2-phenyl-1-(2-thiazolyl)ethyl]amino]propyl]-1-pyrrolidinyl]-1-[(1S)-1-methylpropyl]-4-oxobutyl]-N-methyl-(9Cl)-L-valinamide), mc-MMAF (maleimidocaproyl-monomethylauristatin F or N-[6-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-1-oxohexyl]-N-methyl-L-valyl-L-valyl-(3R,4S,5S)-3-methoxy-5-methyl-4-(methylamino)heptanoyl-( $\alpha$ R,  $\beta$ R,2S)- $\beta$ -methoxy- $\alpha$ -methyl-2-pyrrolidinepropanoyl-L-phenylalanine) and mc-Val-Cit-PABA-MMAE (6-maleimidocaproyl-ValcCit-(p-aminobenzyloxycarbonyl)-monomethylauristatin E or N-[[[4-[N-[6-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-1-oxohexyl]-L-valyl-N5-(aminocarbonyl)-L-ornithyl]amino]phenyl]methoxy]carbonyl]-N-methyl-L-valyl-N-[(1S,2R)-4-[(2S)-2-[(1R,2R)-3-[(1R,2S)-2-hydroxy-1-methyl-2-phenylethyl]amino]-1-methoxy-2-methyl-3-oxopropyl]-1-pyrrolidinyl]-2-methoxy-1-[(1S)-1-methylpropyl]-4-oxobutyl]-N-methyl-L-valinamide). DM1 is a derivative of the tubulin inhibitor maytansine while MMAD, MMAE, and MMAF are auristatin derivatives. In some embodiments, the drug moiety is selected from the group consisting of mc-MMAF and mc-Val-Cit-PABA-MMAE.

[0167] The antibodies or fragments may be conjugated or fused to a therapeutic agent, which may include detectable labels such as radioactive labels, an immunomodulator, a hormone, an enzyme, an oligonucleotide, a photoactive therapeutic or diagnostic agent, a cytotoxic agent, which may be a drug or a toxin, an ultrasound enhancing agent, a non-radioactive label, a combination thereof and other such agents known in the art.

[0168] The antibodies can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antigen-binding polypeptide is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, thionin acridinium ester, imidazole, acridinium salt and oxalate ester.

[0169] The antibodies can also be detectably labeled using fluorescence emitting metals such as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). Techniques for conjugating various moieties to an antibody are well known, *see, e.g.*, Arnon *et al.*, “Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy”, in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. (1985); Hellstrom *et al.*, “Antibodies For Drug Delivery”, in *Controlled Drug Delivery* (2nd Ed.), Robinson *et al.*, (eds.), Marcel Dekker, Inc., pp. 623-53 (1987); Thorpe, “Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review”, in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et al.* (eds.), pp. 475-506 (1985); “Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy”, in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), Academic Press pp. 303-16 (1985), and Thorpe *et al.*, “The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates”, *Immunol. Rev.* (52:119-58 (1982)).

### ***Multi-functional Molecules***

[0170] Multi-functional molecules that include an antibody or antigen-binding fragment specific to CTLA4, such as those disclosed herein, and one or more antibody or antigen-binding fragment having specificity to a second antigen.

[0171] In some embodiments, the second antigen is a protein expressed on an immune cell, such as a T cell, a B cell, a monocyte, a macrophage, a neutrophil, a dendritic cell, a phagocyte, a natural killer cell, an eosinophil, a basophil, and a mast cell.

[0172] In some embodiments, the second antigen is CD3, CD47, PD1, PD-L1, LAG3, TIM3, CTLA4, VISTA, CSFR1, A2AR, CD73, CD39, CD40, CEA, HER2, CMET, 4-1BB, OX40, SIRPA CD16, CD28, ICOS, CTLA4, BTLA, TIGIT, HVEM, CD27, VEGFR, or VEGF.

[0173] Different formats of bispecific antibodies are also provided. In some embodiments, each of the anti-CTLA4 fragment and the second fragment each is independently selected from a Fab fragment, a single-chain variable fragment (scFv), or a single-domain antibody. In some embodiments, the bispecific antibody further includes a Fc fragment.

[0174] Bifunctional molecules that include not just antibody or antigen binding fragment are also provided. As a tumor antigen targeting molecule, an antibody or antigen-binding fragment specific to CTLA4, such as those described here, can be combined with an immune cytokine or ligand optionally through a peptide linker. The linked immune cytokines or ligands include, but not limited to, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, GM-CSF, TNF- $\alpha$ , CD40L, OX40L, CD27L, CD30L, 4-1BBL, LIGHT and GITRL. Such bi-functional molecules can combine the immune checkpoint blocking effect with tumor site local immune modulation.

#### ***Chimeric Antigen Receptors***

[0175] Also provided, in one embodiment, is a chimeric antigen receptor (CAR) that includes the antibody or fragment thereof of the present disclosure as a targeting unit. In some embodiments, the CAR includes an antibody or fragment thereof of the present disclosure, a transmembrane domain, a costimulatory domain, and a CD3 $\xi$  intracellular domain.

[0176] A transmembrane domain can be designed to be fused to the extracellular domain which includes the antibody or fragment, optionally through a hinge domain. It can similarly be fused to an intracellular domain, such as a costimulatory domain. In some embodiments, the transmembrane domain can include the natural transmembrane region of a costimulatory domain (*e.g.*, the TM region of a CD28T or 4-1BB employed as a costimulatory domain) or the natural transmembrane domain of a hinge region (*e.g.*, the TM region of a CD8 alpha or CD28T employed as a hinge domain).

[0177] In some embodiments, the transmembrane domain can include a sequence that spans a cell membrane, but extends into the cytoplasm of a cell and/or into the extracellular space. For example, a transmembrane can include a membrane-spanning sequence which itself can further include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids that extend into the cytoplasm of a cell, and/or the extracellular space. Thus, a transmembrane domain includes a membrane-spanning region, yet can further comprise an amino acid(s) that extend beyond the internal or external

surface of the membrane itself; such sequences can still be considered to be a “transmembrane domain”.

**[0178]** In some embodiments, the transmembrane domain is fused to the cytoplasmic domain through a short linker. Optionally, the short peptide or polypeptide linker, preferably between 2 and 10 amino acids in length can form the linkage between the transmembrane domain and a proximal cytoplasmic signaling domain of the chimeric receptor. A glycine-serine doublet (GS), glycine-serine-glycine triplet (GSG), or alanine- alanine-alanine triplet (AAA) provides a suitable linker.

**[0179]** In some embodiments, the CAR further includes a costimulatory domain. In some embodiments, the costimulatory domain is positioned between the transmembrane domain and an activating domain. Example costimulatory domains include, but are not limited to, CD2, CD3 delta, CD3 epsilon, CD3 gamma, CD4, CD7, CD8a, CD8 , CD11a (ITGAL), CD11b (ITGAM), CD11c (ITGAX), CD11d (ITGAD), CD18 (ITGB2), CD19 (B4), CD27 (TFRSF7), CD28, CD28T, CD29 (ITGB1), CD30 (TNFRSF8), CD40 (TNFRSF5), CD48 (SLAMF2), CD49a (ITGA1), CD49d (ITGA4), CD49f (ITGA6), CD66a (CEACAM1), CD66b (CEACAM8), CD66c (CEACAM6), CD66d (CEACAM3), CD66e (CEACAM5), CD69 (CLEC2), CD79A (B-cell antigen receptor complex-associated alpha chain), CD79B (B-cell antigen receptor complex- associated beta chain), CD84 (SLAMF5), CD96 (Tactile), CD 100 (SEMA4D), CD 103 (ITGAE), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD158A (KIR2DL1), CD158B1 (KIR2DL2), CD158B2 (KIR2DL3), CD158C (KIR3DP1), CD158D (KIR2DL4), CD158F1 (KIR2DL5A), CD158F2 (KIR2DL5B), CD158K (KTR3DL2), CD160 (BY55), CD162 (SELPLG), CD226 (DNAM1), CD229 (SLAMF3), CD244 (SLAMF4), CD247 (CD3-zeta), CD258 (LIGHT), CD268 (BAFFR), CD270 (TFSF14), CD272 (BTLA), CD276 (B7-H3), CD279 (PD-1), CD314 (KG2D), CD319 (SLAMF7), CD335 (K-p46), CD336 (K-p44), CD337 (K-p30), CD352 (SLAMF6), CD353 (SLAMF8), CD355 (CRTAM), CD357 (TNFRSF 18), inducible T cell co-stimulator (ICOS), LFA-1 (CD 11a/CD 18), KG2C, DAP- 10, ICAM-1, Kp80 (KLRF1), IL-2R beta, IL-2R gamma, IL-7R alpha, LFA-1, SLAMF9, LAT, GADS (GrpL), SLP-76 (LCP2), PAG1/CBP, a CD83 ligand, Fc gamma receptor, MHC class 1 molecule, MHC class 2 molecule, a TNF receptor protein, an immunoglobulin protein, a cytokine receptor, an integrin, activating NK cell receptors, a Toll ligand receptor, and fragments or combinations thereof.

[0180] In some embodiments, the cytoplasmic portion of the CAR also includes a signaling/activation domain. In one embodiment, the signaling/activation domain is the CD3 $\xi$  domain, or is an amino acid sequence having at least about 80%, 85%, 90%, 95%, 98% or 99% sequence identity to the CD3 $\xi$  domain.

***Polynucleotides, mRNA, and Methods of Expressing or Preparing Antibodies***

[0181] The present disclosure also provides polynucleotides or nucleic acid molecules encoding the antibodies, variants or derivatives thereof of the disclosure, or the CAR. The polynucleotides of the present disclosure may encode the entire heavy and light chain variable regions of the antigen-binding polypeptides, variants or derivatives thereof on the same polynucleotide molecule or on separate polynucleotide molecules. Additionally, the polynucleotides of the present disclosure may encode portions of the heavy and light chain variable regions of the antigen-binding polypeptides, variants or derivatives thereof on the same polynucleotide molecule or on separate polynucleotide molecules.

[0182] In some embodiments, the polynucleotide is an mRNA molecule. In some embodiments, the mRNA can be introduced into a target cell for expressing the antibody or fragment thereof.

[0183] mRNAs may be synthesized according to any of a variety of known methods. For example, the mRNAs may be synthesized via *in vitro* transcription (IVT). Briefly, IVT is typically performed with a linear or circular DNA template containing a promoter, a pool of ribonucleotide triphosphates, a buffer system that may include DTT and magnesium ions, and an appropriate RNA polymerase (*e.g.*, T3, T7 or SP6 RNA polymerase), DNase I, pyrophosphatase, and/or RNase inhibitor. The exact conditions will vary according to the specific application.

[0184] In some embodiments, for the preparation of antibody-coding mRNA, a DNA template is transcribed *in vitro*. A suitable DNA template typically has a promoter, for example a T3, T7 or SP6 promoter, for *in vitro* transcription, followed by desired nucleotide sequence for desired antibody encoding (*e.g.*, heavy chain or light chain encoding) mRNA and a termination signal.

[0185] Desired antibody encoding (*e.g.*, heavy chain or light chain encoding) mRNA sequence may be determined and incorporated into a DNA template using standard methods. For example, starting from a desired amino acid sequence (*e.g.*, a desired heavy chain or light chain

sequence), a virtual reverse translation is carried out based on the degenerated genetic code. Optimization algorithms may then be used for selection of suitable codons. Typically, the G/C content can be optimized to achieve the highest possible G/C content on one hand, taking into the best possible account the frequency of the tRNAs according to codon usage on the other hand. The optimized RNA sequence can be established and displayed, for example, with the aid of an appropriate display device and compared with the original (wild-type) sequence. A secondary structure can also be analyzed to calculate stabilizing and destabilizing properties or, respectively, regions of the RNA.

[0186] The mRNA may be synthesized as unmodified or modified mRNA. Typically, mRNAs are modified to enhance stability. Modifications of mRNA can include, for example, modifications of the nucleotides of the RNA. A modified mRNA can thus include, for example, backbone modifications, sugar modifications or base modifications. In some embodiments, antibody encoding mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) may be synthesized from naturally occurring nucleotides and/or nucleotide analogues (modified nucleotides) including, but not limited to, purines (adenine (A), guanine (G)) or pyrimidines (thymine (T), cytosine (C), uracil (U)), and as modified nucleotides analogues or derivatives of purines and pyrimidines, such as *e.g.* 1-methyl-adenine, 2-methyl-adenine, 2-methylthio-N-6-isopentenyl-adenine, N6-methyl-adenine, N6-isopentenyl-adenine, 2-thio-cytosine, 3-methyl-cytosine, 4-acetyl-cytosine, 5-methyl-cytosine, 2,6-diaminopurine, 1-methyl-guanine, 2-methyl-guanine, 2,2-dimethyl-guanine, 7-methyl-guanine, inosine, 1-methyl-inosine, pseudouracil (5-uracil), dihydro-uracil, 2-thio-uracil, 4-thio-uracil, 5-carboxymethylaminomethyl-2-thio-uracil, 5-(carboxyhydroxymethyl)-uracil, 5-fluoro-uracil, 5-bromo-uracil, 5-carboxymethylaminomethyl-uracil, 5-methyl-2-thio-uracil, 5-methyl-uracil, N-uracil-5-oxyacetic acid methyl ester, 5-methylaminomethyl-uracil, 5-methoxyaminomethyl-2-thio-uracil, 5'-methoxycarbonylmethyl-uracil, 5-methoxy-uracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 1-methyl-pseudouracil, queosine, 13-D-mannosyl-queosine, wybutoxosine, and phosphoramidates, phosphorothioates, peptide nucleotides, methylphosphonates, 7-deazaguanosine, 5-methylcytosine and inosine. The preparation of such analogues is known to a person skilled in the art *e.g.*, from the U.S. Pat. Nos. 4,373,071, 4,401,796, 4,415,732, 4,458,066, 4,500,707, 4,668,777, 4,973,679, 5,047,524, 5,132,418, 5,153,319, 5,262,530 and 5,700,642, the disclosure of which is included here in its full scope by reference.

[0187] In some embodiments, the mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) may contain RNA backbone modifications. Typically, a backbone modification is a modification in which the phosphates of the backbone of the nucleotides contained in the RNA are modified chemically. Exemplary backbone modifications typically include, but are not limited to, modifications from the group consisting of methylphosphonates, methylphosphoramidates, phosphoramidates, phosphorothioates (*e.g.* cytidine 5'-O-(1-thiophosphate)), boranophosphates, positively charged guanidinium groups etc., which means by replacing the phosphodiester linkage by other anionic, cationic or neutral groups.

