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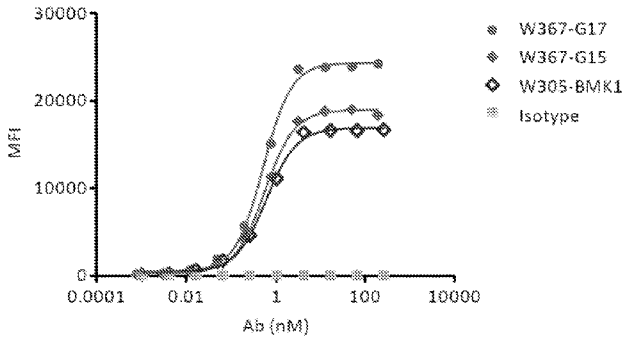

Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	0.52	24331
W367-G15	0.56	18947
W305-BMK1	0.60	16891

Figure 1

(57) Abstract: The present disclosure provides anti-CD47/PD-1 bispecific antibody molecules, isolated polynucleotides encoding the same, pharmaceutical compositions comprising the same, and the uses thereof.

[Continued on next page]

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NOVEL BISPECIFIC PD-1/CD47 ANTIBODY MOLECULES

PRIORITY CLAIM

[0001] The present application claims the priority to PCT Application Number PCT/CN2018/079674, filed on March 20, 2018.

FIELD OF THE INVENTION

[0002] The present disclosure generally relates to novel bispecific antibody molecules directed to human PD-1 and human CD47.

BACKGROUND

[0003] Bispecific antibodies are growing to be the new category of therapeutic antibodies. They can bind two different targets or two different epitopes on a target, creating additive or synergistic effect superior to the effect of individual antibodies. A lot of antibody engineering efforts have been put into designing new bispecific formats, such as DVD-Ig, CrossMab, BiTE etc. (Spiess et al. *Molecular Immunology*, 67(2), pp.95–106 (2015).). However, these formats may potentially have various limitations in stability, solubility, short half-life, and immunogenicity.

[0004] Increasing evidences from preclinical and clinical results have shown that targeting immune checkpoints is becoming the most promising approach to treat patients with cancers. Programmed cell death 1 (PD-1), one of immune-checkpoint proteins, play a major role in limiting the activity of T cells that provide a major immune resistance mechanism by which tumor cells escaped immune surveillance. The interaction of PD-1 expressed on activated T cells, and PD-L1 expressed on tumor cells negatively regulate immune response and damp anti-tumor immunity.

[0005] From Paul Ehrlich proposing 'magic bullet' hypothesis to the approval of rituximab in 1997, monoclonal antibody-based therapeutics had made impressive progress in oncology, autoimmune disease, and many other diseases over the past decades. The major mechanisms

of mAbs effective against cancers are rely on they interacts with components of the immune system through antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP) and alter signal transduction to kill cancer cells. Among these mechanisms, macrophage-mediated cell phagocytosis has been proved an important mechanism of eliminating diseased and damaged cells.

[0006] Cluster of differentiation 47 (CD47), also known as integrin-associated protein (IAP), is an immunoglobulin superfamily membrane protein in the size of approximately 50kDa. CD47 interacts with its ligand on macrophages, signal regulatory protein alpha (SIRPα), to send an anti-phagocytic or "don't eat me" signal to evade immune surveillance. Analysis of patient tumor and matched adjacent normal tissue reveals that CD47 is overexpressed on AML, NHL, breast cancer, NSCC and ovarian cells, and the increased CD47 expression correlated with a worse clinical prognosis. These data showed CD47 may serve as a new immune checkpoint for cancer therapy by blocking CD47-SIRPα interaction. Several anti-CD47 mAbs and SIRPα fusion protein had achieved effective macrophage involved phagocytosis against AML, NHL, breast cells, and ovarian cells. Besides, anti-CD47 monoclonal antibody combined with approved antibodies (anti-tumor-associated antigen) have efficiently enhanced anti-tumor activity. Based on these preclinical studies, two anti-CD47 mAbs (Hu5F9-G4 and CC-90002) and two SIRPα engineered fusion proteins (TTI-621 and ALX148) are in active phase I or II clinical trials, covering hematological malignancies and human solid tumors.

[0007] Despite of the development of therapeutics targeting the targets respectively, there is a significant need for novel bispecific therapeutics that can acts on dual targets.

BRIEF SUMMARY OF THE INVENTION

[0004] Throughout the present disclosure, the articles "a," "an," and "the" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an antibody" means one antibody or more than one antibody.

[0005] The present disclosure provides novel bispecific PD-1/CD47 antibody molecules, amino acid and nucleotide sequences thereof, and uses thereof.

[0006] In one aspect the present disclosure provides herein a bispecific antibody molecule comprising a CD47-binding domain and a PD-1-binding domain, wherein:

the CD47-binding domain comprises:

1, 2, or 3 heavy chain complementarity determining region (CDR) sequences selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 5; and/or

1, 2, or 3 light chain CDR sequences selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 4, and SEQ ID NO: 6;

the PD-1 binding domain comprises:

1, 2, or 3 heavy chain CDR sequences selected from the group consisting of: SEQ ID NO: 15, SEQ ID NO: 17, and SEQ ID NO: 19, and/or

1, 2, or 3 light chain CDR sequences selected from the group consisting of: SEQ ID NO: 16, SEQ ID NO: 18, and SEQ ID NO: 20,

the CD47-binding domain comprises one independently selected from the group consisting of: a Fab and a single chain Fv antibody (scFv); and

the PD-1-binding domain comprises one independently selected from the group consisting of: a Fab and a scFv.

[0007] In certain embodiments, the CD47-binding domain comprises a Fab.

[0008] In certain embodiments, the PD-1-binding domain comprises a Fab.

[0009] In certain embodiments, the CD47-binding domain comprises a scFv.

[00010] In certain embodiments, the PD-1-binding domain comprises a scFv.

[00011] In certain embodiments, the CD47-binding domain comprises a heavy chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 1, 3 and 5, and/or a light chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 2, 4 and 6.

[00012] In certain embodiments, the CD47-binding domain comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 7 and 11, and a

homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to CD47.

[00013] In certain embodiments, the CD47-binding domain further comprises a light chain variable region selected from the group consisting of SEQ ID NOs: 9, 13 and 26, and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to CD47.

[00014] In certain embodiments, the CD47-binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 11 and a light chain variable region comprising the sequence of SEQ ID NO: 13 or SEQ ID NO: 26.

[00015] In certain embodiments, the PD-1-binding domain comprises a heavy chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 15, 17 and 19, and/or a light chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 16, 18 and 20.

[00016] In certain embodiments, the PD-1-binding domain comprises a heavy chain variable region selected from the group consisting of SEQ ID NO: 21 and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to PD-1.

[00017] In certain embodiments, the PD-1-binding domain comprises a light chain variable region selected from the group consisting of SEQ ID NOs: 23 and 28, and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to PD-1.

[00018] In certain embodiments, the PD-1-binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 and a light chain variable region comprising the sequence of SEQ ID NO: 23 or SEQ ID NO: 28.

[00019] In certain embodiments, the CD47-binding domain further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to CD47, and/or the PD-1-binding domain further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to PD-1.

[00020] In certain embodiments, at least one of the substitutions or modifications is in one or more of the CDR sequences, and/or in one or more of the VH or VL sequences but not in any

of the CDR sequences.

[00021] In certain embodiments, the bispecific antibody molecule further comprises an immunoglobulin constant region, optionally a constant region of human Ig, or optionally a constant region of human IgG.

[00022] In certain embodiments, the CD47-binding domain is operably linked to the N terminus or the C terminus of the PD-1-binding domain.

[00023] In certain embodiments, the CD47-binding domain comprises a scFv and the PD-1-binding domain comprises a Fab.

[00024] In certain embodiments, the CD47-binding scFv comprises the sequence of SEQ ID NO: 27, and the PD-1-binding Fab comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 and a light chain variable region comprising the sequence of SEQ ID NO: 23 or SEQ ID NO: 28.

[00025] In certain embodiments, the CD47-binding scFv is operably linked to the C terminus of the heavy chain constant region following the PD-1-binding Fab.

[00026] In certain embodiments, the bispecific antibody molecule comprise a heavy chain in the format of: VH(anti-PD-1)-CH1-Hinge-CH2-CH3-spacer-scFv(anti-CD47), associated with a light chain in the format of VL(anti-PD-1)-CL.

[00027] In certain embodiments, the bispecific antibody molecule comprising a heavy chain comprising the sequence of SEQ ID NO: 31 and a light chain comprising the sequence of SEQ ID NO: 32.

[00028] In certain embodiments, the PD-1-binding domain comprises a scFv and the CD47-binding domain comprises a Fab.

[00029] In certain embodiments, the PD-1-binding scFv comprises the sequence of SEQ ID NO: 25, and the CD47-binding Fab comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 11 and a light chain variable region comprising the sequence of SEQ ID NO: 13 or SEQ ID NO: 26.

[00030] In certain embodiments, the PD-1-binding scFv is operably linked to the N terminus of the heavy chain variable region of the CD47-binding Fab.

[00031] In certain embodiments, the bispecific antibody comprises a heavy chain in the format of: scFv(anti-PD1)-spacer-VH(anti-CD47)-CH1-Hinge-CH2-CH3, which is associated with the light chain VL(anti-CD47)-CL.

[00032] In certain embodiments, the bispecific antibody molecule comprising a heavy chain comprising the sequence of SEQ ID NO: 29 and a light chain comprising the sequence of SEQ ID NO: 30.

[00033] In certain embodiments, the CD47-binding domain and/or the PD-1 antigen-binding domain is humanized.

[00034] In certain embodiments, the bispecific antibody molecule as provided herein is linked to one or more conjugate moieties.

[00035] In certain embodiments, the conjugate moiety comprises a clearance-modifying agent, a chemotherapeutic agent, a toxin, a radioactive isotope, a lanthanide, a luminescent label, a fluorescent label, an enzyme-substrate label, a DNA-alkylators, a topoisomerase inhibitor, a tubulin-binders, or other anticancer drugs.

[00036] In another aspect, the present disclosure provides a pharmaceutical composition comprising the bispecific antibody molecule as provided herein, and a pharmaceutically acceptable carrier.

[00037] In another aspect, the present disclosure provides a polynucleotide encoding the bispecific antibody molecule as provided herein.

[00038] In another aspect, the present disclosure provides a vector comprising the polynucleotide as provided herein.

[00039] In another aspect, the present disclosure provides a host cell comprising the vector as provided herein.

[00040] In another aspect, the present disclosure provides a method of expressing the bispecific antibody molecule as provided herein, comprising culturing the host cell as provided herein under the condition at which the vector as provided herein is expressed.

[00041] In another aspect, the present disclosure provides a method of treating a disease or condition in a subject that would benefit from upregulation of an immune response,

comprising administering to the subject a therapeutically effective amount of the bispecific antibody molecule as provided herein or the pharmaceutical composition as provided herein.

[00042] In certain embodiments, the disease or condition that would benefit from upregulation of an immune response is selected from the group consisting of cancer, a viral infection, a bacterial infection, a protozoan infection, a helminth infection, asthma associated with impaired airway tolerance, a neurological disease, multiple sclerosis, and an immunosuppressive disease.

[00043] In certain embodiments, the disease or condition is PD-1-related and/or CD47-related.

[00044] In certain embodiments, the disease or condition is cancer, autoimmune disease, inflammatory disease, or infectious disease.

[00045] In certain embodiments, the cancer is lung cancer, bronchial cancer, bone cancer, liver and bile duct cancer, pancreatic cancer, breast cancer, liver cancer, ovarian cancer, testicle cancer, kidney cancer, bladder cancer, head and neck cancer, spine cancer, brain cancer, cervix cancer, uterine cancer, endometrial cancer, colon cancer, colorectal cancer, rectal cancer, anal cancer, esophageal cancer, gastrointestinal cancer, skin cancer, prostate cancer, pituitary cancer, stomach cancer, vagina cancer, thyroid cancer, glioblastoma, astrocytoma, melanoma, myelodysplastic syndrome, sarcoma, teratoma, adenocarcinoma, leukemia, myeloma and lymphoma.

[00046] In certain embodiments, the disease or condition is hematological cancer, optionally selected from non-Hodgkin's lymphoma (NHL), acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), multiple myeloma (MM), diffuse large B cell lymphoma (DLBCL), Richter's syndrome, Burkitt's lymphoma or follicular lymphoma.

[00047] In certain embodiments, the subject is human.

[00048] In certain embodiments, the administration is via oral, nasal, intravenous, subcutaneous, sublingual, or intramuscular administration.

[00049] In another aspect, the present disclosure provides a method of modulating CD47 activity in a CD47-expressing cell, comprising exposing the CD47-expressing cell to the

bispecific antibody molecule as provided herein.

[00050] In another aspect, the present disclosure provides use of the bispecific antibody molecule as provided herein in the manufacture of a medicament for treating a disease or condition that would benefit from upregulation of an immune response.

[00051] In another aspect, the present disclosure provides use of the bispecific antibody molecule as provided herein in the manufacture of a medicament for treating a disease or condition that is PD-1 and/or CD47-related.

BRIEF DESCFRIPTION OF FIGURES

[00052] Figure 1 shows the EC_{50} value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to human PD-1 expressing cells by FACS.

[00053] Figure 2 shows the K_D value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to human PD-1 antigen by SPR.

[00054] Figure 3 shows the EC_{50} value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to cynomolgus PD-1 expressing cells by FACS.

[00055] Figure 4 shows the the K_D value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to cynomolgus PD-1 antigen by SPR.

[00056] Figure 5 shows the EC_{50} value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to mouse PD-1 expressing cells by FACS.

[00057] Figure 6 shows the IC_{50} value of both the bispecific antibody molecules W367-G17 and W367-G15 on blocking the binding of human PD-1 to human PD-$L1^+$ cells by FACS.

[00058] Figure 7 shows the EC_{50} value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to human CD47 expressing cells by FACS.

[00059] Figure 8 shows the K_D value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to human CD47 antigen by FACS.

[00060] Figure 9 shows the EC_{50} value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to cynomolgus CD47 expressing cells by FACS.

[00061] Figure 10 shows the K_D value of binding affinity of both the bispecific antibody molecules W367-G17 and W367-G15 to cynomolgus CD47 expressing cells by FACS.

[00062] Figure 11 shows the IC_{50} value of both the bispecific antibody molecules W367-G17 and W367-G15 on blocking the binding of human SIRPα to human CD47 expressing cells by FACS.

[00063] Figure 12A and 12B show the serum stability of W367-G17 (Figure 12B) and W367-G15 (Figure 12A). Both bispecific antibody molecules retain binding to hPD-1 and hCD47 after 14 days incubation in 37 °C serum.

[00064] Figure 13 shows reduced binding to human RBCs for both bispecific antibody molecules W367-G17 and W367-G15 in the human red blood cell binding assay by FACS.

[00065] Figure 14 shows that W367-G17 and W367-G15 do not incur hemagglutination in the hemagglutination assay, while the benchmark mAb W345-BMK2 does.

[00066] Figure 15 shows that both W367-G17 and W367-G15 bind to tumor Raji cells by FACS (represented by EC_{50} value).

[00067] Figure 16 shows the result of ADCP test demonstrating the bispecific antibody W367-G17 stimulates dose-dependent ADCP on tumor Raji cells.

[00068] Figure 17 shows that both W367-G17 and W367-G15 triggers T-cell activation with high potency, as demonstrated by the reporter gene assay (RGA).

[00069] Figure 18 shows that both W367-G17 and W367-G15 stimulate strong and dose-dependent IFN- release from activated $CD4^+$ T cells in mixed lymphocyte reaction (MLR).

[00070] Figure 19 shows that both W367-G17 and W367-G15 show synergistic stimulation of $CD4^+$ T-cells and DC cells.

DETAILED DESCRIPTION OF THE INVENTION

[00071] The following description of the disclosure is merely intended to illustrate various embodiments of the disclosure. As such, the specific modifications discussed are not to be construed as limitations on the scope of the disclosure. It will be apparent to one skilled in the

art that various equivalents, changes, and modifications may be made without departing from the scope of the disclosure, and it is understood that such equivalent embodiments are to be included herein. All references cited herein, including publications, patents and patent applications are incorporated herein by reference in their entirety.

[00072] Definitions

[00073] The term "antibody" as used herein includes any immunoglobulin, monoclonal antibody, polyclonal antibody, multivalent antibody, bivalent antibody, monovalent antibody, multispecific antibody, or bispecific antibody that binds to a specific antigen. A native intact antibody comprises two heavy (H) chains and two light (L) chains. Mammalian heavy chains are classified as alpha, delta, epsilon, gamma, and mu, each heavy chain consists of a variable region (V_H) and a first, second, and third constant region (C_{H1}, C_{H2}, C_{H3}, respectively); mammalian light chains are classified as λ or κ, while each light chain consists of a variable region (V_L) and a constant region. The antibody has a "Y" shape, with the stem of the Y consisting of the second and third constant regions of two heavy chains bound together via disulfide bonding. Each arm of the Y includes the variable region and first constant region of a single heavy chain bound to the variable and constant regions of a single light chain. The variable regions of the light and heavy chains are responsible for antigen binding. The variable regions in both chains generally contain three highly variable loops called the complementarity determining regions (CDRs) (light chain CDRs including LCDR1, LCDR2, and LCDR3, heavy chain CDRs including HCDR1, HCDR2, and HCDR3). CDR boundaries for the antibodies and antigen-binding domains disclosed herein may be defined or identified by the conventions of Kabat, IMGT, AbM, Chothia, or Al-Lazikani (Al-Lazikani, B., Chothia, C., Lesk, A. M., J. Mol. Biol., 273(4), 927 (1997); Chothia, C. et al., J Mol Biol. Dec 5;186(3):651-63 (1985); Chothia, C. and Lesk, A.M., J.Mol.Biol., 196,901 (1987); N. R. Whitelegg et al, Protein Engineering, v13(12), 819-824 (2000); Chothia, C. et al., Nature. Dec 21-28;342(6252):877-83 (1989) ; Kabat E.A. et al., National Institutes of Health, Bethesda, Md. (1991); Marie-Paule Lefranc et al, Developmental and Comparative Immunology, 27: 55-77 (2003); Marie-Paule Lefranc et al, Immunome Research, 1(3), (2005); Marie-Paule Lefranc, Molecular Biology of B cells (second edition), chapter 26, 481-514, (2015)). The

three CDRs are interposed between flanking stretches known as framework regions (FRs), which are more highly conserved than the CDRs and form a scaffold to support the hypervariable loops. The constant regions of the heavy and light chains are not involved in antigen-binding, but exhibit various effector functions. Antibodies are assigned to classes based on the amino acid sequence of the constant region of their heavy chain. The five major classes or isotypes of antibodies are IgA, IgD, IgE, IgG, and IgM, which are characterized by the presence of alpha, delta, epsilon, gamma, and mu heavy chains, respectively. Several of the major antibody classes are divided into subclasses such as IgG1 (gamma1 heavy chain), IgG2 (gamma2 heavy chain), IgG3 (gamma3 heavy chain), IgG4 (gamma4 heavy chain), IgA1 (alpha1 heavy chain), or IgA2 (alpha2 heavy chain).

[00074] The term "antibody molecule" as used herein refers to an antigen-binding protein or polypeptide comprising at least one antibody fragment (such as CDR, and/or variable region sequence). An antibody molecule includes, for example, a monoclonal antibody, an antibody fragment or domain, a fusion protein comprising an antibody fragment or domain, a polypeptide complex comprising an antibody fragment or domain, and so on.

[00075] The term "bivalent" as used herein refers to an antibody or an antigen-binding domain having two antigen-binding sites; the term "monovalent" refers to an antibody or an antigen-binding domain having only one single antigen-binding site; and the term "multivalent" refers to an antibody or an antigen-binding domain having multiple antigen-binding sites. In some embodiments, the antibody or antigen-binding domain thereof is bivalent.

[00076] The term "antigen-binding domain" (e.g. CD47-binding domain or PD-1-binding domain) as used herein refers to an antibody fragment formed from a portion of an antibody comprising one or more CDRs, or any other antibody fragment that binds to an antigen but does not comprise an intact native antibody structure. Examples of antigen-binding domain include, without limitation, a diabody, a Fab, a Fab', a $F(ab')_2$, an Fv fragment, a disulfide stabilized Fv fragment (dsFv), a $(dsFv)_2$, a bispecific dsFv (dsFv-dsFv'), a disulfide stabilized diabody (ds diabody), a single-chain antibody molecule (scFv), an scFv dimer (bivalent diabody), a bispecific antibody, a multispecific antibody, a camelized single domain antibody,

a nanobody, a domain antibody, and a bivalent domain antibody. An antigen-binding domain is capable of binding to the same antigen to which the parent antibody binds. In certain embodiments, an antigen-binding domain may comprise one or more CDRs from a particular human antibody grafted to a framework region from one or more different human antibodies. For more and detailed formats of antigen-binding domain are described in Spiess et al, 2015 (*Supra*), and Brinkman et al., *mAbs*, 9(2), pp.182–212 (2017), which are incorporated herein by entirety reference.

[00077] "Fab" with regard to an antibody refers to that portion of the antibody consisting of a single light chain (both variable and constant regions) bound to the variable region and first constant region of a single heavy chain by a disulfide bond.

[00078] "Fab'" refers to a Fab fragment that includes a portion of the hinge region.

[00079] "$F(ab')_2$"refers to a dimer of Fab'.

[00080] A "fragment difficult (Fd)" with regard to an antibody refers to the amino-terminal half of the heavy chain fragment that can be combined with the light chain to form Fab. For example, Fd fragment may consists of the VH and CH1 domains

[00081] "Fv" with regard to an antibody refers to the smallest fragment of the antibody to bear the complete antigen-binding site. An Fv fragment consists of the variable region of a single light chain bound to the variable region of a single heavy chain. A number of Fv designs have been provided, including dsFvs, in which the association between the two domains is enhanced by an introduced disulphide bond; and scFvs can be formed using a peptide linker to bind the two domains together as a single polypeptide. Fvs constructs containing a variable domain of a heavy or light immunoglobulin chain associated to the variable and constant domain of the corresponding immunoglobulin heavy or light chain have also been produced. Fvs have also been multimerised to form diabodies and triabodies (Maynard et al., Annu Rev Biomed Eng 2 339-376 (2000)).

[00082] "Single-chain Fv antibody" or "scFv" refers to an engineered antibody consisting of a light chain variable region and a heavy chain variable region connected to one another

directly or via a peptide linker sequence (Huston JS *et al.* Proc Natl Acad Sci USA, 85:5879(1988)).

[00083] "ScFab" refers to a fusion polypeptide with an Fd linked to a light chain via a polypeptide linker, resulting in the formation of a single chain Fab fragment (scFab).

[00084] A "dsFv" refers to a disulfide-stabilized Fv fragment that the linkage between the variable region of a single light chain and the variable region of a single heavy chain is a disulfide bond. In some embodiments, a "$(dsFv)_2$" or "(dsFv-dsFv')" comprises three peptide chains: two V_H moieties linked by a peptide linker (*e.g.*, a long flexible linker) and bound to two V_L moieties, respectively, via disulfide bridges. In some embodiments, dsFv-dsFv' is bispecific in which each disulfide paired heavy and light chain has a different antigen specificity.

[00085] "Appended IgG" refers to a fusion protein with a Fab arm fused to an IgG to form the format of bispecific $(Fab)_2$-Fc. It can form a "IgG-Fab" or a "Fab-IgG", with a Fab fused to the C-terminus or N-terminus of an IgG molecule with or without a connector. In certain embodiments, the appended IgG can be further modified to a format of IgG-Fab_4 (see, Brinkman et al., 2017, *Supra*).

[00086] "Fc" with regard to an antibody refers to that portion of the antibody consisting of the second and third constant regions of a first heavy chain bound to the second and third constant regions of a second heavy chain via disulfide bonding. The Fc portion of the antibody is responsible for various effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC), but does not function in antigen binding.

[00087] "Camelized single domain antibody," "heavy chain antibody," or "HCAb" refers to an antibody that contains two V_H domains and no light chains (Riechmann L. and Muyldermans S., J Immunol Methods. Dec 10;231(1-2):25-38 (1999); Muyldermans S., J Biotechnol. Jun;74(4):277-302 (2001); WO94/04678; WO94/25591; U.S. Patent No. 6,005,079). Heavy chain antibodies were originally derived from *Camelidae* (camels, dromedaries, and llamas). Although devoid of light chains, camelized antibodies have an

authentic antigen-binding repertoire (Hamers-Casterman C. *et al.*, Nature. Jun 3;363(6428):446-8 (1993); Nguyen VK. *et al.* "Heavy-chain antibodies in Camelidae; a case of evolutionary innovation," Immunogenetics. Apr;54(1):39-47 (2002); Nguyen VK. *et al.*Immunology. May;109(1):93-101 (2003)). The variable domain of a heavy chain antibody (VHH domain) represents the smallest known antigen-binding unit generated by adaptive immune responses (Koch-Nolte F. *et al.*, FASEB J. Nov;21(13):3490-8. Epub 2007 Jun 15 (2007)).

[00088] A "nanobody" refers to an antibody fragment that consists of a VHH domain from a heavy chain antibody and two constant domains, CH2 and CH3.

[00089] A "domain antibody" refers to an antibody fragment containing only the variable region of a heavy chain or the variable region of a light chain. In certain instances, two or more V_H domains are covalently joined with a peptide linker to create a bivalent or multivalent domain antibody. The two V_H domains of a bivalent domain antibody may target the same or different antigens.

[00090] The term "chimeric" as used herein, means an antibody or antigen-binding domain, having a portion of heavy and/or light chain derived from one species, and the rest of the heavy and/or light chain derived from a different species. In an illustrative example, a chimeric antibody may comprise a constant region derived from human and a variable region from a non-human animal, such as from mouse. In some embodiments, the non-human animal is a mammal, for example, a mouse, a rat, a rabbit, a goat, a sheep, a guinea pig, or a hamster.

[00091] The term "humanized" as used herein means that the antibody or antigen-binding domain comprises CDRs derived from non-human animals, FR regions derived from human, and when applicable, the constant regions derived from human.

[00092] The term "operably link" or "operably linked" refers to a juxtaposition, with or without a spacer or a linker or an intervening sequence, of two or more biological sequences of interest in such a way that they are in a relationship permitting them to function in an intended manner. When used with respect to polypeptides, it is intended to mean that the

polypeptide sequences are linked in such a way that permits the linked product to have the intended biological function. For example, an antibody variable region may be operably linked to a constant region so as to provide for a stable product with antigen-binding activity. For another example, an antigen-binding domain can be operably linked to another antigen-binding domain with an intervening sequence there between, and such intervening sequence can be a spacer or can comprise a much longer sequence such as a constant region of an antibody. The term may also be used with respect to polynucleotides. For one instance, when a polynucleotide encoding a polypeptide is operably linked to a regulatory sequence (e.g., promoter, enhancer, silencer sequence, etc.), it is intended to mean that the polynucleotide sequences are linked in such a way that permits regulated expression of the polypeptide from the polynucleotide.

