(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
01 October 2020 (01.10.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/192709 A1

(51) International Patent Classification:
A61K 39/395 (2006.01) *C07K 16/46* (2006.01)
C07K 16/28 (2006.01) *A61K 39/00* (2006.01)
C07K 14/46 (2006.01) *C07K 16/00* (2006.01)

(21) International Application Number:
PCT/CN2020/081258

(22) International Filing Date:
26 March 2020 (26.03.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PCT/CN2019/079837
27 March 2019 (27.03.2019) CN

(71) Applicant *(for CN only)*: **WUXI BIOLOGICS (SHANGHAI) CO., LTD.** [CN/CN]; No. 299 Fute Zhong Road, Pudong New District, Shanghai 200131 (CN).

(71) Applicant *(for all designated States except CN)*: **WUXI BIOLOGICS IRELAND LIMITED** [IE/IE]; One Spencer Dock, North Wall Quay, Dublin 1 (IE).

(72) Inventors: **WANG, Zhuozhi**; No. 299 Fute Zhong Road, Pudong New District, Shanghai 200131 (CN). **FAN, Xinzhao**; No. 299 Fute Zhong Road, Pudong New District, Shanghai 200131 (CN). **LI, Jing**; No. 299 Fute Zhong Road, Pudong New District, Shanghai 200131 (CN). **XU, Jianqing**; No. 299 Fute Zhong Road, Pudong New District, Shanghai 200131 (CN). **NIE, Siwei**; No. 299 Fute Zhong Road, Pudong New District, Shanghai 200131 (CN).

(74) Agent: **CCPIT PATENT AND TRADEMARK LAW OFFICE**; 8th Floor, Vantone New World Plaza, 2 Fuchengmenwai Street, Xicheng District, Beijing 100037 (CN).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: NOVEL BISPECIFIC POLYPEPTIDE COMPLEXES

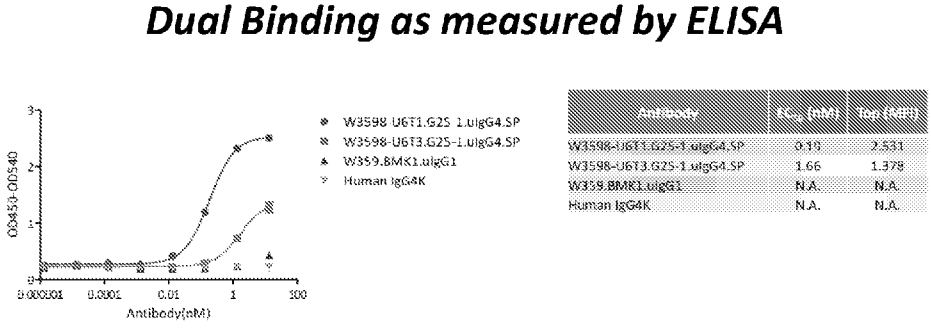


Figure 7

(57) Abstract: Bispecific polypeptide complexes, pharmaceutical compositions comprising the same, and the uses thereof are provided.

WO 2020/192709 A1

Novel bispecific polypeptide complexes

INFORMATION OF PRIORITY

[0000] The present application claims the benefit of PCT/CN2019/079837 filed on March 27, 2019, which is entirely incorporated herein by reference.

FIELD OF THE INVENTION

[0001] The present disclosure generally relates to novel bispecific polypeptide complexes. More specifically, the present disclosure relates to novel bispecific PD-1/TIM-3 antibody molecules.

BACKGROUND

[0002] 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.

[0003] 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, which is also named as "CD279"), is a member of CD28 family expressed on activated T cells and other immune cells. Engagement of PD-1 inhibits function in these immune cells. PD-1 has two known ligands, PD-L1 (also named as "B7-H1" or "CD274") and PD-L2 (also named as "B7-DC" or "CD273"), which are cell surface expressed members of the B7 family (Freeman et al (2000) J Exp Med 192: 1027-34; Latchman et al (2001) Nat Immunol 2:261-8; Carter etal (2002) Eur J Immunol 32:634-43). PD-L1 expression is inducible on a variety of cell types in lymphoid and peripheral tissues, whereas the expression of PD-L2 is more restricted to myeloid cells including dendritic cells. The major role of PD-1 pathway is to tune down inflammatory immune response in tissues and organs.

[0004] T cell immunoglobulin mucin-3 (TIM-3), member of the TIM family, is a type I transmembrane protein that possesses an N-terminal Ig domain of the V type, followed by a mucin domain containing potential sites of glycosylation. TIM-3 is preferentially expressed on activated Th1 cells and cytotoxic CD8 T cells that secrete IFNγ, dendritic cells (DCs),

monocytes and NK cells. It is an activation-induced inhibitory molecule and induces the apoptosis of Th1 cells, resulting in T cell exhaustion in chronic viral infection and cancers.

[0005] Four molecules have been reported as ligands of TIM-3, including carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), phosphatidylserine (PtdSer), High mobility group protein 1 (HMGB1), and Galectine-9 (Gal-9). Among these ligands, CEACAM1, HMGB1 as well as Gal-9 have been reported to negatively regulate immune response. A recent study has showed that CEACAM1, known to be expressed on activated T cells and involved in T cell inhibition, can form cis and trans interaction with TIM-3 to suppress anti-tumor T cell response. HMGB1 binds to DNA released by cells undergoing necrosis, and mediates the activation of innate cells through receptor for advanced glycation end (RAGE) products and / or Toll-like receptors. By binding to HMGB1, TIM-3 prevents the binding of HMGB1 to DNA, and therefore interferes the function of HMGB1 on activating the innate immune response in tumor tissue. Although the role of Gal-9 on human T cells is controversial, Gal-9 has been shown to bind to mouse TIM-3 and negatively regulate Th-1 immune response. Recently, leukocyte immunoglobulin-like receptor subfamily B member 2 (LILRB2) has been reported to interact with TIM-3 to regulate the function of DCs, macrophages and T cells. The blockage of TIM-3 / LILRB2 interaction can enhance the activation of macrophages; increase T cell response and proliferation.

[0006] It has been suggested that TIM-3 may be a key immune checkpoint in tumor-induced immune suppression, as TIM-3 is expressed on the most suppressed or dysfunctional tumor-infiltrating lymphocytes (TILs) in preclinical models of both solid and hematologic malignancy, as well as patients with advanced melanoma, non-small cell lung cancer (NSCLC) or follicular B-cell non-Hodgkin lymphoma. In multiple preclinical tumor models, the treatment of anti-TIM-3 alone or in combination of other immune checkpoint therapies, such as anti-PD-1, can dramatically suppress the tumor growth.

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

BRIEF SUMMARY OF THE INVENTION

[0008] 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.

[0009] The present disclosure provides novel bispecific polypeptide complexes, particularly, novel bispecific PD-1/TIM-3 antibody molecules, and uses thereof.

[00010] Specifically, the present disclosure includes, but not limited to, the following embodiments which are numbered by [Number] in serial. Different embodiments may be combined in any suitable manner.

[00011] [1] A bispecific polypeptide complex comprising a first antigen-binding moiety associated with a second antigen-binding moiety, wherein:

the first antigen-binding moiety comprising:

a first polypeptide comprising, from N-terminus to C-terminus, a first heavy chain variable domain (VH1) of a first antibody operably linked to a first T cell receptor (TCR) constant region (C1), and

a second polypeptide comprising, from N-terminus to C-terminus, a first light chain variable domain (VL1) of the first antibody operably linked to a second TCR constant region (C2),

wherein:

C1 and C2 are capable of forming a dimer comprising at least one non-native interchain bond between C1 and C2, and the non-native interchain bond is capable of stabilizing the dimer;

and

the second antigen-binding moiety comprising:

a second heavy chain variable domain (VH2) of a second antibody operably linked to an antibody heavy chain CH1 domain, and

a second light chain variable domain (VL2) of the second antibody operably linked to an antibody light chain constant (CL) domain,

wherein:

one of the first and the second antigen-binding moiety is an PD-1 binding moiety, and the other one is a TIM-3 binding moiety,

the PD-1 binding moiety is derived from an anti-PD-1 antibody comprising:

a) a heavy chain CDR1 comprising an amino acid sequence of SEQ ID NO: 17,

b) a heavy chain CDR2 comprising an amino acid sequence of SEQ ID NO: 19,

c) a heavy chain CDR3 comprising an amino acid sequence of SEQ ID NO: 21,

d) a light chain CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 18,

e) a light chain CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20, and

f) a light chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 22; and

the TIM-3 binding moiety is derived from an anti-TIM-3 antibody comprising:

a) a heavy chain CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and 7,

b) a heavy chain CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and 9,

c) a heavy chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 5 and 11,

d) a light chain CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 2 and 8,

e) a light chain CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4 and 10, and

f) a light chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 6 and 12.