[0188] In some embodiments, the mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) may contain sugar modifications. A typical sugar modification is a chemical modification of the sugar of the nucleotides it contains including, but not limited to, sugar modifications chosen from the group consisting of 2'-deoxy-2'-fluoro-oligoribonucleotide (2'-fluoro-2'-deoxycytidine 5'-triphosphate, 2'-fluoro-2'-deoxyuridine 5'-triphosphate), 2'-deoxy-2'-deamine-oligoribonucleotide (2'-amino-2'-deoxycytidine 5'-triphosphate, 2'-amino-2'-deoxyuridine 5'-triphosphate), 2'-O-alkyloligoribonucleotide, 2'-deoxy-2'-C-alkyloligoribonucleotide (2'-O-methylcytidine 5'-triphosphate, 2'-methyluridine 5'-triphosphate), 2'-C-alkyloligoribonucleotide, and isomers thereof (2'-aracytidine 5'-triphosphate, 2'-arauridine 5'-triphosphate), or azidotriphosphates (2'-azido-2'-deoxycytidine 5'-triphosphate, 2'-azido-2'-deoxyuridine 5'-triphosphate).

[0189] In some embodiments, the mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) may contain modifications of the bases of the nucleotides (base modifications). A modified nucleotide which contains a base modification is also called a base-modified nucleotide. Examples of such base-modified nucleotides include, but are not limited to, 2-amino-6-chloropurine riboside 5'-triphosphate, 2-aminoadenosine 5'-triphosphate, 2-thiocytidine 5'-triphosphate, 2-thiouridine 5'-triphosphate, 4-thiouridine 5'-triphosphate, 5-aminoallylcytidine 5'-triphosphate, 5-aminoallyluridine 5'-triphosphate, 5-bromocytidine 5'-triphosphate, 5-bromouridine 5'-triphosphate, 5-iodocytidine 5'-triphosphate, 5-iodouridine 5'-triphosphate, 5-methylcytidine 5'-triphosphate, 5-methyluridine 5'-triphosphate, 6-azacytidine 5'-triphosphate, 6-azauridine 5'-triphosphate, 6-chloropurine riboside 5'-triphosphate, 7-deazaadenosine 5'-triphosphate, 7-deazaguanosine 5'-triphosphate, 8-azaadenosine 5'-triphosphate, 8-azidoadenosine 5'-triphosphate, benzimidazole riboside 5'-triphosphate, N1-methyladenosine 5'-triphosphate, N1-methylguanosine 5'-triphosphate, N6-

methyladenosine 5'-triphosphate, O6-methylguanosine 5'-triphosphate, pseudouridine 5'-triphosphate, puromycin 5'-triphosphate or xanthosine 5'-triphosphate.

**[0190]** Typically, mRNA synthesis includes the addition of a “cap” on the N-terminal (5') end, and a “tail” on the C-terminal (3') end. The presence of the cap is important in providing resistance to nucleases found in most eukaryotic cells. The presence of a “tail” serves to protect the mRNA from exonuclease degradation.

**[0191]** Thus, in some embodiments, the mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) include a 5' cap structure. A 5' cap is typically added as follows: first, an RNA terminal phosphatase removes one of the terminal phosphate groups from the 5' nucleotide, leaving two terminal phosphates; guanosine triphosphate (GTP) is then added to the terminal phosphates via a guanylyl transferase, producing a 5'5'5 triphosphate linkage; and the 7-nitrogen of guanine is then methylated by a methyltransferase. Examples of cap structures include, but are not limited to, m7G(5')ppp(5'(A,G(5')ppp(5)A and G(5)ppp(5')G.

**[0192]** In some embodiments, the mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) include a 3' poly(A) tail structure. A poly-A tail on the 3' terminus of mRNA typically includes about 10 to 300 adenosine nucleotides (*e.g.*, about 10 to 200 adenosine nucleotides, about 10 to 175 adenosine nucleotides, about 10 to 150 adenosine nucleotides, about 10 to 125 adenosine nucleotides, 10 to 100 adenosine nucleotides, about 10 to 75 adenosine nucleotides, about 20 to 70 adenosine nucleotides, or about 20 to 60 adenosine nucleotides). In some embodiments, antibody encoding mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) include a 3' poly(C) tail structure. A suitable poly-C tail on the 3' terminus of mRNA typically includes about 10 to 200 cytosine nucleotides (*e.g.*, about 10 to 150 cytosine nucleotides, about 10 to 100 cytosine nucleotides, about 20 to 70 cytosine nucleotides, about 20 to 60 cytosine nucleotides, or about 10 to 40 cytosine nucleotides). The poly-C tail may be added to the poly-A tail or may substitute the poly-A tail.

**[0193]** In some embodiments, the mRNAs (*e.g.*, heavy chain and light chain encoding mRNAs) include a 5' and/or 3' untranslated region. In some embodiments, a 5' untranslated region includes one or more elements that affect an mRNA's stability or translation, for example, an iron responsive element. In some embodiments, a 5' untranslated region may be between about 50 and 500 nucleotides in length (*e.g.*, about 50 and 400 nucleotides in length, about 50 and

300 nucleotides in length, about 50 and 200 nucleotides in length, or about 50 and 100 nucleotides in length).

[0194] In some embodiments, a 5' region of an mRNA (*e.g.*, heavy chain and light chain encoding mRNAs) includes a sequence encoding a signal peptide, such as those described herein. In particular embodiments, a signal peptide derived from human growth hormone (hGH) is incorporated in the 5' region. Typically, a signal peptide encoding sequence is linked, directly or indirectly, to the heavy chain or light chain encoding sequence at the N-terminus.

[0195] The present technology may be used to deliver any antibody known in the art and antibodies that can be produced against desired antigens using standard methods. The present invention may be used to deliver monoclonal antibodies, polyclonal antibodies, antibody mixtures or cocktails, human or humanized antibodies, chimeric antibodies, or bi-specific antibodies.

[0196] Methods of making antibodies are well known in the art and described herein. In certain embodiments, both the variable and constant regions of the antigen-binding polypeptides of the present disclosure are fully human. Fully human antibodies can be made using techniques described in the art and as described herein. For example, fully human antibodies against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Exemplary techniques that can be used to make such antibodies are described in U.S. patents: 6,150,584; 6,458,592; 6,420,140 which are incorporated by reference in their entireties.

### ***Treatment Methods***

[0197] As described herein, the antibodies, variants, derivatives or antibody-drug conjugates of the present disclosure may be used in certain treatment and diagnostic methods.

[0198] The present disclosure is further directed to antibody-based therapies which involve administering the antibodies, fragments, or antibody-drug conjugates of the disclosure to a patient such as an animal, a mammal, and a human for treating one or more of the disorders or conditions described herein. Therapeutic compounds of the disclosure include, but are not limited to, antibodies of the disclosure (including variants and derivatives thereof as described

herein) and nucleic acids or polynucleotides encoding antibodies of the disclosure (including variants and derivatives thereof as described herein).

**[0199]** The antibodies of the disclosure can also be used to treat or inhibit cancer. As provided above, CTLA4 can be overexpressed in tumor cells. Inhibition of CTLA4 has been shown to be useful for treating the tumors.

**[0200]** Accordingly, in some embodiments, provided are methods for treating a cancer in a patient in need thereof. The method, in one embodiment, entails administering to the patient an effective amount of an antibody, fragment, or antibody-drug conjugate of the present disclosure. In some embodiments, at least one of the cancer cells (*e.g.*, stromal cells) in the patient over-express CTLA4.

**[0201]** Cellular therapies, such as chimeric antigen receptor (CAR) T-cell therapies, are also provided in the present disclosure. A suitable cell can be used, that is put in contact with an anti-CTLA4 antibody of the present disclosure (or alternatively engineered to express an anti-CTLA4 antibody of the present disclosure). Upon such contact or engineering, the cell can then be introduced to a cancer patient in need of a treatment. The cancer patient may have a cancer of any of the types as disclosed herein. The cell (*e.g.*, T cell) can be, for instance, a tumor-infiltrating T lymphocyte, a CD4<sup>+</sup> T cell, a CD8<sup>+</sup> T cell, or the combination thereof, without limitation.

**[0202]** In some embodiments, the cell was isolated from the cancer patient him- or her-self. In some embodiments, the cell was provided by a donor or from a cell bank. When the cell is isolated from the cancer patient, undesired immune reactions can be minimized.

**[0203]** Non-limiting examples of cancers include bladder cancer, breast cancer, colorectal cancer, endometrial cancer, esophageal cancer, head and neck cancer, kidney cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, pancreatic cancer, prostate cancer, and thyroid cancer. In some embodiments, the cancer is one or more of gastric, pancreatic, esophageal, ovarian, and lung cancers.

**[0204]** Additional diseases or conditions associated with increased cell survival, that may be treated, prevented, diagnosed and/or prognosed with the antibodies or variants, or derivatives thereof of the disclosure include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (*e.g.*, acute

lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (*e.g.*, chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (*e.g.*, Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyo sarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, 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, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma and retinoblastoma.

**[0205]** A specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the particular antibodies, variant or derivative thereof used, the patient's age, body weight, general health, sex, and diet, and the time of administration, rate of excretion, drug combination, and the severity of the particular disease being treated. Judgment of such factors by medical caregivers is within the ordinary skill in the art. The amount will also depend on the individual patient to be treated, the route of administration, the type of formulation, the characteristics of the compound used, the severity of the disease, and the desired effect. The amount used can be determined by pharmacological and pharmacokinetic principles well known in the art.

**[0206]** Methods of administration of the antibodies, fragments, or antibody-drug conjugates or include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The antigen-binding polypeptides or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active

agents. Thus, pharmaceutical compositions containing the antigen-binding polypeptides of the disclosure may be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray.

**[0207]** The term “parenteral” as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intra-articular injection and infusion.

**[0208]** Administration can be systemic or local. In addition, it may be desirable to introduce the antibodies of the disclosure into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

**[0209]** It may be desirable to administer the antigen-binding polypeptides or compositions of the disclosure locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction, with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the disclosure, care must be taken to use materials to which the protein does not absorb.

**[0210]** The amount of the antibodies, fragments, or antibody-drug conjugates of the disclosure which will be effective in the treatment, inhibition and prevention of an inflammatory, immune or malignant disease, disorder or condition can be determined by standard clinical techniques. In addition, *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 disease, disorder or condition, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[0211] As a general proposition, the dosage administered to a patient of the antibodies, fragments, or antibody-drug conjugates of the present disclosure is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight, between 0.1 mg/kg and 20 mg/kg of the patient's body weight, or 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the disclosure may be reduced by enhancing uptake and tissue penetration (*e.g.*, into the brain) of the antibodies by modifications such as, for example, lipidation.

[0212] In an additional embodiment, the compositions of the disclosure are administered in combination with cytokines. Cytokines that may be administered with the compositions of the disclosure include, but are not limited to, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, anti-CD40, CD40L, and TNF- $\alpha$ .

[0213] In additional embodiments, the compositions of the disclosure are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

### ***Compositions***

[0214] The present disclosure also provides pharmaceutical compositions. Such compositions comprise an effective amount of an antibody, fragment, or antibody-drug conjugate, and an acceptable carrier. In some embodiments, the composition further includes a second anticancer agent (*e.g.*, an immune checkpoint inhibitor).

[0215] In a specific embodiment, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Further, a “pharmaceutically acceptable carrier” will generally be a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

[0216] The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil,

soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents such as acetates, citrates or phosphates. Antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; and agents for the adjustment of tonicity such as sodium chloride or dextrose are also envisioned. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin, incorporated herein by reference. Such compositions will contain a therapeutically effective amount of the antigen-binding polypeptide, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0217] In an embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an

ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0218] The compounds of the disclosure can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

EXAMPLES

Example 1: Analysis of Antibody Binding to ATP

[0219] A human antibody capable of binding to ATP (adenosine triphosphate) was identified from a phage library based on human germline antibody sequences. In addition to ATP, the antibody (R4P4F7) also binds to ADP (adenosine diphosphate) and AMP (adenosine monophosphate). Such bindings were confirmed with an ELISA assay (FIG. 1a) and a competition ELISA assay (FIG. 1b) which also showed that R4P4F7 did not bind ADO (adenosine).

[0220] To identify key amino acid residues involved in the binding, a co-crystal with ATP bound to Fab fragment of R4P4F7 was resolved by X-ray crystallography. As shown in FIG. 2 and Table 1, some of the residues on the VH and VL have suitable distances to the ATP molecule indicating hydrogen bond interactions. In particular, R52, R56, R110 and R111 (numbering based on residue numbers) of the heavy chain, and G91, S96 (numbering based on residue numbers) of the light chain are involved in forming hydrogen bonds with ATP.

Table 1. Hydrogen bond (HB) interactions between ATP and R4P4F7

| ATP  | HSP748     |      | Distance (Å) |
|------|------------|------|--------------|
| Atom | Residue    | Atom |              |
| O1   | VH: ARG110 | NH1  | 2.5          |
|      | VH: ARG111 | NH2  | 3.1          |
| O5   | VH: ARG111 | NE   | 2.9          |
|      | VH: ARG110 | NH1  | 3.3          |
| O10  | VH: ARG52  | NH2  | 2.9          |
|      |            | NH2  | 2.8          |
|      |            | NH1  | 3.4          |
| O8   | VH: ARG56  | NH1  | 2.9          |
| O11  | VH: ARG111 | NH2  | 3.4          |

|     |           |    |     |
|-----|-----------|----|-----|
| O13 | H: NO3    | O1 | 2.5 |
| N4  | VL: SER96 | OG | 2.7 |
| N5  |           |    | 3.4 |
| N3  | VL: GLY91 | O  | 3.2 |

[0221] In a similar experiment, potential water-bridged hydrogen bond interactions were revealed by X-ray crystallography. In particular, D62, M103, R104 and R111 (numbering based on residue numbers) of the heavy chain are involved in forming water-bridged hydrogen bonds with ATP. The results are shown in **FIG. 3** and **Table 2**.

**Table 2. Water bridge hydrogen bond (HB) interactions between ATP and R4P4F7**

| ATP<br>Atom | Distance (Å) | Water ID | R4P4F7     |      | Distance (Å) |
|-------------|--------------|----------|------------|------|--------------|
|             |              |          | Residue    | Atom |              |
| O5          | 3.1          | 309      | VH: ARG111 | N    | 2.8          |
|             |              |          | VH: MET103 | O    | 2.7          |
| O4          | 2.9          | 638      | VH: ARG104 | NH1  | 3.4          |
| O3          | 2.2          |          |            |      |              |
| O7          | 2.4          |          |            |      |              |
| O8          | 3.5          |          |            |      |              |
| O12         | 2.8          | 224      | VH: ASP62  | OD1  | 3.0          |
|             |              |          | VH: ASP62  | OD2  | 3.1          |

[0222] Moreover, **FIG. 4** shows residues that formed pi-pi interactions and amide-pi interactions, in particular G91 and F94 of the light chain and F113 (numbering based on residue numbers) of the heavy chain.

### Example 2. CDR Scanning

[0223] Single-site saturated mutations were made to each residue in the VH and VL CDR regions, as well as some adjacent residues to understand the importance of each residue in the binding to ATP.