[00093] The term "fusion" or "fused" when used with respect to amino acid sequences (e.g. peptide, polypeptide or protein) refers to combination of two or more amino acid sequences, for example by chemical bonding or recombinant means, into a single amino acid sequence which does not exist naturally. A fusion amino acid sequence may be produced by genetic recombination of two encoding polynucleotide sequences, and can be expressed by a method of introducing a construct containing the recombinant polynucleotides into a host cell.

[00094] An "antigen" as used herein refers to a compound, composition, peptide, polypeptide, protein or substance that can stimulate the production of antibodies or a T cell response in cell culture or in an animal, including compositions (such as one that includes a cancer-specific protein) that are added to a cell culture (such as a hybridoma), or injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity (such as an antibody), including those induced by heterologous antigens.

[00095] "CD47" as used herein, refers to the Cluster of Differentiation 47 protein derived from any vertebrate source, including mammals such as primates (e.g. humans, monkeys) and rodents (e.g., mice and rats). Exemplary sequence of human CD47 includes human CD47 protein (NCBI Ref Seq No GI: 728797214). Exemplary sequence of CD47 includes human CD47 protein (NCBI accession number GI: 728797214). Exemplary sequence of CD47 includes Mus musculus (mouse) CD47 protein (NCBI accession number GI: 3036965), Rattus

norvegicus (rat) CD47 protein (NCBI accession number GI 55250722), and Macaca fascicularis (monkey) CD47 protein (NCBI accession number GI:544416831).

[00096] The term "CD47" as used herein is intended to encompass any form of CD47, for example, 1) native unprocessed CD47 molecule, "full-length" CD47 chain or naturally occurring variants of CD47, including, for example, splice variants or allelic variants; 2) any form of CD47 that results from processing in the cell; or 3) full length, a fragment (e.g., a truncated form, an extracellular/transmembrane domain) or a modified form (e.g. a mutated form, a glycosylated/PEGylated, a His-tag/immunofluorescence fused form) of CD47 subunit generated through recombinant method.

[00097] The term "tumoric CD47" as used herein refers to CD47 expressed on a tumor cell. Without wishing to be bound by any theory, it is believed that CD47 expressed on a tumor cells could have a different conformation from CD47 expressed on red blood cells. More details of tumoric CD47 is available in the art, for example, as described in, H. J. Zhao et al, Translational Cancer Research v7(3) 609-621(2018).

[00098] The term "anti-CD47 antibody", "anti-CD47 binding domain" or "CD47-binding domain" refers to an antibody or antigen-binding domain that is capable of specific binding CD47 (e.g. human or monkey or mouse or rat CD47).

[00099] "PD-1" as used herein refers programmed cell death protein, which belongs to the superfamily of immunoglobulin and functions as co-inhibitory receptor to negatively regulate the immune system. PD-1 is a member of the CD28/CD47 family, and has two known ligands including PD-L1 and PD-L2. Representative amino acid sequence of human PD-1 is disclosed under the NCBI accession number: NP_005009.2, and the representative nucleic acid sequence encoding the human PD-1 is shown under the NCBI accession number: NM_005018.2.

[000100] "PD-L1" as used herein refers to programmed cell death ligand 1 (PD-L1, see, for example, Freeman et al. (2000) *J. Exp. Med.* 192:1027). Representative amino acid sequence of human PD-L1 is disclosed under the NCBI accession number: NP_054862.1, and the representative nucleic acid sequence encoding the human PD-L1 is shown under the NCBI

accession number: NM_014143.3. PD-L1 is expressed in placenta, spleen, lymph nodes, thymus, heart, fetal liver, and is also found on many tumor or cancer cells. PD-L1 binds to its receptor PD-1 or B7-1, which is expressed on activated T cells, B cells and myeloid cells. The binding of PD-L1 and its receptor induces signal transduction to suppress TCR-mediated activation of cytokine production and T cell proliferation. Accordingly, PD-L1 plays a major role in suppressing immune system during particular events such as pregnancy, autoimmune diseases, tissue allografts, and is believed to allow tumor or cancer cells to circumvent the immunological checkpoint and evade the immune response.

[000101] "Anti-PD-1 antibody", "anti-PD-1 binding domain" or "PD-1 binding domain" as used herein refers to an antibody or antigen-binding domain that is capable of specific binding to PD-1 (e.g. human or monkey PD-1) with an affinity which is sufficient to provide for diagnostic and/or therapeutic use.

[000102] The term "specific binding" or "specifically binds" as used herein refers to a non-random binding reaction between two molecules, such as for example between an antibody and an antigen. In certain embodiments, the antibody molecules or antigen-binding domains provided herein specifically bind to human PD-1 and/or human CD47 with a binding affinity (K_D) of $\leq 10^{-6}$ M (*e.g.*, $\leq 5x10^{-7}$ M, $\leq 2x10^{-7}$ M, $\leq 10^{-7}$ M, $\leq 5x10^{-8}$ M, $\leq 2x10^{-8}$ M, $\leq 10^{-8}$ M, $\leq 5x10^{-9}$ M, $\leq 4x10^{-9}$M, $\leq 3x10^{-9}$M,$\leq 2x10^{-9}$ M, or $\leq 10^{-9}$ M). K_D used herein refers to the ratio of the dissociation rate to the association rate (k_{off}/k_{on}), which may be determined by using any conventional method known in the art, including but are not limited to surface plasmon resonance method, microscale thermophoresis method, HPLC-MS method and flow cytometry (such as FACS) method. In certain embodiments, the K_D value can be appropriately determined by using flow cytometry.

[000103] The ability to "block binding" or "compete for the same epitope" as used herein refers to the ability of an antibody or antigen-binding domain to inhibit the binding interaction between two molecules (e.g. human CD47 and an anti-CD47 antibody, human PD-1 and an anti-PD-1 antibody) to any detectable degree. In certain embodiments, an antibody or antigen-binding domain that blocks binding between two molecules inhibits the binding

interaction between the two molecules by at least 85%, or at least 90%. In certain embodiments, this inhibition may be greater than 85%, or greater than 90%.

[000104] The term "epitope" as used herein refers to the specific group of atoms or amino acids on an antigen to which an antibody binds. Epitopes can be formed both from contiguous amino acids (also called linear or sequential epitope) or noncontiguous amino acids juxtaposed by tertiary folding of a protein (also called configurational or conformational epitope). Epitopes formed from contiguous amino acids are typically arranged linearly along the primary amino acid residues on the protein and the small segments of the contiguous amino acids can be digested from an antigen binding with major histocompatibility complex (MHC) molecules or retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5, about 7, or about 8-10 amino acids in a unique spatial conformation. Two antibodies may bind the same or a closely related epitope within an antigen if they exhibit competitive binding for the antigen. For example, if an antibody or antigen-binding domain blocks binding of a reference antibody to the antigen by at least 85%, or at least 90%, or at least 95%, then the antibody or antigen-binding domain may be considered to bind the same/closely related epitope as the reference antibody.

[000105] The antibody names as used herein may include one or more suffix symbols which usually indicates the type of the antibody or particular modifications made to the antibody. For example, "uIgG4" means an antibody with human constant region of an IgG4 isotype. "SP" refers to mutation of S228P in the constant region of human IgG4 (i.e. S228P).

[000106] A "conservative substitution" with reference to amino acid sequence refers to replacing an amino acid residue with a different amino acid residue having a side chain with similar physiochemical properties. For example, conservative substitutions can be made among amino acid residues with hydrophobic side chains (e.g. Met, Ala, Val, Leu, and Ile), among residues with neutral hydrophilic side chains (e.g. Cys, Ser, Thr, Asn and Gln), among residues with acidic side chains (e.g. Asp, Glu), among amino acids with basic side chains (e.g. His, Lys, and Arg), or among residues with aromatic side chains (e.g. Trp, Tyr, and Phe).

As known in the art, conservative substitution usually does not cause significant change in the protein conformational structure, and therefore could retain the biological activity of a protein.

[000107] The term "homolog" and "homologous" as used herein are interchangeable and refer to nucleic acid sequences (or its complementary strand) or amino acid sequences that have sequence identity of at least 80% (e.g., at least 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) to another sequences when optimally aligned.

[000108] "Percent (%) sequence identity" with respect to amino acid sequence (or nucleic acid sequence) is defined as the percentage of amino acid (or nucleic acid) residues in a candidate sequence that are identical to the amino acid (or nucleic acid) residues in a reference sequence, after aligning the sequences and, if necessary, introducing gaps, to achieve the maximum number of identical amino acids (or nucleic acids). Conservative substitution of the amino acid residues may or may not be considered as identical residues. Alignment for purposes of determining percent amino acid (or nucleic acid) sequence identity can be achieved, for example, using publicly available tools such as BLASTN, BLASTp (available on the website of U.S. National Center for Biotechnology Information (NCBI), see also, Altschul S.F. et al, J. Mol. Biol., 215:403–410 (1990); Stephen F. et al, Nucleic Acids Res., 25:3389–3402 (1997)), ClustalW2 (available on the website of European Bioinformatics Institute, see also, Higgins D.G. et al, Methods in Enzymology, 266:383-402 (1996); Larkin M.A. et al, Bioinformatics (Oxford, England), 23(21): 2947-8 (2007)), and ALIGN or Megalign (DNASTAR) software. Those skilled in the art may use the default parameters provided by the tool, or may customize the parameters as appropriate for the alignment, such as for example, by selecting a suitable algorithm.

[000109] "Effector functions" as used herein refer to biological activities attributable to the binding of Fc region of an antibody to its effectors such as C1 complex and Fc receptor. Exemplary effector functions include: complement dependent cytotoxicity (CDC) induced by interaction of antibodies and C1q on the C1 complex; antibody-dependent cell-mediated cytotoxicity (ADCC) induced by binding of Fc region of an antibody to Fc receptor on an effector cell; and phagocytosis.

[000110] "Treating" or "treatment" of a condition as used herein includes preventing or alleviating a condition, slowing the onset or rate of development of a condition, reducing the risk of developing a condition, preventing or delaying the development of symptoms associated with a condition, reducing or ending symptoms associated with a condition, generating a complete or partial regression of a condition, curing a condition, or some combination thereof.

[000111] The term "subject" or "individual" or "animal" or "patient" as used herein refers to human or non-human animal, including a mammal or a primate, in need of diagnosis, prognosis, amelioration, prevention and/or treatment of a disease or disorder. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sports, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, swine, cows, bears, and so on.

[000112] The term "vector" as used herein refers to a vehicle into which a polynucleotide encoding a protein may be operably inserted so as to bring about the expression of that protein. A vector may be used to transform, transduce, or transfect a host cell so as to bring about expression of the genetic element it carries within the host cell. Examples of vectors include plasmids, phagemids, cosmids, and artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses. Categories of animal viruses used as vectors include retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.*, herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.*, SV40). A vector may contain a variety of elements for controlling expression, including promoter sequences, transcription initiation sequences, enhancer sequences, selectable elements, and reporter genes. In addition, the vector may contain an origin of replication. A vector may also include materials to aid in its entry into the cell, including but not limited to a viral particle, a liposome, or a protein coating. A vector can be an expression vector or a cloning vector.

[000113] The phrase "host cell" as used herein refers to a cell into which an exogenous polynucleotide and/or a vector has been introduced.

[000114] A "CD47-related" disease or condition as used herein refers to any disease or condition caused by, exacerbated by, or otherwise linked to increased or decreased expression or activities of CD47. In some embodiments, the CD47 related condition is immune-related disorder, such as, for example, cancer, autoimmune disease, inflammatory disease or infectious disease.

[000115] A "PD-1-related" disease or condition as used herein refers to any condition that is caused by, exacerbated by, or otherwise linked to increased or decreased expression or activities of PD-1 (e.g. a human PD-1).

[000116] "Cancer" as used herein refers to any medical condition characterized by malignant cell growth or neoplasm, abnormal proliferation, infiltration or metastasis, and includes both solid tumors and non-solid cancers (hematologic malignancies) such as leukemia. As used herein "solid tumor" refers to a solid mass of neoplastic and/or malignant cells. Examples of cancer or tumors include hematological malignancies, oral carcinomas (for example of the lip, tongue or pharynx), digestive organs (for example esophagus, stomach, small intestine, colon, large intestine, or rectum), peritoneum, liver and biliary passages, pancreas, respiratory system such as larynx or lung (small cell and non-small cell), bone, connective tissue, skin (e.g., melanoma), breast, reproductive organs (fallopian tube, uterus, cervix, testicles, ovary, or prostate), urinary tract (e.g., bladder or kidney), brain and endocrine glands such as the thyroid. In certain embodiments, the cancer is selected from ovarian cancer, breast cancer, head and neck cancer, renal cancer, bladder cancer, hepatocellular cancer, and colorectal cancer. In certain embodiments, the cancer is selected from a lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma and B-cell lymphoma.

[000117] The term "pharmaceutically acceptable" indicates that the designated carrier, vehicle, diluent, excipient(s), and/or salt is generally chemically and/or physically compatible with the other ingredients comprising the formulation, and physiologically compatible with the recipient thereof.

[000118] **A. Bispecific Antibody Molecule**

[000119] In one aspect, the present disclosure provides herein a bispecific antibody molecule. The term "bispecific" as used herein means that, there are at least two antigen-binding domains (i.e. could be dual specific or multispecific), each of which is capable of specifically binding to a different epitope. The bispecific antibody molecule provided herein comprises a CD47-binding domain and a PD-1-binding domain capable of specifically binding to PD-1, the CD47-binding domain comprises one independently selected from the group consisting of: Fab and scFv; and the PD-1-binding domain comprises one independently selected from the group consisting of: Fab and scFv.

[000120] **i. CD47-binding domain**

[000121] In certain embodiments, the CD47-binding domain comprises one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences of an anti-CD47 antibody selected from the group consisting of: W3452-2.683.2 and W3452-2.683.2-z27.

[000122] "W3452-2.683.2" as used herein refers to a rodent monoclonal antibody having a heavy chain variable region of SEQ ID NO: 7, and a lambda light chain variable region of SEQ ID NO: 9.

[000123] "W3452-2.683.2-z27" as used herein refers to a humanized antibody based on W3452-2.683.2 that comprises a heavy chain variable region of SEQ ID NO: 11, and a lambda light chain variable region of SEQ ID NO: 13. W3452-2.683.2-z27 has comparable affinity as compared with its parent antibody W3452-2.683.2.

[000124] In certain embodiments, the CD47-binding domain comprises a heavy chain variable region comprising a heavy chain CDR1 comprising SEQ ID NO: 1, a heavy chain CDR2 comprising SEQ ID NO: 3, and a heavy chain CDR3 comprising SEQ ID NO: 5; and/or a light chain variable region comprising a light chain CDR1 comprising SEQ ID NO: 2, a light chain CDR2 comprising SEQ ID NO: 4, and a light chain CDR3 comprising SEQ ID NO: 6.

[000125] Table 1 shows the CDR sequences of the anti-CD47 antibody. The heavy chain and light chain variable region sequences are also provided below in Table 2 and Table 3.

[000126] Table 1.

		CDR1	CDR2	CDR3
W3452-2.683.2 and W3452-2.683.2-z27	HCDR	SEQ ID NO: 1	SEQ ID NO: 3	SEQ ID NO: 5
		GFTFSTYGMA	SISTSGGSTYYRDSVQG	KGGRTYRLAY
	LCDR	SEQ ID NO: 2	SEQ ID NO: 4	SEQ ID NO: 6
		SGIELSNKYAH	KDSERPS	LSTNSDDDLPV

[000127] Table 2.

	VH	VL
W3452-2.683.2	SEQ ID NO: 7	SEQ ID NO: 9
	EVELVESGGDLVQPGRSMKLSCAASGFTFSTYGMAWVRQTPTKGLEWVASISTSGGSTYYRDSVQGRFTISRDNAKSTLYLQMDSLTSEDTATYYCTTKGGRTYRLAYWGQGTLVTVSS	SYELIQPPSASVTLENTVSLTCSGIELSNKYAHWFQQKPDKTILEVMYKDSERPSGISDRFSGSRSGTTAILTIRDAQAEDEADYYCLSTNSDDDLPVFGGGTELTVL
W3452-2.683.2-z27	SEQ ID NO: 11	SEQ ID NO: 13
	EVQLVESGGGLVQPGRSLRLSCAASGFTFSTYGMAWVRQAPGKGLEWVSSISTSGGSTYYRDSVQGRFTISRDNAKSSLYLQMNSLRAEDTATYYCTTKGGRTYRLAYWGQGTLVTVSS	SYELMQPPSVSVSPGQTARITCSGIELSNKYAHWYQQKPGQAPVEVMYKDSERPSGIPERFSGSRSGTTVTLTISGVQAEDEADYYCLSTNSDDDLPVFGGGTKLTVL

[000128] Table 3.

	VHnu	VLnu
W3452-2.683.2	SEQ ID NO: 8	SEQ ID NO: 10
	gaggtagaactggtggagtctgggggcgacttagtg cagcctggaaggtccatgaaactctcctgtgcagcct caggattcactttcagtacctatggcatggcctgggtc cgccagactccaacgaagggtctggagtgggtcgc atccattagtactagtggtggcagcacttactatcgag actccgtgcagggccgattcactatttccagagataat gcaaagagcaccctatacctacaaatggacagtctg acgtctgaggacacggccacttattactgtacaactaa ggggggggagaacctataggcttgcttactggggcca aggcactctggtcactgtctcttca	agctatgagctgatccaaccaccatcagcatcagtcact ctggaaaatactgtctcactcacttgttctggaattgaatt atcaaacaaatatgctcattggtttcaacaaaagccaga caagaccattttggaagtgatgtacaaagatagtgagc ggccctcaggcatctctgaccgattctctgggtccaggt cagggacaacagccattctgacaatccgtgatgcccag gctgaggatgaggctgattattactgtttgtcaacaaata gtgatgatgatctccctgttttcggtggtggaaccgagct cactgtccta
W3452-2.683.2-z27	SEQ ID NO: 12	SEQ ID NO: 14
	gaggtgcagctggtggagagcggaggaggactgg tgcagcccggaagaagcctgagactgagctgcgcc gccagcggctttaccttcagcacctacggcatggcct gggtgagacaggcccccggcaaaggactggagtg ggtgagcagcatcagcaccagcggcggcagcacct actacagggacagcgtgcagggaaggttcaccatca gcagggacaacgccaagagcagcctgtatctgcag atgaacagcctgagggccgaggacaccgccaccta ctactgcaccaccaagggcggcaggacctacaggc tggcctattggggccagggcaccctggtgacagtga gctcc	agctacgagctgatgcagcctcctagcgtgagcgtga gccccggacagacagccaggatcacctgcagcggca tcgagctgagcaacaagtacgcccactggtaccagca gaagcctggccaggcccctgtggaggtgatgtacaag gacagcgagagacccagcggcatccccgagaggttt agcggcagcaggagcggcaccaccgtgaccctgacc atcagcggagtgcaggccgaggatgaggccgactact actgcctgagcaccaacagcgatgacgacctgcctgt gttcggcggcggcaccaagttaaccgtccta

[000129] CDRs are known to be responsible for antigen binding, however, it has been found that not all of the 6 CDRs are indispensable or unchangeable. In other words, it is possible to replace or change or modify one or more CDRs provided herein for CD47-binding domains, yet substantially retain the specific binding affinity to CD47.

[000130] In certain embodiments, the CD47-binding domains provided herein comprise SEQ ID NO: 5 (i.e. a heavy chain CDR3 sequence of anti-CD47 antibody W3452-2.683.2 or

W3452-2.683.2-z27). In certain embodiments, the anti-CD47 antibodies and the antigen-binding fragments provided herein comprise a heavy chain CDR3 sequence comprising the sequence of SEQ ID NO: 5.

[000131] Heavy chain CDR3 regions are located at the center of the antigen-binding site, and therefore are believed to make the most contact with antigen and provide the most free energy to the affinity of antibody to antigen. It is also believed that the heavy chain CDR3 is by far the most diverse CDR of the antigen-binding site in terms of length, amino acid composition and conformation by multiple diversification mechanisms (Tonegawa S. Nature. 302:575-81). The diversity in the heavy chain CDR3 is sufficient to produce most antibody specificities (Xu JL, Davis MM. Immunity. 13:37-45) as well as desirable antigen-binding affinity (Schier R, etc. J Mol Biol. 263:551-67).

[000132] In certain embodiments, the CD47-binding domains provided herein comprise any suitable framework region (FR) sequences, as long as the antigen-binding domains can specifically bind to CD47. In certain embodiments, the CDR sequences of W3452-2.683.2-z27 are obtained from rodent antibodies W3452-2.683.2, but they can be grafted to any suitable FR sequences of any suitable species such as mouse, human, rat, rabbit, among others, using suitable methods known in the art such as recombinant techniques.

[000133] In certain embodiments, the CD47-binding domains provided herein are humanized. A humanized antigen-binding domain is desirable in its reduced immunogenicity in human. A humanized antigen-binding domain is chimeric in its variable regions, as non-human CDR sequences are grafted to human or substantially human FR sequences. Humanization of an antigen-binding domain can be essentially performed by substituting the non-human (such as murine) CDR genes for the corresponding human CDR genes in a human immunoglobulin gene (see, for example, Jones et al. (1986) Nature 321:522-525; Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536).

[000134] Suitable human heavy chain and light chain variable domains can be selected to achieve this purpose using methods known in the art. In an illustrative example, "best-fit" approach can be used, where a non-human (e.g. rodent) antibody variable domain sequence is screened or BLASTed against a database of known human variable domain sequences, and

the human sequence closest to the non-human query sequence is identified and used as the human scaffold for grafting the non-human CDR sequences (see, for example, Sims et al, (1993) J. Immunol. 151:2296; Chothia et al. (1987) J. Mot. Biol. 196:901). Alternatively, a framework derived from the consensus sequence of all human antibodies may be used for the grafting of the non-human CDRs (see, for example, Carter et at. (1992) Proc. Natl. Acad. Sci. USA, 89:4285; Presta et al. (1993) J. Immunol.,151:2623).

[000135] In certain embodiments, the humanized antigen-binding domains provided herein are composed of substantially all human sequences except for the CDR sequences which are non-human. In some embodiments, the variable region FRs, and constant regions if present, are entirely or substantially from human immunoglobulin sequences. The human FR sequences and human constant region sequences may be derived different human immunoglobulin genes, for example, FR sequences derived from one human antibody and constant region from another human antibody. In some embodiments, the humanized antigen-binding domain comprise human FR1-4.

[000136] In certain embodiments, the humanized CD47-binding domains provided herein comprise one or more FR sequences of W3452-2.683.2-z27. The exemplary humanized anti-CD47 antibody W3452-2.683.2-z27 retained the specific binding affinity to CD47, and is at least comparable to, or even better than, the parent rodent antibody in that aspect.

[000137] In some embodiments, the FR regions derived from human may comprise the same amino acid sequence as the human immunoglobulin from which it is derived. In some embodiments, one or more amino acid residues of the human FR are substituted with the corresponding residues from the parent non-human antibody. This may be desirable in certain embodiments to make the humanized antibody or its fragment closely approximate the non-human parent antibody structure. In certain embodiments, the humanized CD47-binding domain provided herein comprises no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residue substitutions in each of the human FR sequences, or no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residue substitutions in all the FRs of a heavy or a light chain variable domain. In some embodiments, such change in amino acid residue could be present in heavy chain FR regions only, in light chain FR regions only, or in both chains.

[000138] In certain embodiments, the CD47-binding domains provided herein comprise a heavy chain variable domain sequence selected from the group consisting of SEQ ID NO: 7 and SEQ ID NO: 11. In certain embodiments, the CD47-binding domains provided herein comprise a light chain variable domain sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 13 and SEQ ID NO: 26.

[000139] In some embodiments, the CD47-binding domains provided herein comprise all or a portion of the heavy chain variable domain and/or all or a portion of the light chain variable domain. In one embodiment, the CD47-binding domains provided herein are a single domain antibody which consists of all or a portion of the heavy chain variable domain provided herein. More information of such a single domain antibody is available in the art (see, e.g., U.S. Pat. No. 6,248,516).

[000140] **ii. PD-1-binding domain**

[000141] In certain embodiments, the PD-1-binding domain or the bispecific antibody molecule is capable of specifically binding to PD-1 (such as human PD-1), and comprises one independently selected from the group consisting of: Fab and scFv.

[000142] In certain embodiments, the PD-1-binding domain comprises one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences of an anti-PD-1 antibody W3052-2E5.

[000143] "W3052-2E5" as used herein refers to a humanized monoclonal antibody having a heavy chain variable region of SEQ ID NO: 21, and a kappa light chain variable region of SEQ ID NO: 23.

[000144] In certain embodiments, the PD-1-binding domain comprises a heavy chain variable region comprising a heavy chain CDR1 comprising SEQ ID NO: 15, a heavy chain CDR2 comprising SEQ ID NO: 17, and a heavy chain CDR3 comprising SEQ ID NO: 19, and/or a light chain variable region comprising a light chain CDR1 comprising SEQ ID NO: 16, a light chain CDR2 comprising SEQ ID NO: 18, and a light chain CDR3 comprising SEQ ID NO: 20.

[000145] Table 4 shows the CDR sequences of the anti-PD-1 antibody. The heavy chain and light chain variable region sequences are also provided below in Table 5 and Table 6.