[00012] [2] The bispecific polypeptide complex of embodiment 1, wherein the first antigen-binding moiety is the PD-1 binding moiety, and the second antigen-binding moiety is the TIM-3 binding moiety.

[00013] [3] The bispecific polypeptide complex of embodiment 1 or 2, wherein the PD-1 binding moiety comprises a heavy chain variable region comprising or consisting of SEQ ID NO: 34, and a light chain variable region comprising or consisting of SEQ ID NO: 24.

[00014] [4] The bispecific polypeptide complex of embodiment 1 or 2, wherein the TIM-3-binding moiety comprises a heavy chain variable region comprising or consisting of SEQ ID NO: 13 or 15, and a light chain variable region comprising or consisting of SEQ ID NO: 14 or 16.

[00015] [5] The bispecific polypeptide complex of any of the preceding embodiments, wherein C1 has the amino acid sequence as set forth in SEQ ID NO: 25.

[00016] [6] The bispecific polypeptide complex of any of the preceding embodiments, wherein C2has the amino acid sequence as set forth in SEQ ID NO: 26.

[00017] [7] The bispecific polypeptide complex of any of the preceding embodiments, wherein the first polypeptide of the PD-1 binding moiety is linked to the VH2 of the TIM-3 binding moiety via a spacer.

[00018] [8] The bispecific polypeptide complex of embodiment 7, wherein the spacer comprises the amino acid of SEQ ID NO: 27.

[00019] [9] The bispecific polypeptide complex of any of embodiments 1-8, wherein the bispecific polypeptide complex comprises a combination of three polypeptide sequences: SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

[00020] [10] The bispecific polypeptide complex of any of embodiments 1-8, wherein the bispecific polypeptide comprises a combination of three polypeptide sequences: SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33.

[00021] [11] The bispecific polypeptide complex of any of the preceding embodiments, wherein the TIM-3-binding moiety further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to TIM-3, and/or the PD-1 binding moiety further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to PD-1.

[00022] [12] The bispecific polypeptide complex of embodiment 11, 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.

[00023] [13] The bispecific polypeptide complex of any of the preceding embodiments, wherein the TIM-3-binding moiety is operably linked to the N terminus or the C terminus of PD-1 binding moiety.

[00024] [14] The bispecific polypeptide complex of any of the preceding embodiments, wherein the PD-1 binding moiety is operably linked to the N terminus or the C terminus of the TIM-3-binding moiety.

[00025] [15] The bispecific polypeptide complex of any of the preceding embodiments, wherein the bispecific polypeptide complex further comprises an immunoglobulin constant region, optionally a constant region of IgG, or optionally a constant region of human IgG.

[00026] [16] The bispecific polypeptide complex of embodiment 15, wherein the PD-1 moiety or the TIM-3-binding moiety is operably linked to the C terminus of the constant region.

[00027] [17] The bispecific polypeptide complex of any of the preceding embodiments, wherein the TIM-3-binding moiety and/or the antigen-binding moiety is humanized.

[00028] [18] The bispecific polypeptide complex of any of the preceding embodiments, wherein the TIM-3-binding moiety is capable of specifically binding to human TIM-3 at a K_D value of no more than 5×10^{-9}M, 5×10^{-10}M, or 5×10^{-11}M as measured by surface plasmon

resonance (SPR), and/or the PD-1 binding moiety is capable of specifically binding to human PD-1 at a K_D value of no more than 10^{-8} or 5×10^{-8} M as measured by surface plasmon resonance.

[00029] [19] The bispecific polypeptide complex of any of the preceding embodiments, wherein the TIM-3-binding moiety is capable of specifically binding to human TIM-3 expressed on a cell surface at an EC_{50} of no more than 5nM, 1nM, or 0.2 nM as measured by flow cytometry, and/or the antigen-binding moiety is capable of specifically binding to human PD-1 expressed on a cell surface at a K_D value of no more than 10^{-8} M or 5×10^{-10} M as measured by flow cytometry.

[00030] [20] The bispecific polypeptide complex of any of the preceding embodiments, wherein the TIM-3-binding moiety is capable of specifically binding to Cynomolgus monkey TIM-3, rat TIM-3, and/or mouse TIM-3.

[00031] [21] The bispecific polypeptide complex of any of the preceding embodiments, wherein the PD-1 moiety is capable of specifically binding to Cynomolgus monkey PD-1, and/or mouse PD-1.

[00032] [22] The bispecific polypeptide complex of any of the preceding embodiments linked to one or more conjugate moieties.

[00033] [23] The bispecific polypeptide complex of embodiment 22, 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.

[00034] [24] A pharmaceutical composition comprising the bispecific polypeptide complex of any of embodiments 1-23, and a pharmaceutically acceptable carrier.

[00035] [25] An isolated polynucleotide encoding the bispecific polypeptide complex of any of embodiments 1-23.

[00036] [26] A vector comprising the isolated polynucleotide of embodiment 25.

[00037] [27] A host cell comprising the vector of embodiment 26.

[00038] [28] A method of expressing the bispecific polypeptide complex of any of embodiments 1-23, comprising culturing the host cell of embodiment 27 under the condition at which the vector of embodiment 26 is expressed.

[00039] [29] 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 polypeptide complex of any of embodiments 1-23 or the pharmaceutical composition of embodiment 24.

[00040] [30] The method of embodiment 29, wherein 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.

[00041] [31] The method of embodiment 29 or 30, wherein the disease or condition is PD-1-related and/or TIM-3-related.

[00042] [32] The method of any of embodiments 29-31, wherein the disease or condition is cancer, autoimmune disease, inflammatory disease, or infectious disease.

[00043] [33] The method of any of embodiments 29-32, wherein the cancer is lymphoma, basal cell carcinoma, biliary tract cancer, bladder cancer, bone cancer, brain and central nervous system cancer, breast cancer, peritoneal cancer, cervical cancer, uterine or endometrial cancer, choriocarcinoma, colon cancer, colorectal cancer, rectal cancer, connective tissue cancer, esophageal cancer, mesothelioma, nasopharyngeal cancer, eye cancer, head and neck cancer, anal cancer, gastrointestinal cancer, glioblastoma, intra-epithelial neoplasm, kidney or renal cancer, larynx cancer, leukemia, liver cancer, lung cancer, melanoma, myeloma, neuroblastoma, oral cavity cancer, germ cell cancer, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland carcinoma, sarcoma, skin cancer, squamous cell cancer, stomach cancer, testicular cancer, thyroid cancer, or vulval cancer.

[00044] [34] The method of any of embodiments 29-33, wherein the disease or condition is B cell lymphoma, optionally Hodgkin lymphoma or non-Hodgkin lymphoma (NHL), wherein the NHL comprises: diffuse large B-cell lymphoma (DLBCL), small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, mantle cell lymphoma, AIDS-related lymphoma, Waldenstrom's Macroglobulinemia, chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), hairy cell leukemia, chronic myeloblastic leukemia, and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema, and Meigs' syndrome.

[00045] [35] The method of any of embodiments 29-34, wherein the subject is human.

[00046] [36] The method of any of embodiments 29-35, wherein the administration is via oral, nasal, intravenous, subcutaneous, sublingual, or intramuscular administration.