[0224] Individual oligonucleotides were synthesized with NNK codon for each CDR position. Amino acid substitutions with any of the other 19 amino acids for each residue in the VH and VL CDR regions were introduced by PCR based on the R4P4F7 coding sequence in pCDNA3.1. Purified PCR products were directly transformed into DH5a competent cells, the transformant of which was incubated in LB Agar plates containing 100 µg/ml ampicillin overnight. The plasmids were isolated by mini-prep on 96-well plates, PEI transfected into HEK293 cells and cultured for 3-5 days for IgG expression.

[0225] The binding affinity of each single-saturated mutant of R4P4F7 was determined using BLI, Octet RED96. The ATP-biotin complex was immobilized on a SA biosensor. The supernatant of HEK293 cells after 3-5 days transfection was measured in 200-s binding at the association step. The dissociation was obtained by dipping the biosensors into kinetic buffer for 200s. The data were analyzed using ForteBio Octet Data Analysis HT, and mutations with an association response >0.5 nm or similar to the signal of 1 µg/ml R4P4F7 were chosen for sequencing.

[0226] The results, as confirmed by x-ray structure data, show that

- G91 and S96 (Kabat numbering) of the light chain are important for ATP binding. When mutated to T, A, S, L, Y, or H at G91, the antibodies still maintained the ATP binding activity. Also, when mutated to G, T, or A at S96, the antibodies still maintained the ATP binding activity.
- W32, Q90, F94, and P95 of the light chain are important for supporting the structures of the LCDRs.
- R97, R100c and R100d of the heavy chain are important for ATP binding. When mutated to E, K, T, S, Q, G, P, V, M, or W at R97, the antibodies still maintained the ATP binding activity. When mutated to K, H, T, S, N, Q, G, A, P, V, L, M, or W at R100c, the antibodies still maintained the ATP binding activity. At R100d, when mutated to T, L, or M, the antibodies still maintained the ATP binding activity.

[0227] These and the additional results are shown in **Table 3**.

**Table 3. CDR (and adjacent residues) scanning results**

| CDR     | Kabat No. | Residue No. | Scanning result – ATP Binding |                         | Roles in ATP binding |
|---------|-----------|-------------|-------------------------------|-------------------------|----------------------|
|         |           |             | Permissible mutations         | Impermissible mutations |                      |
| VH CDR1 |           |             |                               |                         |                      |
| G       | 26        | 26          | All                           | -                       |                      |
| D       | 27        | 27          | All                           | -                       |                      |
| S       | 28        | 28          | All                           | -                       |                      |
| V       | 29        | 29          | All                           | -                       |                      |
| S       | 30        | 30          | All                           | -                       |                      |
| S       | 31        | 31          | All                           | -                       |                      |
| N       | 32        | 32          | All                           | -                       |                      |
| S       | 33        | 33          | All                           | -                       |                      |
| A       | 34        | 34          | All                           | -                       |                      |
| A       | 35        | 35          | All                           | -                       |                      |
| W       | 35a       | 36          | All                           | -                       |                      |
| N       | 35b       | 37          | All                           | -                       |                      |
| VH CDR2 |           |             |                               |                         |                      |

|                |      |     |                                          |        |                             |
|----------------|------|-----|------------------------------------------|--------|-----------------------------|
| R              | 50   | 52  | -                                        | All    | Hydrogen bond               |
| T              | 51   | 53  | All                                      | -      |                             |
| Y              | 52   | 54  | All                                      | -      |                             |
| Y              | 52a  | 55  | All                                      | -      |                             |
| R              | 52b  | 56  | -                                        | All    | Hydrogen bond               |
| S              | 53   | 57  | All                                      | -      |                             |
| K              | 54   | 58  | All                                      | -      |                             |
| W              | 55   | 59  | All                                      | -      |                             |
| Y              | 56   | 60  | All                                      | -      |                             |
| N              | 57   | 61  | All                                      | -      |                             |
| D              | 58   | 62  | All                                      | -      |                             |
| Y              | 59   | 63  | All                                      | -      |                             |
| A              | 60   | 64  | All                                      | -      |                             |
| V              | 61   | 65  | All                                      | -      |                             |
| S              | 62   | 66  | All                                      | -      |                             |
| V              | 63   | 67  | All                                      | -      |                             |
| K              | 64   | 68  | All                                      | -      |                             |
| S              | 65   | 69  | All                                      | -      |                             |
| <b>VH CDR3</b> |      |     |                                          |        |                             |
| G              | 95   | 102 | All                                      | -      |                             |
| M              | 96   | 103 | All                                      | -      |                             |
| R              | 97   | 104 | E, K, T, S, Q, G, P, V, M, or W          | Others | Water bridged-hydrogen bond |
| G              | 98   | 105 | All                                      | -      |                             |
| I              | 99   | 106 | All                                      | -      |                             |
| A              | 100  | 107 | All                                      | -      |                             |
| V              | 100a | 108 | All                                      | -      |                             |
| A              | 100b | 109 | All                                      | -      |                             |
| R              | 100c | 110 | K, H, T, S, N, Q, G, A, P, V, L, M, or W | Others | Hydrogen bond               |
| R              | 100d | 111 | T, L, or M                               | Others | Hydrogen bond               |
| A              | 100e | 112 | All                                      | -      |                             |
| F              | 100f | 113 | All                                      | -      |                             |
| D              | 101  | 114 | All                                      | -      |                             |
| I              | 102  | 115 | All                                      | -      |                             |
| <b>VL CDR1</b> |      |     |                                          |        |                             |
| C              | 23   | 23  | All                                      | -      |                             |
| R              | 24   | 24  | All                                      | -      |                             |
| A              | 25   | 25  | All                                      | -      |                             |
| S              | 26   | 26  | All                                      | -      |                             |
| Q              | 27   | 27  | All                                      | -      |                             |
| G              | 28   | 28  | All                                      | -      |                             |
| V              | 29   | 29  | All                                      | -      |                             |
| S              | 30   | 30  | All                                      | -      |                             |
| S              | 31   | 31  | All                                      | -      |                             |
| W              | 32   | 32  | All                                      | -      |                             |
| L              | 33   | 33  | All                                      | -      |                             |
| A              | 34   | 34  | All                                      | -      |                             |
| W              | 35   | 35  | All                                      | -      |                             |
| <b>VL CDR2</b> |      |     |                                          |        |                             |
| Y              | 49   | 49  | All                                      | -      |                             |
| S              | 50   | 50  | All                                      | -      |                             |
| A              | 51   | 51  | All                                      | -      |                             |
| S              | 52   | 52  | All                                      | -      |                             |
| S              | 53   | 53  | All                                      | -      |                             |
| L              | 54   | 54  | All                                      | -      |                             |

|                |    |    |                     |        |                                                    |
|----------------|----|----|---------------------|--------|----------------------------------------------------|
| Q              | 55 | 55 | All                 | -      |                                                    |
| S              | 56 | 56 | All                 | -      |                                                    |
| G              | 57 | 57 | All                 | -      |                                                    |
| <b>VL CDR3</b> |    |    |                     |        |                                                    |
| C              | 88 | 88 | All                 | -      |                                                    |
| Q              | 89 | 89 | All                 | -      |                                                    |
| Q              | 90 | 90 | -                   | All    | Structural support                                 |
| <b>G</b>       | 91 | 91 | T, A, S, L, Y, or H | Others | Hydrogen bond and pi/amide stacking interactions   |
| H              | 92 | 92 | All                 | -      |                                                    |
| S              | 93 | 93 | All                 | -      |                                                    |
| F              | 94 | 94 | -                   | All    | Structural support, pi/amide stacking interactions |
| P              | 95 | 95 | -                   | All    | Structural support                                 |
| <b>S</b>       | 96 | 96 | G, T, or A          | Others | Hydrogen bond                                      |
| T              | 97 | 97 | All                 | -      |                                                    |

### Example 3. Generation of Anti-CTLA4 Antibodies

**[0228]** With the CDR scanning results as obtained in Example 2, the residues important for ATP binding were used to search, in human antibody libraries, for antibodies that contained permissible amino acids at those residues and were capable of binding to the human CTLA4 protein.

**[0229]** Three of the identified ATP-binding, CTLA4-specific antibodies were subjected to additional testing. Their sequences (P106A2, P106A7, and P119H7), along with those of R4P4F7, are shown in **Table 4**. The sequences of the relevant CDR and framework regions are shown in **Tables 5-6**. As noted in **Tables 5 and 6**, the CDR regions herein are “extended” relative to the standard CDR regions according to Kabat, to include certain adjacent residues that are potentially involved in ATP binding (*e.g.*, VH R50, according to Kabat number). The starting and ending residues for each of these regions are annotated (according to Kabat numbering) in the tables.

**Table 4. Antibody sequences**

| Antibody  | Sequence                                                                                                                              | SEQ ID NO: |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------|------------|
| R4P4F7 VH | QVQLQQSGPGLVKPSQTLSTCAISGDSVSSNSAAWNWIHQSPSRGLEWLGRTYYRSK<br>WYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARGMRGIAVARRAFDIW<br>GQGTTVTVSS | 1          |
| R4P4F7 VL | DIQMTQSPSSVSASVGDRVITTCRASQGVSSWLAWYQQKPGKAPKLLIYSASSLQSGV<br>PSRFGSGSGTDFTLTITISLQPEDFATYYCQQGHSEFPSTFGQGTKVEIK                      | 2          |
| P106A2 VH | QVQLQQSGPGLVKPSQTLSTCAISGESVSSNSAAWNWIHQSPSRGLEWLGRTYYRSK<br>WYNDYAESVKSRTINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSRRGFDIW<br>GQGTTVTVSS  | 3          |

|           |                                                                                                                                         |   |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------|---|
| P106A2 VL | DIQMTQSPSSVSASVGDRVITTCRASQGISSWLAWYQQKPGKAPKLLIYAASSLQSGV<br>PSRFGSGSGTDFTLTISLQPEDFATYYCQQGNVFPSTFGQGTKVEIK                           | 4 |
| P106A7 VH | QVQLQQSGPGLVKPSQTLSTLCAISGDSVSSNSAAWNWIQSPSRGLEWLGRTYYRSK<br>WYNDYAVSVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAISARRGFDYW<br>GQGTTIVTVSS | 5 |
| P106A7 VL | DIQMTQSPSSVSASVGDRVITTCRASQGVSSWLAWYQQKPGKAPKLLIYAASGLQSGV<br>PSRFGSGSGTDFTLTISLQPEDFATYYCQQGNSFPSTFGQGTKVEIK                           | 6 |
| P119H7 VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSSNSAAWNWIQSPSRGLEWLGRTYYRSK<br>WYNDYAVSVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERYAYSARRAFDYW<br>GQGTTIVTVSS | 7 |
| P119H7 VL | DIQMTQSPSSVSASVGDRVITTCRASQGISSWLAWYQQKPGKAPKLLIYAASSLQSGV<br>PSRFGSGSGTDFTLTISLQPEDFATYYCQQGNSFPSTFGQGTKVEIK                           | 8 |

Table 5. Extended CDR sequences

| Antibody  | Extended CDR (Kabat numbering) | Sequence                                     | SEQ ID NO: |
|-----------|--------------------------------|----------------------------------------------|------------|
| R4P4F7 VH | VH CDR1 (26-35b)               | GDSVSSNSAAWN                                 | 9          |
|           | VH CDR2 (50-65)                | <u>R</u> TY <u>Y</u> R <u>S</u> KWYNDYAVSVKS | 10         |
|           | VH CDR3 (95-102)               | GM <u>R</u> GIAVA <u>RR</u> AFDI             | 11         |
| R4P4F7 VL | VL CDR1 (23-35)                | CRASQGVSSWLAW                                | 12         |
|           | VL CDR2 (49-57)                | YSASSLQSG                                    | 13         |
|           | VL CDR3 (88-97)                | CQ <u>Q</u> G <u>H</u> S <u>F</u> PST        | 14         |
| P106A2 VH | See above                      | GESVSSNSAAWN                                 | 15         |
|           |                                | <u>R</u> TY <u>Y</u> R <u>S</u> KWYNDYAESVKS | 16         |
|           |                                | GE <u>R</u> RAVTS <u>RR</u> GFDI             | 17         |
| P106A2 VL | See above                      | CRASQGISSWLAW                                | 18         |
|           |                                | YAASSLQSG                                    | 19         |
|           |                                | CQ <u>Q</u> G <u>N</u> V <u>F</u> PST        | 20         |
| P106A7 VH | See above                      | GDSVSSNSAAWN                                 | 21         |
|           |                                | <u>R</u> TY <u>Y</u> R <u>S</u> KWYNDYAVSVKS | 22         |
|           |                                | GE <u>R</u> RAIS <u>ARR</u> GFDY             | 23         |
| P106A7 VL | See above                      | CRASQGVSSWLAW                                | 24         |
|           |                                | YAASGLQSG                                    | 25         |
|           |                                | CQ <u>Q</u> G <u>N</u> S <u>F</u> PST        | 26         |
| P119H7 VH | See above                      | GESVSSNSAAWN                                 | 27         |
|           |                                | <u>R</u> TY <u>Y</u> R <u>S</u> KWYNDYAVSVKS | 28         |
|           |                                | GE <u>R</u> YAYS <u>ARR</u> AFDY             | 29         |
| P119H7 VL | See above                      | CRASQGISSWLAW                                | 30         |
|           |                                | YAASSLQSG                                    | 31         |
|           |                                | CQ <u>Q</u> G <u>N</u> S <u>F</u> PST        | 32         |

Table 6. Framework regions

| Chain  | Kabat Numbering | Sequence                         | SEQ ID NO: |
|--------|-----------------|----------------------------------|------------|
| VH FM1 | 1-25            | QVQLQQSGPGLVKPSQTLSTLCAIS        | 33         |
| VH FM2 | 36-49           | WIRQSPSRGLEWLG                   | 34         |
| VH FM3 | 66-94           | RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR | 35         |
| VH FM4 | 103-113         | WGQGTIVTVSS                      | 36         |
| VL FM1 | 1-22            | DIQMTQSPSSVSASVGDRVITIT          | 37         |
| VL FM2 | 36-48           | YQQKPGKAPKLLI                    | 38         |
| VL FM3 | 58-87           | VPSRFGSGSGTDFTLTISLQPEDFATYY     | 39         |

|        |        |            |    |
|--------|--------|------------|----|
| VL FM4 | 98-107 | FGQGTKVEIK | 40 |
|--------|--------|------------|----|

[0230] All of the anti-CTLA4 antibodies retained the CDR residues revealed to be important for ATP binding, including R50, R52b, R97, R100c, and R100d of the VH, and Q90, G91, F94, P95 and S96 of the VL (all according to Kabat numbering). All of these antibodies also include the entire framework (residues beyond the extended CDR regions) of R4P4F7.

#### Example 4. CTLA4-Binding was ANP-Dependent

[0231] The binding affinity of P106A2 to CTLA4 was tested, in particular in the presence or absence of ATP, ADP and/or AMP (collectively referred to as “ANP”). Anti-CTLA4 antibody Ipilimumab was used as benchmark (BMK001-I).