[000146] Table 4 CDR amino acid sequences

		CDR1	CDR2	CDR3
W3052-2E5	HCDR	SEQ ID NO: 15	SEQ ID NO: 17	SEQ ID NO: 19
		GFTFTTYYIS	YINMGSGGTNYNEKFKG	IGYFDY
	LCDR	SEQ ID NO: 16	SEQ ID NO: 18	SEQ ID NO: 20
		RSSQSLLDSDGGTYLY	LVSTLGS	MQLTHWPYT

[000147] Table 5. Variable region amino acid sequences

	VH	VL
W3052-2E5	SEQ ID NO: 21	SEQ ID NO: 23
	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISWVRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGTMVTVSS	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTYLYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLEIK

[000148] Table 6. Variable region nucleotide sequences

	VHnu	VLnu
W3052-2E5	SEQ ID NO: 22	SEQ ID NO: 24
	caggtgcagctggtccagtctggagctgaggtgaagaaacccggcagctccgtgaaggtcagttgcaaagcatcaggcttcacttttaccacatactatatctcttgggtgaggcaggcacctggacagggcctggagtacctgggctatattaacatggggtccggcgggaccaactacaatgaaaagttcaaagggcgggtgactatcaccgcagacaagtccacatctactgcctatatggagctgtctagtctgagatccgaagacacagccgtctactattgtgctattatcggctactttgattatt	gacgtggtcatgactcagtctcccctgtccctgcctgtgaccctgggacagccagcctctatcagttgccgaagctcccagtcactgctggacagcgatgggggtacatacctgtattggtttcagcagagaccaggacagagcccccggcggctgatctacctggtgtccacctgggatctggagtccctgacaggttctcaggaagcggctccgggaccgacttcaccctgaagattagccgcgtggaggccgaagatgtgggggtctactattgtatgcagctgactcactggccatatacctttggacag

	gggggcagggaacgatggtgacagtctca agc	ggcacaaagctggagatcaag

[000149] CDRs are known to be responsible for antigen binding, however, it has been found that not all of the 6 CDRs are indispensable or unchangeable. In other words, it is possible to replace or change or modify one or more CDRs provided herein for PD-1-binding domains, yet substantially retain the specific binding affinity to PD-1 (e.g. human PD-1).

[000150] In certain embodiments, the PD-1-binding domains provided herein comprise SEQ ID NO: 19 (i.e. a heavy chain CDR3 sequence of the anti-PD-1 antibody W3052-2E5).

[000151] In certain embodiments, the PD-1-binding domains provided herein comprise suitable framework region (FR) sequences, as long as the antigen-binding domains can specifically bind to PD-1, respectively. In certain embodiments, the CDR sequences of W3052-2E5 is obtained from rat antibodies, but they can be grafted to any suitable FR sequences of any suitable species such as mouse, human, rat, rabbit, among others, using suitable methods known in the art such as recombinant techniques.

[000152] In certain embodiments, the PD-1-binding domains provided herein are humanized. The exemplary humanized anti-PD-1 antibodies W3052-2E5 retained the specific binding affinity to PD-1, and are at least comparable to, or even better than, the parent rat antibodies in that aspect.

[000153] In some embodiments, the FR regions derived from human may comprise the same amino acid sequence as the human immunoglobulin from which it is derived. In some embodiments, one or more amino acid residues of the human FR are substituted with the corresponding residues from the parent non-human antibody. This may be desirable in certain embodiments to make the humanized antibody or its fragment closely approximate the non-human parent antibody structure. In certain embodiments, the humanized PD-1 binding domain provided herein comprises no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residue substitutions in each of the human FR sequences, or no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residue substitutions in all the FRs of a heavy or a light chain variable domain. In some embodiments, such change in amino acid residue could be present in heavy chain FR regions only, in light chain FR regions only, or in both chains.

[000154] In certain embodiments, the PD-1-binding domains provided herein comprise a heavy chain variable domain sequence comprising SEQ ID NO: 21. In certain embodiments, PD-1-binding domains provided herein comprise a light chain variable domain sequence selected from SEQ ID NO: 23 and SEQ ID NO: 28.

[000155] In some embodiments, the PD-1-binding domains provided herein comprise all or a portion of the heavy chain variable domain and/or all or a portion of the light chain variable domain. In one embodiment, the PD-1-binding domains provided herein are a single domain antibody which consists of all or a portion of the heavy chain variable domain provided herein. More information of such a single domain antibody is available in the art (see, e.g., U.S. Pat. No. 6,248,516).

[000156] **iii. Bispecific Antibody Molecule**

[000157] In certain embodiments, the bispecific antibody molecule provided herein comprises a CD47-binding domain comprising one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences of SEQ ID NOs: 1-6 (i.e. derived from W3452-2.683.2 or W3452-2.683.2-z27), and a PD-1 binding domain comprising one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences of SEQ ID NOs: 15-20 (i.e. derived from W3052-2E5), and the CD47-binding domain comprises one independently selected from the group consisting of: Fab and scFv, the PD-1-binding domain comprises one independently selected from the group consisting of: Fab and scFv.

[000158] In certain embodiments, the CD47-binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 7, 11, or a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to CD47 (e.g. human CD47), and/or a light chain variable region comprising the sequence of SEQ ID NO: 9, 13, 26, or a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to CD47 (e.g. human CD47).

[000159] In certain embodiments, the PD-1 binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 or a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to PD-1 (e.g. human PD-1), and/or a light chain variable region comprising the sequence of SEQ ID NO: 23, 28 or a

homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to PD-1 (e.g. human PD-1).

[000160] In certain embodiments, the CD47-binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 11 and a light chain variable region comprising the sequence of SEQ ID NO: 13 or SEQ ID NO: 26 (derived from W3452-2.683.2-z27), and the PD-1 binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 and and a light chain variable region comprising the sequence of SEQ ID NO: 23 or SEQ ID NO: 28 (W3052-2E5) (such bispecific antibody molecules are also referred to as "W367D" herein).

[000161] The CD47-binding domains and/or the PD-1-binding domains provided herein comprise one independently selected from the group consisting: Fab and scFv.

[000162] Various techniques can be used for the production of such antigen-binding domains. Illustrative methods include, enzymatic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and Brennan et al., Science, 229:81 (1985)), recombinant expression by host cells such as E. Coli (e.g. for Fab, Fv and ScFv antibody fragments), and screening from a phage display library as discussed above (e.g. for ScFv). Other techniques for the production of antibody fragments will be apparent to a skilled practitioner.

[000163] In certain embodiments, the CD47-binding domain and/or the PD-1-binding domain is or comprises a scFv. Generation of scFv is described in, for example, WO 93/16185; U.S. Pat. Nos. 5,571,894; and 5,587,458. scFv may be fused to an effector protein at either the amino or the carboxyl terminus to provide for a fusion protein (see, for example, Antibody Engineering, ed. Borrebaeck). An scFv can comprise from a VH linked directly or via a peptide linker to a VL. In certain embodiments, the VH can be at the N-terminus and the VL can be at the C terminus of the scFv. In certain embodiments, the VL can be at the N-terminus and the VH can be at the C terminus of the scFv.

[000164] In certain embodiments, the PD-1-binding domain comprises scFv comprising a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 21 (W3052-2E5

VH) linked to a light chain variable region (VL) comprising the sequence of SEQ ID NO: 23 (W3052-2E5 VL) via a peptide linker. In certain embodiments, the CD47-binding domain comprises scFv comprising a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 11 (W3452-2.683.2-z27 VH) linked to a light chain variable region (VL) comprising the sequence of SEQ ID NO: 13 or 26 (derived from W3452-2.683.2-z27 VL) via a peptide linker.

[000165] The peptide linker can comprise a single or repeated sequences composed of threonine/serine and glycine, such as TGGGG (SEQ ID NO: 39), GGGGS (SEQ ID NO: 36), GGGGSGGGGS (SEQ ID NO: 37) or SGGGG (SEQ ID NO: 40) or its tandem repeats (e.g. 2, 3, 4, or more repeats). In certain embodiments, the peptide linker comprises GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 38). In certain embodiments, the PD-1-binding scFv comprises SEQ ID NO: 25.

[000166] In certain embodiments, the PD-1-binding domain comprises scFv comprising a VH comprising the sequence of SEQ ID NO: 21 (W3052-2E5 VH) linked to the C-terminus of a VL comprising the sequence of SEQ ID NO: 23 (W3052-2E5 VL) via a peptide linker. In certain embodiments, the peptide linker comprises or is SEQ ID NO: 38. In certain embodiments, the PD-1-binding domain comprises scFv comprising SEQ ID NO: 25.

[000167] In certain embodiments, the CD47-binding domain comprises scFv comprising a heavy chain variable region (VH) comprising the sequence of SEQ ID NO: 11 (W3452-2.683.2-z27 VH) linked to the C-terminus of a light chain variable region (VL) comprising the sequence of SEQ ID NO: 13 or 26 (derived from W3452-2.683.2-z27 VL) via a peptide linker. In certain embodiments, the peptide linker comprises or is SEQ ID NO: 38. In certain embodiments, the CD47-binding domain comprises scFv comprising SEQ ID NO: 27.

[000168] In certain embodiments, the CD47-binding domain and/or the PD-1-binding domain is or comprises a Fab. In certain embodiments, the PD-1-binding Fab comprises a heavy chain variable region comprising SEQ ID NO: 21 (derived from W3052-2E5 VH) and a light chain variable region comprising SEQ ID NO: 28 or SEQ ID NO: 23 (derived from W3052-2E5 VL). In certain embodiments, the CD-47-binding Fab comprises a heavy chain variable

region comprising SEQ ID NO: 11 (derived from W3452-2.683.2-z27 VH) and a light chain variable region comprising SEQ ID NO: 26 or SEQ ID NO: 13 (derived from W3452-2.683.2-z27 VL). The heavy chain variable region and the light chain variable region can be disulfidely bonded. The term "disulfidely bonded" refers to linkage via one or more disulfide bond (optionally in addition to another bond). A disulfide bond can be formed between, for example, one cysteine residue of an antibody heavy chain and another cysteine residue of the light chain.

[000169] In certain embodiments, the CD47-binding and/or the PD-1-binding domains are multivalent, such as bivalent, trivalent, tetravalent. The term "valent" as used herein refers to the presence of a specified number of antigen binding sites in a given molecule. As such, the terms "bivalent", "tetravalent", and "hexavalent" denote the presence of two binding site, four binding sites, and six binding sites, respectively, in an antigen-binding molecule. A bivalent molecule can be monospecific if the two binding sites are both for specific binding of the same antigen or the same epitope. Similarly, a trivalent molecule can be bispecific, for example, when two binding sites are monospecific for a first antigen (or epitope) and the third binding site is specific for a second antigen (or epitope). In certain embodiments, the CD47-binding and/or the PD-1-binding domains in the bispecific antibody molecule provided herein can be bivalent, trivalent, or tetravalent, with at least two binding sites specific for the same antigen or epitope. This, in certain embodiments, provides for stronger binding to the antigen or the epitope than a monovalent counterpart. In certain embodiments, in a bivalent antigen-binding moiety, the first valent of binding site and the second valent of binding site are structurally identical (i.e. having the same sequences), or structurally different (i.e. having different sequences albeit with the same specificity). In certain embodiments, CD47-binding and/or the PD-1-binding domains comprises two or more antigen binding sites (e.g. scFv or Fab) operably linked together, with or without a spacer.

[000170] In certain embodiments, the CD47-binding domain is operably linked to the N terminus or the C terminus of the PD-1-binding domain. In certain embodiments, the PD-1-binding domain is operably linked to the N terminus or the C terminus of the CD47-binding domain.

[000171] The operable linkage can be a direct chemical bond linkage or an indirect linkage via a spacer or via an intervening sequence. The term "spacer" as used herein refers to an artificial amino acid sequence having 1, 2, 3, 4 or 5 amino acid residues, or a length of between 5 and 15, 20, 30, 50 or more amino acid residues, joined by peptide bonds and are used to link one or more binding domains, such as a scFv and a Fab or IgG. The spacer can be identical to or different from the peptide linker in the scFv. In certain embodiment, the spacer comprises 1, 2, 3, 4 or more sequential or tandem repeats of SEQ ID NOs: 36, 37, 39 and 40. In certain embodiments, the spacer comprises GGGGS (SEQ ID NO: 36). In certain embodiments, the spacer comprises GGGGSGGGGS (SEQ ID NO: 37), GGGGSGGGGSGGGGS (SEQ ID NO: 41), GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 38). The intervening sequence as used herein can be any amino acid sequence located between the CD47-binding domain and the PD-1-binding domain, as long as both the CD47-binding domain and the PD-1-binding domain are capable of binding to its respective antigen. In an illustrative example, the intervening sequence can comprise a heavy chain constant region, or a light chain constant region.

[000172] In certain embodiments, the CD47-binding domain comprises scFv and the PD-1-binding domain comprises Fab or IgG. In certain embodiments, the CD47-binding scFv can be operably linked to the N terminus or the C-terminus of the heavy chain of the PD-1-binding Fab or IgG (e.g. the C-terminus of the heavy chain constant region following the PD-1-binding Fab), or to the N terminus or the C-terminus of the light chain of the PD-1-binding Fab or IgG, or any combination thereof, and vice versa.

[000173] In an illustrative example, the bispecific antibody molecule can comprise a heavy chain in the format of: VH(anti-PD-1)-CH1-Hinge-CH2-CH3-spacer-scFv(anti-CD47) or scFv (anti-CD47)-spacer-VH(anti-PD-1)-CH1-Hinge-CH2-CH3, and a light chain in the format of VL(anti-PD-1)-CL. As used herein, VH(anti-PD-1) and VL(anti-PD-1) refer respectively to the heavy and light chain variable domain of the PD-1 binding domain; scFv(anti-CD47) refers to scFv of the CD47-binding domain, CL refers to the light chain constant region; and CH1-Hinge-CH2-CH3 are collectively heavy chain constant region.

[000174] In another illustrative example, the bispecific antibody molecule can comprise a

light chain in the format of: scFv(anti-CD47)-spacer-VL(anti-PD-1)-CL or VL(anti-PD-1)-CL-spacer-scFv (anti-CD47), and a heavy chain in the format of VH(anti-PD-1)-CH1-Hinge -CH2-CH3, by the same token.

[000175] In certain embodiments, the PD-1-binding domain comprises scFv and the CD47-binding domain comprises Fab or IgG. In certain embodiments, the PD-1-binding domain scFv can be operably linked to the N terminus or the C-terminus of the heavy chain of the anti-CD47 Fab or IgG (e.g. the C-terminus of the heavy chain constant region following the PD-1-binding Fab), or to the N terminus or the C-terminus of the light chain of the anti-CD47 Fab or IgG, or any combination thereof, and vice versa.

[000176] In an illustrative example, the bispecific antibody molecule can comprise a heavy chain in the format of: VH(anti-CD47)-CH1-Hinge-CH2-CH3-spacer-scFv(anti-PD-1) or scFv(anti-PD-1)-spacer-VH(anti-CD47)-CH1-Hinge-CH2-CH3, and a light chain in the format of VL(anti-CD47)-CL, by the same token. In another illustrative example, the bispecific antibody molecule can comprise a light chain in the format of: scFv (anti-PD-1)-spacer-VL(anti-CD47)-CL or VL(anti-CD47)-CL-spacer-scFv (anti-PD-1), and a heavy chain VH(anti-CD47)-CH1-Hinge-CH2-CH3, by the same token.

[000177] In the bispecific antibody molecule provided herein, the CD47-binding domain may be monovalent (i.e. one scFv or Fab) or multivalent (e.g. more than one scFv or Fab), and/or the PD-1-binding domain may be monovalent or multivalent.

[000178] In certain embodiments, the bispecific antibody molecule comprises a heavy chain in the format of: scFv(anti-PD1)-spacer-VH(anti-CD47)-CH1-Hinge-CH2-CH3, and a light chain in the format of: VL(anti-CD47)-CL, wherein the scFv (anti-PD-1) comprises the sequence of SEQ ID NO: 25, the VH (anti-CD47) comprises the sequence of SEQ ID NO: 11, and the VL (anti-CD47) comprises an amino acid sequence of SEQ ID NO: 26. In certain embodiments, the spacer comprises the sequence of SEQ ID NO: 36. In certain embodiments the heavy chain constant region is of human IgG4 isotype, and optionally contains mutations of S228P and/or L235E. In certain embodiments, the heavy chain constant region comprises the sequence of SEQ ID NO: 35. In certain embodiments, the light chain constant region

comprises the sequence of SEQ ID NO: 33. In certain embodiments, the bispecific antibody molecule comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 29 and a light chain comprising the amino acid sequence of SEQ ID NO: 30. This antibody is also called W367-G17 in the present disclosure.

[000179] In certain embodiments, the bispecific antibody molecule comprises a heavy chain in the format of VH(anti-PD-1)-CH1-Hinge-CH2-CH3-spacer-scFv(anti-CD47), and a light chain in the format of VL(anti-PD-1)-CL, wherein the VH(anti-PD-1) comprises an amino acid sequence of SEQ ID NO: 21, the scFv (anti-CD47) comprises the sequence of SEQ ID NO: 27, and the VL(anti-PD-1) comprises the sequence of SEQ ID NO: 28. In certain embodiments, the spacer comprises the sequence of SEQ ID NO: 37. In certain embodiments the heavy chain constant region is of human IgG4 isotype, and optionally contains mutations of S228P and L235E. In certain embodiments, the heavy chain constant region comprises the sequence of SEQ ID NO: 35. In certain embodiments, the light chain constant region comprises the sequence of SEQ ID NO: 34. In certain embodiments, the bispecific antibody molecule comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 31 and a light chain comprising the amino acid sequence of SEQ ID NO: 32. This antibody is also called W367-G15 in the present disclosure.

[000180] Tables 7 and 8 show the combination of heavy chain and light chain sequences of the bispecific antibody molecules of W367D (specifically W367-G17 and W367-G15).

[000181] Table 7

<table>
<tr><td rowspan="2">W367-U13T5.G17-1.uIgG4.SP
(W367-G17)</td><td>Light chain
(SEQ ID NO: 30)</td><td>VL(anti-CD47)
(W3452-2.683.2-z27)
SEQ ID NO: 26</td><td colspan="4">CL
SEQ ID NO: 33</td></tr>
<tr><td>Heavy chain
(SEQ ID</td><td colspan="3">scFv (Anti-PD-1) (SEQ ID NO: 25)
VL(anti-PD-1) (W3052-2E5) | Linker SEQ ID | VH(anti-PD-1) (W3052-</td><td>Spacer SEQ ID NO: 36</td><td>VH(anti-CD47) (W3452-2.683.2-</td><td>CH SEQ ID NO: 35</td></tr>
</table>

	NO: 29)	SEQ ID NO: 23	NO: 38	2E5) SEQ ID NO: 21		z27) SEQ ID NO: 11	
W367-U13T5.G15-2.uIgG4.SP (W367-G15)	Light chain (SEQ ID NO: 32)	VL(anti-PD-1) (W3052-2E5) SEQ ID NO: 28	CL SEQ ID NO: 34				
	Heavy chain (SEQ ID NO: 31)	VH(anti-PD-1) (W3052-2E5) SEQ ID NO: 21	CH SEQ ID NO: 35	Spacer SEQ ID NO: 37	scFv(Anti-CD47) (SEQ ID NO: 27)		
					VL(anti-CD47) (W3452-2.683.2-z27) SEQ ID NO: 26	Linker SEQ ID NO: 38	VH(anti-CD47) (W3452-2.683.2-z27) SEQ ID NO: 11

"CL" refers to light chain constant region; "CH" refers to heavy chain constant region; "VL" refers to light chain variable region; "VH" refers to heavy chain variable region;

"Anti-PD-1" refers to anti-PD-1 antibody, in particular, the sequence provided in the table is the sequence derived from anti-PD-1 antibody W3052-2E5.

"Anti-CD47" refers to anti-CD47 antibody, in particular, the sequence provided in the table is the sequence derived from anti-CD47 antibody W3452-2.683.2-z27.

[000182] Table 8

scFv (anti-PD1)	SEQ ID NO: 25
	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTYLYWFQQRPGQSPR RLIYLVSTLGSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQLTHW PYTFGQGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVKKPG SSVKVSCKASGFTFTTYYISWVRQAPGQGLEYLGYINMGSGGTNYNEKF KGRVTITADKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGTMVTV SS
VL (anti-CD47)	SEQ ID NO: 26

	SYELTQPPSVSVSPGQTARITCSGIELSNKYAHWYQQKPGQAPVEVMYK DSERPSGIPERFSGSRSGTTVTLTISGVQAEDEADYYCLSTNSDDDLPVFG GGTKLTVL
scFv (anti-CD47)	SEQ ID NO: 27
	SYELTQPPSVSVSPGQTARITCSGIELSNKYAHWYQQKPGQAPVEVMYK DSERPSGIPERFSGSRSGTTVTLTISGVQAEDEADYYCLSTNSDDDLPVFG GGTKLTVLGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGRSLRL SCAASGFTFSTYGMAWVRQAPGKGLEWVSSISTSGGSTYYRDSVQGRFT ISRDNAKSSLYLQMNSLRAEDTATYYCTTKGGRTYRLAYWGQGTLVTV SS
VL (anti-PD-1)	SEQ ID NO: 28
	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTYLYWYQQRPGQSP RRLIYLVSTLGSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQLTH WPYTFGQGTKLEIK
W367-G17 HC: scFv (anti- PD1)-spacer-VH (anti-CD47)-CH1-Hinge-CH2-CH3	SEQ ID NO: 29
	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTYLYWFQQRPGQSPR RLIYLVSTLGSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQLTHW PYTFGQGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLVQSGAEVKKPG SSVKVSCKASGFTFTTYYISWVRQAPGQGLEYLGYINMGSGGTNYNEKF KGRVTITADKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGTMVTV SSGGGGSEVQLVESGGGLVQPGRSLRLSCAASGFTFSTYGMAWVRQAP GKGLEWVSSISTSGGSTYYRDSVQGRFTISRDNAKSSLYLQMNSLRAED TATYYCTTKGGRTYRLAYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSE STAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA KTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI SKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHN HYTQKSLSLSLG
W367-G17 LC: LC(anti-CD47)	SEQ ID NO: 30
	SYELTQPPSVSVSPGQTARITCSGIELSNKYAHWYQQKPGQAPVEVMYK DSERPSGIPERFSGSRSGTTVTLTISGVQAEDEADYYCLSTNSDDDLPVFG GGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVA

	WKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHKSYSCQV THEGSTVEKTVAPTECS
W367-G15 HC: VH(anti-PD-1) - CH1-Hinge -CH2-CH3-spacer-scFv(anti- CD47)	SEQ ID NO: 31
	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISWVRQAPGQGLEYL GYINMGSGGTNYNEKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCA IIGYFDYWGQGTMVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYT CNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLM ISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVY TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGG GGGSGGGGSSYELTQPPSVSVSPGQTARITCSGIELSNKYAHWYQQKPG QAPVEVMYKDSERPSGIPERFSGSRSGTTVTLTISGVQAEDEADYYCLST NSDDDLPVFGGGTKLTVLGGGGSGGGGSGGGGSGGGGSEVQLVESGGG LVQPGRSLRLSCAASGFTFSTYGMAWVRQAPGKGLEWVSSISTSGGSTY YRDSVQGRFTISRDNAKSSLYLQMNSLRAEDTATYYCTTKGGRTYRLA YWGQGTLVTVSS
W367-G15 LC: LC(anti-PD-1)	SEQ ID NO: 32
	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTYLYWYQQRPGQSP RRLIYLVSTLGSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQLTH WPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC
W367-G17 CL(light chain constant domain)	SEQ ID NO: 33
	GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPV KAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHKSYSCQVTHEGSTVEK TVAPTECS
W367-G15 CL(light chain constant domain)	SEQ ID NO: 34
	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPV TKSFNRGEC

W367 CH (heavy chain constant domain)	SEQ ID NO: 35
	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVES KYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLG

[000183] In certain embodiments, the bispecific antibody molecules provided herein may further comprise an immunoglobulin constant region. In some embodiments, an immunoglobulin constant region comprises a heavy chain and/or a light chain constant region. The heavy chain constant region comprises CH1, hinge, and/or CH2-CH3 regions. In certain embodiments, the heavy chain constant region comprises an Fc region. In certain embodiments, the light chain constant region comprises Cκ or Cλ.

[000184] The bispecific antibody molecules provided herein can have a constant region of an immunoglobulin (Ig), optionally, a human Ig constant region, or a human IgG constant region. The constant region can be in any suitable isotype. In certain embodiments, the bispecific antibody molecules provided herein comprises a constant region of IgG1 isotype, which could induce ADCC or CDC, or a constant region of IgG4 or IgG2 isotype, which has reduced or depleted effector function.

[000185] In some embodiments, the bispecific antibody molecules provided herein have reduced or depleted effector function. In some embodiments, the bispecific antibody molecules provided herein have a constant region of IgG4 isotype, which has reduced or depleted effector function. Effector functions such as ADCC and CDC can lead to cytotoxicity to cells expressing PD-1. Many cells such as T cells normally express PD-1. In order to avoid potential unwanted toxicity to those normal cells, certain embodiments of the antibodies and antigen-binding fragments provided herein can possess reduced or even depleted effector functions. Various assays are known to evaluate ADCC or CDC activities, for example, Fc receptor binding assay, C1q binding assay, and cell lysis assay, and can be readily selected by people in the art. Without wishing to be bound to theory, but it is believed

that antibodies with reduced or depleted effector functions such as ADCC or CDC would cause no or minimal cytotoxicity to PD-1-expressing cells, for example those T cells, and therefore spare them from unwanted side effects, whereas in the meantime, blocking of PD-1 would boost immune system for the treatment of conditions such as cancer or chronic infection.

[000186] In certain embodiments, the bispecific antibody molecules provided herein have reduced side effects. For example, the bispecific antibody molecules provided herein can comprise at least one fully human antigen-binding domain and Fc region and therefore reduced immunogenicity than a humanized antibody counterpart.

[000187] B. Characerization of the Bispecific Antibody Molecule

[000188] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically binding to both human PD-1 and human CD47. The bispecific antibody molecules provided herein retain the specific binding affinity to both PD-1 and CD47, in certain embodiments are at least comparable to, or even better than, the parent antibodies in that aspect.

[000189] Binding affinity of the bispecific antibody molecules provided herein can be represented by K_D value, which represents the ratio of dissociation rate to association rate (k_{off}/k_{on}) when the binding between the antigen and the bispecific antibody molecule reaches equilibrium. The antigen-binding affinity (e.g. K_D) can be appropriately determined using suitable methods known in the art, including, for example, flow cytometry assay. In some embodiments, binding of the bispecific antibody molecule to the antigen at different concentrations can be determined by flow cytometry, the determined mean fluorescence intensity (MFI) can be firstly plotted against antibody concentration, K_D value can then be calculated by fitting the dependence of specific binding fluorescence intensity (Y) and the concentration of antibodies (X) into the one site saturation equation: $Y=B_{max}*X/(K_D + X)$ using Prism version 5 (GraphPad Software, San Diego, CA), wherein B_{max} refers to the maximum specific binding of the tested antibody to the antigen.

[000190] In certain embodiments, the bispecific antibody molecules provided herein have a specific binding affinity to human CD47 which is sufficient to provide for diagnostic and/or therapeutic use.