[00047] [37] A method of modulating TIM-3 activity in a TIM-3-expressing cell, comprising exposing the TIM-3-expressing cell to the bispecific polypeptide complex of any of embodiments 1-23.

[00048] [38] Use of the bispecific polypeptide complex of any of embodiments 1-23 in the manufacture of a medicament for treating a disease or condition that would benefit from upregulation of an immune response.

[00049] [39] Use of the bispecific polypeptide complex of any of embodiments 1-23 in the manufacture of a medicament for treating a disease or condition that is PD-1 and/or TIM-3-related.

BRIEF DESCFRIPTION OF FIGURES

[00050] Figure 1 shows the analysis of purified antibodies using SEC-HPLC.

[00051] Figure 2 shows the result of DSF test.

[00052] Figure 3 shows the binding of the antibodies to human PD-1, as measured by FACS.

[00053] Figure 4 shows the binding of the antibodies to cyno PD-1, as measured by FACS.

[00054] Figure 5 shows the binding of the antibodies to human TIM-3, as measured by FACS.

[00055] Figure 6 shows the binding of the antibodies to cyno TIM-3, as measured by FACS.

[00056] Figure 7 shows the dual binding of the antibodies to TIM-3 and PD-1, as measured by ELISA.

[00057] Figure 8 shows the binding capacity of the antibodies to PD1 or TIM-3, as measured by SPR.

[00058] Figure 9 shows that the competitive binding of the antibodies to human PD1, as measured by FACS.

[00059] Figure 10 shows that the competitive binding of the antibodies to PS-TIM-3, as measured by FACS.

[00060] Figure 11 illustrates the effect of the antibodies on IFNγ production in MLR.

[00061] Figure 12 shows the results of reporter gene assay.

[00062] Figure 13 shows schematic description of a symmetric format G25. In the format G25, two TCR-containing chimeric Fab-like domains were grafted at the C-terminus and N-terminus of a normal antibody, respectively. The rectangles indicate TCR constant domains, and the ovals

indicate variable and constant domains of an antibody. This format can accommodate different variable regions from different antibody pairs and usually have a molecular weight around 240-250 kD.

DETAILED DESCRIPTION OF THE INVENTION

[00063] 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.

[00064] **Definitions**

[00065] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues, or an assembly of multiple polymers of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, gamma-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an alpha-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. An alpha-carbon refers to the first carbon atom that attaches to a functional group, such as a carbonyl. A beta-carbon refers to the second carbon atom linked to the alpha-carbon, and the system continues naming the carbons in alphabetical order with Greek letters. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. Polypeptide sequences are usually described as the left-hand end of a polypeptide sequence is the amino-terminus (N-terminus); the

right-hand end of a polypeptide sequence is the carboxyl-terminus (C-terminus). "Polypeptide complex" as used herein refers to a complex comprising one or more polypeptides that are associated to perform certain functions. In certain embodiments, the polypeptides are immune-related.

[00066] The term "antibody" as used herein encompasses any immunoglobulin, monoclonal antibody, polyclonal antibody, multispecific antibody, or bispecific (bivalent) antibody that binds to a specific antigen. A native intact antibody comprises two heavy chains and two light chains. Each heavy chain consists of a variable region ("HCVR") and a first, second, and third constant region (CH1, CH2 and CH3), while each light chain consists of a variable region ("LCVR") and a constant region (CL). Mammalian heavy chains are classified as α, δ, ε, γ, and μ, and mammalian light chains are classified as λ or κ. 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 disulphide 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 (L) chain CDRs including LCDR1, LCDR2, and LCDR3, heavy (H) chain CDRs including HCDR1, HCDR2, HCDR3). CDR boundaries for antibodies may be defined or identified by the conventions of Kabat, Chothia, AbM 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); 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)). AbM method is available from the website of Abysis. 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. Each HCVR and LCVR comprises four FRs, and the CDRs and FRs are arranged from amino terminus to carboxy terminus in the order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. 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 α, δ, ε, γ, and μ heavy chains, respectively. Several of the major antibody classes are divided into subclasses such as IgG1 (γ1 heavy chain), IgG2 (γ2 heavy chain), IgG3 (γ3 heavy chain), IgG4 (γ4 heavy chain), IgA1 (α1 heavy chain), or IgA2 (α2 heavy chain).

[00067] 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.

[00068] The term "bivalent" as used herein refers to an antibody or an antigen-binding moiety having two antigen-binding sites; the term "monovalent" refers to an antibody or an antigen-binding moiety having only one single antigen-binding site; and the term "multivalent" refers to an antibody or an antigen-binding moiety having multiple antigen-binding sites. In some embodiments, the antibody or antigen-binding moiety thereof is bivalent.

[00069] The term "antigen-binding moiety" (e.g. TIM-3-binding moiety or PD-1-binding moiety) 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 moiety 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 moiety is capable of binding to the same antigen to which the parent antibody binds. In certain embodiments, an antigen-binding moiety 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 moiety 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.

[00070] "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.

[00071] "Fab'" refers to a Fab fragment that includes a portion of the hinge region.

[00072] "$F(ab')_2$"refers to a dimer of Fab'.

[00073] 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

[00074] "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)).

[00075] "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)).

[00076] "ScFab" refers to a fusion polypeptide with a Fd linked to a light chain via a polypeptide linker, resulting in the formation of a single chain Fab fragment (scFab).

[00077] 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.

[00078] "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*).

[00079] "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.

[00080] "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)).

[00081] 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.

[00082] In certain embodiments, an "scFv dimer" is a bivalent diabody or bivalent ScFv (BsFv) comprising V_H-V_L (linked by a peptide linker) dimerized with another V_H-V_L moiety such that V_H's of one moiety coordinate with the V_L's of the other moiety and form two binding sites which can target the same antigens (or epitopes) or different antigens (or epitopes).

[00083] In other embodiments, an "scFv dimer" is a bispecific diabody comprising V_{H1}-V_{L2} (linked by a peptide linker) associated with V_{L1}-V_{H2} (also linked by a peptide linker) such that V_{H1} and V_{L1} coordinate and V_{H2} and V_{L2} coordinate and each coordinated pair has a different antigen specificity.

[00084] 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.

[00085] The term "chimeric" as used herein, means an antibody or antigen-binding moiety, 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.

[00086] The term "humanized" as used herein means that the antibody or antigen-binding moiety comprises CDRs derived from non-human animals, FR regions derived from human, and when applicable, the constant regions derived from human.

[00087] The term "fully human" as used herein, with reference to antibody or antigen-binding moiety, means that the antibody or the antigen-binding moiety has or consists of amino acid sequence(s) corresponding to that of an antibody produced by a human or a human immune cell, or derived from a non-human source such as a transgenic non-human animal that utilizes human antibody repertoires or other human antibody-encoding sequences. In certain embodiments, a fully human antibody does not comprise amino acid residues (in particular antigen-binding residues) derived from a non-human antibody.

[00088] 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 polypeptides. A spacer may or may not have a secondary structure. Spacer sequences are known in the art, see, for example, Holliger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993); Poljak et al. Structure 2:1121-1123 (1994). Any suitable spacers known in the art can be used. For example, a useful spacer in the present disclosure may be rich in glycine and proline residues. Examples include spacers having a single or repeated sequences composed of threonine/serine and glycine, such as TGGGG, GGGGS or SGGGG or its tandem repeats (e.g. 2, 3, 4, or more repeats). In certain embodiment, the spacer has an amino acid sequence as set forth in SEQ ID NO: 27.

[00089] The term "operably link" or "operably linked" refers to a juxtaposition, with or without a spacer or linker, 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. 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.

[00090] 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.

[00091] "TIM-3" as used herein, is 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 TIM-3 includes human TIM-3 protein (NCBI accession number GI: 18182535). Exemplary sequence of TIM-3 includes Mus musculus (mouse) TIM-3 protein (NCBI accession number GI: 18182531), Rattus norvegicus (rat) TIM-3 protein (NCBI accession number GI 39725405), and Macaca fascicularis (monkey) TIM-3 protein (NCBI accession number GI: 355750365).