[0232] The testing was conducted with CHOK-1 cells expressing human CTLA4 and those that did not. The results are shown in **FIG. 5** and **Table 7** below.

**Table 7. CTLA4 binding – P106A2**

| Ab       | w ANP(300μM) |         | w/o ANP  |         | Non-specific to CHOK1 |         |
|----------|--------------|---------|----------|---------|-----------------------|---------|
|          | EC50(nM)     | Max MFI | EC50(nM) | Max MFI | w ANP(300μM)          | w/o ANP |
| P106A2   | 1.604        | 18955   | 15.66    | 2195    | —                     | —       |
| BMK001-I | 1.751        | 39744   | 1.91     | 40774   | —                     | —       |
| IgG1     | NA           | 182     | NA       | 172     | —                     | —       |

[0233] As shown, unlike the benchmark antibody which bound to CTLA4 regardless of the presence of ANP (ATP + ADP + AMP), P106A2 only bound to CTLA4 in the presence of ANP (300 μM). However, while both antibodies had similar EC50, P106A2’s maximum MFI was about half of that for the benchmark antibody.

[0234] Likewise, when P106A7 was tested, it exhibited similar binding pattern as P106A2. See **FIG. 6** and **Table 8**.

**Table 8. CTLA4 binding – P106A7**

| Ab       | w ANP(300μM) |         | w/o ANP  |         | Non-specific to CHOK1 |         |
|----------|--------------|---------|----------|---------|-----------------------|---------|
|          | EC50(nM)     | Max MFI | EC50(nM) | Max MFI | w ANP(300μM)          | w/o ANP |
| P106A7   | <b>1.523</b> | 8086    | 11.82    | 1596    | —                     | —       |
| BMK001-I | <b>1.906</b> | 18308   | 1.989    | 20226   | —                     | —       |
| IgG1     | NA           | 181     | NA       | 174     | —                     | —       |

[0235] FIG. 7 and Table 9 compare antibodies P119H7 and P106A2 to the benchmark antibody, and show that P119H7 and P106A2's binding to CTLA4 is ANP-dependent and had lower maximum MFI as compared to the benchmark.

**Table 9. CTLA4 binding – P106A2 and P119H7**

| Ab       | w ANP (300μM) |         | w/o ANP  |         |
|----------|---------------|---------|----------|---------|
|          | EC50(nM)      | Max MFI | EC50(nM) | Max MFI |
| P106A2   | 1.382         | 41628   | 103.7    | 7236    |
| P119H7   | 3.483         | 29361   | 26.13    | 8452    |
| BMK001-I | 0.98          | 70659   | 1.641    | 63158   |
| IgG1     | NA            | 174     | 0.908    | 182     |

[0236] In a further experiment, the antibody-CTLA4 binding by P106A2 at different concentrations of ATP, ADP and AMP was measured. As shown in FIG. 8 and Table 10, the binding of P106A2, unlike for the benchmark antibody, was dose-dependent.

**Table 10. ATP/ADP/AMP-dependent binding of P106A2**

| Ab       | w ATP (1000μM) |         | w ATP (100μM) |         | w ATP (10μM) |         | w ATP (1μM) |         | w/o ATP   |         | Non-specific to CHOK1 |         |
|----------|----------------|---------|---------------|---------|--------------|---------|-------------|---------|-----------|---------|-----------------------|---------|
|          | EC50 (nM)      | Max MFI | EC50 (nM)     | Max MFI | EC50 (nM)    | Max MFI | EC50 (nM)   | Max MFI | EC50 (nM) | Max MFI | w ATP                 | w/o ATP |
| P106A2   | 3.346          | 28172   | 2.473         | 29377   | 5.668        | 18780   | 35.62       | 8567    | 89.12     | 5487    | —                     | —       |
| BMK001-I | 1.926          | 70184   | 1.694         | 68710   | 1.763        | 67623   | 1.508       | 67700   | 0.9847    | 80775   | —                     | —       |
| IgG1     | NA             | 189     | NA            | 179     | NA           | 172     | NA          | 171     | NA        | 182     | —                     | —       |

| Ab       | w ADP (1000μM) |         | w ADP (100μM) |         | w ADP (10μM) |         | w ADP (1μM) |         | w/o ADP   |         | Non-specific to CHOK1 |         |
|----------|----------------|---------|---------------|---------|--------------|---------|-------------|---------|-----------|---------|-----------------------|---------|
|          | EC50 (nM)      | Max MFI | EC50 (nM)     | Max MFI | EC50 (nM)    | Max MFI | EC50 (nM)   | Max MFI | EC50 (nM) | Max MFI | w ADP                 | w/o ADP |
| P106A2   | 2.138          | 39154   | 2.181         | 47558   | 2.757        | 33675   | 15.27       | 12845   | 89.12     | 5487    | —                     | —       |
| BMK001-I | 1.818          | 73320   | 1.557         | 75547   | 1.675        | 77743   | 1.351       | 78086   | 0.9847    | 80775   | —                     | —       |
| IgG1     | NA             | 216     | NA            | 182     | NA           | 182     | NA          | 171     | NA        | 182     | —                     | —       |

| Ab       | w AMP (1000μM) |         | w AMP (100μM) |         | w AMP (10μM) |         | w AMP (1μM) |         | w/o AMP   |         | Non-specific to CHOK1 |         |
|----------|----------------|---------|---------------|---------|--------------|---------|-------------|---------|-----------|---------|-----------------------|---------|
|          | EC50 (nM)      | Max MFI | EC50 (nM)     | Max MFI | EC50 (nM)    | Max MFI | EC50 (nM)   | Max MFI | EC50 (nM) | Max MFI | w AMP                 | w/o AMP |
| P106A2   | 1.952          | 51289   | 1.868         | 47945   | 3.077        | 29482   | 17.39       | 17998   | 89.12     | 5487    | —                     | —       |
| BMK001-I | 1.613          | 75351   | 1.601         | 74091   | 1.658        | 77107   | 1.38        | 83770   | 0.9847    | 80775   | —                     | —       |
| IgG1     | NA             | 168     | NA            | 177     | NA           | 168     | NA          | 166     | NA        | 182     | —                     | —       |

[0237] Similar dose-dependency was observed for P106A7. See FIG. 9 and Table 11.

**Table 11. ATP/ADP/AMP-dependent binding of P106A7**

| Ab       | w ATP (1000μM) |         | w ATP (100μM) |         | w ATP (10μM) |         | w ATP (1μM) |         | w/o ATP   |         | Non-specific to CHOK1 |         |
|----------|----------------|---------|---------------|---------|--------------|---------|-------------|---------|-----------|---------|-----------------------|---------|
|          | EC50 (nM)      | Max MFI | EC50 (nM)     | Max MFI | EC50 (nM)    | Max MFI | EC50 (nM)   | Max MFI | EC50 (nM) | Max MFI | w ATP                 | w/o ATP |
| P106A7   | 4.379          | 15873   | 5.154         | 17318   | NA           | 10838   | 42.84       | 5271    | 54.44     | 4104    | —                     | —       |
| BMK001-I | 1.926          | 70184   | 1.694         | 68710   | 1.763        | 67623   | 1.508       | 67700   | 0.9847    | 80775   | —                     | —       |
| IgG1     | NA             | 189     | NA            | 179     | NA           | 172     | NA          | 171     | NA        | 182     | —                     | —       |

| Ab       | w ADP (1000μM) |         | w ADP (100μM) |         | w ADP (10μM) |         | w ADP (1μM) |         | w/o ADP   |         | Non-specific to CHOK1 |         |
|----------|----------------|---------|---------------|---------|--------------|---------|-------------|---------|-----------|---------|-----------------------|---------|
|          | EC50 (nM)      | Max MFI | EC50 (nM)     | Max MFI | EC50 (nM)    | Max MFI | EC50 (nM)   | Max MFI | EC50 (nM) | Max MFI | w ADP                 | w/o ADP |
| P106A7   | 1.92           | 30748   | 2.043         | 31276   | 3.967        | 18919   | 26.45       | 6853    | 54.44     | 4104    | —                     | —       |
| BMK001-I | 1.818          | 73320   | 1.557         | 75547   | 1.675        | 77743   | 1.351       | 78086   | 0.9847    | 80775   | —                     | —       |
| IgG1     | NA             | 216     | NA            | 182     | NA           | 182     | NA          | 171     | NA        | 182     | —                     | —       |

| Ab       | w AMP (1000μM) |         | w AMP (100μM) |         | w AMP (10μM) |         | w AMP (1μM) |         | w/o AMP   |         | Non-specific to CHOK1 |         |
|----------|----------------|---------|---------------|---------|--------------|---------|-------------|---------|-----------|---------|-----------------------|---------|
|          | EC50 (nM)      | Max MFI | EC50 (nM)     | Max MFI | EC50 (nM)    | Max MFI | EC50 (nM)   | Max MFI | EC50 (nM) | Max MFI | w AMP                 | w/o AMP |
| P106A7   | 1.942          | 45282   | 1.841         | 39275   | 5.344        | 22893   | 53.65       | 12866   | 54.44     | 4104    | —                     | —       |
| BMK001-I | 1.613          | 75351   | 1.601         | 74091   | 1.658        | 77107   | 1.38        | 83770   | 0.9847    | 80775   | —                     | —       |
| IgG1     | NA             | 168     | NA            | 177     | NA           | 168     | NA          | 166     | NA        | 182     | —                     | —       |

[0238] Such ATP/ADP/AMP dose-dependency of P106A2 and P106A7 was further confirmed with Octet® measurements at pH 7.4 (FIG. 10).

### Example 5. Affinity mutations of P106A2

[0239] Single-site saturated mutations were made the extended CDR regions of the P106A2 antibody to understand the importance of each residue for the activity, which was evaluated by ELISA and FACS. The important mutations which could improve the activity of P106A2 were produced and tested alone or in combinations.

[0240] Individual oligonucleotides were synthesized with NNK codon for each CDR position. Amino acid substitutions with any of the other 19 amino acids for each residue in the VH and VL CDR regions were introduced by PCR based on the P106A2 coding sequence in pCDNA3.1. Purified PCR products were directly transformed into DH5a competent cells, the transformant of which was incubated in LB Agar plates containing 100 μg/ml ampicillin overnight. The plasmids were isolated by mini-prep on 96-well plates, PEI transfected into HEK293 cells and cultured for 3-5 days for IgG expression.

[0241] The binding affinity of each single-saturated mutation of P106A2 was determined using ELISA. Briefly, the test sample (HEK293 cell supernatant) was added to hCTLA-4.ECD His biotin coated NeutrAvidin 96-well microtiter plate. ATP/ADP/AMP was added into corresponding wells, and was then incubated at RT for 1h. Anti-hFab-HRP or anti-hFc-HRP was used as the 2<sup>nd</sup> antibody for the detection. Mutations with an OD450 signal >2 fold P106A2 were chosen for FACS and sequence.

[0242] The results show that L53 (position S53 of the light chain, according to Kabat numbering), L55 and H32 of the CDRs are important for CTLA4 binding. When VL S53 was mutated to G, the affinity to CTLA4 was improved. When VL Q55 was mutated to M, F, Y, or L, the affinity to CTLA4 was also improved; Also, when VH N32 was mutated to L, E, F, A, M, W, Q, Y, H, K, or R, the affinity to CTLA4 was improved.

[0243] The mutations at VH N32, VL Q55, and VL S53 were produced alone or in combinations, and the affinity of the resulting mutants to CTLA4 was tested in the present or absent of ATP/ADP/AMP.

[0244] In a first testing, mutations at VH N32 and VL Q55, alone or in combination, were tested. As shown in FIG. 11A-E and Table 12, except for VL Q55E alone and VL Q55E combine with VH N32E, the other mutations improved the CTLA4 binding activity and maintain ANP-dependency significantly. VL Q55M appears to be a good mutation for improving the CTLA4 binding activity.

**Table 12. CTLA4-binding by mutant P106A2**

| Name                   | Mutation |      | w ANP (300μM) |         | w/o ANP  |         |
|------------------------|----------|------|---------------|---------|----------|---------|
|                        | VH       | VL   | EC50(nM)      | Max MFI | EC50(nM) | Max MFI |
| P106A2                 | -        | -    | 5.13          | 4500    | 96.88    | 808     |
| P106A2-N(H32)L         | N32L     | -    | 3.833         | 12119   | 8.593    | 6513    |
| P106A2-N(H32)R         | N32R     | -    | 5.515         | 8657    | 21.85    | 3543    |
| P106A2-N(H32)E         | N32E     | -    | 5.514         | 8004    | 11.93    | 3672    |
| P106A2-Q(L55)M         | -        | Q55M | 1.657         | 15897   | 2.358    | 13837   |
| P106A2-Q(L55)E         | -        | Q55E | 183.3         | 511     | 221.4    | 221     |
| P106A2-Q(L55)Y         | -        | Q55Y | 7.074         | 12414   | 10.09    | 5806    |
| P106A2-N(H32)R,Q(L55)M | N32R     | Q55M | 1.278         | 17315   | 1.145    | 17073   |
| P106A2-N(H32)R,Q(L55)E | N32R     | Q55E | 4819          | 1864    | 204010   | 1191    |
| P106A2-N(H32)R,Q(L55)Y | N32R     | Q55Y | 3.59          | 15762   | 4.881    | 10858   |
| P106A2-N(H32)E,Q(L55)M | N32E     | Q55M | 1.961         | 19050   | 1.801    | 17964   |
| P106A2-N(H32)E,Q(L55)E | N32E     | Q55E | 56.91         | 1019    | 143.4    | 185     |
| P106A2-N(H32)E,Q(L55)Y | N32E     | Q55Y | 4.312         | 15780   | 9.472    | 10859   |
| BMK001-I               |          |      | 1.975         | 16912   | 1.928    | 17057   |

|      |  |  |    |     |    |     |
|------|--|--|----|-----|----|-----|
| IgG1 |  |  | NA | 242 | NA | 227 |
|------|--|--|----|-----|----|-----|

| Name                   | Mutations |      | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         |
|------------------------|-----------|------|-----------------------------|---------|-----------------------|---------|
|                        | VH        | VL   | EC50 (nM)                   | Max MFI | EC50 (nM)             | Max MFI |
| P106A2                 |           |      | 1.382                       | 41628   | 103.7                 | 7236    |
| P106A2-N(H32)L,Q(L55)L | N32L      | Q55L | 0.9343                      | 64215   | 0.745                 | 49577   |
| P106A2-N(H32)L,Q(L55)Y | N32L      | Q55Y | 1.11                        | 77704   | 1.03                  | 62475   |
| P106A2-N(H32)F,Q(L55)L | N32F      | Q55L | 1.008                       | 64925   | 1.409                 | 40279   |
| P106A2-N(H32)F,Q(L55)Y | N32F      | Q55Y | 1.222                       | 76471   | 1.329                 | 59157   |
| P106A2-N(H32)E,Q(L55)L | N32E      | Q55L | 0.9692                      | 69444   | 1.737                 | 50037   |
| BMK001-I               |           |      | 0.98                        | 70659   | 1.641                 | 63158   |
| IgG1                   |           |      | NA                          | 174     | 0.908                 | 182     |