[000191] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically binding to human CD47 with a binding affinity (K_D) of no more than 5×10^{-8}M, no more than 4×10^{-8}M, no more than 3×10^{-8}M, no more than 2×10^{-8}M, no more than 1×10^{-8}M, no more than 9×10^{-9}M, no more than 8×10^{-9}M, no more than 7×10^{-9}M, no more than 6×10^{-9}M, no more than 5×10^{-9}M, no more than 4×10^{-9}M, no more than 3×10^{-9}M, no more than 2×10^{-9}M, as measured by flow cytometry.

[000192] In certain embodiments, the bispecific antibody molecules provided herein cross-react with Cynomolgus monkey CD47.

[000193] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically binding to Cynomolgus monkey CD47 with a binding affinity (K_D) of no more than 3×10^{-8}M, no more than 2×10^{-8}M, no more than 10^{-8}M, no more than 9×10^{-9}M, no more than 8×10^{-9}M, no more than 7×10^{-9}M, no more than 6×10^{-9}M, no more than 5×10^{-9}M, no more than 4×10^{-9}M, no more than 3×10^{-9}M, no more than 2×10^{-9}M, or no more than 10^{-9}M as measured by flow cytometry.

[000194] Binding of the bispecific antibody molecules can also be represented by "half maximal effective concentration" (EC_{50}) value, which refers to the concentration of the bispecific antibody molecule where 50% of its maximal effect (e.g., binding or inhibition etc.) is observed. The EC_{50} value can be measured by methods known in the art, for example, sandwich assay such as ELISA, Western Blot, flow cytometry assay, and other binding assay.

[000195] In certain embodiments, the bispecific antibody molecules provided herein specifically bind to human CD47 at an EC_{50} (i.e. 50% binding concentration) of no more than 16 nM, no more than 15 nM, no more than 14 nM, no more than 13 nM, no more than 12 nM, no more than 11 nM, no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM as menasured by flow cytometry.

[000196] In certain embodiments, the bispecific antibody molecules provided herein specifically bind to recombinant Cynomolgus monkey CD47 with an EC_{50} of no more than 16 nM, no more than 15 nM, no more than 14 nM, no more than 13 nM, no more than 12 nM, no more than 11 nM, no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM as menasured by flow cytometry. In certain embodiments, the bispecific antibody molecules bind to Cynomolgus monkey CD47 with a binding affinity similar to that of human CD47. For example, the exemplary antibodies W367-G17 and W367-G15 bind to Cynomolgus monkey CD47 at an EC_{50} value comparable to that to human CD47.

[000197] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically blocking the binding between human SIRPα and human CD47. The blocking activity can be represented by the half maximal inhibitory concentration (IC_{50}) value, which refers to the concentration of an antibody that is required for 50% inhibition effect is observed. The IC_{50} value can be measured by methods known in the art, for example, sandwich assay such as ELISA, Western Blot, flow cytometry assay, and other binding assay. In certain embodiments, the bispecific antibody molecules provided herein specifically block the binding between human SIRPα and human CD47 at an IC_{50} (i.e. 50% blocking concentration) of no more than 16 nM, no more than 15 nM, no more than 14 nM, no more than 13 nM, no more than 12 nM, no more than 11 nM, no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM as measured by flow cytometry.

[000198] In certain embodiments, the bispecific antibody molecules selectively bind to the tumoric CD47 with high affinity, but with much reduced binding affinity to the CD47 on the human red blood cells (RBCs). In certain embodiments, the binding of tumoric CD47 is 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 15-, 20-, 25-, or 30-fold higher than that to the human RBC CD47.

[000199] In certain embodiments, the bispecific antibody molecules are capable of specifically binding to tumoric CD47 on Raji cell with an EC_{50} of no more than 5 nM, no

more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM, no more than 0.9 nM, no more than 0.8 nM, no more than 0.7 nM, no more than 0.6 nM, no more than 0.5 nM, no more than 0.4 nM, no more than 0.3 nM, no more than 0.2 nM as determined by flow cytometry.

[000200] In certain embodiments, the bispecific antibody molecules are capable of specifically binding to RBCs CD47 with an EC_{50} of no less than 1 nM, no less than 2 nM, no less than 3 nM, no less than 4 nM, no less than 5 nM, no less than 6 nM, no less than 7 nM, or no less than 8 nM, as determined by flow cytometry. In certain embodiments, the bispecific antibody molecules are capable of specifically binding to RBCs CD47 at a maximum binding capacity at least 50% lower than, at least 60% lower than, at least 70% lower than, or at least 80% lower than that of the counterpart anti-CD47 monoclonal antibody having the same set of 6 CDR sequence or having the same pair of heavy and light chain variable region sequences. . "Maximum binding capacity" as used herein with respect to antibody binding to antigen, refers to the maximum level (or pleatau) of the binding signal as determined in the binding assay. The maximum binding capacity no longer increases despite of the increase in the antibody concentration.

[000201] In certain embodiments, the bispecific antibody molecules do not induce hemagglutination. Hemagglutination is a form of agglutination that involves red blood cells (RBCs). Hemagglutination can be determined by formation of a clumped deposit of RBC on a surface area, whereas non-agglutinated RBC do not form aggregates and are distributed evenly over the well surface area.

[000202] In certain embodiments, the bispecific antibody molecules provided herein block binding of human tumoric CD47 to its ligand and thereby promoting (e.g., inducing or increasing) phagocytosis to tumor cells but not promote hemagglutination of normal cells such as erythrocytes. In certain embodiments, the bispecific antibody molecules provided herein are capable of triggering phagocytosis on at least 10%, at least 15%, at least 20%, or at least 23# of tumoric Raji cells by macrophages.

[000203] In certain embodiments, the the bispecific antibody molecules provided herein have a specific binding affinity to human PD-1 which is sufficient to provide for diagnostic and/or therapeutic use.

[000204] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically binding to purified human PD-1 antigen with a binding affinity (K_D) of no more than 10^{-7}M, no more than $9x10^{-8}$M, no more than $8x10^{-8}$M, no more than $7x10^{-8}$M, no more than $6x10^{-8}$M, no more than $5x10^{-8}$M, no more than $4x10^{-8}$M, no more than $3x10^{-8}$M, no more than $2.5x10^{-8}$M, no more than $2x10^{-8}$M, no more than 10^{-8}M, no more than $6x10^{-9}$M, or no more than $5.5x10^{-9}$M, as measured by surface plasmon resonance (SPR).

[000205] In certain embodiments, the bispecific antibody molecules provided herein cross-react with Cynomolgus monkey PD-1 and/or mouse PD-1. In certain embodiments, the bispecific antibody molecules provided herein bind to Cynomolgus monkey PD-1 or mouse PD-1 with a binding affinity similar to that of human PD-1.

[000206] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically binding to purified cynomolgus monkey PD-1 antigen with a binding affinity (K_D) of no more than no more than 100nM, no more than 90nM, no more than 80nM, no more than 70nM, no more than 60nM, no more than 50nM, no more than 45nM, no more than 40nM, no more than 35nM, no more than 30nM, no more than 25nM, no more than 20nM, no more than 15nM, no more than 12nM, or no more than 10nM, as measured by SPR.

[000207] In certain embodiments, the bispecific antibody molecules provided herein specifically bind to human PD-1 at an EC_{50} (i.e. 50% binding concentration) of no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM, no more than 0.9 nM, no more than 0.8 nM, no more than 0.7 nM, no more than 0.6 nM, or no more than 0.5 nM as menasured by FACS.

[000208] In certain embodiments, the bispecific antibody molecules provided herein specifically bind to Cynomolgus monkey PD-1 at an EC_{50} of no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM,

no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM, no more than 0.9 nM, no more than 0.8 nM, no more than 0.7 nM, no more than 0.6 nM, or no more than 0.5 nM, no more than 0.4 nM as menasured by FACS.

[000209] In certain embodiments, the bispecific antibody molecules provided herein specifically bind to mouse PD-1 expressing cells at an EC_{50} of no more than 30 nM, no more than 28 nM, no more than 26 nM, no more than 24 nM, no more than 22 nM, no more than 20 nM, no more than 18 nM, no more than 16 nM, no more than 14 nM, no more than 12 nM, no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM as menasured by FACS.

[000210] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically blocking the binding between human PD-1 and human PD-L1. In certain embodiments, the bispecific antibody molecules provided herein specifically block the binding between human PD-1 and human PD-L1 at an IC_{50} (i.e. 50% blocking concentration) of no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM, no more than 0.9 nM, no more than 0.8 nM, no more than 0.7 nM, no more than 0.6 nM as menasured by flow cytometry.

[000211] In certain embodiments, the bispecific antibody molecules provided herein block binding of human PD-1 to its ligand and thereby providing biological activity including, for example, inducing cytokine production from the activated T cells (such as CD4+ T cells and CD8+ T cells), inducing proliferation of activated T cells (such as CD4+ T cells and CD8+ T cells), and reversing T reg's suppressive function. Exemplary cytokines include IL-2 and IFNγ. The term "IL-2" refers to interleukin 2, a type of cytokine signaling molecule in the immune system that regulates the activities of white blood cells (e.g. leukocytes). The term "Interferon gamma (IFNγ)" is a cytokine that is produced by natural killer (NK), NK T cells, CD4+ and CD8+T cells, which is a critical activator of macrophages and inducer of major histocompatibility complex (MHC) molecule expression. The cytokine production can be determined using methods known in the art, for example, by ELISA. Methods can also be used to detect proliferation of T cells, including [^{3}H] thymidine incorporation assay.

[000212] In certain embodiments, the bispecific antibody molecules provided herein are capable of specifically triggering T cell activation at an EC_{50} of no more than 10 nM, no more than 9 nM, no more than 8 nM, no more than 7 nM, no more than 6 nM, no more than 5 nM, no more than 4 nM, no more than 3 nM, no more than 2 nM, no more than 1 nM, no more than 0.9 nM, no more than 0.8 nM, no more than 0.7 nM, no more than 0.6 nM, or no more than 0.5 nM, no more than 0.4 nM as menasured by reporter gene assay.

[000213] In certain embodiments, the bispecific antibody molecules provided herein are capable of specifically activating T cells and stimulating IFNγ release in the activated T cells in a dose-dependent way, as measured by mixed lymphocyte reaction (MLR).

[000214] In certain embodiments, the bispecific antibody molecules provided herein are capable of simultaneous stimulating cells from both the innate and the asdaptive immune system.

[000215] The bispecific antibody molecules are stable in serum in binding to both PD-1 and CD47. In certain embodiments, the bispecific antibody molecules are capable of binding to both human PD-1 and human CD47 with binding affinity at an EC_{50} of no more than 1nM, no more than 0.9 nM, no more than 0.8 nM, no more than 0.7 nM, no more than 0.6 nM, no more than 0.5 nM, no more than 0.4 nM, no more than 0.3 nM, no more than 0.25 nM, no more than 0.2 nM, or no more than 0.15 nM after incubating in serum for no less than one week (e.g. no less than 10 days, or no less than two weeks etc).

[000216] **C. Format of the Bispecific Antibody Molecule**

[000217] Bispecific antibody fragments are antigen-binding fragments that are derived from an antibody but lack some or all of the antibody constant domains. Examples of such a bispecific antibody fragment include, for example, such as single domain antibody, Fv, Fab and diabody etc.

[000218] In certain embodiments, the bispecific antibody molecules as provided herein are based on the format of a "whole" antibody, such as whole IgG or IgG-like molecules, and small recombinant formats.

[000219] The bispecific antibody molecules provided herein can be made with any suitable methods known in the art. In a conventional approach, two immunoglobulin heavy chain-light chain pairs having different antigen-binding specificities can be co-expressed in a host cell to produce bispecific antibodies in a recombinant way (see, for example, Milstein and Cuello, Nature, 305: 537 (1983)), followed by purification by affinity chromatography.

[000220] Recombinant approach may also be used, where sequences encoding the antibody heavy chain variable domains for the two specificities are respectively fused to immunoglobulin constant domain sequences, followed by insertion to an expression vector which is co-transfected with an expression vector for the light chain sequences to a suitable host cell for recombinant expression of the bispecific antibody (see, for example, WO 94/04690; Suresh et al., Methods in Enzymology, 121:210 (1986)). Similarly, scFv dimers can also be recombinantly constructed and expressed from a host cell (see, e.g. Gruber et al., J. Immunol., 152:5368 (1994)).

[000221] **D. Variants**

[000222] The antigen-binding domains and bispecific antibody molecules provided herein also encompass various variants thereof. In certain embodiments, the variants comprise one or more modifications or substitutions in one or more CDR sequences as provided in Table 1 or Table 4, one or more variable region sequences (but not in any of the CDR sequences) provided in Table 2 or Table 5 and/or the constant region (e.g. Fc region). Such variants retain specific binding affinity to CD47 and/or PD-1 of their parent antibodies, but have one or more desirable properties conferred by the modification(s) or substitution(s). For example, the variants may have improved antigen-binding affinity, improved productivity, improved stability, improved glycosylation pattern, reduced risk of glycosylation, reduced deamination, reduced or depleted effector function(s), improved FcRn receptor binding, increased pharmacokinetic half-life, pH sensitivity, and/or compatibility to conjugation (e.g. one or more introduced cysteine residues).

[000223] The parent antibody sequence may be screened to identify suitable or preferred residues to be modified or substituted, using methods known in the art, for example “alanine

scanning mutagenesis" (see, for example, Cunningham and Wells (1989) Science, 244:1081-1085). Briefly, target residues (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) can be identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine), and the modified antibodies are produced and screened for the interested property. If substitution at a particular amino acid location demonstrates an interested functional change, then the position can be identified as a potential residue for modification or substitution. The potential residues may be further assessed by substituting with a different type of residue (e.g. cysteine residue, positively charged residue, etc.).

[000224] In certain embodiments, the CD47-binding domains and/or the PD-1 binding domains provided herein comprise one or more amino acid residue substitutions in one or more CDR sequences, and/or one or more FR sequences, and/or one or more variable region sequences. In certain embodiments, a variant comprises no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 substitutions in the CDR sequences and/or FR sequences and/or one or more variable region sequences in total. In certain embodiments, the light chain FR1 of the CD47-binding domain contains a M5T mutation (i.e. as can be identified by aligning SEQ ID NO: 13 against SEQ ID NO: 26). This mutation is believed to enhance productivity of the bispecific antibody. In certain embodiments, the light chain FR1 of thePD-1-binding domain contains a F36Y mutation (i.e. as can be identified by aligning SEQ ID NO: 23 against SEQ ID NO: 28). This mutation is believed to enhance stability of the bispecific antibody.

[000225] In certain embodiments, the CD47-binding domains comprise 1, 2, or 3 CDR sequences having at least 80% (e.g. at least 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to a sequence selected from SEQ ID NO: 1-6, and in the meantime retain the binding affinity to CD47 at a level similar to or even higher than its parent antibody.

[000226] In certain embodiments, the anti-CD47-binding domains comprise one or more variable region sequences having at least 80% (e.g. at least 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to SEQ ID NOs: 7, 9, 11, 13, or 26, and in the meantime retain the binding affinity to CD47 at a level similar to or even higher than its parent antibody. In some embodiments, a total of 1 to 10 amino acids have been substituted,

inserted, or deleted in a variable region sequence comprising SEQ ID NO: 7, 9, 11, 13, or 26. In some embodiments, the substitutions, insertions, or deletions occur in regions outside the CDRs (e.g., in the FRs).

[000227] In certain embodiments, the PD-1-binding domains comprise 1, 2, or 3 CDR sequences having at least 80% (e.g. at least 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to a sequence selected from SEQ ID NO: 15-20, , and in the meantime retain the binding affinity to PD-1 at a level similar to or even higher than its parent antibody.

[000228] In certain embodiments, the PD-1-binding domains comprise one or more variable region sequences having at least 80% (e.g. at least 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to a sequence selected from SEQ ID NO: 21, 23, or 28, and in the meantime retain the binding affinity to PD-1 at a level similar to or even higher than its parent antibody. In some embodiments, a total of 1 to 10 amino acids have been substituted, inserted, or deleted in a variable region sequence comprising SEQ ID NO: 21, 23, or 28. In some embodiments, the substitutions, insertions, or deletions occur in regions outside the CDRs (e.g., in the FRs).

[000229] **i. Glycosylation variant**

[000230] The antigen-binding domains and bispecific antibody molecules provided herein also encompass a glycosylation variant, which can be obtained to either increase or decrease the extent of glycosylation of the bispecific antibody molecules.

[000231] The antigen-binding domains and bispecific antibody molecules provided herein may comprise one or more amino acid residues with a side chain to which a carbohydrate moiety (e.g. an oligosaccharide structure) can be attached. Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue, for example, an asparagine residue in a tripeptide sequence such as asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly

to serine or threonine. Removal of a native glycosylation site can be conveniently accomplished, for example, by altering the amino acid sequence such that one of the above-described tripeptide sequences (for N-linked glycosylation sites) or serine or threonine residues (for O-linked glycosylation sites) present in the sequence in the is substituted. A new glycosylation site can be created in a similar way by introducing such a tripeptide sequence or serine or threonine residue.

[000232] **ii. Cysteine-engineered variant**

[000233] The antigen-binding domains and bispecific antibody molecules also encompass a cysteine-engineered variant, which comprises one or more introduced free cysteine amino acid residues.

[000234] A free cysteine residue is one which is not part of a disulfide bridge. A cysteine-engineered variant is useful for conjugation with for example, a cytotoxic and/or imaging compound, a label, or a radioisoptype among others, at the site of the engineered cysteine, through for example a maleimide or haloacetyl. Methods for engineering antibody polypeptides to introduce free cysteine residues are known in the art, see, for example, WO2006/034488.

[000235] **iii. Fc Variant**

[000236] The antigen-binding domains and bispecific antibody molecules provided herein also encompass an Fc variant, which comprises one or more amino acid residue modifications or substitutions at its Fc region and/or hinge region.

[000237] In certain embodiments, the antigen-binding domains and bispecific antibody molecules comprise one or more amino acid substitution(s) that improves pH-dependent binding to neonatal Fc receptor (FcRn). Such a variant can have an extended pharmacokinetic half-life, as it binds to FcRn at acidic pH which allows it to escape from degradation in the lysosome and then be translocated and released out of the cell. Methods of engineering an antibody molecule to improve binding affinity with FcRn are well-known in the art, see, for example, Vaughn, D. et al, Structure, 6(1): 63-73, 1998; Kontermann, R. et al, Antibody Engineering, Volume 1, Chapter 27: Engineering of the Fc region for improved PK, published

by Springer, 2010; Yeung, Y. et al, Cancer Research, 70: 3269-3277 (2010); and Hinton, P. et al, J. Immunology, 176:346-356 (2006).

[000238] In certain embodiments, the antigen-binding domains and bispecific antibody molecules comprise one or more amino acid substitution(s) that alters the antibody-dependent cellular cytotoxicity (ADCC). Certain amino acid residues at the Fc region (e.g. at the CH2 domain) can be substituted to provide for altered (e.g. enhanced, decreased, or depleted) ADCC activity. Alternatively or additionally, carbohydrate structures on the antibody can be changed to alter (e.g. enhance, decrease, or deplete) ADCC activity. Methods of altering ADCC activity by antibody engineering have been described in the art, see for example, Shields RL. et al., J Biol Chem. 2001. 276(9): 6591-604; Idusogie EE. et al., J Immunol. 2000.164(8):4178-84; Steurer W. et al., J Immunol. 1995, 155(3): 1165- 74; Idusogie EE. et al., J Immunol. 2001, 166(4): 2571-5; Lazar GA. et al., PNAS, 2006, 103(11): 4005-4010; Ryan MC. et al., Mol. Cancer Ther., 2007, 6: 3009-3018; Richards JO,. et al., Mol Cancer Ther. 2008, 7(8): 2517-27; Shields R. L. et al, J. Biol. Chem, 2002, 277: 26733-26740; Shinkawa T. et al, J. Biol. Chem, 2003, 278: 3466-3473.

[000239] In certain embodiments, the antigen-binding domains and bispecific antibody molecules comprise one or more amino acid substitution(s) that alters Complement Dependent Cytotoxicity (CDC), for example, by improving or diminishing C1q binding and/or CDC (see, for example, WO99/51642; Duncan & Winter Nature 322:738-40 (1988); U.S. Pat. No. 5,648,260; U.S. Pat. No. 5,624,821); and WO94/29351 concerning other examples of Fc region variants.

[000240] In certain embodiments, the antigen-binding domains and bispecific antibody molecules comprise a human IgG4 constant region in which the 228th amino acid residue is altered, for example from Ser228Pro (S228P, which may prevent or reduce strand exchange), and/or the 235th amino acid residue is altered, for example from Leu235Glu (L235E, which may alter Fc receptor interactions.

[000241] In certain embodiments, the antigen-binding domains and bispecific antibody molecules comprise one or more amino acid substitution(s) in the interface of the Fc region to

facilitate and/or promote heterodimerization. These modifications comprise introduction of a protuberance into a first Fc polypeptide and a cavity into a second Fc polypeptide, wherein the protuberance can be positioned in the cavity so as to promote interaction of the first and second Fc polypeptides to form a heterodimer or a complex. Methods of generating antibodies with these modifications are known in the art, e.g., as described in U.S. Pat. No. 5,731,168.

[000242] **E. Conjugates**

[000243] In some embodiments, the bispecific antibody molecules further comprise a conjugate moiety. The conjugate moiety can be linked to the bispecific antibody molecules. A conjugate moiety is a non-proteinaceous moiety that can be attached to the bispecific antibody molecules. It is contemplated that a variety of conjugate moieties may be linked to the bispecific antibody molecules provided herein (see, for example, "Conjugate Vaccines", Contributions to Microbiology and Immunology, J. M. Cruse and R. E. Lewis, Jr. (eds.), Carger Press, New York, (1989)). These conjugate moieties may be linked to the bispecific antibody molecules by covalent binding, affinity binding, intercalation, coordinate binding, complexation, association, blending, or addition, among other methods.

[000244] In certain embodiments, the bispecific antibody molecules disclosed herein may be engineered to contain specific sites outside the epitope binding portion that may be utilized for binding to one or more conjugates. For example, such a site may include one or more reactive amino acid residues, such as for example cysteine or histidine residues, to facilitate covalent linkage to a conjugate.

[000245] In certain embodiments, the bispecific antibody molecules may be linked to a conjugate moiety indirectly, or through another conjugate moieties. For example, the bispecific antibody molecules may be conjugated to biotin, then indirectly conjugated to a second conjugate moiety that is conjugated to avidin. The conjugate moieties can be a clearance-modifying agent, a toxin (e.g., a chemotherapeutic agent), a detectable label (e.g., a radioactive isotope, a lanthanide, a luminescent label, a fluorescent label, or an enzyme-substrate label), or purification moiety.

[000246] A "toxin" can be any agent that is detrimental to cells or that can damage or kill

cells. Examples of toxin include, without limitation, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, MMAE, MMAF, DM1, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin and analogs thereof, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), anti-mitotic agents (e.g., vincristine and vinblastine), a topoisomerase inhibitor, and a tubulin-binders.

[000247] Examples of detectable label may include a fluorescent labels (e.g. fluorescein, rhodamine, dansyl, phycoerythrin, or Texas Red), enzyme-substrate labels (e.g. horseradish peroxidase, alkaline phosphatase, luceriferases, glucoamylase, lysozyme, saccharide oxidases or β-D-galactosidase), radioisotopes (e.g. ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{35}S, ^{3}H, ^{111}In, ^{112}In, ^{14}C, ^{64}Cu, ^{67}Cu, ^{86}Y, ^{88}Y, ^{90}Y, ^{177}Lu, ^{211}At, ^{186}Re, ^{188}Re, ^{153}Sm, ^{212}Bi, and ^{32}P, other lanthanides), luminescent labels, chromophoric moiety, digoxigenin, biotin/avidin, a DNA molecule or gold for detection.

[000248] In certain embodiments, the conjugate moiety can be a clearance-modifying agent which helps increase half-life of the antibody. Illustrative example include water-soluble polymers, such as PEG, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, copolymers of ethylene glycol/propylene glycol, and the like. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules.

[000249] In certain embodiments, the conjugate moiety can be a purification moiety such as a magnetic bead.

[000250] In certain embodiments, the bispecific antibody molecule provided herein is used for a base for a conjugate.

[000251] F. Polynucleotides and Recombinant Methods

[000252] The present disclosure provides polynucleotides that encode the bispecific antibody molecules provided herein.

[000253] The term "nucleic acid" or "polynucleotide" as used herein refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses polynucleotides containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular polynucleotide sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (see Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

[000254] In certain embodiments, the polynucleotides comprise one or more nucleotide sequences as shown in SEQ ID NOs: 8, 10, 12, 14, 22 and/or 24, and/or a homologous sequence thereof having at least 80% (e.g. at least 85%, 88%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity, and/or a variant thereof having only degenerate substitutions, and encodes the variable region of the exemplary antibodies provided herein. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). The encoding DNA may also be obtained by synthetic methods.

[000255] The isolated polynucleotide that encodes the bispecific antibody molecule can be inserted into a vector for further cloning (amplification of the DNA) or for expression, using recombinant techniques known in the art. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter (e.g. SV40, CMV, EF-1α), and a transcription termination sequence.

[000256] The present disclosure provides vectors (e.g., expression vectors) containing the nucleic acid sequence provided herein encoding the bispecific antibody molecules, at least one promoter (e.g., SV40, CMV, EF-1α) operably linked to the nucleic acid sequence, and at least one selection marker. Examples of vectors include, but are not limited to, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (e.g., herpes simplex virus), poxvirus, baculovirus, papillomavirus, papovavirus (e.g., SV40), lambda phage, and M13 phage, plasmid pcDNA3.3, pMD18-T, pOptivec, pCMV, pEGFP, pIRES, pQD-Hyg-GSeu, pALTER, pBAD, pcDNA, pCal, pL, pET, pGEMEX, pGEX, pCI, pEGFT, pSV2, pFUSE, pVITRO, pVIVO, pMAL, pMONO, pSELECT, pUNO, pDUO, Psg5L, pBABE, pWPXL, pBI, p15TV-L, pPro18, pTD, pRS10, pLexA, pACT2.2, pCMV-SCRIPT.RTM., pCDM8, pCDNA1.1/amp, pcDNA3.1, pRc/RSV, PCR 2.1, pEF-1, pFB, pSG5, pXT1, pCDEF3, pSVSPORT, pEF-Bos etc.

[000257] Vectors comprising the polynucleotide sequence encoding the bispecific antibody molecule can be introduced to a host cell for cloning or gene expression. Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis*, *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*.

[000258] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for the vectors provided. *Saccharomyces cerevisiae*,

or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, e.g., *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

[000259] Suitable host cells for the expression of glycosylated bispecific antibody molecules provided herein are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruiffly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx* mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

[000260] However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, *Mather, Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL

1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2). In some preferable embodiments, the host cell is 293F cell.