[00092] The term "TIM-3" as used herein is intended to encompass any form of TIM-3, for example, 1) native unprocessed TIM-3 molecule, "full-length" TIM-3 chain or naturally occurring variants of TIM-3, including, for example, splice variants or allelic variants; 2) any form of TIM-3 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 TIM-3 subunit generated through recombinant method.

[00093] The term "anti-TIM-3 antibody" refers to an antibody that is capable of specific binding TIM-3 (e.g. human or monkey or mouse or rat TIM-3).

[00094] "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/TIM-3-4 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.

[00095] "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.

[00096] "Anti-PD-1 antibody" as used herein refers to an antibody 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.

[00097] 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 moieties provided herein specifically bind to human PD-1 and/or human TIM-3 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.

[00098] The ability to "block binding" or "compete for the same epitope" as used herein refers to the ability of an antibody or antigen-binding moiety to inhibit the binding interaction between two molecules (e.g. human TIM-3 and an anti-TIM-3 antibody, human PD-1 and an anti-PD-1 antibody) to any detectable degree. In certain embodiments, an antibody or antigen-binding moiety 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%.

[00099] 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 moiety 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 moiety may be considered to bind the same/closely related epitope as the reference antibody.

[000100] 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, "uIgG1" or "uIgG4" means an antibody with human constant region of IgG1 isotype or a IgG4 isotype, "hAb" or "uAb" means human antibody, "z" means humanized antibody, "K" means Kappa light chain, "L" means Lambda light chain.

[000101] 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.

[000102] The term "homologue" 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.

[000103] "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.

[000104] "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.

[000105] "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.

[000106] 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.

[000107] 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, 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.

[000108] The phrase "host cell" as used herein refers to a cell into which an exogenous polynucleotide and/or a vector has been introduced.

[000109] A "TIM-3-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 TIM-3. In some embodiments, the TIM-3 related condition is immune-related disorder, such as, for example, cancer, autoimmune disease, inflammatory disease or infectious disease.

[000110] 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).

[000111] "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.

[000112] 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.

[000113] **A. Bispecific Antibody Molecule**

[000114] Provided herein are novel polypeptide complexes that comprise an antibody heavy chain variable domain operably linked to a first T cell receptor (TCR) constant region, and an

antibody light chain variable domain operably linked to a second TCR constant region, wherein the first TCR constant region and the second TCR constant region are associated via at least one non-native interchain bond. The polypeptide complex comprises at least two polypeptide chains, each of which comprises a variable domain derived from an antibody and a constant region derived from a TCR. The two polypeptide chains of the polypeptide complexes comprise a pair of heavy chain variable domain and a light chain variable domain, which are operably linked to a pair of TCR constant regions respectively. Examples of pairs of TCR constant regions include, for example, alpha/beta, pre-alpha/beta, and gamma/delta TCR constant regions. The TCR constant regions in the polypeptide complexes provided herein can be in full length or in a fragment, and can be engineered, as long as the pair of TCR constant regions are capable of associating with each other to form a dimer.

[000115] It is surprisingly found that the polypeptide complexes provided herein with at least one non-native interchain bond (in particular a non-native disulphlide bond) can be recombinantly expressed and assembled into the desired conformation, which stabilizes the TCR constant region dimer while providing for good antigen-binding activity of the antibody variable regions. Moreover, the polypeptide complexes are found to well tolerate routine antibody engineering, for example, modification of glycosylation sites, and removal of some natural sequences. Furthermore, the polypeptide complexes provided herein can be incorporated into a bispecific format which can be readily expressed and assembled with minimal or substantially no mispairing of the antigen-binding sequences due to the presence of the TCR constant regions in the polypeptide complexes. Additional advantages of the polypeptide complexes and constructs provided herein will become more evident in the following disclosure below.

[000116] The bispecific polypeptide complex provided herein is significantly less prone to have mis-paired heavy chain and light chain variable domains. Without wishing to be bound by any theory, it is believed that the stabilized TCR constant regions in the first antigen-binding moiety can specifically associate with each other and therefore contribute to the highly specific pairing of the intended VH1 and VL1, while discouraging unwanted mispairings of VH1 or VL1 with other variable regions that do not provide for the intended antigen-binding sites.

[000117] In certain embodiments, the second antigen-binding moiety further comprises an antibody constant CH1 domain operably linked to VH2, and an antibody light chain constant domain operably linked to VL2. Thus, the second antigen-binding moiety comprises a Fab.

[000118] Where the first, second, third, and fourth variable domains (e.g. VH1, VH2, VL1 and VL2) are expressed in one cell, it is highly desired that VH1 specifically pairs with VL1, and VH2 specifically pairs with VL2, such that the resulting bispecific protein product would have

the correct antigen-binding specificities. However, in existing technologies such as hybrid-hybridoma (or quadroma), random pairing of VH1, VH2, VL1 and VL2 occurs and consequently results in generation of up to ten different species, of which only one is the functional bispecific antigen-binding molecule. This not only reduces production yields but also complicates the purification of the target product.

[000119] The bispecific polypeptide complexes provided herein are exceptional in that the variable domains are less prone to mispair than otherwise would have been if both the first and the second antigen-binding moieties are counterparts of natural Fab. In an illustrative example, the first antigen-binding domain comprises VH1-C1 paired with VL1-C2, and the second antigen-binding domain comprises VH2-CH1 paired with VL2-CL. It has been surprisingly found that C1 and C2 preferentially associates with each other, and are less prone to associate with CL or CH1, thereby formation of unwanted pairs such as C1-CH, C1-CL, C2-CH, and C2-CL are discouraged and significantly reduced. As a result of specific association of C1-C2, VH1 specifically pairs with VL1, thereby rendering the first antigen binding site, and CH1 specifically pairs with CL, thereby allowing specific pairing of VH2-VL2 which provides for the second antigen binding site. Accordingly, the first antigen binding moiety and the second antigen binding moiety are less prone to mismatch, and mispairings between for example VH1-VL2, VH2-VL1, VH1-VH2, VL1-VL2 are significantly reduced than otherwise could have been if both the first and the second antigen-binding moieties are counterparts of natural Fabs, e.g. in the form of VH1-CH1, VL1-CL, VH2-CH1, and VL2-CL.

[000120] In certain embodiments, the bispecific polypeptide complex provided herein, when expressed from a cell, has significantly less mispairing products (e.g., at least 1, 2, 3, 4, 5 or more mispairing products less) and/or significantly higher production yield (e.g., at least 10%, 20%, 30%, 40%, 50%, 60% or more higher yield), than a reference molecule expressed under comparable conditions, wherein the reference molecule is otherwise identical to the bispecific polypeptide complex except having a native CH1 in the place of C1 and a native CL in the place of C2.

[000121] In one aspect, the present disclosure provides herein a bispecific polypeptide complex, more specifically, a bispecific antibody molecule. The term "bispecific" as used herein means that, there are at least two antigen-binding moieties (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 TIM-3 binding moiety and a PD-1 binding moiety.

[000122] In certain embodiments, the bispecific polypeptide complex provided herein comprises two sets of tri-polypeptide chains comprising: i) VH1-C1-VH2-CH1-Hinge-CH2-CH3; ii) VL1-

C2; and iii) VL2-CL, wherein the C1 and C2 are capable of forming a dimer comprising at least one non-native interchain bond. As used herein, VH1 refers to the first heavy chain variable domain of the first antigen-binding moiety, C1 refers to the first TCR constant region of the first antigen-binding moiety, VH2 refers to the second heavy chain variable domain of the second antigen-binding moiety, CH1 refers to the antibody CH1 domain of the second antigen-binding moiety, Hinge-CH2-CH3 refers to the hinge, CH2 and CH3 domains of an antibody, VL1 refers to the first light chain variable domain of the first antigen-binding moiety, C2 refers to the second TCR constant region of the first antigen-binding moiety, VL2 refers the second light chain variable domain of the second antigen-binding moiety, and CL refers to the antibody light chain constant region.