| Name             | Mutations |      | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         |
|------------------|-----------|------|-----------------------------|---------|-----------------------|---------|
|                  | VH        | VL   | EC50 (nM)                   | Max MFI | EC50 (nM)             | Max MFI |
| P106A2-N32L-Q55F | N32L      | Q55F | 1.372                       | 60254   | 1.639                 | 49597   |
| P106A2-N32E-Q55F | N32E      | Q55F | 1.609                       | 59911   | 2.109                 | 48575   |
| P106A2-N32A-Q55M | N32A      | Q55M | 0.8774                      | 50169   | 0.927                 | 43867   |
| P106A2-N32A-Q55F | N32A      | Q55F | 1.443                       | 58194   | 2.551                 | 44977   |
| P106A2-N32A-Q55L | N32A      | Q55L | 1.113                       | 44543   | 1.786                 | 28894   |
| P106A2-N32A-Q55Y | N32A      | Q55Y | 2.074                       | 61508   | 3.171                 | 41731   |
| P106A2-N32M-Q55F | N32M      | Q55F | 1.428                       | 58130   | 2.003                 | 46509   |
| P106A2-N32M-Q55Y | N32M      | Q55Y | 1.692                       | 58572   | 2.242                 | 46708   |
| P106A2-N32M-S53G | N32M      | S53G | 1.671                       | 55240   | 2.132                 | 32104   |
| P106A2-N32Q-Q55M | N32Q      | Q55M | 0.9285                      | 52497   | 1.014                 | 46720   |
| BMK001-I         |           |      | 1.247                       | 51319   | 1.127                 | 47837   |
| IgG1             |           |      | 448.3                       | 252.5   | 143.9                 | 183.1   |

| Name                  | Mutations |            | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         |
|-----------------------|-----------|------------|-----------------------------|---------|-----------------------|---------|
|                       | VH        | VL         | EC50 (nM)                   | Max MFI | EC50 (nM)             | Max MFI |
| P106A2-N32Q-Q55F      | N32Q      | Q55F       | 1.124                       | 48772   | 2.066                 | 38859   |
| P106A2-N32Q-Q55Y      | N32Q      | Q55Y       | 1.381                       | 48712   | 2.227                 | 35059   |
| P106A2-N32Q-S53G      | N32Q      | S53G       | 1.262                       | 43900   | 1.737                 | 21760   |
| P106A2-N32Q-S53G-Q55F | N32Q      | S53G, Q55F | 1.027                       | 52695   | 1.137                 | 42212   |
| P106A2-N32Y-Q55F      | N32Y      | Q55F       | 1.176                       | 50134   | 1.618                 | 43357   |
| P106A2-N32H-Q55F      | N32H      | Q55F       | 1.291                       | 49652   | 2.061                 | 41745   |
| P106A2-N32H-Q55Y      | N32H      | Q55Y       | 1.48                        | 49890   | 2.423                 | 37885   |
| BMK001-I              |           |            | 0.9022                      | 42626   | 0.9176                | 43751   |
| IgG1                  |           |            | 1.124                       | 48772   | 2.066                 | 38859   |

[0245] In another experiment, mutations at VH N32 and VL S53/Q55, alone or in combination, were tested. As shown in **FIG. 12A-B** and **Table 13**, these mutations could improve the CTLA4 binding activity and maintain ANP-dependency significantly.

**Table 13. CTLA4-binding by mutant P106A2**

| Name                    | Mutation |      | w ANP (300μM) |         | w/o ANP  |         |
|-------------------------|----------|------|---------------|---------|----------|---------|
|                         | VH       | VL   | EC50(nM)      | Max MFI | EC50(nM) | Max MFI |
| P106A2                  | -        | -    | 4.22          | 4664    | 50.91    | 870     |
| P106A2 -N(H32)L,S(L53)G | N32L     | S53G | 2.95          | 13864   | 4.169    | 8247    |
| P106A2 -N(H32)L,Q(L55)M | N32L     | Q55M | 1.505         | 14575   | 1.371    | 12654   |
| P106A2 -N(H32)F,S(L53)G | N32F     | S53G | 3.171         | 11724   | 4.2      | 5553    |
| P106A2 -N(H32)F,Q(L55)M | N32F     | Q55M | 0.6576        | 14730   | 0.7313   | 12779   |
| P106A2-N(H32)R,S(L53)G  | N32R     | S53G | 2.362         | 11773   | 6.634    | 5752    |
| P106A2 -N(H32)E,S(L53)G | N32E     | S53G | 4.35          | 11116   | 5.17     | 5615    |
| BMK001-I                |          |      | 2.698         | 14481   | 2.459    | 14022   |
| IgG1                    |          |      | NA            | 216     | NA       | 218     |

| Name                         | Mutations |           | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         |
|------------------------------|-----------|-----------|-----------------------------|---------|-----------------------|---------|
|                              | VH        | VL        | EC50 (nM)                   | Max MFI | EC50 (nM)             | Max MFI |
| P106A2-N(H32)E,S(L53)G       | N32E      | S53G      | 2.372                       | 19124   | 3.932                 | 11451   |
| P106A2-N(H32)L,S(L53)G       | N32L      | S53G      | 2.114                       | 23172   | 2.279                 | 16507   |
| P106A2-N(H32)A,S(L53)G       | N32A      | S53G      | 2.156                       | 19821   | 3.917                 | 9497    |
| P106A2-N(H32)E,S(L53)G, Q55L | N32E      | S53G Q55L | 1.803                       | 18973   | 1.924                 | 15077   |
| P106A2-N(H32)E,S(L53)G, Q55Y | N32E      | S53G Q55Y | 1.757                       | 24869   | 1.886                 | 20932   |
| BMK001-I                     |           |           | 1.504                       | 24268   | 1.161                 | 24367   |
| IgG1                         |           |           | NA                          | 215     | NA                    | 223     |

[0246] Next, various mutations at VH N32 and VL S53/Q55 were tested. As shown in **FIG. 13** and **Table 14**, these mutations could improve the CTLA4 binding activity and maintain ANP-dependency significantly.

**Table 14. CTLA4-binding by mutant P106A2**

| Name               | Mutations |      | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         |
|--------------------|-----------|------|-----------------------------|---------|-----------------------|---------|
|                    | VH        | VL   | EC50 (nM)                   | Max MFI | EC50 (nM)             | Max MFI |
| P106A2-N32A-Q55L   | N32A      | Q55L | 1.494                       | 49798   | 3.025                 | 34008   |
| P106A2-N32M-S53G   | N32M      | S53G | 1.919                       | 57524   | 2.215                 | 33422   |
| P106A2-N32Q-S53G   | N32Q      | S53G | 2.049                       | 52861   | 3.119                 | 29739   |
| P106A2- N32E -S53G | N32E      | S53G | 2.272                       | 54183   | 2.138                 | 35238   |
| P106A2_ N32A -S53G | N32A      | S53G | 2.163                       | 54533   | 3.309                 | 31166   |
| BMK001-I           |           |      | 1.623                       | 47694   | 1.317                 | 46237   |
| IgG1               |           |      | NA                          | 160     | NA                    | 164     |

[0247] Cross-reactivity of the various mutant P106A2 to human, cyno and mouse CTLA4 was tested with CHOK1 cells expressing the human, cyno and mouse CTLA4 proteins. As shown

in **FIG. 14** and **Table 15**, these mutants were able to bind all three species of CTLA4 proteins with high affinity, in an ANP-dependent manner.

**Table 15. Cross-reactivity to human, cyno and mouse CTLA4 proteins on CHOK1**

| Name               | Mutations |      | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         | (-ANP)/(+ANP)  | (+ANP)/(-ANP)     |
|--------------------|-----------|------|-----------------------------|---------|-----------------------|---------|----------------|-------------------|
|                    | VH        | VL   | EC50(nM)                    | Max MFI | EC50(nM)              | Max MFI | Fold of (EC50) | Fold of (Max MFI) |
| P106A2- N32E -S53G | N32E      | S53G | 1.686                       | 93472   | 1.862                 | 56988   | 1.1            | 1.6               |
| P106A2_ N32A -S53G | N32A      | S53G | 1.508                       | 93478   | 2.374                 | 52306   | 1.6            | 1.8               |
| BMK001-I           |           |      | 1.492                       | 76695   | 1.423                 | 82362   | 1.0            | 0.9               |
| IgG1               |           |      | NA                          | 193     | NA                    | 209     |                | 0.9               |

| Name               | CHOK1-cynoCTLA4, w ANP (300μM) |         | CHOK1-cynoCTLA4, w/o ANP |         | (-ANP)/(+ANP)  | (+ANP)/(-ANP)     |
|--------------------|--------------------------------|---------|--------------------------|---------|----------------|-------------------|
|                    | EC50(nM)                       | Max MFI | EC50(nM)                 | Max MFI | Fold of (EC50) | Fold of (Max MFI) |
| P106A2- N32E -S53G | 1.878                          | 10509   | 2.68                     | 6563    | 1.4            | 1.6               |
| P106A2_ N32A -S53G | 1.545                          | 11234   | 3.377                    | 6158    | 2.2            | 1.8               |
| BMK001-I           | 1.264                          | 10593   | 1.257                    | 11774   | 1.0            | 0.9               |
| IgG1               | NA                             | 205     | NA                       | 200     |                | 1.0               |

| Name               | CHOK1-mCTLA4, w ANP (300μM) |         | CHOK1-mCTLA4, w/o ANP |         | (-ANP)/(+ANP)  | (+ANP)/(-ANP)     | 300nM Ab Non-specific to CHOK1 |         |
|--------------------|-----------------------------|---------|-----------------------|---------|----------------|-------------------|--------------------------------|---------|
|                    | EC50(nM)                    | Max MFI | EC50(nM)              | Max MFI | Fold of (EC50) | Fold of (Max MFI) | w ANP (300μM)                  | w/o ANP |
| P106A2- N32E -S53G | 4.612                       | 1280451 | 3.176                 | 821339  | 0.7            | 1.6               | 179                            | 223     |
| P106A2_ N32A -S53G | 4.427                       | 1442570 | 2.319                 | 665418  | 0.5            | 2.2               | 166                            | 498     |
| BMK001-I           | 1.81                        | 236989  | 2.607                 | 242428  | 1.4            | 1.0               | 298                            | 287     |
| IgG1               | NA                          | 248     | NA                    | 226     |                | 1.1               | 215                            | 211     |

**[0248]** The cross-reactivity was also tested with human, cyno and mouse CTLA4 extracellular domain fused to the Fc fragment, by ELISA. The results (**FIG. 15** and **Table 16**) are consistent with the cell-based testing.

**Table 16. Cross-reactivity to human, cyno and mouse CTLA4 proteins by ELISA**

| Name               | hCTLA-4.ECD.hFc, w ANP (300μM) |          | hCTLA-4.ECD.hFc, w/o ANP |          | (-ANP)/(+ANP)  |
|--------------------|--------------------------------|----------|--------------------------|----------|----------------|
|                    | EC50(nM)                       | Max A450 | EC50(nM)                 | Max A450 | Fold of (EC50) |
| P106A2- N32E -S53G | 0.1673                         | 2.536    | 3.502                    | 2.431    | 20.9           |
| P106A2_ N32A -S53G | 0.1612                         | 2.498    | 12.4                     | 2.061    | 76.9           |
| BMK001-I           | 0.1239                         | 2.457    | 0.1178                   | 2.535    | 1.0            |
| IgG1               | NA                             | 0.117    | NA                       | 0.082    | NA             |

| Name | cynoCTLA-4.ECD.hFc, w ANP (300μM) | cynoCTLA-4.ECD.hFc, w/o ANP | (-ANP)/(+ANP) |
|------|-----------------------------------|-----------------------------|---------------|
|------|-----------------------------------|-----------------------------|---------------|

|                    | EC50(nM) | Max A450 | EC50(nM) | Max A450 | Fold of (EC50) |
|--------------------|----------|----------|----------|----------|----------------|
| P106A2- N32E -S53G | 0.1353   | 2.572    | 0.4803   | 2.485    | 3.5            |
| P106A2_ N32A -S53G | 0.1188   | 2.529    | 1.207    | 2.297    | 10.2           |
| BMK001-I           | 0.1254   | 2.472    | 0.1233   | 2.494    | 1.0            |
| IgG1               | NA       | 0.067    | NA       | 0.066    | NA             |

| Name               | mCTLA-4.ECD.hFc, w ANP (300μM) |          | mCTLA-4.ECD.hFc, w/o ANP |          | (-ANP)/(+ANP)  | 300nM Ab Non-specific to hFc |         |
|--------------------|--------------------------------|----------|--------------------------|----------|----------------|------------------------------|---------|
|                    | EC50(nM)                       | Max A450 | EC50(nM)                 | Max A450 | Fold of (EC50) | w ANP (300μM)                | w/o ANP |
| P106A2- N32E -S53G | 0.2364                         | 2.6      | 3.053                    | 2.557    | 12.9           | 0.093                        | 0.119   |
| P106A2_ N32A -S53G | 0.2027                         | 2.563    | 18.76                    | 2.306    | 92.6           | 0.063                        | 0.086   |
| BMK001-I           | 68.03                          | 1.731    | 66.15                    | 1.912    | 1.0            | 0.082                        | 0.082   |
| IgG1               | NA                             | 0.069    | NA                       | 0.068    | NA             | 0.103                        | 0.107   |

[0249] Based on the above experiments and similar additional testing, the importance of certain residues in the extended CDR regions of P106A2 was assessed, which is summarized in **Table 17**.