[000261] Host cells are transformed with the above-described expression or cloning vectors for production of the bispecific antibody molecules and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In another embodiment, the bispecific antibody molecules may be produced by homologous recombination known in the art.

[000262] The host cells used to produce the bispecific antibody molecule provided herein may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.* 58:44 (1979), Barnes *et al.*, *Anal. Biochem.* 102:255 (1980), U.S. Pat. No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN^{TM} drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[000263] When using recombinant techniques, the bispecific antibody molecules can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or

lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the bispecific antibody molecules are secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[000264] The bispecific antibody molecules thereof prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, DEAE-cellulose ion exchange chromatography, ammonium sulfate precipitation, salting out, and affinity chromatography, with affinity chromatography being the preferred purification technique.

[000265] In certain embodiments, Protein A immobilized on a solid phase is used for immunoaffinity purification of the bispecific antibody molecules. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the bispecific antibody molecules. Protein A can be used to purify antibodies that are based on human gamma1, gamma2, or gamma4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human gamma3 (Guss et al., *EMBO J.* 5:1567 1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the bispecific antibody molecule comprises a CH3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™

chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[000266] Following any preliminary purification step(s), the mixture comprising the antibody molecule of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

[000267] G. Pharmaceutical Composition

[000268] The present disclosure further provides pharmaceutical compositions comprising the bispecific antibody molecule and one or more pharmaceutically acceptable carriers.

[000269] Pharmaceutical acceptable carriers for use in the pharmaceutical compositions disclosed herein may include, for example, pharmaceutically acceptable liquid, gel, or solid carriers, aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispending agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, other components known in the art, or various combinations thereof.

[000270] Suitable components may include, for example, antioxidants, fillers, binders, disintegrants, buffers, preservatives, lubricants, flavorings, thickeners, coloring agents, emulsifiers or stabilizers such as sugars and cyclodextrins. Suitable antioxidants may include, for example, methionine, ascorbic acid, EDTA, sodium thiosulfate, platinum, catalase, citric acid, cysteine, thioglycerol, thioglycolic acid, thiosorbitol, butylated hydroxanisol, butylated hydroxytoluene, and/or propyl gallate. As disclosed herein, inclusion of one or more antioxidants such as methionine in a composition comprising a bispecific antibody molecule and conjugates as provided herein decreases oxidation of the bispecific antibody molecule. This reduction in oxidation prevents or reduces loss of binding affinity, thereby improving antibody stability and maximizing shelf-life. Therefore, in certain embodiments compositions are provided that comprise one or more bispecific antibody molecules as disclosed herein and one or more antioxidants such as methionine. Further provided are methods for preventing

oxidation of, extending the shelf-life of, and/or improving the efficacy of a bispecific antibody molecule as provided herein by mixing the bispecific antibody molecule with one or more antioxidants such as methionine.

[000271] To further illustrate, pharmaceutical acceptable carriers may include, for example, aqueous vehicles such as sodium chloride injection, Ringer's injection, isotonic dextrose injection, sterile water injection, or dextrose and lactated Ringer's injection, nonaqueous vehicles such as fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil, or peanut oil, antimicrobial agents at bacteriostatic or fungistatic concentrations, isotonic agents such as sodium chloride or dextrose, buffers such as phosphate or citrate buffers, antioxidants such as sodium bisulfate, local anesthetics such as procaine hydrochloride, suspending and dispersing agents such as sodium carboxymethylcelluose, hydroxypropyl methylcellulose, or polyvinylpyrrolidone, emulsifying agents such as Polysorbate 80 (TWEEN-80), sequestering or chelating agents such as EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol tetraacetic acid), ethyl alcohol, polyethylene glycol, propylene glycol, sodium hydroxide, hydrochloric acid, citric acid, or lactic acid. Antimicrobial agents utilized as carriers may be added to pharmaceutical compositions in multiple-dose containers that include phenols or cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and benzethonium chloride. Suitable excipients may include, for example, water, saline, dextrose, glycerol, or ethanol. Suitable non-toxic auxiliary substances may include, for example, wetting or emulsifying agents, pH buffering agents, stabilizers, solubility enhancers, or agents such as sodium acetate, sorbitan monolaurate, triethanolamine oleate, or cyclodextrin.

[000272] The pharmaceutical compositions can be a liquid solution, suspension, emulsion, pill, capsule, tablet, sustained release formulation, or powder. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, etc.

[000273] In certain embodiments, the pharmaceutical compositions are formulated into an injectable composition. The injectable pharmaceutical compositions may be prepared in any conventional form, such as for example liquid solution, suspension, emulsion, or solid forms

suitable for generating liquid solution, suspension, or emulsion. Preparations for injection may include sterile and/or non-pyretic solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use, including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready to be combined with a vehicle just prior to use, and sterile and/or non-pyretic emulsions. The solutions may be either aqueous or nonaqueous.

[000274] In certain embodiments, unit-dose parenteral preparations are packaged in an ampoule, a vial or a syringe with a needle. All preparations for parenteral administration should be sterile and not pyretic, as is known and practiced in the art.

[000275] In certain embodiments, a sterile, lyophilized powder is prepared by dissolving a bispecific antibody molecule as disclosed herein in a suitable solvent. The solvent may contain an excipient which improves the stability or other pharmacological components of the powder or reconstituted solution, prepared from the powder. Excipients that may be used include, but are not limited to, water, dextrose, sorbital, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agent. The solvent may contain a buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art at, in one embodiment, about neutral pH. Subsequent sterile filtration of the solution followed by lyophilization under standard conditions known to those of skill in the art provides a desirable formulation. In one embodiment, the resulting solution will be apportioned into vials for lyophilization. Each vial can contain a single dosage or multiple dosages of the bispecific antibody molecule or composition thereof. Overfilling vials with a small amount above that needed for a dose or set of doses (e.g., about 10%) is acceptable so as to facilitate accurate sample withdrawal and accurate dosing. The lyophilized powder can be stored under appropriate conditions, such as at about 4 °C to room temperature.

[000276] Reconstitution of a lyophilized powder with water for injection provides a formulation for use in parenteral administration. In one embodiment, for reconstitution the sterile and/or non-pyretic water or other liquid suitable carrier is added to lyophilized powder. The precise amount depends upon the selected therapy being given, and can be empirically determined.

[000277] **H. Methods of Use**

[000278] In another aspect, methods are provided to treat a condition in a subject that would benefit from up-regulation of immune response, comprising administering a therapeutically effective amount of the bispecific antibody molecule as provided herein to a subject in need thereof. The disease or condition that would benefit from up-regulation of an immune response is selected from the group consisting of cancer, a viral infection, a bacterial infection, a protozoan infection, a helminth infection, asthma associated with impaired airway tolerance, a neurological disease, multiple sclerosis, and an immunosuppressive disease.

[000279] Therapeutic methods are also provided, comprising: administering a therapeutically effective amount of the bispecific antibody molecule as provided herein to a subject in need thereof, thereby treating or preventing a PD-1 related and/or a CD47-related condition or a disorder.

[000280] PD-1-related conditions and disorders can be immune related disease or disorder, tumors and cancers, autoimmune diseases, or infectious disease. In certain embodiments, the PD-1-related conditions and disorders include tumors and cancers, for example, non-small cell lung cancer, small cell lung cancer, renal cell cancer, colorectal cancer, ovarian cancer, breast cancer, pancreatic cancer, gastric carcinoma, bladder cancer, esophageal cancer, mesothelioma, melanoma, head and neck cancer, thyroid cancer, sarcoma, prostate cancer, glioblastoma, cervical cancer, thymic carcinoma, leukemia, lymphomas, myelomas, mycoses fungoids, merkel cell cancer, and other hematologic malignancies, such as classical Hodgkin lymphoma (CHL), primary mediastinal large B-cell lymphoma, T-cell/histiocyte-rich B-cell lymphoma, EBV-positive and -negative PTLD, and EBV-associated diffuse large B-cell lymphoma (DLBCL), plasmablastic lymphoma, extranodal NK/T-cell lymphoma, nasopharyngeal carcinoma, and HHV8-associated primary effusion lymphoma, Hodgkin's lymphoma, neoplasm of the central nervous system (CNS), such as primary CNS lymphoma, spinal axis tumor, brain stem glioma. In certain embodiments, the tumors and cancers are metastatic, especially metastatic tumors expressing PD-L1.

[000281] In certain embodiments, the PD-1-related conditions and disorders include autoimmune diseases. Autoimmune diseases include, but are not limited to, Acquired Immunodeficiency Syndrome (AIDS, which is a viral disease with an autoimmune component), alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diabetes, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease (AIED), autoimmune lymphoproliferative syndrome (ALPS), autoimmune thrombocytopenic purpura (ATP), Behcet's disease, cardiomyopathy, celiac sprue-dermatitis hepetiformis; chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy (CIPD), cicatricial pemphigold, cold agglutinin disease, crest syndrome, Crohn's disease, Degos' disease, dermatomyositis-juvenile, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, Graves' disease, Guillain-Barre syndrome, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin-dependent diabetes mellitus, juvenile chronic arthritis (Still's disease), juvenile rheumatoid arthritis, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, pemacious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynaud's phenomena, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma (progressive systemic sclerosis (PSS), also known as systemic sclerosis (SS)), Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, Takayasu arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis, vitiligo and Wegener's granulomatosis.

[000282] In certain embodiments, the PD-1-related conditions and disorders include infectious disease. Infectious disease include, for example, chronic viral infection, for example, fungus infection, parasite/protozoan infection or chronic viral infection, for example, malaria, coccidioiodmycosis immitis, histoplasmosis, onychomycosis, aspergilosis, blastomycosis, candidiasis albicans, paracoccidioiomycosis, microsporidiosis, Acanthamoeba keratitis, Amoebiasis, Ascariasis, Babesiosis, Balantidiasis, Baylisascariasis, Chagas disease, Clonorchiasis, Cochliomyia, Cryptosporidiosis, Diphyllobothriasis, Dracunculiasis,

Echinococcosis, Elephantiasis, Enterobiasis, Fascioliasis, Fasciolopsiasis, Filariasis, Giardiasis, Gnathostomiasis, Hymenolepiasis, Isosporiasis, Katayama fever, Leishmaniasis, Lyme disease, Metagonimiasis, Myiasis, Onchocerciasis, Pediculosis, Scabies, Schistosomiasis, Sleeping sickness, Strongyloidiasis, Taeniasis, Toxocariasis, Toxoplasmosis, Trichinosis, Trichuriasis, Trypanosomiasis, helminth infection, infection of hepatitis B (HBV), hepatitis C (HCV), herpes virus, Epstein-Barr virus, HIV-1, HIV-2, cytomegalovirus, herpes simplex virus type I, herpes simplex virus type II, human papilloma virus, adenovirus, Kaposi West sarcoma associated herpes virus epidemics, thin ring virus (Torquetenovirus), human T lymphotrophic viruse I, human T lymphotrophic viruse II, varicella zoster, JC virus or BK virus.

[000283] In some embodiments, the subject has been identified as being likely to respond to a PD-1 antagonist. The presence or level of PD-L1 on an interested biological sample can be indicative of whether the subject from whom the biological sample is derived could likely respond to a PD-1 antagonist. Various methods can be used to determine the presence or level of PD-L1 in a test biological sample from the subject. For example, the test biological sample can be exposed to anti-PD-L1 antibody or antigen-binding fragment thereof, which binds to and detects the expressed PD-L1 protein. Alternatively, PD-L1 can also be detected at nucleic acid expression level, using methods such as quantitative Polymerase Chain Reaction (qPCR), reverse transcriptase PCR, microarray, Serial analysis of gene expression (SAGE), Fluorescence in situ hybridization (FISH), and the like. In some embodiments, the test sample is derived from a cancer cell or tissue, or tumor infiltrating immune cells. In certain embodiments, presence or upregulated level of the PD-L1 in the test biological sample indicates likelihood of responsiveness. The term “upregulated” as used herein, refers to an overall increase of no less than 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% or greater, in the protein level of PD-L1 in the test sample, as compared to the PD-L1 protein level in a reference sample as detected using the same antibody. The reference sample can be a control sample obtained from a healthy or non-diseased individual, or a healthy or non-diseased sample obtained from the same individual from whom the test

sample is obtained. For example, the reference sample can be a non-diseased sample adjacent to or in the neighborhood of the test sample (e.g. tumor).

[000284] In some embodiments, the subject is resistant or has developed resistance to PD-1 antagonist therapy or PD-L1 inhibitor therapy. For example, the subject can be one who progressed (e.g., experienced tumor growth) during therapy with a PD-1 inhibitor (e.g., an antibody molecule as described herein) and/or a PD-L1 inhibitor (e.g., antibody molecule).

[000285] The present disclosure also provides therapeutic methods comprising: administering a therapeutically effective amount of the bispecific antibody molecule as provided herein to a subject in need thereof, thereby treating or preventing a CD47-related condition or a disorder. In some embodiment, the CD47-related condition or a disorder is cancer, autoimmune disease, inflammatory disease, or infectious disease.

[000286] Examples of cancer include but are not limited to, lymphoma, bladder cancer, bone cancer, brain and central nervous system cancer, breast cancer, uterine or endometrial cancer, rectal cancer, esophageal cancer, head and neck cancer, anal cancer, gastrointestinal cancer, intra-epithelial neoplasm, kidney or renal cancer, leukemia, liver cancer, lung cancer (e.g. non-small cell lung cancer and small cell lung cancer), melanoma, myeloma, pancreatic cancer, prostate cancer, sarcoma, skin cancer, squamous cell cancer, stomach cancer, testicular cancer, vulval cancer, cancer of the endocrine system, cancer of the parathyroid gland, cancer of the adrenal gland, penile carcinoma, solid tumors of childhood, tumor angiogenesis, spinal axis tumor, pituitary adenoma, or epidermoid cancer.

[000287] Examples of autoimmune diseases include, but are not limited to, Acquired Immunodeficiency Syndrome (AIDS, which is a viral disease with an autoimmune component), alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease (AIED), autoimmune lymphoproliferative syndrome (ALPS), autoimmune thrombocytopenic purpura (ATP), Behcet's disease, cardiomyopathy, celiac sprue-dermatitis hepetiformis; chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy (CIPD), cicatricial pemphigold, cold agglutinin disease, crest

syndrome, Crohn's disease, Degos' disease, dermatomyositis-juvenile, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, Graves' disease, Guillain-Barre syndrome, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin-dependent diabetes mellitus, juvenile chronic arthritis (Still's disease), juvenile rheumatoid arthritis, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, pemacious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynaud's phenomena, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma (progressive systemic sclerosis (PSS), also known as systemic sclerosis (SS)), Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, Takayasu arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis, vitiligo and Wegener's granulomatosis.

[000288] Inflammatory disorders, include, for example, chronic and acute inflammatory disorders. Examples of inflammatory disorders include Alzheimer's disease, asthma, atopic allergy, allergy, atherosclerosis, bronchial asthma, eczema, glomerulonephritis, graft vs. host disease, hemolytic anemias, osteoarthritis, sepsis, stroke, transplantation of tissue and organs, vasculitis, diabetic retinopathy and ventilator induced lung injury.

[000289] Examples of infectious disease include, but are not limited to, fungus infection, parasite/protozoan infection or chronic viral infection, for example, malaria, coccidioiodmycosis immitis, histoplasmosis, onychomycosis, aspergilosis, blastomycosis, candidiasis albicans, paracoccidioiomycosis, microsporidiosis, Acanthamoeba keratitis, Amoebiasis, Ascariasis, Babesiosis, Balantidiasis, Baylisascariasis, Chagas disease, Clonorchiasis, Cochliomyia, Cryptosporidiosis, Diphyllobothriasis, Dracunculiasis, Echinococcosis, Elephantiasis, Enterobiasis, Fascioliasis, Fasciolopsiasis, Filariasis, Giardiasis, Gnathostomiasis, Hymenolepiasis, Isosporiasis, Katayama fever, Leishmaniasis, Lyme disease, Metagonimiasis, Myiasis, Onchocerciasis, Pediculosis, Scabies, Schistosomiasis, Sleeping sickness, Strongyloidiasis, Taeniasis, Toxocariasis, Toxoplasmosis, Trichinosis, Trichuriasis, Trypanosomiasis, helminth infection, infection of hepatitis B (HBV),

hepatitis C (HCV), herpes virus, Epstein-Barr virus, HIV, cytomegalovirus, herpes simplex virus type I, herpes simplex virus type II, human papilloma virus, adenovirus, human immunodeficiency virus I, human immunodeficiency virus II, Kaposi West sarcoma associated herpes virus epidemics, thin ring virus (Torquetenovirus), human T lymphotrophic viruse I, human T lymphotrophic viruse II, varicella zoster, JC virus or BK virus.

[000290] The therapeutically effective amount of an bispecific antibody molecule as provided herein will depend on various factors known in the art, such as for example body weight, age, past medical history, present medications, state of health of the subject and potential for cross-reaction, allergies, sensitivities and adverse side-effects, as well as the administration route and extent of disease development. Dosages may be proportionally reduced or increased by one of ordinary skill in the art (e.g., physician or veterinarian) as indicated by these and other circumstances or requirements.

[000291] In certain embodiments, the bispecific antibody molecule as provided herein may be administered at a therapeutically effective dosage of about 0.01 mg/kg to about 100 mg/kg. In certain of these embodiments, the bispecific antibody molecule is administered at a dosage of about 50 mg/kg or less, and in certain of these embodiments the dosage is 10 mg/kg or less, 5 mg/kg or less, 3 mg/kg or less, 1 mg/kg or less, 0.5 mg/kg or less, or 0.1 mg/kg or less. In certain embodiments, the administration dosage may change over the course of treatment. For example, in certain embodiments the initial administration dosage may be higher than subsequent administration dosages. In certain embodiments, the administration dosage may vary over the course of treatment depending on the reaction of the subject.

[000292] Dosage regimens may be adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single dose may be administered, or several divided doses may be administered over time.

[000293] The bispecific antibody molecule disclosed herein may be administered by any route known in the art, such as for example parenteral (e.g., subcutaneous, intraperitoneal, intravenous, including intravenous infusion, intramuscular, or intradermal injection) or non-parenteral (e.g., oral, intranasal, intraocular, sublingual, rectal, or topical) routes.

[000294] In some embodiments, the bispecific antibody molecules disclosed herein may be administered alone or in combination with one or more additional therapeutic means or agents. For example, the bispecific antibody molecules disclosed herein may be administered in combination with another therapeutic agent, for example, a chemotherapeutic agent or an anti-cancer drug.

[000295] In certain of these embodiments, an bispecific antibody molecule as disclosed herein that is administered in combination with one or more additional therapeutic agents may be administered simultaneously with the one or more additional therapeutic agents, and in certain of these embodiments the bispecific antibody molecule and the additional therapeutic agent(s) may be administered as part of the same pharmaceutical composition. However, a bispecific antibody molecule administered "in combination" with another therapeutic agent does not have to be administered simultaneously with or in the same composition as the agent. A bispecific antibody molecule administered prior to or after another agent is considered to be administered "in combination" with that agent as the phrase is used herein, even if the bispecific antibody molecule and second agent are administered via different routes. Where possible, additional therapeutic agents administered in combination with the bispecific antibody molecule disclosed herein are administered according to the schedule listed in the product information sheet of the additional therapeutic agent, or according to the Physicians' Desk Reference 2003 (Physicians' Desk Reference, 57th Ed; Medical Economics Company; ISBN: 1563634457; 57th edition (November 2002)) or protocols well known in the art.

[000296] The present disclosure further provides methods of using the bispecific antibody molecule thereof.

[000297] In some embodiments, the present disclosure also provides use of the bispecific antibody molecule provided herein in the manufacture of a medicament for treating a PD-1 and/or CD47 related disease or condition in a subject.

[0004] I. Advantages

[000298] The bispecific antibodies provided herein are advantageous over existing therapies in many aspects. For example, the bispecific antibodies provided herein can block both PD1

and CD47 pathways, and they particularly resume T cell function and macrophage function. The bispecific antibodies provided herein are superior to monospecific anti-PD1 antibodies, or monospecific anti- CD47 antibodies, or combination of monospecific anti-PD1 antibodies and monospecific anti- CD47 antibodies. The bispecific antibodies provided herein are also advantageous in that they are cross-reactive to human, monkey PD1 and CD47, and in some embodiments, cross-reactive to murine PD-1. In addition, the bispecific antibodies provided herein could be advantageous in that: 1) it is stably expressed in the antibody generation cell line that can be produced in large scale of industrial antibody production; 2) it enhance T cell and macrophage function without causing hemagglutination and platelet depletion due its reduced binding affinity to red blood cells while its binding to tumor cells is maintained, and therefore has better selectivity and safety that may not cause side effect and have advantages in clinical trials. The bispecific antibodies may be used to treat the patients who are resistant to or relapse from anti-PD1 therapy. The bispecific antibodies may also increase the response rate comparing with anti-PD1 therapy. The bispecific antibodies may also reduce the toxicity of anti-PD1 therapy.

[000299] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. All specific compositions, materials, and methods described below, in whole or in part, fall within the scope of the present invention. These specific compositions, materials, and methods are not intended to limit the invention, but merely to illustrate specific embodiments falling within the scope of the invention. One skilled in the art may develop equivalent compositions, materials, and methods without the exercise of inventive capacity and without departing from the scope of the invention. It will be understood that many variations can be made in the procedures herein described while still remaining within the bounds of the present invention. It is the intention of the inventors that such variations are included within the scope of the invention.

EXAMPLES

[000300] EXAMPLE 1: Generation and characterization of PD-1 monoclonal antibody of W3052-2E5

[000301] The humanized monoclonal PD-1 antibody W3052-2E5 was generated as described in WO2018053709A1. Generally, SD rats were immunized with human PD-1 extracellular domain (ECD) protein and the B lymphocytes isolated from lymph node of the immunized SD rats were combined with myeloma cells to obtain a hybridoma, which was isolated, selected and sub-cloned. The total RNA of the hybridoma was extracted and the cDNA was synthesized and amplified. The framework region of the rat VH and VL genes were replaced with human frameworks by CDR-grafting technique and were cloned into expression vectors to create corresponding clones of humanized antibodies. The monoclonal antibody W3052-2E5 was obtained after affinity maturation by point mutation(s) in the CDR and/or framework regions.

[000302] The humanzied W3052-2E5 antibody has a heavy chain variable region of SEQ ID NO: 21, a kappa light chain variable region of SEQ ID NO: 23, and a human IgG4 constant region. As described in PCT application No.: WO2018053709A1, W3052-2E5 binds to human PD-1 transfected CHO-S cells with EC50 of 2.20 nM, to mouse PD-1 transfected 293F cells with EC50 of 12.9 nM and to activated cynomolgus PBMC in a dose dependent way, as determined by flow cytometry. W3052-2E5 binds to human PD-1 with EC50 of 0.18 nM, to mouse PD-1 with EC50 of 0.37 nM and to cynomolgus PD-1 with EC50 of 0.25 nM by ELISA. W3052-2E5 binds specifically to human PD-1, but not to CD28 and CD47, as measured by flow cytometry. W3052-2E5 blocks human PD-L1 binding to PD-1 transfected CHO-S cells with IC50 of 2.14 nM, blocks mouse PD-L1 binding to PD-1 transfected 293F cells with IC50 of 13 nM, and blocks human PD-L2 binding to PD-1 in a dose-dependent manner, as determined by ELISA. The affinity ability of W3052-2E5 to human PD-1 by SPR assay was 6.13 nM. The affinity ability of W3052-2E5 to mouse PD-1 by SPR assay was 3.99 nM. The affinity ability of W3052-2E5 to human PD-1 by FACS assay was 0.23 nM. The affinity ability of W3052-2E5 to mouse PD-1 by FACS assay was 29 nM.

[000303] Result of epitope binning test showed that the parent antibody of W3052-2E5 (i.e. W3052_r16.88.9), is in the same or close epitope bin as benchmark antibodies nivolumab (clone of 5C4 from BMS patent US9084776B2) and pembrolizumab (disclosed as clone hPD-1.09A in US8354509B2 and WO2008156712A1). However, after setting an additional cutoff

to the binding fold change (<0.55), the final determined epitope residues revealed that W3052_r16.88.9 binds to both human and murine PD-1 while pembrolizumab only bound to the human PD-1. Result of epitope mapping showed that although both are functional in binding human PD-1 and blocking human PD-L1, they have obviously different epitopes.

[000304] W3052-2E5 increased IL-2 secretion, IFN-γ secretion in a dose-dependent manner, as measured by ELISA in both human and mouse T cell function assays. W3052-2E5 increased $CD4^+$ T cells proliferation in a dose-dependent manner, as measured by 3H-thymidine incorporation assay in both human and mouse T cell function assays. Tests in W3052-2E5 on cell proliferation and cytokine production by autologous antigen specific immune response showed that W3052-2E5 can enhance the function of human $CD4^+$ T cell by increase IFN-γ secretion and $CD4^+$ T cells proliferation in a dose-dependent manner. W3052-2E5 also can reverse the suppressive function of Tregs by restoring the IFN-γ secretion and the T-cell proliferation.

[000305] W3052-2E5 does not mediated ADCC or CDC activity on activated $CD4^+$ T cells.

[000306] In vivo efficacy of W3052-2E5 were studied in CloudmanS91 syngeneic tumor model. A weak inhibitory effect was observed in 1 mg/kg W3052-2E5 group compared with the control group, with the tumor volume of 1,089 mm^3 (T/C=68.1%, TGI=34.4%, p=0.367) and tumor growth delay of 0 days. A significant anti-tumor effect was observed in 3 mg/kg W3052-2E5 group compared with the solvent control group, with the tumor volume of 361 mm^3 (T/C=22.9%, TGI=81.0%, p=0.008) and tumor growth delay of 5 days. A significant anti-tumor effect was also observed in 10 mg/kg W3052-2E5 group compared with the solvent control group, with the tumor volume of 614 mm^3 (T/C=39.4%, TGI=64.7%, p=0.036) and tumor growth delay of 5 days.

[000307] Compared to the solvent control group, W3052-2E5 prolonged the median survival time of tumor-bearing mice by 25% (p=0.077) at 1 mg/kg, by 66.7% (p=0.001) at 3 mg/kg, and by 100% (p=0.022) at 10 mg/kg. W3052-2E5 was also shown to have good tolerability in all tumor-bearing mice.