[000123] In certain embodiments, the bispecific polypeptide complex provided herein comprises two sets of tri-polypeptide chains comprising: i) VH2-CH1-Hinge-CH2-CH3-VH1-C1; ii) VL1-C2; and iii) VL2-CL, wherein C1 and C2 are capable of forming a dimer comprising at least one non-native interchain bond.

[000124] In another illustrative example, the bispecific polypeptide complex provided herein comprise a first heavy chain in the format of: VH1(anti-PD-1)-engineered CBeta-VH2(anti-TIM-3)-CH1-Hinge-CH2-CH3; a first light chain in the format of: VL1(anti-PD-1)-engineered CAlpha; and a second light chain in the format of VL2(anti-TIM-3)-CL. In certain embodiments, the VH1(anti-PD-1) comprises the sequence of SEQ ID NO: 34, the engineered CBeta comprises the sequence of SEQ ID NO: 25, the VH2(anti-TIM-3) comprises the sequence of SEQ ID NO: 13 or 15. In certain embodiments, the engineered CBeta is operably linked to VH2(anti-TIM-3) via a spacer comprising the sequence of SEQ ID NO: 27. In certain embodiments, the first heavy chain comprises the sequence of SEQ ID NO: 30 or 33. In certain embodiments, the VL1(anti-PD-1) comprises the sequence of SEQ ID NO: 24, the engineered CAlpha comprises the sequence of SEQ ID NO: 26. In certain embodiments, the first light chain comprises the sequence of SEQ ID NO: 28 or 31. In certain embodiments, the VL2(anti-TIM-3) comprises the sequence of SEQ ID NO: 14 or 16. In certain embodiments, the second light chain comprises the sequence of SEQ ID NO: 29 or 32.

[000125] **<u>i. TIM-3 binding moiety</u>**

[000126] In certain embodiments, the TIM-3 binding moiety comprises one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences of an anti-TIM-3 antibody selected from the group consisting of: W3402 and W3405.

[000127] Table 1 shows the CDR sequences of these 2 anti-TIM-3 antibodies. The variable region amino acid sequences of these 2 anti-TIM-3 antibodies are provided below in Table 2.

[000128] Table 1. CDR amino acid sequences

		CDR1	CDR2	CDR3
W3402	HCDR	SEQ ID NO: 1	SEQ ID NO: 3	SEQ ID NO: 5
		GFSLTNYGVG	IMTSGGSTYYNSALRA	DGTTVETLFDY
	LCDR	SEQ ID NO: 2	SEQ ID NO: 4	SEQ ID NO: 6
		RSSQSLSDSAGITYLY	LASNLGS	MQGIHVPLT
W3405	HCDR	SEQ ID NO: 7	SEQ ID NO: 9	SEQ ID NO: 11
		GFTFSNYAMS	SISDQGGTAYHADSVQG	DFGDSPGY
	LCDR	SEQ ID NO: 8	SEQ ID NO: 10	SEQ ID NO: 12
		KSSQSVLYSFKNKNYLA	WASTRES	QQYYSSPLT

[000129] Table 2. Variable region amino acid sequences

	VH	VL
W3402	SEQ ID NO: 13	SEQ ID NO: 14
	QVQLQESGPGLVKPSETLSLTCTVSGFSLTNYGVGWIRQPPGKGLEWIGIMTSGGSTYYNSALRARVTINRDTSKNQFSLKLSSVTAADTAVYYCTRDGTTVETLFDYWGQGTMVTVSS	DIVMTQTPLSLSVTPGQPASISCRSSQSLSDSAGITYLYWYLQKPGQSPQLLIYLASNLGSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQGIHVPLTFGQGTKLEIK
W3405	SEQ ID NO: 15	SEQ ID NO: 16
	EVQLLEAGGGPVQPGGSLRLSCAAAGFTFSNYAMSWVRQAPGKGLEWVSSISDQGGTAYHADSVQGRFTISRDNSKSTLYLQMNSLRAEDTAVYYCAKDFGDSPGYWGQGTLVTVSS	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSFKNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSSPLTFGGGTKVEIK

[000130] 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 TIM-3-binding moieties, yet substantially retain the specific binding affinity to TIM-3.

[000131] In certain embodiments, the TIM-3-binding moieties provided herein comprise a heavy chain CDR3 sequence of one of the anti-TIM-3 antibodies W3402 or W3405.

[000132] 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).

[000133] In certain embodiments, the TIM-3-binding moieties provided herein comprise any suitable framework region (FR) sequences, as long as the antigen-binding moieties can specifically bind to TIM-3.

[000134] In certain embodiments, the TIM-3-binding moieties provided herein are humanized. A humanized antigen-binding moiety is desirable in its reduced immunogenicity in human. A humanized antigen-binding moiety 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 moiety 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).

[000135] 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).

[000136] In certain embodiments, the humanized antigen-binding moieties 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 moiety comprise human FR1-4.

[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 TIM-3-binding moiety 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] Binding affinity of the antigen-binding moieties 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 antigen-binding 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 antibody 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.

[000139] In some embodiments, the TIM-3-binding moieties provided herein are capable of specifically binding to human TIM-3 with a binding affinity (K_D) of 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, no more than 10^{-9} M, no more than 5×10^{-10} M, no more than 4×10^{-10} M, no more than 3×10^{-10} M, no more than 2×10^{-10} M, no more than 10^{-10} M, no more than 5×10^{-11} M, or no more than 4×10^{-11} M as measured by surface plasmon resonance (SPR).

[000140] In certain embodiments, the TIM-3-binding moieties provided herein cross-react with Cynomolgus monkey TIM-3, for example, Cynomolgus monkey TIM-3 expressed on a cell surface, or a soluble recombinant Cynomolgus monkey TIM-3.

[000141] Binding of TIM-3-binding moieties can also be represented by "half maximal effective concentration" (EC_{50}) value, which refers to the concentration of an antibody 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. In certain embodiments, the TIM-3-binding moieties provided herein specifically bind to human TIM-3 at an EC_{50} (i.e. 50% binding concentration) of no more than 0.25 nM, no more than 0.3 nM, no more than 0.35 nM, no more than 0.4 nM no more than 0.45 nM, no more than 0.5 nM, no more than 0.6 nM, no more than 0.7 nM, no more than 0.8 nM no more than 0.9 nM, no more than 1 nM, no more than 1.5 nM, no more than 2 nM, no more than 2.5 nM no more than 5 nM by flow cytometery assay.

[000142] In certain embodiments, the antibodies and antigen-binding fragments thereof bind to cynomolgus monkey TIM-3 with a binding affinity similar to that of human TIM-3. For example, binding of the exemplary antibody W3402 or W3405 to cynomolgus monkey TIM-3 is at a similar EC_{50} value to that of human TIM-3.

[000143] In certain embodiments, the antibodies and the fragments thereof provided herein specifically bind to cynomolgus monkey TIM-3 with an EC_{50} of no more than 0.35 nM, no more than 0.4 nM, no more than 0.45 nM, no more than 0.5 nM, no more than 0.6 nM, no more than 0.7 nM, no more than 0.8 nM no more than 0.9 nM, no more than 1 nM, no more than 1.5 nM, no more than 2 nM, no more than 2.5 nM no more than 5 nM by flow cytometery assay.

[000144] In certain embodiments, the TIM-3-binding moieties provided herein have a specific binding affinity to human TIM-3 which is sufficient to provide for diagnostic and/or therapeutic use.

[000145] In certain embodiments, the TIM-3-binding moieties provided herein inhibit binding of TIM-3 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.

[000146] **ii. PD-1-binding moiety**

[000147] In certain embodiments, the antigen-binding moiety is a PD-1-binding moiety capable of specifically binding to PD-1 (such as human PD-1).

[000148] In certain embodiments, the PD-1-binding moiety comprises one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences of the anti-PD-1 antibody W3055.

[000149] Table 3 shows the CDR sequences of the anti-PD-1 antibody. The variable region amino acid sequences are provided below in Table 4.