**Table 17. Residue assessment**

| R4P4F7  | Kabat | AA | ATP Binding           |                         | P106A2 | CTLA4 Binding                      |                         |
|---------|-------|----|-----------------------|-------------------------|--------|------------------------------------|-------------------------|
|         |       |    | Permissible mutations | Impermissible mutations |        | Permissible mutations              | Impermissible mutations |
| VH CDR1 |       |    |                       |                         |        |                                    |                         |
| G       | 26    | 26 | All                   | -                       | G      |                                    |                         |
| D       | 27    | 27 | All                   | -                       | E      |                                    |                         |
| S       | 28    | 28 | All                   | -                       | S      |                                    |                         |
| V       | 29    | 29 | All                   | -                       | V      |                                    |                         |
| S       | 30    | 30 | All                   | -                       | S      |                                    |                         |
| S       | 31    | 31 | All                   | -                       | S      | -                                  | All                     |
| N       | 32    | 32 | All                   | -                       | N      | L, E, F, A, M, W, Q, Y, H, K, or R | Others                  |
| S       | 33    | 33 | All                   | -                       | S      | -                                  | All                     |
| A       | 34    | 34 | All                   | -                       | A      | -                                  | All                     |
| A       | 35    | 35 | All                   | -                       | A      |                                    |                         |
| W       | 35a   | 36 | All                   | -                       | W      |                                    |                         |
| N       | 35b   | 37 | All                   | -                       | N      |                                    |                         |
| VH CDR2 |       |    |                       |                         |        |                                    |                         |
| R       | 50    | 52 | -                     | All                     | R      |                                    |                         |
| T       | 51    | 53 | All                   | -                       | T      |                                    |                         |
| Y       | 52    | 54 | All                   | -                       | Y      |                                    |                         |
| Y       | 52a   | 55 | All                   | -                       | Y      |                                    |                         |
| R       | 52b   | 56 | -                     | All                     | R      |                                    |                         |
| S       | 53    | 57 | All                   | -                       | S      |                                    |                         |
| K       | 54    | 58 | All                   | -                       | K      |                                    |                         |
| W       | 55    | 59 | All                   | -                       | W      |                                    |                         |
| Y       | 56    | 60 | All                   | -                       | Y      |                                    |                         |
| N       | 57    | 61 | All                   | -                       | N      |                                    |                         |
| D       | 58    | 62 | All                   | -                       | D      |                                    |                         |
| Y       | 59    | 63 | All                   | -                       | Y      |                                    |                         |
| A       | 60    | 64 | All                   | -                       | A      |                                    |                         |
| V       | 61    | 65 | All                   | -                       | E      |                                    |                         |

|                |      |     |                                                |        |   |               |        |
|----------------|------|-----|------------------------------------------------|--------|---|---------------|--------|
| S              | 62   | 66  | All                                            | -      | S |               |        |
| V              | 63   | 67  | All                                            | -      | V |               |        |
| K              | 64   | 68  | All                                            | -      | K |               |        |
| S              | 65   | 69  | All                                            | -      | S |               |        |
| <b>VH CDR3</b> |      |     |                                                |        |   |               |        |
| G              | 95   | 102 | All                                            | -      | G |               |        |
| M              | 96   | 103 | All                                            | -      | E | -             | All    |
| R              | 97   | 104 | E, K, T, S, Q,<br>G, P, V, M, or<br>W          | Others | R | -             | All    |
| G              | 98   | 105 | All                                            | -      | R | -             | All    |
| I              | 99   | 106 | All                                            | -      | A |               |        |
| A              | 100  | 107 | All                                            | -      | V |               |        |
| V              | 100a | 108 | All                                            | -      | T |               |        |
| A              | 100b | 109 | All                                            | -      | S |               |        |
| R              | 100c | 110 | K, H, T, S, N,<br>Q, G, A, P, V,<br>L, M, or W | Others | R | -             | All    |
| R              | 100d | 111 | T, L, or M                                     | Others | R | -             | All    |
| A              | 100e | 112 | All                                            | -      | G |               |        |
| F              | 100f | 113 | All                                            | -      | F |               |        |
| D              | 101  | 114 | All                                            | -      | D |               |        |
| I              | 102  | 115 | All                                            | -      | I |               |        |
| <b>VL CDR1</b> |      |     |                                                |        |   |               |        |
| C              | 23   | 23  | All                                            | -      | C |               |        |
| R              | 24   | 24  | All                                            | -      | R |               |        |
| A              | 25   | 25  | All                                            | -      | A |               |        |
| S              | 26   | 26  | All                                            | -      | S |               |        |
| Q              | 27   | 27  | All                                            | -      | Q |               |        |
| G              | 28   | 28  | All                                            | -      | G |               |        |
| V              | 29   | 29  | All                                            | -      | I |               |        |
| S              | 30   | 30  | All                                            | -      | S |               |        |
| S              | 31   | 31  | All                                            | -      | S |               |        |
| W              | 32   | 32  | All                                            | -      | W |               |        |
| L              | 33   | 33  | All                                            | -      | L |               |        |
| A              | 34   | 34  | All                                            | -      | A |               |        |
| W              | 35   | 35  | All                                            | -      | W |               |        |
| <b>VL CDR2</b> |      |     |                                                |        |   |               |        |
| Y              | 49   | 49  | All                                            | -      | Y |               |        |
| S              | 50   | 50  | All                                            | -      | A |               |        |
| A              | 51   | 51  | All                                            | -      | A |               |        |
| S              | 52   | 52  | All                                            | -      | S |               |        |
| S              | 53   | 53  | All                                            | -      | S | G             | Others |
| L              | 54   | 54  | All                                            | -      | L |               |        |
| Q              | 55   | 55  | All                                            | -      | Q | M, F, Y, or L | Others |
| S              | 56   | 56  | All                                            | -      | S |               |        |
| G              | 57   | 49  | All                                            | -      | G |               |        |
| <b>VL CDR3</b> |      |     |                                                |        |   |               |        |
| C              | 88   | 88  | All                                            | -      | C |               |        |
| Q              | 89   | 89  | All                                            | -      | Q |               |        |
| Q              | 90   | 90  | -                                              | All    | Q |               |        |
| G              | 91   | 91  | T, A, S, L, Y,<br>or H                         | Others | G |               |        |
| H              | 92   | 92  | All                                            | -      | N |               |        |
| S              | 93   | 93  | All                                            | -      | V |               |        |
| F              | 94   | 94  | -                                              | All    | F |               |        |
| P              | 95   | 95  | -                                              | All    | P |               |        |
| S              | 96   | 96  | G, T, or A                                     | Others | S |               |        |
| T              | 97   | 97  | All                                            | -      | T |               |        |

**Example 6. Antibody-dependent cellular cytotoxicity**

[0250] The ability of P106A2 and various mutant versions to induce ADCC (antibody-dependent cellular cytotoxicity) was evaluated with an ADCC reporter assay. As shown in **FIG. 16** and **Table 18**, P106A2 mutants in huIgG1 format or with Fc mutations enhanced ADCC had ADCC function in an ANP-dependent manner.

**Table 18. ADCC activities**

| Antibody                              | w ANP (300μM) |         | w/o ANP  |         |
|---------------------------------------|---------------|---------|----------|---------|
|                                       | EC50(nM)      | Max RLU | EC50(nM) | Max RLU |
| P106A2-N32E-S53G-hlgG1/κ              | 3.246         | 432960  | 9.174    | 360680  |
| P106A2-N32E-S53G- hlgG1, ehanced ADCC | 0.162         | 542880  | 0.4878   | 595280  |
| P106A2_ N32A-S53G                     | 4.178         | 459280  | 20.23    | 362400  |
| P106A2-N32A-S53G- hlgG1, ehanced ADCC | 0.1654        | 583440  | 0.7345   | 688200  |
| BMK001-I                              | 0.8796        | 389600  | 0.7932   | 361840  |
| IgG1                                  | NA            | 53960   | NA       | 48480   |

| Antibody          | w ANP (300μM) |         | w/o ANP  |         |
|-------------------|---------------|---------|----------|---------|
|                   | EC50(nM)      | Max RLU | EC50(nM) | Max RLU |
| P106A2-N32A-Q55L  | 4.938         | 366360  | 10.35    | 234960  |
| P106A2-N32M-S53G  | 5.5           | 391600  | 15.79    | 243640  |
| P106A2-N32Q-S53G  | 6.7           | 393080  | 33.22    | 241200  |
| P106A2_ N32A-S53G | 5.638         | 516320  | 12.35    | 300520  |
| BMK001-I          | 0.8254        | 380080  | 0.7926   | 357640  |
| IgG1              | NA            | 47200   | NA       | 48400   |

**Example 7. Blocking of CTLA4 interaction with CD80/CD86**

[0251] The ability of the antibodies to block CTLA4 binding to its ligands CD80 and CD86 was measured with an ELISA blocking assay. As shown in **FIG. 17** and **Table 19**, P106A2 and its mutants had reduced blocking efficacy as compared to the benchmark antibody. Nevertheless, the efficacy was still dependent upon the presence of ANP.

**Table 19. Blocking of CTLA4 binding to CD80/CD86**

| Antibody         | CD80, w ANP (300μM) |          |                    | CD80, w/o ANP |          |                    | (-ANP)/<br>(+ANP) |
|------------------|---------------------|----------|--------------------|---------------|----------|--------------------|-------------------|
|                  | IC50 (nM)           | Min A450 | Max Inhibition (%) | IC50 (nM)     | Min A450 | Max Inhibition (%) | Fold of (IC50)    |
| P106A2-N32E-S53G | 10.98               | 0.14     | 90.91%             | 75.55         | 0.566    | 64.54%             | 6.9               |
| P106A2-N32A-S53G | 11.05               | 0.184    | 88.05%             | 65            | 1.005    | 37.03%             | 5.9               |

|          |        |       |        |        |       |        |     |
|----------|--------|-------|--------|--------|-------|--------|-----|
| BMK001-I | 0.7397 | 0.05  | 96.77% | 0.7696 | 0.051 | 96.82% | 1.0 |
| IgG1     | NA     | 1.408 | 0.00%  | NA     | 1.47  | 0.00%  |     |

| Antibody         | CD86, w ANP (300μM) |          |                    | CD86, w/o ANP |          |                    | (-ANP)/<br>(+ANP) |
|------------------|---------------------|----------|--------------------|---------------|----------|--------------------|-------------------|
|                  | IC50 (nM)           | Min A450 | Max Inhibition (%) | IC50 (nM)     | Min A450 | Max Inhibition (%) | Fold of (IC50)    |
| P106A2-N32E-S53G | 6.375               | 0.236    | 82.24%             | 37.99         | 0.577    | 55.27%             | 6.0               |
| P106A2-N32A-S53G | 6.916               | 0.243    | 81.72%             | 65.99         | 0.779    | 39.61%             | 9.5               |
| BMK001-I         | 1.068               | 0.077    | 94.70%             | 1.074         | 0.076    | 94.22%             | 1.0               |
| IgG1             | NA                  | 1.257    | 0.00%              | NA            | 1.262    | 0.00%              |                   |

[0252] In some of the antibodies tested above, the Fc fragment included certain ADCC-enhancing mutations, such as S239D and I332E in the human IgG1 (hIgG1 Fc-DE). The impact of various Fc versions on the antibody binding activity was assessed next, including afucosylation, DE (S239D and I332E, EU numbering), AAA (S298A, E333A and K334A, EU numbering), and YTE (M252Y, S254T and T256E, EU numbering).

[0253] As shown in FIG. 18 and Table 20, the various Fc formats did not impact the binding activity of these antibodies.

**Table 20. Impact of Fc variants on antibody binding**

| Antibody                              | CHOK1-hCTLA4, w ANP (300μM) |         | CHOK1-hCTLA4, w/o ANP |         | (-ANP)/<br>(+ANP) | (+ANP)/<br>(-ANP) | 300nM Ab Non-specific to CHOK1 |         |
|---------------------------------------|-----------------------------|---------|-----------------------|---------|-------------------|-------------------|--------------------------------|---------|
|                                       | EC50(nM)                    | Max MFI | EC50(nM)              | Max MFI | Fold of (EC50)    | Fold of (Max MFI) | w ANP (300μM)                  | w/o ANP |
| P106A2-N32E-S53G-hIgG1/κ              | 2.56                        | 14927   | 3.267                 | 7917    | 1.3               | 1.9               | 214                            | 261     |
| P106A2-N32E-S53G-hIgG1, enhanced ADCC | 2.519                       | 15767   | 2.907                 | 8242    | 1.2               | 1.9               | 190                            | 235     |
| BMK001-I                              | 1.579                       | 18026   | 1.269                 | 16293   | 0.8               | 1.1               | 279                            | 286     |
| IgG1                                  | NA                          | 209     | NA                    | 203     |                   | 1.0               | 251                            | 257     |

### Example 8. *In vivo* efficacy and safety testing

[0254] The *in vivo* efficacy and safety of P106A2-N32E-S53G-hIgG1/κ (“NESG”, VH N32E, VL S53G, Kabat numbering, Table 21) and variants with ADCC-enhanced Fc mutations were tested in an MC38 mouse model in B-hPD-1/hPD-L1/hCTLA4 mice (Biocytogen JiangSu Co.,Ltd).. Ipilimumab was still used as the benchmark. P106A2-N32E-S53G-hIgG1/κ has good anti-tumor activity at 1mpk. With the ADCC-enhanced Fc, the anti-tumor activity was

further improved, achieving higher efficacy at both doses (1 mg/kg and 3 mg/kg) than the benchmark (FIG. 19).

**Table 21. Sequences of P106A2 Variants**

| Antibody                      | Sequence                                                                                                                                          | SEQ ID NO: |
|-------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| P106A2-NESG VH                | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>ESA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS | 41         |
| P106A2-NESG VL                | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>GLQ</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 42         |
| P106A2-N(H32)L<br>VH          | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>LSA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS | 43         |
| P106A2-N(H32)L<br>VL          | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLQ</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 4          |
| P106A2-N(H32)R<br>VH          | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>RSA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS | 44         |
| P106A2-N(H32)R<br>VL          | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLQ</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 4          |
| P106A2-N(H32)E<br>VH          | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>ESA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS | 41         |
| P106A2-N(H32)E<br>VL          | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLQ</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 4          |
| P106A2, Q(L55)M<br>VH         | QVQLQQSGPGLVKPSQTLSTCAISGESVSSNSAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS          | 3          |
| P106A2, Q(L55)M<br>VL         | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLM</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 45         |
| P106A2, Q(L55)Y<br>VH         | QVQLQQSGPGLVKPSQTLSTCAISGESVSSNSAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS          | 3          |
| P106A2, Q(L55)Y<br>VL         | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLY</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 46         |
| P106A2-N(H32)R,<br>Q(L55)M VH | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>RSA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS | 44         |
| P106A2-N(H32)R,<br>Q(L55)M VL | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLM</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 45         |
| P106A2-N(H32)R,<br>Q(L55)Y VH | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>RSA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGGTTTVTVSS | 44         |
| P106A2-N(H32)R,<br>Q(L55)Y VL | DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKPGKAPKLLIYAAS <b>SLY</b><br>SGVPSRFSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                          | 46         |