[000308] EXAMPLE 2: Generation and characterization of monoclonal antibody of W3452-2.683.2-z27

[000309] The humanized monoclonal anti-CD47 antibody W3452-2.683.2-z27 was generated as described in PCT/CN2016/094624. Generally, SD rats were immunized with human CD47 extracellular domain (ECD) protein and the B lymphocytes isolated from spleen and lymph node of the immunized rats were combined with myeloma cells to obtain a hybridoma, which was isolated, selected and sub-cloned. The total RNA of the hybridoma was extracted and the cDNA was synthesized and amplified. The framework region of the rat VH and VL genes were replaced with human frameworks by CDR-grafting technique and were cloned into expression vectors to create corresponding clones of humanized antibodies. The genes encoding for the humanized VH and VL were codon-optimized for expression.

[000310] Humanized antibody W3452-2.683.2-z27 has a heavy chain variable region of SEQ ID NO: 11, a lambda light chain variable region of SEQ ID NO: 13, and a human IgG4 constant region containing containing mutations of S228P and L235E (i.e. in IgG4PE,L isotype).

[000311] As described in PCT application No.: PCT/CN2018/079673, W3452-2.683.2-z27 binds to cyno PBMC at an EC50 of 0.21 nM and Max MFI of 14500 as measured by FACS. W3452-2.683.2-z27-IgG1L (i.e. W3452-2.683.2-z27's IgG1L isotype counterpart) binds to CHOK1 cells stably transfected with human CD47 at K_D of 0.42 nM, and W3452-2.683.2-z27 in IgG4PE,L isotype binds to CHOK1 cells stably transfected with human CD47 at K_D of 0.49 nM, as determined by FACS. W3452-2.683.2-z27 blocks binding of human CD47-expressing CHO-K1 cells to SIRPα by more than 90%, as measured by competition binding assay. W3452-2.683.2-z27 does not exhibit hemagglutinating activity at any tested concentration. In comparison, the benchmark control antibody W345-BMK2.uIgG4P.K (Liu J et al, *PloS One* 2015; 10: e0137345) showed hemagglutinating activity. W3452-2.683.2-z27 induced potent phagocytosis of tumor cells in cell-based phagocytosis assay.

[000312] W3452-2.683.2-z27 induces no or weak ADCC and CDC activity.

[000313] W3452-2.683.2-z27 shows good thermal stability compared to human mAb and with values as good as or better than 4 benchmark control antibodies (namely: WBP345-BMK1 (sequence disclosed in WO 2016/109415 A1), WBP345-BMK2 (disclosed as Hu5F9-G4 in Liu J. et al., *PLoS One*, 2015, 10: e0137345), WBP345-BMK3 (C47B222-IgG1, the sequence of C47B222 is disclosed in WO 2016/081423 A1), WBP345-BMK4 (disclosed as 2.3D11 IgG4 in US 2017/0081407 A1).). W3452-2.683.2-z27 shows good serum stability, as 14 days serum culturing has no effect on the human CD47 binding of the antibodies by FACS.

[000314] W3452-2.683.2-z27 demonstrates the most potent anti-tumor activity in an animal model of lymphoma. 3 mg/kg treatment shows the best anti-tumor ability, much better than benchmark control antibody (3 mg/kg). W3452-2.683.2-z27 shows better anti-tumor ability than W3452-2.683.2-z27-IgG1L at 0.5 mg/kg. Since CD47 is ubiquitously expressed in normal human cells, especially in human red blood cells, IgG1 format may induce severe side effect such as anemia. The IgG4 isotype with site mutation (e.g. IgG4PE,L) would be helpful to reduce Fc-based effector function.

[000315] W3452-2.683.2-z27 were studied in non-naïve male cynomolgus monkeys to determine pharmacokinetics and toxicity. The animals in W3452-2.683.2-z27 group (IgG4 format) shows normal sign and normal RBC count in clinical observation. But on Day 7, RBC and HGB counts are reduced, indicating a milder anemia in both low and high dose groups. However, RBC and HGB counts start to recover as early as Day 7 and reach to normal range around Day 28. In addition, reticulocyte (RET) counts are significantly increased as early as day 3 and compensats the RBC loss in blood (data not shown). Thus, it is concluded that the anemia is transient and well-tolerated, or compensated. Compared with IgG4 format, the parameters of RBC, HGB and hematocrit (HCT) in hematology study for W3452-2.683.2-z27-IgG1L at 30 and 150 mg/kg all decreased at Day 7, and caused an anemia in all animals leading to one animal death in Day 8. Two animals are euthanized on Day 12 due to severe anemia (data not shown). This severe anemia is probably due to the IgG1 format or Fc function.

[000316] The average half-life of W3452-2.683.2-z27 is 147 and 79 hours for 30 mg/kg and 150 mg/kg groups, respectively. The half-life for W3452-2.683.2-z27 is longer than that of

Hu5F9-G4 (as described in Liu, J. et al. (2015), *PLoS ONE 10*, e0137345), indicating W3452-2.683.2-z27 PK is comparable to or better than Hu5F9-G4.

[000317] EXAMPLE 3. Generation of bispecific antibodies

[000318] 1 Generation of soluble antigens

[000319] DNA sequences encoding the extracellular domain (ECD) sequence of human CD47 (Uniprot No.: Q08722), human PD-1 (Uniport No.: Q15116), mouse PD-1 (Uniport No.: Q02242), human PD-L1 (Uniport No.: Q9NZQ7), mouse PD-L1 (Uniport No.: Q9EP73) were synthesized by Sangon Biotech (Shanghai, China), then subcloned into modified pcDNA3.3 expression vectors with different tags (such as 6xHis, human Fc, or mouse Fc) at C-terminal.

[000320] Expi293 cells (Invitrogen-A14527) were transfected with the purified expression vector pcDNA3.3. Cells were cultured for 5 days. Supernatant was collected for protein purification using the Ni-NTA column (GE Healthcare, 175248), the Protein A column (GE Healthcare, 175438) or the Protein G column (GE Healthcare, 170618). The produced human CD47 ECD, human PD-1, mouse PD-1, human PD-L1, mouse PD-L1 were tested by SDS-PAGE and SEC, then stored at -80 ^{o}C.

[000321] 2. Generation of target-expressing cell lines

[000322] Using Lipofectamine 2000, CHO-S or 293F cells were transfected with the expression vectors containing gene encoding full length human PD-1, cynomolgus PD-1 or mouse PD-1. The cells were cultured in medium containing proper selection pressure. Human PD-1 high expression stable cell line, cynomolgus PD-1 high expression stable cell line and mouse PD-1 high expression stable cell lines were obtained by limiting dilution.

[000323] The genes of human CD47, cynomolgus CD47 were subcloned into the expression vector pcDNA 3.3. The plasmids were then transfected to CHO-K1 cells by the following protocol. One day prior to transfection, 5x10^{5} CHO-K1 cells were plated into one well of 6-well tissue culture plate and incubated at 5% CO_2 and 37^{o}C. The cells were fed with 3 mL of fresh non-selective media (F12-K, 10% FBS). Transfection reagents were prepared in a 1.5 mL tube, including 4 μg of DNA mixed with 10 μg of Lipofectamine 2000 to make the final volume 200 μL in Opti-MEM medium. The solution was pipetted to the cells drop by drop. 6-

8 hours after transfection, cells were washed with PBS and fed with 3mL of fresh non-selective media. Expressing cells were harvested with trypsin 24-48 hours post-transfection and plated to T75 flask in selective media (F12-K, 10% FBS, 10 μg /mL Blasticidin). After two or three passages of selection, the cells were enriched by an anti-CD47 antibody tagged with phycoerythrin (PE) and Anti-PE Microbeads (Miltenyi-013-048-801). Stable single cell clones were isolated by limiting dilution and screened by FACS using anti-CD47 antibodies.

[000324] 3 Generation of benchmark antibodies

[000325] DNA sequence encoding the variable region of the benchmark anti-CD47 antibody (W345-BMK2, disclosed in US9017675B2 and WO2011143624A2, also disclosed as Hu5F9-G4 in Liu J. et al., *PLoS One*, 2015, 10: e0137345) and anti-PD-1 benchmark antibodies nivolumab (disclosed as clone of 5C4 from BMS patent US9084776B2) were synthesized by Sangon Biotech (Shanghai, China), then subcloned into modified pcDNA3.3 expression vectors with constant region of human IgG4 or human IgG4(S228P). The vectors were co-transfected into Expi293 cells. Cells were cultured for 5 days. Supernatant was collected for protein purification using the Protein A column (GE Healthcare, 175438) or the Protein G column (GE Healthcare, 170618). The produced antibodies were tested by SDS-PAGE and SEC then stored at -80°C.

[000326] 4. Generation of the bispecific antibodies

[000327] 4.1 Construct expression vectors of bispecific antibodies

[000328] Two CD47xPD1 bispecific antibodies were constructed: W367-G17 (having a heavy chain sequence of SEQ ID NO: 29 and light chain sequence of SEQ ID NO: 30) and W367-G15 (having a heavy chain sequence of SEQ ID NO: 31 and light chain sequence of SEQ ID NO: 32). In order to construct W367-G17, DNA sequence encoding scFv of the anti-PD1 antibody W3052-2E5 was linked to the N-terminus of the heavy chain of anti-CD47 antibody W3452-2.683.2-z27. In order to construct W367-G15, DNA sequence encoding scFv of the anti-CD47 antibody W3452-2.683.2-z27 was linked to the C-terminus of the heacy chain of anti-PD1 antibody W3052-2E5. The genes coding the above-mentioned

bispecific antibody molecules were cloned into a modified pcDNA3.3 expression vector with constant region of human IgG4 containing the S228P mutation.

[000329] 4.2 Transfection, expression and purification of bispecific antibodies

[000330] Heavy chain and light chain expression plasmid were co-transfected into ExpiCHO cells using ExpiCHO expression system kit (ThermoFisher-A29133) according to the manufacturer's instructions. Ten days after transfection, the supernatants were collected and used for protein purification with Protein A column (GE Healthcare-17543802) and size exclusion chromatography (GE Healthcare-17104301). Antibody concentration was measured by NanoDrop. The purity of proteins was evaluated by SDS-PAGE, HPLC-SEC and endotoxin.

[000331] The obtained two test antibodies (W367-G17 and W367-G15) and two benchmark antibodies (W345-BMK1 and W345-BMK2) were used in the following studies.

[000332] EXAMPLE 4. *In vitro* Characterization of the bispecific antibodies

[000333] 0. Dynamic light scattering (DLS):

[000334] kD measurement was investigated with DynaPro Plate Reader III (Wyatt DynaproTM). 5 acquisitions were collected for each protein sample while each acquisition time was 5 sec. Each well contained 7.5 L of solution at protein concentrations of 1.25, 2.5, 5, 10 mg/mL and the original dose (10 - 20 mg/mL) in a 1536-well plate (Aurora microplate). For each measurement, the diffusion coefficient was determined and plotted against protein concentration. kD values were calculated automatically by the operation software (DYNAMICS 7.8.1.3).

[000335] Both W367-G17 and W367-G15 have good biophysical properties. When transiently transfected to Expi293 cells and purified by Protein A, the yield of the two bispecific antibodies was 109.4 mg/L for W367-G17 and 103.0 mg/L for W367-G15. The two antibodies were not prone to aggregation, based on their dispersion rates of -6.98 mL/g and -6.03 mL/g, respectively, as measured by dynamic light scattering (DLS).

[000336] 1. Differential scanning fluorimetry (DSF)

[000337] A DSF assay was performed using 7500 Fast Real-Time PCR system (Applied Biosystems). Briefly, 19 μL of bispecific antibody solution was mixed with 1 μL of 62.5x

SYPRO Orange solution (TheromFisher-S6650) and added to a 96-well plate. The plate was heated from 26°C to 95°C at a rate of 2°C/min. The resulting fluorescence data was analyzed by the operation software of the system. The melting temperature (*Tm*) was calculated by taking the maximal value of the negative derivative of the fluorescence data with respect to temperature. T_{on} can be roughly determined as the temperature of negative derivative plot beginning to decrease from a pre-transition baseline.

[000338] The melting temperatures of the two antibodies were 54.0°C and 64.9°C, respectively.

[000339] **2. CD47 binding by FACS**

[000340] Binding of W367-G17 and W367-G15 to human CD47 expressing cells or cynomolgus CD47-expressing cells was determined by flow cytometry. Briefly, 1 x 10^5 cells were added to each well of a 96-well plate and centrifuged at 1500 rpm for 5 minutes at 4°C before the supernatant was discarded. Serial dilutions of test antibodies, as well as positive and negative control antibodies were added to the pelleted cells and incubated for 1 hour at 4°C. The cells were washed two times with 200 μL PBS with 1% BSA. PE conjugated goat anti-human IgG Fc (Jackson, #109-115-098) diluted 100x in FACS buffer were added to the cells and incubated at 4°C for 30 minutes. Additional washing steps were performed two times with 200 μL FACS buffer followed by centrifugation at 1500 rpm for 5 minutes at 4°C. The cells were then resuspended in 100 μL FACS buffer. Fluorescence data was then measured by flow cytometry and analyzed by FlowJo.

[000341] Both W367-G17 and W367-G15 bind to human CD47. In the FACS based assay (Figure 7), EC_{50} of binding of W367-G17, W367-G15 and W345-BMK2 to human CD47 expressing cells are 0.54 nM, 1.28 nM and 0.37 nM, respectively, indicating strong binding to human CD47 expressing cells.

[000342] Both W367-G17 and W367-G15 bind to cynomolgus CD47. In the FACS based assay (Figure 9), EC_{50} of binding of W367-G17, W367-G15 and W345-BMK2 to cynomolgus CD47 expressing cells are 0.82 nM, 1.58 nM and 0.45 nM, respectively, indicating strong binding to cynomolgus CD47 expressing cells.

[000343] **3. PD-1 binding by FACS**

[000344] For FACS binding, engineered human PD-1 expressing cells, engineered mouse PD-1 expressing cells, and engineered cynomolgus PD-1 expressing cells respectively were seeded at 1×10^5 cells/well in U-bottom 96-well plates. Test antibodies titrated 3-folds from 83.3 nM to 0.001 nM were added to the human PD-1 expressing cells; test antibodies titrated 3-folds from 133.3 nM to 0.06 nM were added to the mouse PD-1 expressing cells; and test antibodies titrated 3-folds from 133.3 nM to 0.06 nM were added to the cynomolgus PD-1 expressing cells. Plates were incubated at 4°C for 1 hour. After wash, PE-labeled goat anti-human antibody was added to each well and the plates were incubated at 4°C for 1 hour. The binding of the antibodies onto the cells was tested by flow cytometry. The mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000345] Both W367-G17 and W367-G15 bind to human PD-1 (Figure 1). The EC_{50} of W367-G17, W367-G15 and the WBP305-BMK1 binding to human PD-1 expressing cells in a FACS assay are 0.52 nM, 0.56 nM and 0.60 nM, respectively, indicating bispecific antibodies' stronger binding to PD-1+ cells than the benchmark monoclonal antibody.

[000346] Both W367-G17 and W367-G15 bind to mouse PD-1. The EC_{50} of W367-G17 and W367-G15 binding to mouse PD-1 expressing cells determined by FACS are 3.51 nM and 3.97 nM, respectively (Figure 5). This is comparable to that of the monoclonal anti-PD-1 parental antibody W3052-2E5 (with an EC_{50} of 3.07 nM).

[000347] Both W367-G17 and W367-G15 bind to cynomolgus PD-1. In the FACS assay (Figure 3), W367-G17 and W367-G15 bind to cynomolgus PD-1 expressing cells with EC_{50} of 0.31 nM and 1.21 nM, respectively. These EC_{50} values are as good as that of the benchmark monoclonal antibody WBP305-BMK1 in the same assay, which is 0.51 nM.

[000348] **4. CD47 and PD-1 binding affinity (SPR/FACS)**

[000349] Surface plasmon resonance (SPR) technology was used to measure the association (k_{on}) and disassociation (k_{off}) rates the antibodies' binding to excellular domain (ECD) of PD-1 (human, or cynomolgus). The affinity constant (K_D) was determined as the ratio between k_{off} and k_{on}.

[000350] Biacore T200, Series S Sensor Chip CM5, Amine Coupling Kit, and 10x HBS-EP were purchased from GE Healthcare. Goat anti-human IgG Fc antibody was purchased from

Jackson ImmunoResearch Lab (catalog number 109-005-098). In the immobilization step, the activation buffer was prepared by mixing 400 mM EDC and 100 mM NHS immediately prior to injection. The CM5 sensor chip was activated for 420 seconds with the activation buffer. 30 μg/mL of goat anti-human IgG Fc antibody in 10 mM NaAc (pH 4.5) was then injected to Fc1-Fc4 channels for 200 seconds at a flow rate of 5 μL/min. The chip was deactivated by 1 M ethanolamine-HCl (GE). 4 μg/mL of test antibodies in the running buffer (HBS-EP+) was injected individually to Fc3 channel for 30 seconds at a flow rate of 10 μL/min. Eight different concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 nM) of the analyte, which is ECD of CD47 or ECD of PD-1 or the blank running buffer were injected orderly to Fc1-Fc4 channels at a flow rate of 30 μL/min for an association phase of 120 seconds, followed by the dissociation phase of 2400 seconds. The regeneration buffer (10 mM Glycine pH 1.5) was injected at 10 μL/min for 30 seconds following every dissociation phase. The SPR sensorgrams were then fitted to the 1:1 Langmuir binding model by the software of the SPR system.

[000351] The SPR affinities (K_D) of W367-G17, W367-G15 and WBP305-BMK1 to human PD-1 antigen produced in-house are 5.87 nM, 22.2 nM and 2.32 nM, respectively (Figure 2). The K_D values of W367-G17 and W367-G15 are comparable to that of the PD-1 monoclonal antibody counterpart W3052-2E5 (with a K_D value of 3.5 nM).

[000352] Both W367-G17, W367-G15 also bind to purified cynomolgus PD-1 antigen in the SPR assay, with binding affinities (K_D) of 9.57 nM and 54.2 nM (Figure 4). These are comparable to that of the PD-1 monoclonal antibody counterpart W3052-2E5 (with a K_D value of 5.45 nM) and also that of the benchmark control W305-BMK1 (with a K_D value of 1.55 nM).

[000353] A FACS-based assay was used to measure affinity of the test antibodies' binding to human or cynomolgus CD47 on cell surface. HEK293 cells expressing human or cynomolgus CD47 were transferred in to 96-well U-bottom plates (BD) at a density of $5x10^4$ cells/well. Test antibodies were diluted 2-folds serially in PBS with 1% BSA and incubated with cells at 4^oC for 1 hour. After centrifugation at 1500 rpm for 4 minutes, the supernatant was discarded. The secondary antibody, FITC conjugated goat anti-human IgG Fc (3.2 FITC per IgG,

Jackson ImmunoResearch Lab, catalog number 109-095-098), was added to re-suspend cells to final concentration at 14 μg/ml, and incubated at 4°C in the dark for 30 minutes. The cells were then washed once and re-suspended in PBS with 1% BSA, and analyzed by flow cytometry on a BD Canto™ II equipment. Fluorescence intensity was converted to bound molecules/cell based on the quantitative beads (QuantumTM MESF Kits, Bangs Laboratories). K_D was calculated by Graphpad Prism 5.

[000354] In the cell-binding affinity FACS assay, the K_D of W367-G17, W367-G15 and W345-BMK2 to human CD47 expressing cells were 1.60 nM, 3.70 nM and 0.36 nM, respectively (Figure 8), indicating similar conclusion of strong binding to human CD47 expressing cells. The K_D of W367-G17 and W367-G15 are also comparable to that of the CD47 monoclonal antibody W3452-2.683.2.z27 (with a K_D value of 0.59 nM).

[000355] The K_D of W367-G17, W367-G15 and WBP345-BMK2 to cynomolgus expressing cells are 0.95 nM, 2.50 nM and 0.23 nM, respectively (Figure 10). The K_D of W367-G17 and W367-G15 are also comparable to that of the CD47 monoclonal antibody W3452-2.683.2.z27 (with a K_D value of 0.44 nM).

[000356] <u>5. PD-1 and CD47 dual binding ELISA</u>

[000357] An ELISA assay was developed to test whether the bispecific antibodies could simultaneously bind to both PD-1 and CD47. A 96-well ELISA plate (Nunc MaxiSorp, ThermoFisher) was coated overnight at 4°C with 0.5 μg/ml CD47-ECD in carbonate-bicarbonate buffer. After one hour blocking with 2% (w/v) BSA (Pierce) in PBS, serial dilutions of W367-G17 or W367-G15 in PBS containing 2% BSA are added then incubated for 1 hour at ambient temperature. Following incubation, plates were washed three times with 300 μL per well of PBS containing 0.5% (v/v) Tween 20. 0.1 μg/mL PD-1-ECD-biotin was added to plates and incubated for 1 hour. After washing the plates three times, Streptavidin-HRP (Invitrogen, #SNN1004, 1:25000 diluted) was added and incubated for 1 hour at ambient temperature. After washing six times with 300 μL per well of PBS containing 0.5% (v/v) Tween 20, Tetramethylbenzidine (TMB) Substrate (Sigma-860336-5G) was added for detection. The reaction was stopped after approximate 10 minutes through the addition of 100

μL per well of 2 M HCl. The absorbance of the wells was measured at 450 nm with a multiwall plate reader (SpectraMax M5).

[000358] Both bispecific antibodies simultaneously bind to both PD-1 and CD47. The dual binding ELISA was used to test serum stablility of W367-G17 and W367-G15, and the results are shown in Figure 12A and Figure 12B.

[000359] **6. Cell based CD47 ligand competition assay**

[000360] Human CD47-expressing cell lines and CCRF-CEM (ATCC#CRM-CCL-119) were prepared by WuXi Biologics. Human SIRPα was purchased from Sino Biological (Cat# 11612-H08H-50).

[000361] Competition of W367-G17 and W367-G15 with the human SIRPα ligand on CD47 expressing cells was determined by flow cytometry. Briefly, cells were added to a 96-well round bottom plate at the density of $1x10^5$ cells/well. Supernatant was discarded after centrifugation at 1500rpm for 5 minutes at 4°C. Serial dilutions of test antibodies, as well as positive and negative control antibodies with 1μg/mL human SIRPα were added to the pelleted cells and incubated for 2 hours at 4°C. The cells were washed two times with 200 μL PBS with 1% BSA. Biotin conjugated anti-His Antibody (GenScript, # A00613, 1:400 dilution) were added to the cells and incubated at 4°C for 30 minutes. PE conjugated Streptavidin (Affymetrix, #12-4317, 1:200 dilution) were added to the cells and incubated at 4°C for 30 minutes. Additional washing steps were performed two times with 200 μL FACS buffer followed by centrifugation at 1500 rpm for 5 minutes at 4°C. Finally, the cells were resuspended in 100 μL FACS buffer. Fluorescence data was measured by flow cytometry and analyzed by FlowJo.

[000362] Both W367-G17 and W367-G15 block the binding between human SIRPα and human CD47. As shown in the blocking assay, the IC_{50} of CD47-SIRPα inhibition by W367-G17, W367-G15 and W345-BMK2 are 1.38 nM, 2.55 nM and 0.44 nM, respectively (Figure 11).

[000363] **7. Human RBC hemagglutination assay (HA)**

[000364] Human red blood cells (RBCs) were isolated from fresh human blood. Cells were washed three times with PBS, 2000 rpm for 5 minutes. Cells were then diluted to 4% in PBS

and incubated at 37°C for 1 hour with a titration of CD47 antibodies, as well as positive and negative control antibodies in a round bottom 96 well plate. The graph of results was recorded by camera.

[000365] Hemagglutination is an adverse effect that should be avoided if possible. As shown in the hemagglutination assay (Figure 14), W367-G17 and W367-G15 did not induce hemagglutination, whereas W345-BMK2 did. The CD47 monoclonal antibody W3452-2.683.2.z27 also did not induce hemagglutination. This assay demonstrates potential advantage in safety of the two bispecific antibodies over benchmark monoclonal antibodies.

[000366] **8. Antibody dependent cellular phagocytosis (ADCP)**

[000367] PBMCs were isolated from human blood. The CD14 positive monocytes were isolated from PBMC by hCD14 Microbeads (Miltenyi Biotec, #130-050-201). Monocytes were then induced to macrophages by 100 ng/mL rhM-CSF (R&D Systems, #216-MC/CF) in 10% FBS RPIM1640 medium for 7days. These monocyte derived macrophages (MDMs) become adherent allowing other cells to be washed away. MDMs were scraped and re-plated in 96 well dishes. The human tumor cell line Jurkat.2B8, CCRF-CEM, Raji etc were chosen as target cells due to their high CD47 expression. Target cells were labeled with 1 μM CFSE (Life-technology, #C34570) at 37°C for 30 minutes, then washed and added to MDMs at a ratio of 1:1. W367-G17 and W367-G15 were added at various concentrations. After two hours of incubation, cells were stained with an anti-CD14 antibody conjugated with APC (BD Pharmingen, #561708) then analyzed by flow cytometry. Phagocytosis was measured as the percentage of CFSE+ cells among the total CD14+ cells.

[000368] One of the bispecific antibodies, W367-G17, stimulates strong and dose dependent ADCP reactions. At the same molar dose, this bispecific molecule triggers phagocytosis on 23% of the tumoric Raji cells by macrophages. Its phagocytosis effect is more pronounced than that of the benchmark monoclonal antibody, W345-BMK2. ADCP effect by the other bispecific antibody, W367-G15, is weaker (Figure 16).

[000369] **9. Antibody competition with PD-1/PDL1 assays**

[000370] In an FACS based blocking assay, engineered human PD-1 expressing cells were seeded in a U-bottom 96-well plate at the density of 1×10^5 cells/well. 3-Fold titrated

bispecific antibodies from 250 nM to 0.001 nM coupled with 5 μg/mL human PD-L1 protein were added to the cells. Plates were incubated at 4°C for 1 hour. After wash, the binding of human PD-L1 protein to cells expressing human PD-1 was detected by a PE-labeled goat anti-mouse antibody. The competition binding of antibodies to the cells was tested by flow cytometry. The mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000371] Both W367-G15 and W367-G17 block the interaction between human PD-1 and human PD-L1. The IC_{50} of the blocking by W367-G17, W367-G15 and WBP305-BMK1 are 0.67 nM, 0.68 nM and 0.64 nM, respectively, indicating that the two bispecific antibodies block the PD-1 and PD-L1 interaction as strong as the benchmark monoclonal antibody (Figure 6).

[000372] 10. Antibody Dependnet Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC)

[000373] Human PD-1 or CD47 expressing cells and various concentrations of the test antibodies were pre-incubated in a 96-well U-plate. NK cells isolated from PBMC were then added at the effector/target ratio of 2.5:1. The plate was kept at 37°C in a 5% CO2 incubator for 4 hours. Target cell lysis was determined by the LDH-based cytotoxicity detection kit (cat# Roche-11644793001). Raji cells and Rituximab was used as positive control.