[000150] Table 3. CDR amino acid sequences

		CDR1	**CDR2**	**CDR3**
W3055	**HCDR**	SEQ ID NO: 17	SEQ ID NO: 19	SEQ ID NO: 21
		GFTFSSHAMS	TITGGGGSIYY ADSVKG	NRAGEGYFDY
	LCDR	SEQ ID NO: 18	SEQ ID NO: 20	SEQ ID NO: 22
		GGDNIGNKDVH	RDSNRPS	QVWDSIWV

[000151] Table 4. Variable region amino acid sequences

	VH	**VL**
W3055	SEQ ID NO: 23	SEQ ID NO: 24
	EVQLLESGGGLVQPGGSLRL SCAAS**GFTFSSHAMS**WVRQ APGKGLEWVS**TITGGGGSI YYADSVKG**RFTISRDNSKNT LYLQMNSLRAEDTAVYYCA K**NRAGEGYFDY**WGQGTLV TVSS	SYELTQPLSVSVALGQTARITC**G GDNIGNKDVH**WYQQKPGQAPV LVIY**RDSNRPS**GIPEGFSGSNSG NTATLTISRAQAGDEADYYC**QV WDSIWV**FGGGTKLTVL

[000152] 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 moieties, yet substantially retain the specific binding affinity to PD-1 (e.g. human PD-1).

[000153] In certain embodiments, the PD-1-binding moieties provided herein comprise a heavy chain CDR3 sequence of W3055.

[000154] In certain embodiments, the PD-1-binding moieties provided herein are not fully human. In certain embodiments, the PD-1-binding moieties provided herein comprise suitable framework region (FR) sequences, as long as the antigen-binding moieties can specifically bind to PD-1, respectively.

[000155] 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 moiety 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.

[000156] In some embodiments, the PD-1-binding moieties provided herein are capable of specifically binding to human PD-1 with a binding affinity (K_D) of no more than $5x10^{-8}$M, no more than $4x10^{-8}$M, no more than $3x10^{-8}$M, no more than $2x10^{-8}$M, no more than 10^{-8}M, no more than $5x10^{-9}$M, no more than $4x10^{-9}$M, no more than $3x10^{-9}$M, no more than $2x10^{-9}$M, no more than 10^{-9}M, no more than $5x10^{-10}$M, no more than $4x10^{-10}$M, no more than $3x10^{-10}$M, no more than $2x10^{-10}$M, no more than 10^{-10}M, no more than $5x10^{-11}$ M, or no more than $4x10^{-11}$ M, no more than $3x10^{-11}$ M, or no more than $2x10^{-11}$ M, no more than 10^{-11} M, no more than $5x10^{-12}$ M, or no more than $4x10^{-12}$ M, no more than $3x10^{-12}$ M, or no more than $2x10^{-12}$ M, or no more than 10^{-12} M as measured by surface plasmon resonance (SPR).

[000157] In certain embodiments, the PD-1-binding moieties provided herein cross-react with Cynomolgus monkey PD-1 and/or mouse PD-1. In certain embodiments, the PD-1-binding moieties bind to Cynomolgus monkey PD-1 or mouse PD-1 with a binding affinity similar to that of human PD-1.

[000158] In some embodiments, the PD-1-binding moieties provided herein are capable of specifically binding to Cynomolgus monkey PD-1 with a binding affinity (K_D) of no more than $5x10^{-8}$M, no more than $4x10^{-8}$M, no more than $3x10^{-8}$M, no more than $2x10^{-8}$M, no more than 10^{-8}M, no more than $5x10^{-9}$M, no more than $4x10^{-9}$M, no more than $3x10^{-9}$M, no more than $2x10^{-9}$M, no more than 10^{-9}M, no more than $5x10^{-10}$M, no more than $4x10^{-10}$M, no more than $3x10^{-10}$M, no more than $2x10^{-10}$M, or no more than 10^{-10}M as measured by SPR.

[000159] Binding of PD-1-binding moieties can also be represented by "half maximal effective concentration" (EC_{50}) value, which refers to the concentration of an antibody 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. In certain embodiments, the PD-1-binding moieties provided herein specifically bind to human PD-1 at an EC_{50} (i.e. 50% binding concentration) of no more than 0.1 nM, no more than 0.15 nM, no more than 0.2 nM, no more than 0.25 nM, no more than 0.3 nM, no more than 0.35 nM, no more than 0.4 nM, no more than 0.45 nM or no more than 0.5 nM by ELISA, or no more than 1 nM, no more than 1.5 nM, no more than 2 nM, no more than 2.5 nM, no more than 3 nM, no more than 3.5 nM, no more than 4 nM, or no more than 4.5 nM by FACS.

[000160] In certain embodiments, the PD-1-binding moieties provided herein specifically bind to monkey PD-1 at an EC_{50} of no more than 0.1 nM, no more than 0.15 nM, no more than 0.2 nM, no more than 0.25 nM, no more than 0.3 nM, no more than 0.35 nM, no more than 0.4 nM, no more than 0.45 nM or no more than 0.5 nM by ELISA, or no more than 1 nM, no more than 1.5 nM, no more than 2 nM, no more than 2.5 nM, no more than 3 nM, no more than 3.5 nM, no more than 4 nM, or no more than 4.5 nM by FACS.

[000161] In certain embodiments, the PD-1-binding moieties provided herein have a specific binding affinity to human PD-1 which is sufficient to provide for diagnostic and/or therapeutic use.

[000162] iii. Variants

[000163] The antigen-binding moieties 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 3, one or more full-length sequences (but not in any of the CDR sequences) provided in Table 2 or Table 4, and/or the constant region (e.g. Fc region). Such variants retain specific binding affinity to TIM-3 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 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).

[000164] 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.).

[000165] In certain embodiments, the TIM-3-binding moieties and/or the PD-1 binding moieties 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.

[000166] In certain embodiments, the TIM-3-binding moieties 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 that (or those) listed in Table 1, and in the meantime retain the binding affinity to TIM-3 at a level similar to or even higher than its parent antibody.

[000167] In certain embodiments, the anti-TIM-3-binding moieties 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 that (or those) listed in Table 2, and in the meantime retain the binding affinity to TIM-3 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 listed in Table 2. In some embodiments, the substitutions, insertions, or deletions occur in regions outside the CDRs (e.g., in the FRs).

[000168] In certain embodiments, the PD-1-binding moieties 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 that (or those) listed in Table 3, and in the meantime retain the binding affinity to PD-1 at a level similar to or even higher than its parent antibody.

[000169] In certain embodiments, the PD-1-binding moieties 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 that (or those) listed in Table 4, 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 listed in Table 4. In some embodiments, the substitutions, insertions, or deletions occur in regions outside the CDRs (e.g., in the FRs).

[000170] **Glycosylation variant**

[000171] The antigen-binding moieties 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.

[000172] The antigen-binding moieties 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.

[000173] **Cysteine-engineered variant**

[000174] The antigen-binding moieties and bispecific antibody molecules also encompass a cysteine-engineered variant, which comprises one or more introduced free cysteine amino acid residues.

[000175] 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.

[000176] **Fc Variant**

[000177] The antigen-binding moieties 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.

[000178] In certain embodiments, the antigen-binding moieties 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).

[000179] In certain embodiments, the antigen-binding moieties 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 CH2 domain of the Fc region can be substituted to provide for enhanced ADCC activity. Alternatively or additionally, carbohydrate structures on the antibody can be changed to enhance 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. In certain embodiments, the antigen-binding moieties 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.

[000180] In certain embodiments, the antigen-binding moieties 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 Fe region variants.

[000181] In certain embodiments, the antigen-binding moieties 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.

[000182] **iv. Bispecific Antigen-Binding moieties**

[000183] In certain embodiments, the bispecific antibody molecules provided herein comprises an TIM-3-binding moiety comprising one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences (or a variant thereof) of anti-TIM-3 antibody W3402 or W3405, and a PD-1-binding moiety comprising one or more (e.g. 1, 2, 3, 4, 5, or 6) CDR sequences (or a variant thereof) of an anti-PD-1 antibody W3055.