|                                           |                                                                                                                                                                                                                                                               |    |
|-------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| P106A2-N(H32)R,<br>S(L53)G VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>RS</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 44 |
| P106A2-N(H32)R,<br>S(L53)G VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>GL</del> Q<br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                             | 42 |
| P106A2-N(H32)E,<br>Q(L55)M VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>ES</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 41 |
| P106A2-N(H32)E,<br>Q(L55)M VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>SLM</del><br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                              | 45 |
| P106A2-N(H32)E,<br>Q(L55)Y VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>ES</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 41 |
| P106A2-N(H32)E,<br>Q(L55)Y VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>SLY</del><br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                              | 46 |
| P106A2-N(H32)E,<br>Q(L55)L VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>ES</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 41 |
| P106A2-N(H32)E,<br>Q(L55)L VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>SL</del> L<br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                             | 47 |
| P106A2-N(H32)E,<br>Q(L55)F VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>ES</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 41 |
| P106A2-N(H32)E,<br>Q(L55)F VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>SLF</del><br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                              | 48 |
| P106A2-N(H32)E,<br>S(L53)G, Q55L VH       | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>ES</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 41 |
| P106A2-N(H32)E,<br>S(L53)G, Q55L VL       | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>GL</del> L<br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                             | 49 |
| P106A2-N(H32)E,<br>S(L53)G, Q(L55)Y<br>VH | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>ES</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 41 |
| P106A2-N(H32)E,<br>S(L53)G, Q(L55)Y<br>VL | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>GLY</del><br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                              | 50 |
| P106A2-N(H32)L,<br>Q(L55)L VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>LS</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 43 |
| P106A2-N(H32)L,<br>Q(L55)L VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>SL</del> L<br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                             | 47 |
| P106A2-N(H32)L,<br>Q(L55)Y VH             | QVQLQQSGPGLVKPSQTL <del>SL</del> TC <del>AI</del> SGESVSS <del>LS</del> SAAWN <del>WIR</del> QSPSRGLEWLGR <del>TY</del> Y<br>RSK <del>WY</del> NDYA <del>ES</del> VKSRI <del>T</del> INPDT <del>SK</del> NQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 43 |
| P106A2-N(H32)L,<br>Q(L55)Y VL             | DIQMTQSPSSVSASVGDRVTITCRASQG <del>ISS</del> WLAWYQQKPGKAPKLLIYAAS <del>SLY</del><br>SGVPSR <del>F</del> SGSGSGTDFTLT <del>TI</del> SS <del>LQ</del> PEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                              | 46 |

|                               |                                                                                                                                                    |    |
|-------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|----|
| P106A2-N(H32)L,<br>Q(L55)F VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSSLSAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS          | 43 |
| P106A2-N(H32)L,<br>Q(L55)F VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLF</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 48 |
| P106A2-N(H32)L,<br>S(L53)G VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSSLSAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS          | 43 |
| P106A2-N(H32)L,<br>S(L53)G VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>GLQ</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 42 |
| P106A2-N(H32)L,<br>Q(L55)M VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSSLSAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS          | 43 |
| P106A2-N(H32)L,<br>Q(L55)M VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLM</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 45 |
| P106A2-N(H32)F,<br>Q(L55)L VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSS <b>FS</b> AAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 51 |
| P106A2-N(H32)F,<br>Q(L55)L VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLL</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 47 |
| P106A2-N(H32)F,<br>Q(L55)Y VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSS <b>FS</b> AAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 51 |
| P106A2-N(H32)F,<br>Q(L55)Y VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLY</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 46 |
| P106A2-N(H32)F,<br>S(L53)G VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSS <b>FS</b> AAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 51 |
| P106A2-N(H32)F,<br>S(L53)G VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>GLQ</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 42 |
| P106A2-N(H32)F,<br>Q(L55)M VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSS <b>FS</b> AAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 51 |
| P106A2-N(H32)F,<br>Q(L55)M VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLM</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 45 |
| P106A2-N(H32)A,<br>Q(L55)M VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSS <b>ASA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 52 |
| P106A2-N(H32)A,<br>Q(L55)M VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLM</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 45 |
| P106A2-N(H32)A,<br>Q(L55)F VH | QVQLQQSGPGLVKPSQTLSTLCAISGESVSS <b>ASA</b> AWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRIITINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGTTTVTVSS | 52 |
| P106A2-N(H32)A,<br>Q(L55)F VL | DIQMTQSPSSVSASVGDRVITITCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLF</b><br>SGVPSRFRSGSGSGTDFTLTITSSLPEDFATYYCQQGNVFPSTFGQGTKVEIK                         | 48 |

|                               |                                                                                                                                                                                                        |    |
|-------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| P106A2-N(H32)A,<br>Q(L55)L VH | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>ASA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 52 |
| P106A2-N(H32)A,<br>Q(L55)L VL | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SL</del> <del>L</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                      | 47 |
| P106A2-N(H32)A,<br>Q(L55)Y VH | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>ASA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 52 |
| P106A2-N(H32)A,<br>Q(L55)Y VL | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SLY</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                  | 46 |
| P106A2-N(H32)A,<br>S(L53)G VH | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>ASA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 52 |
| P106A2-N(H32)A,<br>S(L53)G VL | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>G</del> LQ<br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                 | 42 |
| P106A2-(H32)M,<br>Q(L55)F VH  | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>SMA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 53 |
| P106A2-(H32)M,<br>Q(L55)F VL  | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SLF</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                  | 48 |
| P106A2-(H32)M,<br>Q(L55)Y VH  | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>SMA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 53 |
| P106A2-(H32)M,<br>Q(L55)Y VL  | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SLY</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                  | 46 |
| P106A2-(H32)M,<br>S(L55)G VH  | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>SMA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 53 |
| P106A2-(H32)M,<br>S(L55)G VL  | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>G</del> LQ<br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                 | 42 |
| P106A2-N(H32)Q,<br>Q(L55)M VH | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>QSA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 54 |
| P106A2-N(H32)Q,<br>Q(L55)M VL | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SLM</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                  | 45 |
| P106A2-N(H32)Q,<br>Q(L55)F VH | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>QSA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 54 |
| P106A2-N(H32)Q,<br>Q(L55)F VL | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SLF</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                  | 48 |
| P106A2-N(H32)Q,<br>Q(L55)Y VH | QVQLQQSGPGLVKPSQTL <del>SLTCAISGESVSS</del> <del>QSA</del> AWN <del>WIRQSPSRGLEWLGR</del> ITYY<br>RSKWYNDYAESVKSRI <del>TINPD</del> TSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGQGT <del>TVTVSS</del> | 54 |
| P106A2-N(H32)Q,<br>Q(L55)Y VL | DIQMTQSPSSVSASVGDRV <del>ITTCRASQGISSWLA</del> WYQQKPGKAPKLLIYAAS <del>SLY</del><br>SGVPSRFSGSGSGTDFLT <del>TISS</del> LQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                  | 46 |

|                                           |                                                                                                                                                                                                                                                                                                 |                                                                      |
|-------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|
| P106A2-N(H32)Q,<br>S(L53)G VH             | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>Q</b> SAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRTINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGTTTVTVSS                                                                                                                                                  | 54                                                                   |
| P106A2-N(H32)Q,<br>S(L53)G VL             | DIQMTQSPSSVSASVGDRVITTCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>GLQ</b><br>SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                                                                                                                        | 42                                                                   |
| P106A2-N(H32)Q,<br>S(L53)G, Q(L55)F<br>VH | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>Q</b> SAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRTINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGTTTVTVSS                                                                                                                                                  | 54                                                                   |
| P106A2-N(H32)Q,<br>S(L53)G, Q(L55)F<br>VL | DIQMTQSPSSVSASVGDRVITTCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>GLF</b><br>SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                                                                                                                        | 55                                                                   |
| P106A2-N(H32)Y,<br>Q(L55)F VH             | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>Y</b> SAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRTINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGTTTVTVSS                                                                                                                                                  | 56                                                                   |
| P106A2-N(H32)Y,<br>Q(L55)F VL             | DIQMTQSPSSVSASVGDRVITTCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLF</b><br>SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                                                                                                                        | 48                                                                   |
| P106A2-N(H32)H,<br>Q(L55)F VH             | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>H</b> SAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRTINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGTTTVTVSS                                                                                                                                                  | 57                                                                   |
| P106A2-N(H32)H,<br>Q(L55)F VL             | DIQMTQSPSSVSASVGDRVITTCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLF</b><br>SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                                                                                                                        | 48                                                                   |
| P106A2-N(H32)H,<br>Q(L55)Y VH             | QVQLQQSGPGLVKPSQTLSTCAISGESVSS <b>H</b> SAAWNWIRQSPSRGLEWLGRITYY<br>RSKWYNDYAESVKSRTINPDTSKNQFSLQLNSVTPEDTAVYYCARGERRAVTSR<br>RGFDIWGGTTTVTVSS                                                                                                                                                  | 57                                                                   |
| P106A2-N(H32)H,<br>Q(L55)Y VL             | DIQMTQSPSSVSASVGDRVITTCRASQGIISSWLAWYQQKPGKAPKLLIYAAS <b>SLY</b><br>SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGNVFPSTFGQGTKVEIK                                                                                                                                                                        | 46                                                                   |
| VH CDR1                                   | GESVSSNSAAWN<br>GESVSS <b>E</b> SAAWN<br>GESVSS <b>L</b> SAAWN<br>GESVSS <b>F</b> SAAWN<br>GESVSS <b>A</b> SAAWN<br>GESVSS <b>M</b> SAAWN<br>GESVSS <b>Q</b> SAAWN<br>GESVSS <b>Y</b> SAAWN<br>GESVSS <b>H</b> SAAWN<br>GESVSS <b>R</b> SAAWN<br>GESVSS <b>W</b> SAAWN<br>GESVSS <b>K</b> SAAWN | 15<br>58<br>59<br>60<br>61<br>62<br>63<br>64<br>65<br>66<br>67<br>68 |
| VH CDR2                                   | RTYYRSKWYNDYAESVKS                                                                                                                                                                                                                                                                              | 16                                                                   |
| VH CDR3                                   | GERRAVTSRRGFDI<br>GERR <b>S</b> VTSSRRGFDI<br>GERRAV <b>S</b> SSRRGFDI<br>GERRAVT <b>A</b> RRRGFDI<br>GERRAVT <b>Y</b> RRRGFDI<br>GERRAVT <b>V</b> RRRGFDI<br>GERRAVT <b>T</b> RRRGFDI<br>GERRAVTARR <b>A</b> FDI                                                                               | 17<br>69<br>70<br>71<br>72<br>73<br>74<br>75                         |
| VL CDR1                                   | CRASQGISSWLAW                                                                                                                                                                                                                                                                                   | 18                                                                   |
| VL CDR2                                   | YAASSLQSG<br>YAAS <b>G</b> LQSG<br>YAASSL <b>M</b> SG<br>YAASSL <b>E</b> SG                                                                                                                                                                                                                     | 19<br>76<br>77<br>78                                                 |

|         |                             |    |
|---------|-----------------------------|----|
|         | YAASSL <b>Y</b> SG          | 79 |
|         | YAASSL <b>L</b> SG          | 80 |
|         | YAAS <b>G</b> L <b>F</b> SG | 81 |
|         | YAAS <b>G</b> L <b>Y</b> SG | 82 |
|         | YAAS <b>G</b> L <b>L</b> SG | 83 |
|         | YAAS <b>G</b> L <b>M</b> SG | 84 |
| VL CDR3 | CQQGNVFPST                  | 20 |

### Example 9. Manufacturability of the antibodies

[0255] The manufacturability of the antibodies was assessed by UNcle, which combines three different measurement modes, fluorescence, Static Light Scattering (SLS) and Dynamic Light Scattering (DLS).

**Table 22. Thermo- and colloidal stability assessment by UNcle**

| Antibody                | Conc.<br>(mg/ml) | T <sub>m1</sub><br>(°C) | T <sub>m2</sub><br>(°C) | T <sub>onset</sub><br>(°C) | T <sub>agg</sub> at<br>266nm<br>(°C) | Initial<br>diameter<br>at 25°C (nm) | Initial<br>PDI<br>at 25°C | Pk1<br>Mass<br>(%) |
|-------------------------|------------------|-------------------------|-------------------------|----------------------------|--------------------------------------|-------------------------------------|---------------------------|--------------------|
| P106A2-N32E-S53G, hIgG1 | 5.0              | 69.34                   | 82.40                   | 56.36                      | 67.32                                | 11.30                               | 0.047                     | 100                |

[0256] As shown in **Table 22**, these antibodies exhibited excellent thermal stability and colloidal stability.

### Example 10. ANP-Dependent Anti-FolR Antibodies

[0257] This example applied the antibody screening technology evaluated in the preceding examples in screening for anti-FolR (folate receptor alpha) antibodies.

[0258] With the CDR scanning results as obtained in **Example 2**, the residues important for ATP binding were used to search, in the human antibody libraries as used in **Example 3**, for antibodies that contained permissible amino acids at those residues and were capable of binding to the human FolR protein. The resulting anti-FolR antibody, FP010-F01, included the CDR residues as shown in **Table 23**, and the same framework sequences as the anti-CTLA4 antibodies of **Example 3** (see, **Table 6**).

**Table 23. FP010-F01 CDR residues**

| Domain | Kabat | FP010-F01 |
|--------|-------|-----------|
| HCDR1  | H26   | G         |
|        | H27   | D         |
|        | H28   | S         |

|       |      |          |
|-------|------|----------|
|       | H29  | V        |
|       | H30  | S        |
|       | H31  | S        |
|       | H32  | N        |
|       | H33  | T        |
|       | H34  | V        |
|       | H35  | A        |
|       | H35a | W        |
|       | H35b | N        |
| HCDR2 | H50  | R        |
|       | H51  | T        |
|       | H52  | Y        |
|       | H52a | Y        |
|       | H52b | R        |
|       | H53  | S        |
|       | H54  | K        |
|       | H55  | W        |
|       | H56  | Y        |
|       | H57  | N        |
|       | H58  | D        |
|       | H59  | Y        |
|       | H60  | A        |
|       | H61  | V        |
|       | H62  | S        |
|       | H63  | V        |
|       | H64  | K        |
|       | H65  | S        |
| HCDR3 | H95  | T        |
|       | H96  | M        |
|       | H97  | <b>R</b> |
|       | H98  | W        |
|       | H99  | Y        |
|       | H100 | G        |
|       | i    | F        |
|       | j    | G        |
|       | k    | K        |
|       | l    | <b>R</b> |
|       | m    | A        |
|       | n    | F        |
|       | H101 | D        |
|       | H102 | Y        |

| Domain | Kabat | FP010-F01 |
|--------|-------|-----------|
| LCDR1  | L24   | R         |
|        | L25   | A         |
|        | L26   | S         |
|        | L27   | Q         |
|        | L28   | G         |
|        | L29   | I         |
|        | L30   | S         |
|        | L31   | R         |
|        | L32   | W         |

|       |     |   |
|-------|-----|---|
|       | L33 | L |
|       | L34 | A |
| LCDR2 | L50 | S |
|       | L51 | A |
|       | L52 | S |
|       | L53 | S |
|       | L54 | L |
|       | L55 | Q |
|       | L56 | S |
|       |     |   |
| LCDR3 | L89 | Q |
|       | L90 | Q |
|       | L91 | G |
|       | L92 | H |
|       | L93 | S |
|       | L94 | F |
|       | L95 | P |
|       | L96 | S |
|       | L97 | T |

[0259] The ability of the anti-FolR antibody FP010-F01 to bind to the target FolR protein was tested with FolR-positive and -negative cells. As shown in **FIG. 20**, similar to a benchmark antibody (BMK002), FP010-F01 bound to FolR-positive cells in an ATP-dependent manner (i.e., considerably higher binding in the presence of ATP).

[0260] The ATP-dependency of FP010-F01 in FolR binding was further analyzed at different concentrations of ATP (0 nM, 10 nM, 1  $\mu$ M, 10  $\mu$ M, and 100  $\mu$ M). As shown in **FIG. 21**, the FolR binding affinity correlated with ATP concentrations.