[000374] Human PD-1 or CD47 expressing cells and various concentrations of the test antibodies were pre-incubated in a flat bottom 96-well plate. Human complement was added at the dilution ratio of 1:50. The plate was kept at 37°C in a 5% CO_2 incubator for 4 hours. Target cell lysis was determined by the CellTiter-Glo kit (Promega). Raji cells and Rituximab were used as positive control.

[000375] Due to their effector-null Fc segments, both W367-G15 and W367-G17 do not induce ADCC or CDC responses, as demonstrated by corresponding assays (data not shown).

[000376] 11. Red Blood Cells (RBCs) binding FACS

[000377] RBCs from fresh blood were seeded at 1×10^5 cells/well in a U-bottom 96-well plate. The supernatant was discarded after spinning at 1500 rpm for 5 minutes. Antibodies diluted with 1% BSA DPBS were added. The plate was then incubated for 4°C for 1 hour. After wash, 100 μL 1:150 diluted PE-labeled goat anti-human antibody was added to each well. The plate

was incubated at 4°C for 1 hour. The binding of the antibodies onto the cells was measured by flow cytometry. The mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000378] One bispecific antibody, W367-G17, binds to human red blood cells (RBCs) with EC_{50} of 1.00 nM determined by FACS. As a contrast, W367-G15 has significantly reduced binding to RBCs as demonstrated by the much weaker EC_{50} of 8.09 nM. In addition, W367-G15 has a much lower maximum binding capacity (as shown by top MFI value determined by FACS) to RBCs than W367-G17, where W367-G15 has top MFI value which is about 20% of the top MFI value of the binding between W367-G17 and RBCs (Figure 13). In contrast, the monoclonal anti-CD47 antibody counterpart W3452-2.683.2-z27 is shown to have an EC_{50} of 0.43 nM and a top MFI value of 11972, which indicates much stronger binding to RBCs than its bispecific derivative W367-G15.

[000379] 12. Raji cell-binding FACS

[000380] Raji cells were seeded at 1×10^5 cells/well in a U-bottom 96-well plate. The supernatant was discarded after spinning at 1500 rpm for 5 minutes. Antibodies diluted with 1% BSA DPBS were added. The plate was then incubated for 4°C for 1 hour. After wash, 100 μL 1:150 diluted PE-labeled goat anti-human antibody was added to each well. The plate was incubated at 4°C for 1 hour. The binding of the antibodies onto the cells was measured by flow cytometry. The mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000381] Both W367-G15 and W367-G17 bind to tumoric Raji cells with high affinity. In the FACS based assay, EC_{50} of binding of W367-G17, W367-G15 and W345-BMK2 to Raji cells are 0.20 nM, 0.42 nM and 0.12 nM, respectively (Figure 15). The EC_{50} of W367-G15 and W367-G17 are similar to that of the monoclonal anti-CD47 antibody W3452-62.683.2-z27 (with an EC_{50} of 0.20nM).

[000382] 13. T cell activation by reporter gene assay

[000383] Cells engineered in house to express human PD-L1, APC-CHOK1-OKT3-PDL1, were seeded at flat bottom 96-well plates at 2×10^4 cells per well one day before experiment. NFAT-luciferase integrated Jurkat cells transiently transfected with human PD-1 were added to each well at the density of $4x10^4$ cells per well. Antibodies of different concentrations were

added. Reconstituted luciferase substrate was added after incubation at 37°C for 6 hours with 5% CO_2. Luciferase intensity signal was measured by a microplate spectrophotometer.

[000384] Both W367-G15 and W367-G17 trigger T-cell activation with high potency, as demonstrated by the reporter gene assay (Figure 17). EC_{50} of T-cell activation by W367-G17, W367-G15 and WBP305-BMK1 are 0.31 nM, 0.33 nM and 0.36 nM, respectively.

[000385] 14. Allogeneic mixed lymphocyte reaction (MLR)

[000386] Human peripheral blood mononuclear cells (PBMCs) were freshly isolated from healthy donors with Ficoll-Paque PLUS gradient centrifugation. Isolated PBMCs were cultured in complete RPMI-1640 supplemented with 10% FBS, 1% PS and 100 units of recombinant human IL-2. Human monocytes were isolated using Human Monocyte Enrichment Kit according to the manufacturer's instructions. Cell concentration was adjusted in complete RPMI-1640 medium supplemented with 800 U/mL of recombinant human GM-CSF and 50 ng/mL of rhIL-4. Isolated monocytes were seeded to a flat-bottomed 6-well plate at the density of 2×10^6 cells per mL. Cells were cultured for 5 to 7 days to differentiate into immature dendritic cells (iDCs). Human CD4+ T cells were isolated with the Human CD4+ T cell Enrichment kit according to the manufacturer's protocol.

[000387] Isolated CD4+ T-cells, iDCs and antibodies of various concentrations were then co-cultured in a round-bottomed 96-well plate in complete RPMI-1640 medium at 37°C in a 5% CO_2 incubator for 5 days. IFN- production was then read with the IFN- detection kit from Peprotech (300-02).

[000388] Activation of T-cells by W367-G15 and W367-G17 is also demonstrated by the MLR assay (Figure 18), in which both antibodies stimulate strong and dose-dependent IFN- release from activated T-cells. Combo in Figure 18 means the combination of two benchmark antibodies W345-BMK2 and W305-BMK1.

[000389] 15. Synergic stimulation of CD4+ T cells and DC cells

[000390] To demonstrate that the test antibodies can synergistically stimulate the adaptive and the innate immune systems, CD14+ monocytes were first separated from human PBMC and then induced to iDCs with GM-CSF and IL-4 at 37°C in a 5% CO_2 incubator for 2 days. The iDCs were then co-cultured with Raji cells, CD4+ T-cells and bispecific antibodies in

complete RPMI-1640 medium in 96-well U-bottom plates. After incubation at 37°C with 5% CO2, IFN- production was read on day 3 with the IFN- detection kit from Peprotech (300-02).

[000391] Simultaneous stimulation of cells from the innate and adaptive immune systems has synergistic effect on T-cell activation. As demonstrated in the dual function assay in which T-cells, iDC cells and tumor cells are co-cultured with antibodies, the release of IFN- is minimal by the anti-CD47 benchmark monoclonal antibody W345-BMK2, or moderate by the anti-PD-1 benchmark monoclonal antibody W305-BMK1. However, release of IFN- is significantly enhanced by the combination of the two benchmark antibodies, and even more strongly enhanced by W367-G15 or W367-G17. This assay shows synergistic effect between iDC activation and T-cell activation. It also demonstrates superiority of the bispecific antibodies over mono-specific benchmark antibodies (Figure 19). Combo in Figure 19 means the combination of both benchmark antibodies W305-BMK1 and W345-BMK2.

[000392] **16. Serum stability**

[000393] Antibodies were incubated in freshly isolated human serum (serum content > 90%) at 37°C. On indicated time points, an aliquot of sample was removed from the incubator and snap frozen in liquid nitrogen then stored at -80°C until test. One day before the test, a plate was pre-coated with 1.0 μg/mL of His-tagged human PD-1 antigen produced in house, then incubated at 4°C overnight. On the day of the test, the coated plate was blocked for one hour. Antibody samples were quick thawed, then added to the plate at various concentrations. After the plate was incubated at ambient temperature for 1 hour, the plate was washed three times with 300 μL per well of PBS containing 0.5% (v/v) Tween 20. Human CD47 antigen fused with mouse Fc (in house) was added to the plate at the concentration of 1.0 μg/mL. After incubation at ambient temperature for 1 hour, the plate was washed three times. HRP-conjugated goat anti-mouse Fc antibody was added. After incubation for 1 hour at ambient temperature, the plate was washed six times. 100 μL tetramethylbenzidine (TMB) Substrate was then added to each well for detection. The reaction was stopped after 5 minutes with the addition of 100 μL 2M HCl per well. The absorbance of the wells was measured at 450 nm with a plate reader (SpectraMax M5e).

[000394] Both bispecific antibodies are stable in serum. When incubated in serum for two weeks at 37°C, the EC_{50} of W367-G17's simultaneous binding to human PD-1 and human CD47 remain unchanged at 0.19 nM. Likewise, the EC_{50} of W367-G15's simultaneous binding to human PD-1 and human CD47 remain unchanged at around 0.15 nM (Figure 12A and 12B).

WHAT IS CLAIMED IS:

1. A bispecific antibody molecule comprising a CD47-binding domain and a PD-1-binding domain, wherein:

the CD47-binding domain comprises:

1, 2, or 3 heavy chain complementarity determining region (CDR) sequences selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 5; and/or

1, 2, or 3 light chain CDR sequences selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 4, and SEQ ID NO: 6;

the PD-1 binding domain comprises:

1, 2, or 3 heavy chain CDR sequences selected from the group consisting of: SEQ ID NO: 15, SEQ ID NO: 17, and SEQ ID NO: 19, and/or

1, 2, or 3 light chain CDR sequences selected from the group consisting of: SEQ ID NO: 16, SEQ ID NO: 18, and SEQ ID NO: 20,

the CD47-binding domain comprises one independently selected from the group consisting of: a Fab and a single chain Fv antibody (scFv); and

the PD-1-binding domain comprises one independently selected from the group consisting of: a Fab and a scFv.

2. The bispecific antibody molecule of claim 1, wherein the CD47-binding domain comprises a Fab.

3. The bispecific antibody molecule of claim 1, wherein the PD-1-binding domain comprises a Fab.

4. The bispecific antibody molecule of claim 1 or 3, wherein the CD47-binding domain comprises a scFv.

5. The bispecific antibody molecule of claim 1 or 2, wherein the PD-1-binding domain comprises a scFv.

6. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain comprises a heavy chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 1, 3 and 5, and/or a light chain variable region

comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 2, 4 and 6.

7. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain comprises a heavy chain variable region selected from the group consisting of SEQ ID NOs: 7 and 11, and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to CD47.

8. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain further comprises a light chain variable region selected from the group consisting of SEQ ID NOs: 9, 13 and 26, and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to CD47.

9. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 11 and a light chain variable region comprising the sequence of SEQ ID NO: 13 or SEQ ID NO: 26.

10. The bispecific antibody molecule of any of the preceding claims, wherein the PD-1-binding domain comprises a heavy chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 15, 17 and 19, and/or a light chain variable region comprising 1, 2, or 3 CDR sequences selected from SEQ ID NOs: 16, 18 and 20.

11. The bispecific antibody molecule of any of the preceding claims, wherein the PD-1-binding domain comprises a heavy chain variable region selected from the group consisting of SEQ ID NO: 21 and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to PD-1.

12. The bispecific antibody molecule of any of the preceding claims, wherein the PD-1-binding domain comprises a light chain variable region selected from the group consisting of SEQ ID NOs: 23 and 28, and a homologous sequence thereof having at least 80% sequence identity yet retaining specific binding affinity to PD-1.

13. The bispecific antibody molecule of any of the preceding claims, wherein the PD-1-binding domain comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 and a light chain variable region comprising the sequence of SEQ ID NO: 23 or SEQ ID NO: 28.

14. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to CD47, and/or the PD-1-binding domain further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to PD-1.

15. The bispecific antibody molecule of claim 14, wherein at least one of the substitutions or modifications is in one or more of the CDR sequences, and/or in one or more of the VH or VL sequences but not in any of the CDR sequences.

16. The bispecific antibody molecule of any of the preceding claims, wherein the bispecific antibody molecule further comprises an immunoglobulin constant region, optionally a constant region of human Ig, or optionally a constant region of human IgG.

17. The bispecific antibody molecule of any of preceding claims, wherein the CD47-binding domain is operably linked to the N terminus or the C terminus of the PD-1-binding domain.

18. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain comprises a scFv and the PD-1-binding domain comprises a Fab.

19. The bispecific antibody molecule of claim 18, wherein the CD47-binding scFv comprises the sequence of SEQ ID NO: 27, and the PD-1-binding Fab comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 21 and a light chain variable region comprising the sequence of SEQ ID NO: 23 or SEQ ID NO: 28.

20. The bispecific antibody molecule of claim 18 or 19, wherein the CD47-binding scFv is operably linked to the C terminus of the heavy chain constant region following the PD-1-binding Fab.

21. The bispecific antibody molecule of claim 20, wherein the bispecific antibody molecule comprise a heavy chain in the format of: VH(anti-PD-1)-CH1-Hinge-CH2-CH3-spacer-scFv(anti-CD47), associated with a light chain in the format of VL(anti-PD-1)-CL.

22. The bispecific antibody molecule of any of claims 18-21, comprising a heavy chain comprising the sequence of SEQ ID NO: 31 and a light chain comprising the sequence of SEQ ID NO: 32.

23. The bispecific antibody molecule of any of claims 1-17, wherein the PD-1-binding

domain comprises a scFv and the CD47-binding domain comprises a Fab.

24. The bispecific antibody molecule of claim 23, wherein the PD-1-binding scFv comprises the sequence of SEQ ID NO: 25, and the CD47-binding Fab comprises a heavy chain variable region comprising the sequence of SEQ ID NO: 11 and a light chain variable region comprising the sequence of SEQ ID NO: 13 or SEQ ID NO: 26.

25. The bispecific antibody molecule of claim 23 or 24, wherein the PD-1-binding scFv is operably linked to the N terminus of the heavy chain variable region of the CD47-binding Fab.

26. The bispecific antibody molecule of claim 25, wherein the bispecific antibody comprises a heavy chain in the format of: scFv(anti-PD1)-spacer-VH(anti-CD47)-CH1-Hinge-CH2-CH3, which is associated with the light chain VL(anti-CD47)-CL.

27. The bispecific antibody molecule of claim 26, comprising a heavy chain comprising the sequence of SEQ ID NO: 29 and a light chain comprising the sequence of SEQ ID NO: 30.

28. The bispecific antibody molecule of any of the preceding claims, wherein the CD47-binding domain and/or the PD-1 antigen-binding domain is humanized.

29. The bispecific antibody molecule of any of the preceding claims, which is linked to one or more conjugate moieties.

30. The bispecific antibody molecule of claim 29, wherein the conjugate moiety comprises a clearance-modifying agent, a chemotherapeutic agent, a toxin, a radioactive isotope, a lanthanide, a luminescent label, a fluorescent label, an enzyme-substrate label, a DNA-alkylators, a topoisomerase inhibitor, a tubulin-binders, or other anticancer drugs.

31. A pharmaceutical composition comprising the bispecific antibody molecule of any of the preceding claims, and a pharmaceutically acceptable carrier.

32. An isolated polynucleotide encoding the bispecific antibody molecule of any of claims 1-28.

33. A vector comprising the isolated polynucleotide of claim 32.

34. A host cell comprising the vector of claim 33.

35. A method of expressing the bispecific antibody molecule of any of claims 1-28, comprising culturing the host cell of claim 34 under the condition at which the vector of claim

33 is expressed.

36. A method of treating a disease or condition in a subject that would benefit from upregulation of an immune response, comprising administering to the subject a therapeutically effective amount of the bispecific antibody molecule of any of claims 1-28 or the pharmaceutical composition of claim 31.

37. The method of claim 36, wherein the disease or condition that would benefit from upregulation of an immune response is selected from the group consisting of cancer, a viral infection, a bacterial infection, a protozoan infection, a helminth infection, asthma associated with impaired airway tolerance, a neurological disease, multiple sclerosis, and an immunosuppressive disease.

38. The method of claim 36 or 37, wherein the disease or condition is PD-1-related and/or CD47-related.

39. The method of any of claims 36-38, wherein the disease or condition is cancer, autoimmune disease, inflammatory disease, or infectious disease.

40. The method of claim 39, wherein the cancer is lung cancer, bronchial cancer, bone cancer, liver and bile duct cancer, pancreatic cancer, breast cancer, liver cancer, ovarian cancer, testicle cancer, kidney cancer, bladder cancer, head and neck cancer, spine cancer, brain cancer, cervix cancer, uterine cancer, endometrial cancer, colon cancer, colorectal cancer, rectal cancer, anal cancer, esophageal cancer, gastrointestinal cancer, skin cancer, prostate cancer, pituitary cancer, stomach cancer, vagina cancer, thyroid cancer, glioblastoma, astrocytoma, melanoma, myelodysplastic syndrome, sarcoma, teratoma, adenocarcinoma, leukemia, myeloma and lymphoma.

41. The method of any of claims 36-40, wherein the disease or condition is hematological cancer, optionally selected from non-Hodgkin's lymphoma (NHL), acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), multiple myeloma (MM), diffuse large B cell lymphoma (DLBCL), Richter's syndrome, Burkitt's lymphoma or follicular lymphoma.

42. The method of any of claims 36-41, wherein the subject is human.

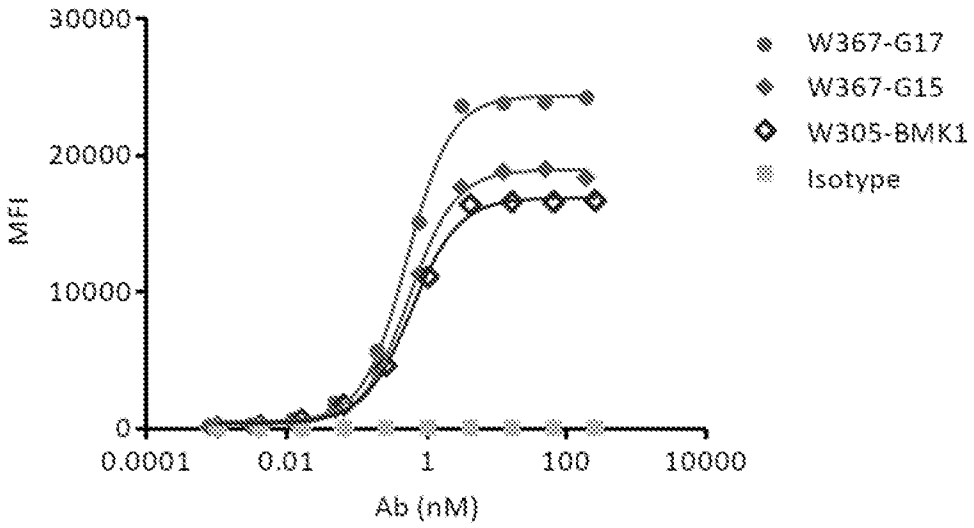
43. The method of any of claims 36-42, wherein the administration is via oral, nasal,

intravenous, subcutaneous, sublingual, or intramuscular administration.

44. A method of modulating CD47 activity in a CD47-expressing cell, comprising exposing the CD47-expressing cell to the bispecific antibody molecule of any of claims 1-30.

45. Use of the bispecific antibody molecule of any of claims 1-30 in the manufacture of a medicament for treating a disease or condition that would benefit from upregulation of an immune response.

46. Use of the bispecific antibody molecule of any of claims 1-30 in the manufacture of a medicament for treating a disease or condition that is PD-1 and/or CD47-related.

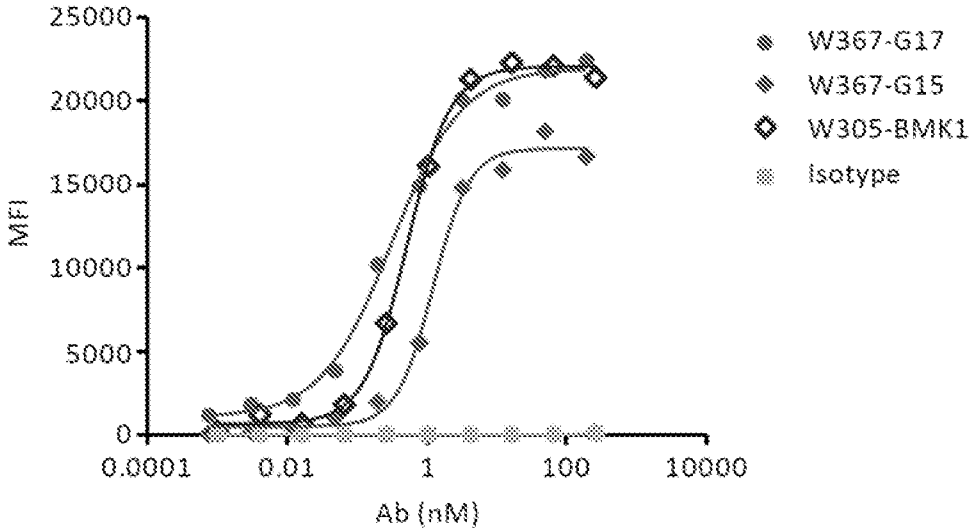


Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	0.52	24331
W367-G15	0.56	18947
W305-BMK1	0.60	16891

Figure 1

Antibody	K_{on} (1/Ms)	K_{off} (1/s)	KD (nM)
W367-G17	1.18E+06	6.92E-03	5.87
W367-G15	9.93E+05	2.21E-02	22.2
W3052-2E5	1.29E+06	4.52E-03	3.50
W305-BMK1	5.82E+05	1.35E-03	2.32

Figure 2

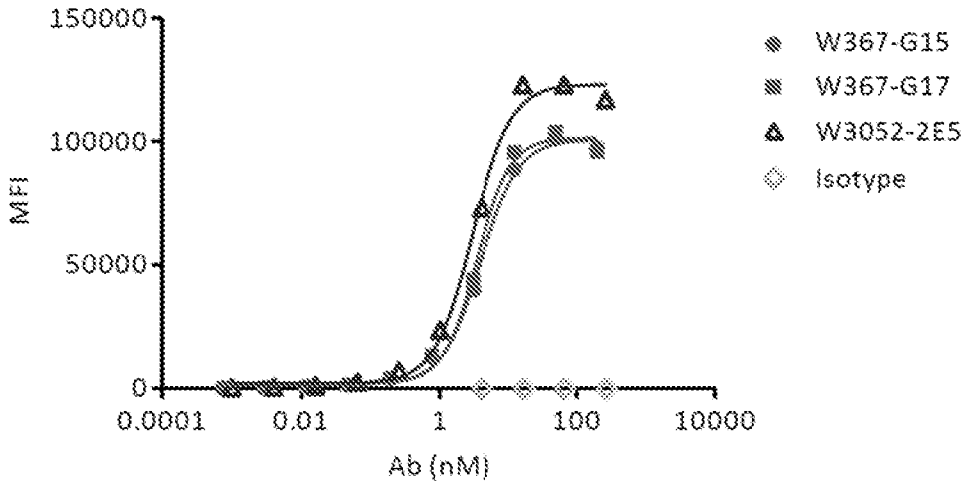


Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	0.31	21946
W367-G15	1.21	17159
W305-BMK1	0.51	22056

Figure 3

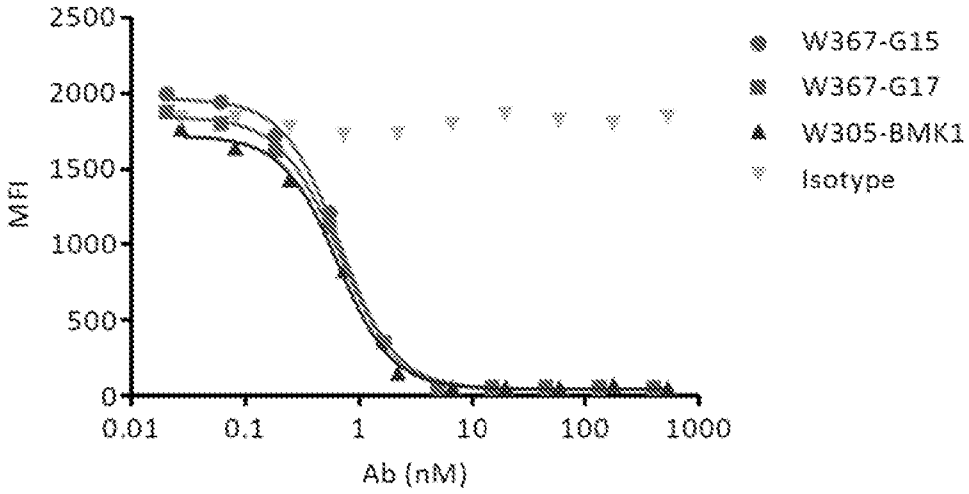
Antibody	K_{on} (1/Ms)	K_{off} (1/s)	K_D (nM)
W367-G17	1.30E+06	1.24E-02	9.57
W367-G15	1.11E+06	6.00E-02	54.2
W3052-2E5	1.26E+06	6.89E-03	5.45
W305-BMK1	3.71E+05	5.76E-04	1.55

Figure 4



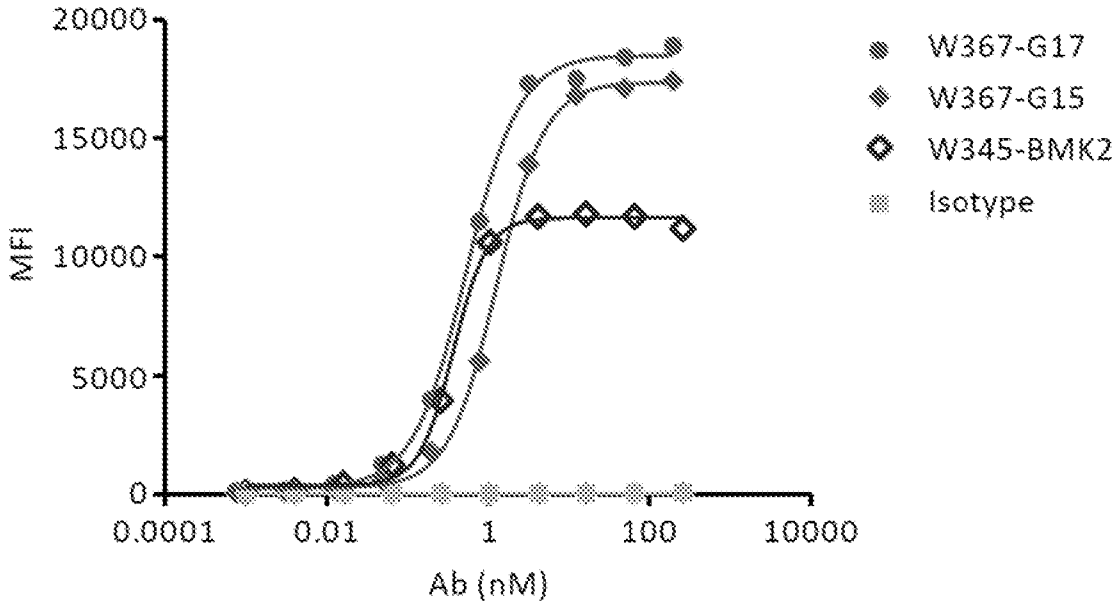
Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	3.51	101408
W367-G15	3.97	100737
W3052-2E5	3.07	123223