[000184] Table 5 shows the CDR sequences of two representative bispecific antibody molecules W3598-U6T1.G25-1.hIgG4.SP (also named as "W3598B") and W3598-U6T3.G25-1.uIgG4.SP (also named as "W3598C").

[000185] Table 5.

BsAb	mAb		**CDR1**	**CDR2**	**CDR3**
W3598-U6T1.G25-1.hIgG4.SP	**W3055**	**HCDR**	SEQ ID NO: 17	SEQ ID NO: 19	SEQ ID NO: 21
			GFTFSSHAMS	TITGGGGSIYYADSVKG	NRAGEGYFDY
		LCDR	SEQ ID NO: 18	SEQ ID NO: 20	SEQ ID NO: 22
			GGDNIGNKDVH	RDSNRPS	QVWDSIWV
	W3402	**HCDR**	SEQ ID NO: 1	SEQ ID NO: 3	SEQ ID NO: 5
			GFSLTNYGVG	IMTSGGSTYYNSALRA	DGTTVETLFDY
		LCDR	SEQ ID NO: 2	SEQ ID NO: 4	SEQ ID NO: 6
			RSSQSLSDSAGITYLY	LASNLGS	MQGIHVPLT
W3598-U6T3.G25-1.uIgG4.SP	**W3055**	**HCDR**	SEQ ID NO: 17	SEQ ID NO: 19	SEQ ID NO: 21
			GFTFSSHAMS	TITGGGGSIYYADSVKG	NRAGEGYFDY
		LCDR	SEQ ID NO: 18	SEQ ID NO: 20	SEQ ID NO: 22
			GGDNIGNKDVH	RDSNRPS	QVWDSIWV
	W3405	**HCDR**	SEQ ID NO: 7	SEQ ID NO: 9	SEQ ID NO: 11
			GFTFSNYAMS	SISDQGGTAYHADSVQG	DFGDSPGY
		LCDR	SEQ ID NO: 8	SEQ ID NO: 10	SEQ ID NO: 12
			KSSQSVLYSFKNKNYLA	WASTRES	QQYYSSPLT

[000186] In some embodiments, the bispecific antibody molecules provided herein are capable of specifically binding to both human PD-1 and human TIM-3. The bispecific antibody molecules provided herein retain the specific binding affinity to both PD-1 and TIM-3, in certain embodiments are at least comparable to, or even better than, the parent antibodies in that aspect.

[000187] 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.

[000188] 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.

[000189] The bispecific antibody molecules provided herein can have a constant region, for example 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.

[000190] 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.

[000191] 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 moiety and Fc region and therefore reduced immunogenicity than a humanized antibody counterpart.

[000192] **v. Bispecific Antibody Molecule**

[000193] The TIM-3-binding moiety and the PD-1-binding moiety of the bispecific antibody molecules provided herein can be in any suitable format.

[000194] Various techniques can be used for the production of such antigen-binding moieties. 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), screening from a phage display library as discussed above (e.g. for ScFv), and chemical coupling of two Fab'-SH fragments to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). Other techniques for the production of antibody fragments will be apparent to a skilled practitioner.

[000195] In certain embodiments, the TIM-3-binding and/or the PD-1-binding moieties 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 TIM-3-binding and/or the PD-1-binding moieties 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, TIM-3-binding and/or the PD-1-binding moieties

comprises two or more antigen binding sites (e.g. scFv or Fab) operably linked together, with or without a spacer.

[000196] In certain embodiments, the TIM-3-binding moiety is operably linked to the N terminus or the C terminus of the PD-1-binding moiety. In certain embodiments, the PD-1-binding moiety is operably linked to the N terminus or the C terminus of the TIM-3-binding moiety.

[000197] In certain embodiments, the TIM-3-binding moiety or the PD-1-binding moiety is operably linked to the C-terminus of the constant region of the bispecific antibody molecule.

[000198] 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.

[000199] 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, such as tandem single chain variable fragment molecules (taFvs), diabodies (Dbs), single chain diabodies (scDbs) and various other derivatives of these (cf. bispecific antibody formats as described by Byrne H. et al. (2013) Trends Biotech, 31 (11): 621-632. Examples of bispecific antibody is based on a format which include, but is not limited to, quadroma, chemically coupled Fab (fragment antigen binding), and BiTE (bispecific T cell engager).

[000200] 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.

[000201] 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).)

[000202] **vi. Bispecific format**

[000203] In the polypeptide complex provided herein, the first antigen-binding moiety and the second binding moiety are associated into an Ig-like structure. An Ig-like structure is like a natural antibody having a Y shaped construct, with two arms for antigen-binding and one stem for association and stabilization. The resemblance to natural antibody can provide for various advantages such as good in vivo pharmakinetics, desired immunological response and stability etc. It has been found that the Ig-like structure comprising the first antigen-binding moiety provided herein associated with the second antigen-binding moiety provided herein has thermal stability which is comparable to that of an Ig (e.g. an IgG). In certain embodiments, the Ig-like structure provided herein is at least 70%, 80%, 90%, 95% or 100% of that of a natural IgG.

[000204] The bispecific polypeptide complexes disclosed herein have longer *in vivo* half-life and are relatively easier to manufacture when compared to bispecific polypeptide complexes in other formats.

[000205] **vii. Antigen-Binding Moiety Comprising Engineered CAlpha and CBeta**

[000206] The first antigen-binding moiety provided herein comprises a first antibody heavy chain variable domain operably linked to a first T cell receptor (TCR) constant region, and a first antibody light chain variable domain operably linked to a second TCR constant region, wherein the first TCR constant region and the second TCR constant region are associated via at least one non-native interchain disulphide bond. The first antigen-binding moiety comprises at least two polypeptide chains, each of which comprises a variable domain derived from an antibody and a constant region derived from a TCR. Thus, the first antigen-binding moiety comprises a heavy chain variable domain and a light chain variable domain, which are operably linked to a pair of TCR constant regions, respectively. In certain embodiments, the pair of TCR constant regions in the first antigen-binding moiety are alpha/beta TCR constant regions. The TCR constant regions in the polypeptide complexes provided herein are capable of associating with each other to form a dimer through at least one non-native disulphide bond.

[000207] It is surprisingly found that the first antigen-binding moiety provided herein with at least one non-native disulphide bond can be recombinantly expressed and assembled into the desired conformation, which stabilizes the TCR constant region dimer while providing for good antigen-binding activity of the antibody variable regions. Moreover, the first antigen-binding moiety is found to well tolerate routine antibody engineering, for example, modification of glycosylation sites, and removal of some natural sequences. Furthermore, the polypeptide complexes provided herein can be incorporated into a bispecific format which can be readily expressed and assembled with minimal or substantially no mispairing of the antigen-binding sequences due to the presence of the TCR constant regions in the first antigen-binding moiety.

Additional advantages of the first antigen-binding moiety and constructs provided herein will become more evident in the following disclosure below.

[000208] In summary, the first antigen-binding moiety provided herein comprises a first polypeptide comprising, from N-terminus to C-terminus, a first heavy chain variable domain (VH) of a first antibody operably linked to a first T cell receptor (TCR) constant region (C1), and a second polypeptide comprising, from N-terminus to C-terminus, a first light chain variable domain (VL) of the first antibody operably linked to a second TCR constant region (C2), wherein C1 and C2 are capable of forming a dimer, and the non-native interchain disulphide bond between C1 and C2 is capable of stabilizing the dimer.

[000209] **viii. TCR constant region**

[000210] The first antigen-binding moiety provided herein comprises an alpha or beta constant region derived from a TCR.

[000211] Human TCR alpha chain constant region is known as TRAC, with the NCBI accession number of P01848.

[000212] Human TCR beta chain constant region has two different variants, known as TRBC1 and TRBC2 (IMGT nomenclature) (see also Toyonaga B, *et al.*, PNAs, Vol. 82, pp.8624-8628, Immunology (1985)).

[000213] In the present disclosure, the first and the second TCR constant regions of the first antigen-binding moiety provided herein are capable of forming a dimer comprising, between the TCR constant regions, at least one non-native interchain disulphide bond that is capable of stabilizing the dimer.