[0261] The ATP dependency of FP010-F01 did not hamper its cross-species binding. As shown in **FIG. 22**, FP010-F01 bound to the FolR proteins from both humans and cynomolgus monkeys.

[0262] Finally, the dependency dynamics of FP010-F01 binding to FolR on AMP, ADP and ATP was evaluated. As shown in the results (**Table 24**), such dependency had large windows.

**Table 24. FP010-F01 exhibited ATP ADP AMP dependent binding with large windows**

| Sample                        | Response | KD (M)    | ka (1/Ms) | kdis (1/s) |
|-------------------------------|----------|-----------|-----------|------------|
| FP010-F01 PBS 100 $\mu$ M ATP | 0.3773   | 6.755E-09 | 5.303E05  | 3.582E-03  |
| FP010-F01 PBS 100 $\mu$ M ADP | 0.402    | 3.531E-09 | 5.743E05  | 2.028E-03  |
| FP010-F01 PBS 100 $\mu$ M AMP | 0.4034   | 3.030E-09 | 1.052E06  | 3.187E-03  |
| FP010-F01 PBS                 | 0.2064   | 1.277E-07 | 1.694E05  | 2.164E-02  |

\* \* \*

**[0263]** The present disclosure is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the disclosure, and any compositions or methods which are functionally equivalent are within the scope of this disclosure. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present disclosure without departing from the spirit or scope of the disclosure. Thus, it is intended that the present disclosure cover the modifications and variations of this disclosure provided they come within the scope of the appended claims and their equivalents.

**[0264]** All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## CLAIMS

**What is claimed is:**

1. An antibody or antigen-binding fragment thereof capable of binding to ATP (adenosine triphosphate) ADP (adenosine diphosphate), or AMP (adenosine monophosphate), wherein the antibody or the fragment thereof comprises a heavy chain variable region (VH) and a light chain variable region (VL), wherein the VH has

R at position 50;

R at position 52b;

R, E, K, T, S, Q, G, P, V, M or W at position 97;

R, K, H, T, S, N, Q, G, A, P, V, L, M or W at position 100c; and

R, T, L or M at position 100d,

and the VL has

Q at position 90;

G, T, A, S, L, Y or H at position 91;

F at position 94;

P at position 95; and

S, G, T or A at position 96,

all positions according to Kabat numbering.

2. The antibody or antigen-binding fragment thereof of claim 1, which has binding specificity to a tumor associated antigen or immune checkpoint molecule, and binds to the tumor associated antigen or immune checkpoint molecule at higher affinity in the presence of the ATP, ADP or AMP than in the absence of the ATP, ADP or AMP.

3. The antibody or antigen-binding fragment thereof of claim 1 or 2, wherein the VH has

R at position 50;

R at position 52b;

R at position 97;

R at position 100c; and  
R at position 100d,  
and the VL has

Q at position 90;  
G at position 91;  
F at position 94;  
P at position 95; and  
S at position 96.

4. The antibody or antigen-binding fragment thereof of any preceding claim, wherein the VH comprises heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and wherein

the VH CDR1 comprises the amino acid sequence of SEQ ID NO:9 or SEQ ID NO:9  
with 1, 2, 3, 4 or 5 amino acid substitutions; and  
the VH CDR2 comprises the amino acid sequence of SEQ ID NO:10 or SEQ ID  
NO:10 with 1, 2, 3, 4 or 5 amino acid substitutions.

5. The antibody or antigen-binding fragment thereof of claim 4, wherein  
the VH CDR1 comprises the amino acid sequence of SEQ ID NO:9 or SEQ ID NO:9  
with 1, 2, or 3 amino acid substitutions; and  
the VH CDR2 comprises the amino acid sequence of SEQ ID NO:10 or SEQ ID  
NO:10 with 1, 2, or 3 amino acid substitutions.

6. The antibody or antigen-binding fragment thereof of any preceding claim, wherein the VL comprises light chain complementarity determining regions (CDR) VL CDR1, VL CDR2, and VL CDR3, and wherein

the VL CDR1 comprises the amino acid sequence of SEQ ID NO:12 or SEQ ID  
NO:12 with 1, 2, 3, 4 or 5 amino acid substitutions; and

the VL CDR2 comprises the amino acid sequence of SEQ ID NO:13 or SEQ ID NO:14 with 1, 2, 3, 4 or 5 amino acid substitutions.

7. The antibody or antigen-binding fragment thereof of claim 6, wherein the VL CDR1 comprises the amino acid sequence of SEQ ID NO:12 or SEQ ID NO:12 with 1, 2, or 3 amino acid substitutions; and the VL CDR2 comprises the amino acid sequence of SEQ ID NO:13 or SEQ ID NO:13 with 1, 2, or 3 amino acid substitutions.
8. The antibody or antigen-binding fragment thereof of any preceding claim, wherein the VH comprises a first framework region, a second framework region, a third framework region, and a fourth framework region which, collectively have at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:33, 34, 35 and 36, and the VL comprises a first framework region, a second framework region, a third framework region, and a fourth framework region which, collectively have at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:37, 38, 39 and 40.
9. The antibody or antigen-binding fragment thereof of claim 8, wherein the first, second, third and fourth framework regions of the VH, respectively, has at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:33, 34, 35 and 36.
10. The antibody or antigen-binding fragment thereof of claim 8 or 9, wherein the first, second, third and fourth framework regions of the VL, respectively, has at least 70%, 80%, 90% or 95% sequence identity to SEQ ID NO: 37, 38, 39 and 40.
11. The antibody or antigen-binding fragment thereof of any one of claims 2-10, wherein the antigen is CTLA4.

12. The antibody or antigen-binding fragment thereof of claim 11, which comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3, and wherein

the VH CDR1 comprises the amino acid sequence of SEQ ID NO:15, or SEQ ID NO:15 in which position 32 is an amino acid selected from L, E, F, A, M, W, Q, Y, H, K or R;

the VH CDR2 comprises the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having at least 60%, 70%, 80%, 90% or 95% sequence identity to SEQ ID NO:16;

the VH CDR3 comprises the amino acid sequence of SEQ ID NO:17, or SEQ ID NO:17 with an amino acid substitution at any one of the positions 95, 99, 100, 100a, 100b, 100e, 100f, 101, or 102;

the VL CDR1 comprises the amino acid sequence of SEQ ID NO:18, or SEQ ID NO:18 with an amino acid substitution;

the VL CDR2 comprises the amino acid sequence of SEQ ID NO:19, or SEQ ID NO:19 in which position 53 is G, or position 55 is M, F, Y or L; and

the VL CDR3 comprises the amino acid sequence of SEQ ID NO:20, or SEQ ID NO:20 an amino acid substitution at any one of the positions 88, 89, 92, 93 or 97,

all positions according to Kabat numbering.

13. The antibody or antigen-binding fragment thereof of claim 12, wherein

the VH CDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:15 and 58-68;

the VH CDR2 comprises the amino acid sequence of SEQ ID NO:16;

the VH CDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NO:17 and 69-75;

the VL CDR1 comprises the amino acid sequence of SEQ ID NO:18;  
the VL CDR2 comprises an amino acid sequence selected from the group consisting  
of SEQ ID NO:19 and 76-84; or  
the VL CDR3 comprises the amino acid sequence of SEQ ID NO:20.

14. The antibody or antigen-binding fragment thereof of claim 12, wherein  
the VH CDR1 comprises an amino acid sequence selected from the group consisting  
of SEQ ID NO:15 and 58-66;  
the VH CDR2 comprises the amino acid sequence of SEQ ID NO:16;  
the VH CDR3 comprises the amino acid sequence of SEQ ID NO:17;  
the VL CDR1 comprises the amino acid sequence of SEQ ID NO:18;  
the VL CDR2 comprises an amino acid sequence selected from the group consisting  
of SEQ ID NO:19 and 76-83; or  
the VL CDR3 comprises the amino acid sequence of SEQ ID NO:20.

15. The antibody or antigen-binding fragment thereof of claim 12, wherein the VH  
CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively,  
the amino acid sequences of

SEQ ID NO:15, 16, 17, 18, 19 and 20,  
SEQ ID NO:58, 16, 17, 18, 76 and 20,  
SEQ ID NO:59, 16, 17, 18, 19 and 20,  
SEQ ID NO:66, 16, 17, 18, 19 and 20,  
SEQ ID NO:58, 16, 17, 18, 19 and 20,  
SEQ ID NO:15, 16, 17, 18, 77 and 20,  
SEQ ID NO:15, 16, 17, 18, 79 and 20,  
SEQ ID NO:66, 16, 17, 18, 77 and 20,  
SEQ ID NO:66, 16, 17, 18, 79 and 20,  
SEQ ID NO:66, 16, 17, 18, 76 and 20,

SEQ ID NO:58, 16, 17, 18, 77 and 20,  
SEQ ID NO:58, 16, 17, 18, 79 and 20,  
SEQ ID NO:58, 16, 17, 18, 80 and 20,  
SEQ ID NO:58, 16, 17, 18, 78 and 20,  
SEQ ID NO:58, 16, 17, 18, 83 and 20,  
SEQ ID NO:58, 16, 17, 18, 82 and 20,  
SEQ ID NO:59, 16, 17, 18, 80 and 20,  
SEQ ID NO:59, 16, 17, 18, 79 and 20,  
SEQ ID NO:59, 16, 17, 18, 78 and 20,  
SEQ ID NO:59, 16, 17, 18, 76 and 20,  
SEQ ID NO:59, 16, 17, 18, 77 and 20,  
SEQ ID NO:60, 16, 17, 18, 80 and 20,  
SEQ ID NO:60, 16, 17, 18, 79 and 20,  
SEQ ID NO:60, 16, 17, 18, 76 and 20,  
SEQ ID NO:60, 16, 17, 18, 77 and 20,  
SEQ ID NO:61, 16, 17, 18, 77 and 20,  
SEQ ID NO:61, 16, 17, 18, 78 and 20,  
SEQ ID NO:61, 16, 17, 18, 80 and 20,  
SEQ ID NO:61, 16, 17, 18, 79 and 20,  
SEQ ID NO:61, 16, 17, 18, 76 and 20,  
SEQ ID NO:62, 16, 17, 18, 78 and 20,  
SEQ ID NO:62, 16, 17, 18, 79 and 20,  
SEQ ID NO:62, 16, 17, 18, 76 and 20,  
SEQ ID NO:63, 16, 17, 18, 77 and 20,  
SEQ ID NO:63, 16, 17, 18, 78 and 20,  
SEQ ID NO:63, 16, 17, 18, 79 and 20,  
SEQ ID NO:63, 16, 17, 18, 76 and 20,  
SEQ ID NO:63, 16, 17, 18, 81 and 20,

SEQ ID NO:64, 16, 17, 18, 78 and 20,  
SEQ ID NO:65, 16, 17, 18, 78 and 20, or  
SEQ ID NO:65, 16, 17, 18, 79 and 20.

16. The antibody or antigen-binding fragment thereof of claim 12, wherein the VH and the VL comprise, respectively, the amino acid sequences of

SEQ ID NO:3 and 4,  
SEQ ID NO:41 and 42,  
SEQ ID NO:43 and 4,  
SEQ ID NO:44 and 4,  
SEQ ID NO:41 and 4,  
SEQ ID NO:3 and 45,  
SEQ ID NO:3 and 46,  
SEQ ID NO:44 and 45,  
SEQ ID NO:44 and 46,  
SEQ ID NO:44 and 42,  
SEQ ID NO:41 and 45,  
SEQ ID NO:41 and 46,  
SEQ ID NO:41 and 47,  
SEQ ID NO:41 and 48,  
SEQ ID NO:41 and 49,  
SEQ ID NO:41 and 50,  
SEQ ID NO:43 and 47,  
SEQ ID NO:43 and 46,  
SEQ ID NO:43 and 48,  
SEQ ID NO:43 and 42,  
SEQ ID NO:43 and 45,  
SEQ ID NO:51 and 47,

SEQ ID NO:51 and 46,  
SEQ ID NO:51 and 42,  
SEQ ID NO:51 and 45,  
SEQ ID NO:52 and 45,  
SEQ ID NO:52 and 48,  
SEQ ID NO:52 and 47,  
SEQ ID NO:52 and 46,  
SEQ ID NO:52 and 42,  
SEQ ID NO:53 and 48,  
SEQ ID NO:53 and 46,  
SEQ ID NO:53 and 42,  
SEQ ID NO:54 and 45,  
SEQ ID NO:54 and 48,  
SEQ ID NO:54 and 46,  
SEQ ID NO:54 and 42,  
SEQ ID NO:54 and 55,  
SEQ ID NO:56 and 48,  
SEQ ID NO:57 and 48, or  
SEQ ID NO:57 and 46.

17. The antibody or antigen-binding fragment thereof of claim 12, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:58, 16, 17, 18, 76 and 20.

18. The antibody or antigen-binding fragment thereof of claim 12, wherein the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:41 and 42.

19. The antibody or antigen-binding fragment thereof of claim 11, which comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3, and wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:21, 22, 23, 24, 25 and 26.

20. The antibody or antigen-binding fragment thereof of claim 19, wherein the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:5 and 6.

21. The antibody or antigen-binding fragment thereof of claim 11, which comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions (CDR) VH CDR1, VH CDR2, and VH CDR3, and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3, and wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2 and VL CDR3 comprise, respectively, the amino acid sequences of SEQ ID NO:27, 28, 29, 30, 31 and 32.

22. The antibody or antigen-binding fragment thereof of claim 21, wherein the VH and the VL comprise, respectively, the amino acid sequences of SEQ ID NO:7 and 8.

23. The antibody or antigen-binding fragment thereof of any preceding claim, which comprises an IgG Fc fragment that is ADCC competent.

24. A multispecific antibody comprising an antigen-binding fragment of any one of claims 1-23 and one or more antibody or antigen-binding fragment having binding specificity to a target antigen that is not CTLA4.

25. One or more polynucleotide(s) encoding the antibody or antigen-binding fragment thereof of any one of claims 1-24.
26. A cell comprising the polynucleotide(s) of claim 25.
27. A method of treating cancer in a patient in need thereof, comprising administering to the patient the antibody or antigen-binding fragment thereof of any one of claims 1-24, the polynucleotide(s) of claim 25, or the cell of claim 26.
28. Use of the antibody or antigen-binding fragment thereof of any one of claims 1-24, the polynucleotide(s) of claim 25, or the cell of claim 26 for the manufacture of a medicament for the treatment of cancer.
29. The method of claim 27 or the use of claim 28, wherein the cancer is selected from the group consisting of bladder cancer, liver cancer, colon cancer, rectal cancer, endometrial cancer, leukemia, lymphoma, pancreatic cancer, small cell lung cancer, non-small cell lung cancer, breast cancer, urethral cancer, head and neck cancer, gastrointestinal cancer, stomach cancer, oesophageal cancer, ovarian cancer, renal cancer, melanoma, prostate cancer and thyroid cancer.