Figure 5



Antibody	EC_{50} (nM)
W367-G17	0.67
W367-G15	0.68
W305-BMK1	0.64

Figure 6

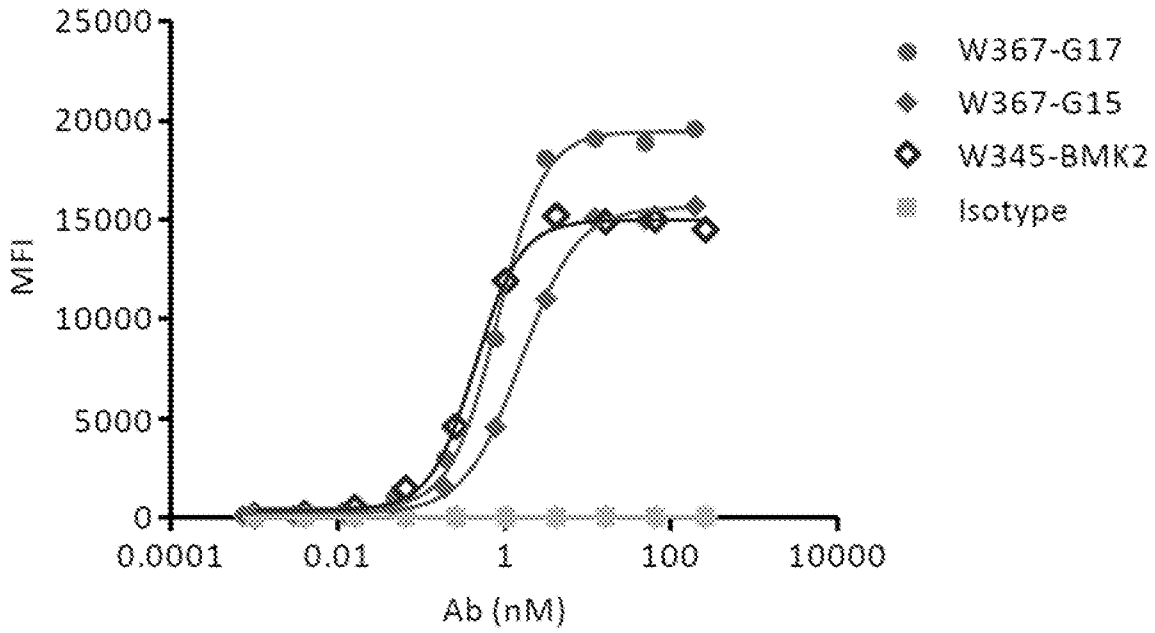


Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	0.54	18484
W367-G15	1.28	17353
W345-BMK2	0.37	11643

Figure 7

Antibody	K_D (nM)	Bmax (nM)	r^2
W367-G17	1.60	1.10	1.00
W367-G15	3.70	1.30	0.95
W3452-2.683.2-z27	0.59	1.20	0.99
W345-BMK2	0.36	0.86	1.00

Figure 8

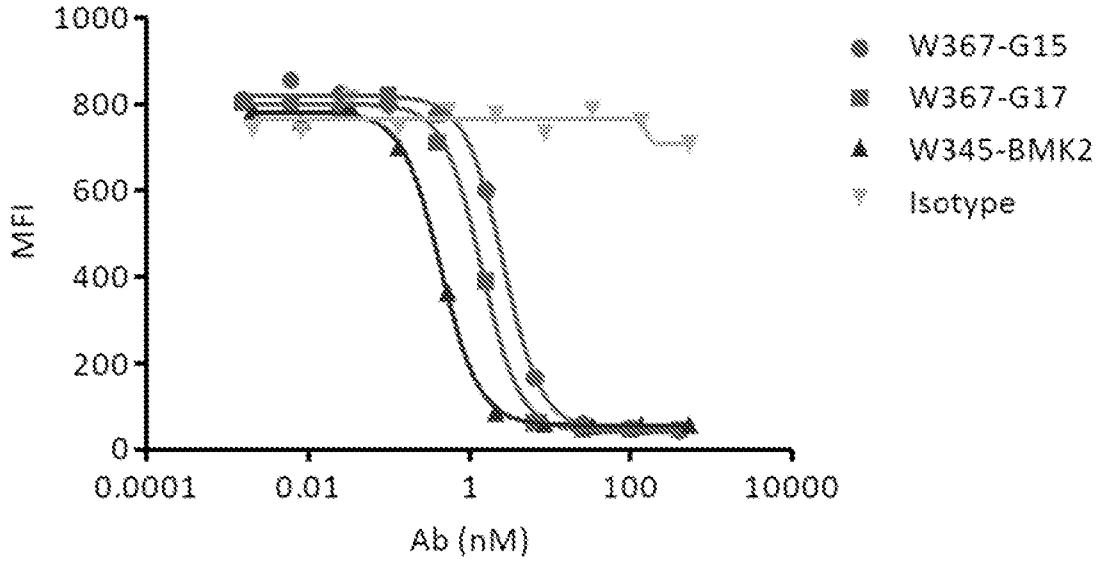


Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	0.82	19456
W367-G15	1.58	15634
W345-BMK2	0.45	14990

Figure 9

Antibody	K_D (nM)	Bmax (nM)	r^2
W367-G17	0.95	0.56	1.00
W367-G15	2.50	0.59	0.99
W3452-2.683.2-z27	0.44	0.59	0.98
W345-BMK2	0.23	0.52	1.00

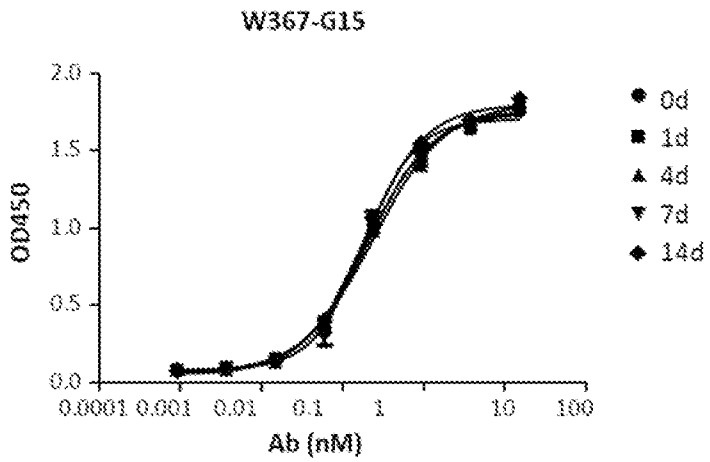
Figure 10



Antibody	IC_{50} (nM)
W367-G17	1.38
W367-G15	2.55
W345-BMK2	0.44

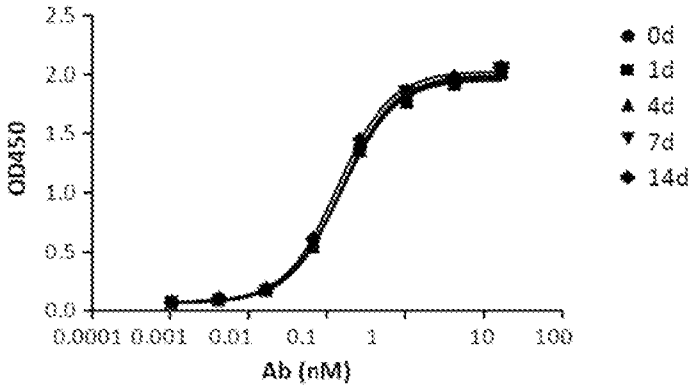
Figure 11

A



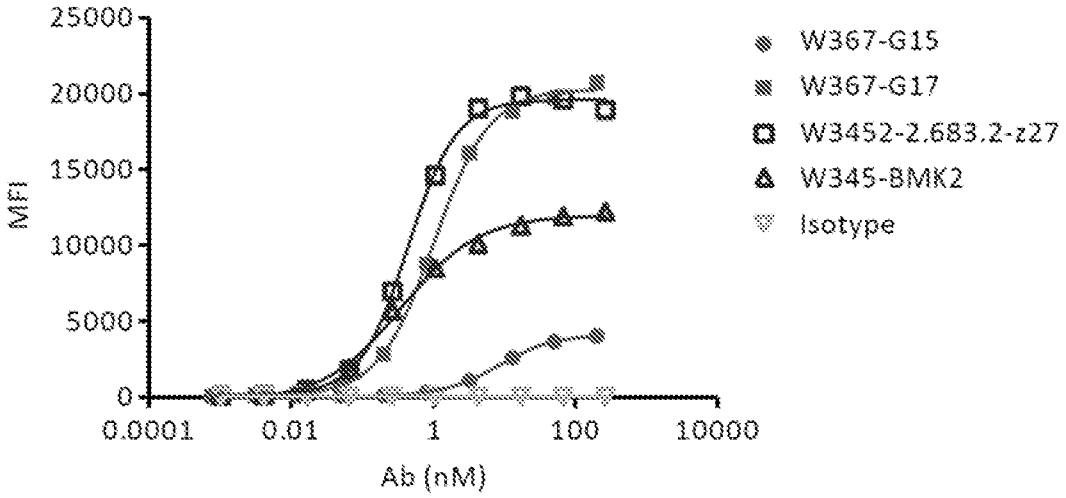
W367-G15	EC_{50} (nM)
Day 0	0.19
Day 1	0.20
Day 4	0.24
Day 7	0.21
Day 14	0.19

B



W367-G17	EC_{50} (nM)
Day 0	0.16
Day 1	0.15
Day 4	0.16
Day 7	0.16
Day 14	0.14

Figure 12



Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	1.00	20268
W367-G15	8.09	4147
W3452-2.683.2-z27	0.43	19583
W345-BMK2	0.34	11972

Figure 13

Lane	Antibody	HA
1	W367-G15	No
2	W367-G17	No
3	W3452-2.683.2-27	No
4	W345-BMK2	Yes
5	Isotype	No
6	W305-BMK1	No
7	No Ab	No

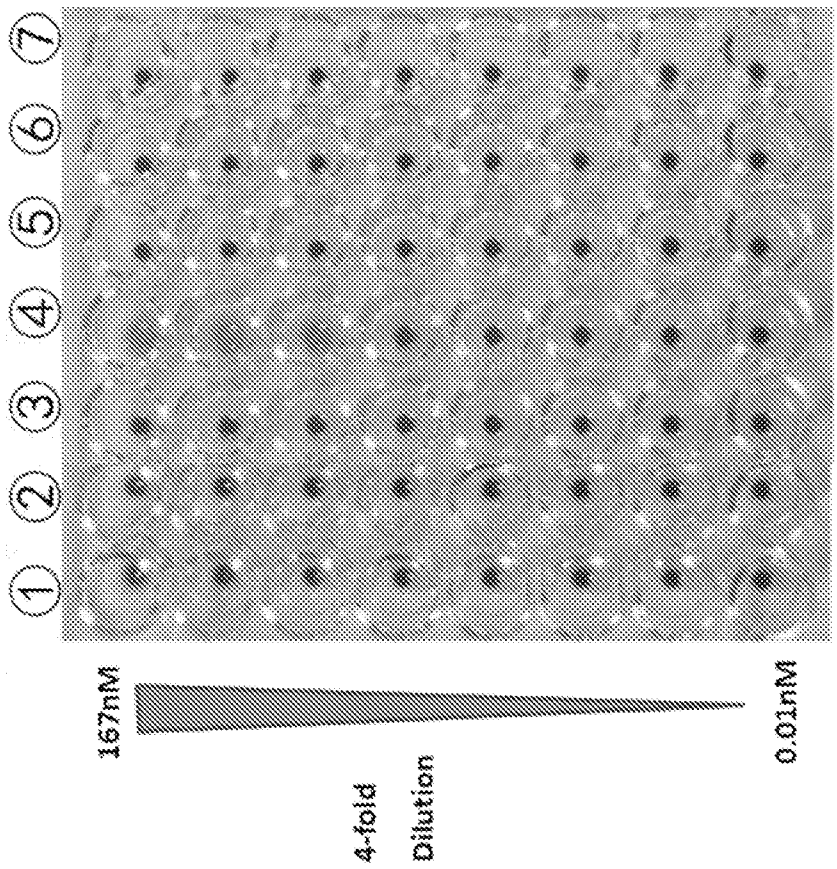
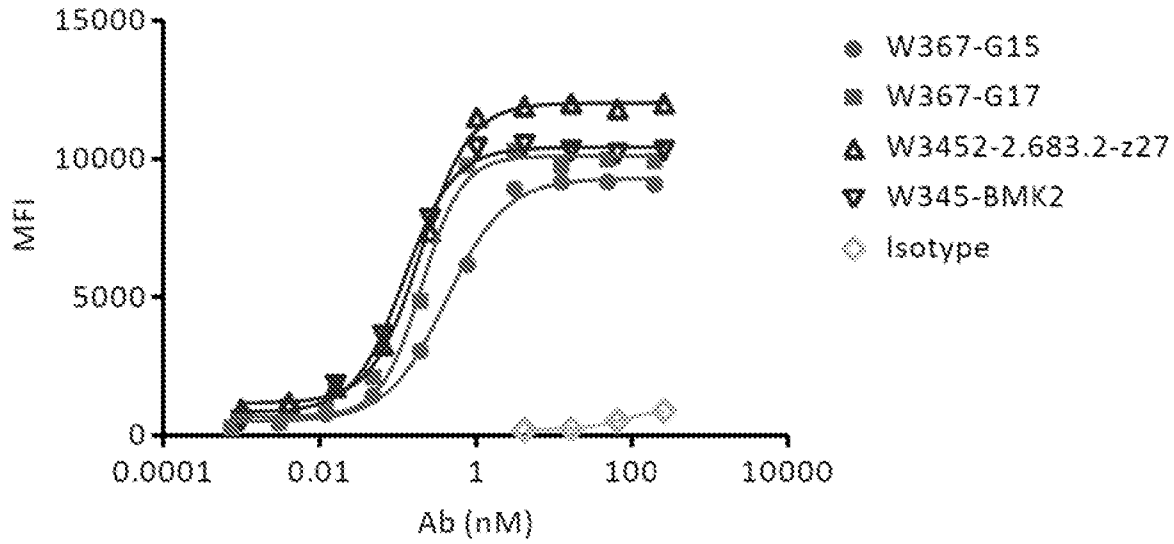


Figure 14



Antibody	EC_{50} (nM)	Top (MFI)
W367-G17	0.20	10109
W367-G15	0.42	9297
W3452-2.683.2-z27	0.20	12038
W345-BMK2	0.12	10429

Figure 15

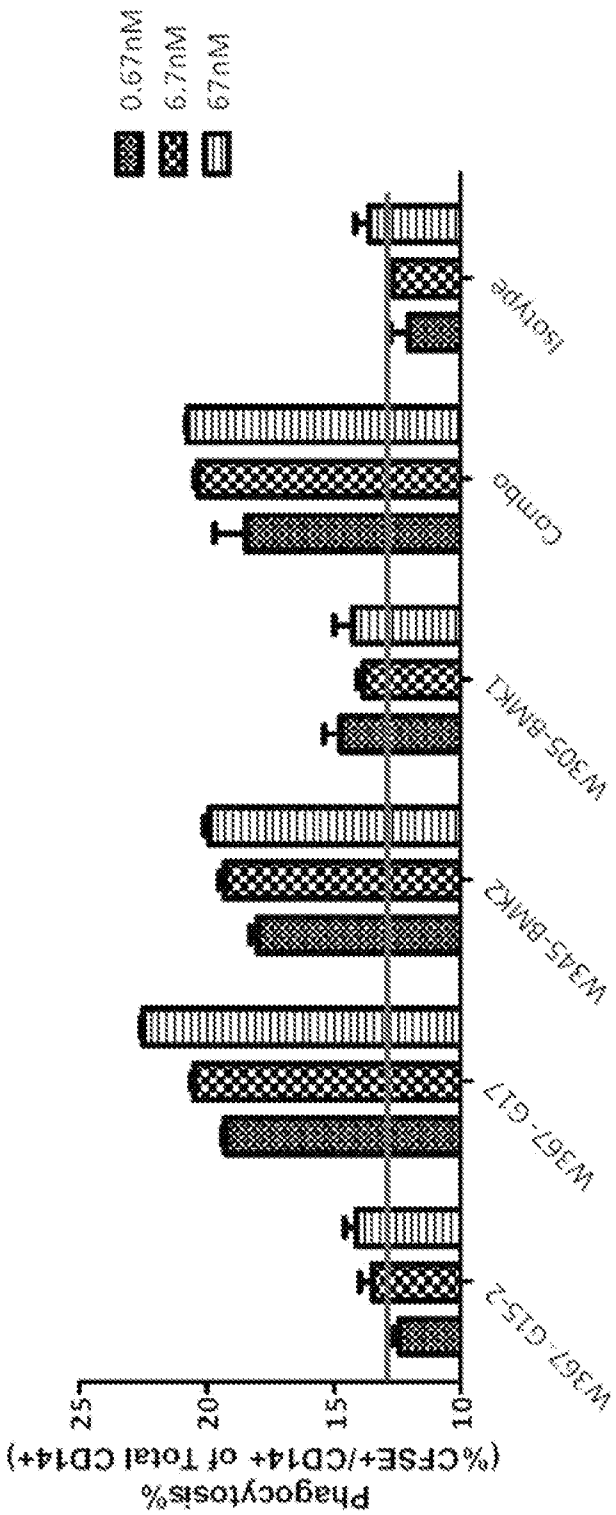
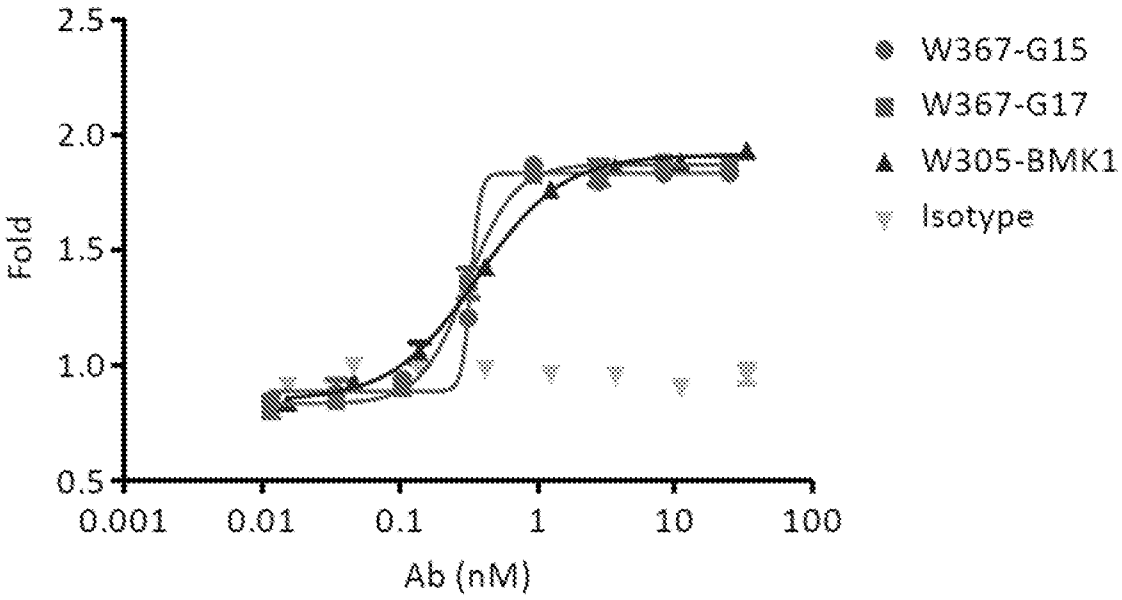

0.67nM
6.7nM
67nM
Isotype
Combo
W305-BAMA1
W345-BAMA2
W367-GL7
W367-GL5-2
10
15
20
25
Phagocytosis%
(%CFSE+/CD14+ of Total CD14+)

Figure 16



Antibody	EC_{50} (nM)
W367-G17	0.31
W367-G15	0.33
W305-BMK1	0.36

Figure 17

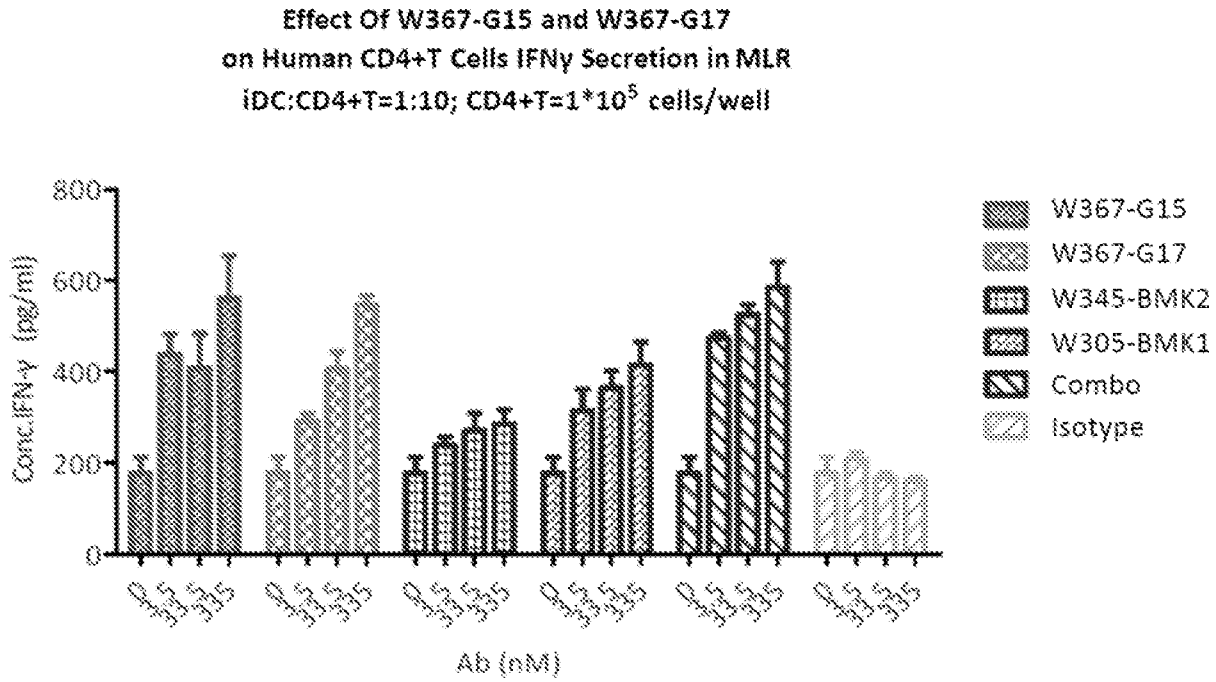

Effect Of W367-G15 and W367-G17
on Human CD4+T Cells IFNγ Secretion in MLR
iDC:CD4+T=1:10; CD4+T=1*10^5 cells/well
Conc.IFN-γ (pg/ml)
800
600
400
200
0
Ab (nM)
W367-G15
W367-G17
W345-BMK2
W305-BMK1
Combo
Isotype

Figure 18

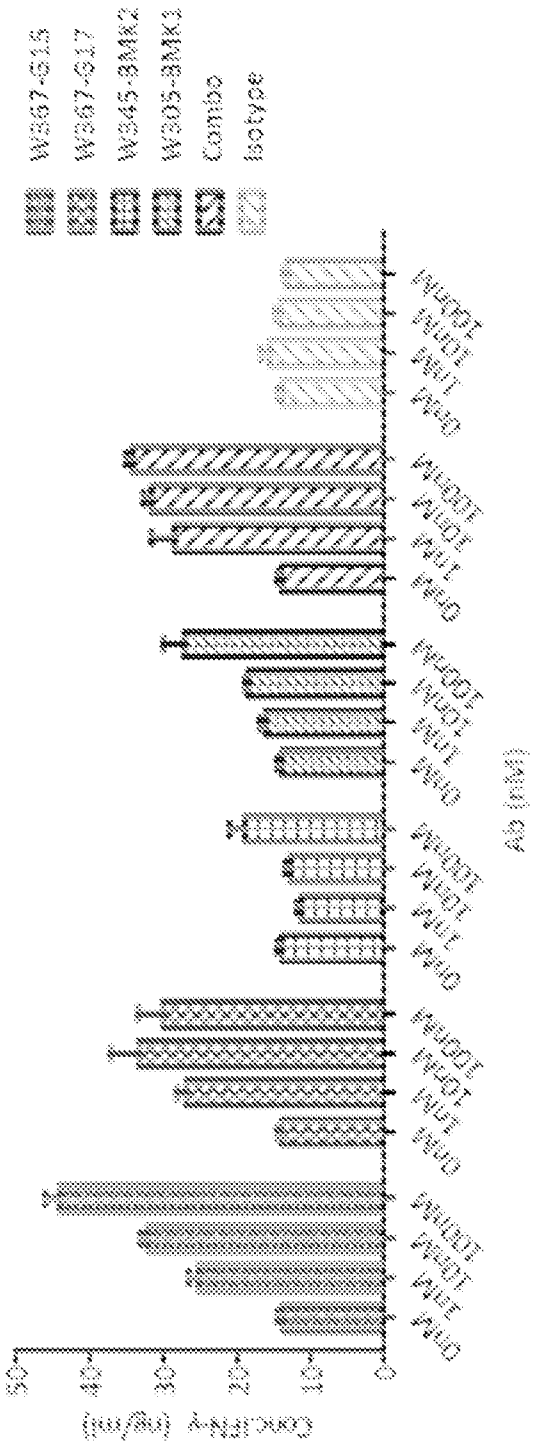

W367-G15
W367-G17
W345-BMK2
W305-BMK3
Combo
Isotype
Conc.IFN-γ (ng/ml)
0
10
20
30
40
50
Ab (nM)

Figure 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/078708

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i; C12N 15/13(2006.01)i; A61K 39/395(2006.01)i; A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K; C12N; A61K; A61P

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

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

CNABS, CPRS, DWPI, SIPOABS, CNKI, NCBI, ISI Web of Science, GenBank:CD, 47, cluster, differentiation, PD, 1, programmed, death, bispecific, antibody, human,SEQ ID NOs: 1-28 of the present application

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIU, Boning. "Construction and Anti-tumor Effects of a Novel Bispecific Fusion Protein Targeting PD-L1 and CD47" *Dissertation Submitted for the Degree of Doctor of Philosophy of South China University of Technology*, 31 December 2016 (2016-12-31), Retrieved from: CDFD (http://www.cnki.net). the whole document, especially section 1.2 and the abstract	1-46

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 2019	**28 May 2019**
Name and mailing address of the ISA/CN **National Intellectual Property Administration, PRC** **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing** **100088** **China** Facsimile No. **(86-10)62019451**	Authorized officer **WU,Yongqing** Telephone No. **+86-10-62089161**