[000214] The term "dimer" as used herein refers to an associated structure formed by two molecules, such as polypeptides or proteins, via covalent or non-covalent interactions. A homodimer or homodimerization is formed by two identical molecules, and a heterodimer or heterodimerization is formed by two different molecules. The dimer formed by the first and the second TCR constant regions is a heterodimer.

[000215] A "mutated" amino acid residue refers to one which is substituted, inserted or added and is different from its native counterpart residue in a corresponding native TCR constant region. For example, if an amino acid residue at a particular position in the wild-type TCR constant region is referred to as the "native" residue, then its mutated counterpart is any residue that is different from the native residue but resides at the same position on the TCR constant region. A mutated residue can be a different residue which substitutes the native residue at the

same position, or which is inserted before the native residue and therefore takes up its original position.

[000216] In the polypeptide complexes provided herein, the first and/or the second TCR constant regions have been engineered to comprise one or more mutated amino acid residues that are responsible for forming the non-native interchain disulphide bond. To introduce such a mutated residue to the TCR constant region, an encoding sequence of a TCR region can be manipulated to for example, substitute a codon encoding a native residue for the codon encoding the mutated residue, or to insert a codon encoding the mutated residue before the codon of the native residue.

[000217] In the polypeptide complexes provided herein, the first and/or the second TCR constant regions have been engineered to comprise one or more mutated cysteine residues such that, after replacement to cysteine residues, a non-native interchain disulphide bond could be formed between the two TCR constant regions.

[000218] The non-native interchain disulphide bond is capable of stabilizing the first antigen-binding moiety. Such effects in stablization can be embodied in various ways. For example, the presence of the mutated amino acid residue or the non-native interchain disulphide bond can enable the polypeptide complex to stably express, and/or to express in a high level, and/or to associate into a stable complex having the desired biological activity (e.g. antigen binding activity), and/or to express and assemble into a high level of desired stable complex having the desired biological activity. The capability of the interchain disulphide bond to stabilize the first and the second TCR constant regions can be assessed using proper methods known in the art, such as the molecular weight displayed on SDS-PAGE, or thermostability measured by differential scanning calorimetry (DSC) or differential scanning fluorimetry (DSF). In an illustrative example, formation of a stable first antigen-binding moiety provided herein can be confirmed by SDS-PAGE, if a product shows a molecular weight comparable to the combined molecular weight of the first and the second polypeptides. In certain embodiments, the first antigen-binding moiety provided herein is stable in that its thermal stability is no less than 50%, 60%, 70%, 80%, or 90% of that of a natural Fab. In certain embodiments, the first antigen-binding moiety provided herein is stable in that its thermal stability is comparable to that of a natural Fab.

[000219] Without wishing to be bound by any theory, it is believed that the non-native interchain disulphide bond formed between the first and the second TCR constant regions in the first antigen-binding moiety are capable of stabilizing the heterodimer of TCR constant regions, thereby enhancing the level of correct folding, the structural stability and/or the expression level

of the heterodimer and of the first antigen-binding moiety. Unlike native TCR anchored on the membrane of T cell surface, heterodimers of native TCR extracellular domains are found to be much less stable, despite of its similarity to antibody Fab in 3D structure. As a matter of fact, the instability of native TCR in soluble condition used to be a significant obstacle that prevents elucidation of its crystal structure (see Wang, *Protein Cell*, 5(9), pp.649–652 (2014)). By introducing a pair of Cysteine (Cys) mutations in TCR constant regions and thereby enabling formation of interchain non-native disulphide bond, the first antigen-binding moiety can be stably expressed while in the meantime the antigen-binding capabilities of the antibody variable region are retained.

[000220] The TCR constant region comprising a mutated residue is also referred to herein as an "engineered" TCR constant region. In certain embodiments, the first TCR constant region (C1) of the polypeptide complex comprises an engineered TCR Alpha chain (CAlpha), and the second TCR constant region (C2) comprises an engineered TCR Beta chain (CBeta). In the polypeptide complexes provided herein, C1 comprises an engineered CBeta, and C2 comprises an engineered CAlpha.

[000221] In the polypeptide complexes provided herein, the engineered TCR constant region comprises one or more mutated cysteine residue within a contact interface of the first and/or the second engineered TCR constant regions.

[000222] The term "contact interface" as used herein refers to the particular region(s) on the polypeptides where the polypeptides interact/associate with each other. A contact interface comprises one or more amino acid residues that are capable of interacting with the corresponding amino acid residue(s) that comes into contact or association when interaction occurs. The amino acid residues in a contact interface may or may not be in a consecutive sequence. For example, when the interface is three-dimensional, the amino acid residues within the interface may be separated at different positions on the linear sequence.

[000223] In certain embodiments, one or more disulphide bonds can be formed between the engineered CAlpha and the engineered CBeta. In certain embodiments, the pair of cysteine residues are capable of forming a non-native interchain disulphide bond.

[000224] As used herein throughout the application, "XnY" with respect to a TCR constant region is intended to mean that the n^{th} amino acid residue X on the TCR constant region is replaced by amino acid residue Y, where X and Y are respectively the one-letter abbreviation of a particular amino acid residue.

[000225] In the polypeptide complexes provided herein, the engineered CBeta comprises or is SEQ ID NO: 25, and the engineered CAlpha comprises or is SEQ ID NO: 26.

The sequences represented by SEQ ID NO: 25 and SEQ ID NO: 26 are provided below:

SEQ ID NO: 25

LEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCT DPQPLKEQPALQDSRYALSSRLRVSATFWQNPRNHFRCQVQFYGLSENDEWTQDRAKP VTQIVSAEAWGR

SEQ ID NO: 26

PDIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTQVSQSKDSDVYITDKCVLDMRSMDFK SNSAVAWSQKSDFACANAFQNSIIPEDTFFPSPESS

[000226] In the polypeptide complexes provided herein, one or more native glycosylation site present in the native TCR constant regions has been modified (e.g. removed) in the first antigen-binding moiety provided in the present disclosure. The term "glycosylation site" as used herein with respect to a polypeptide sequence refers to an amino acid residue with a side chain to which a carbohydrate moiety (e.g. an oligosaccharide structure) can be attached. Glycosylation of polypeptides like 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 native glycosylation sites can be conveniently accomplished by altering the amino acid sequence such that one or more of the above-described tripeptide sequences (for N-linked glycosylation sites) or one or more serine or threonine residues (for O-linked glycosylation sites) are substituted.

[000227] In the first antigen-binding moiety provided herein, at least one native glycosylation site is absent in the engineered TCR constant regions, for example, in the first and/or the second TCR constant regions. Without wishing to be bound by any theory, but it is believed that the first antigen-binding moiety provided herein can tolerate removal of all or part of the glycosylation sites without affecting the protein expression and stability, in contrast to existing teachings that presence of N-linked glycosylation sites on TCR constant region, such as CAlpha (i.e. N34, N68, and N79) and CBeta (i.e. N69) are necessary for protein expression and stability (see Wu et al., Mabs, 7:2, 364-376, 2015).

[000228] In the first antigen-binding moiety provided herein, the constant regions derived from a TCR are operably linked to the variable regions derived from an antibody.

[000229] In certain embodiments, the first antibody variable domain (VH) is fused to the first TCR constant region (C1) at a first conjunction domain, and the first antibody variable domain (VL) is fused to the second TCR constant region (C2) at a second conjunction domain.

[000230] "Conjunction domain" as used herein refers to a boundary or border region where two amino acid sequences are fused or combined. In certain embodiments, the first conjunction domain comprises at least a portion of the C terminal fragment of an antibody V/C conjunction, and the second conjunction domain comprises at least a portion of the N-terminal fragment of a TCR V/C conjunction.

[000231] The term "antibody V/C conjunction" as used herein refers to the boundary of antibody variable domain and constant domain, for example, the boundary between heavy chain variable domain and the CH1 domain, or between light chain variable domain and the light chain constant domain. Similarly, the term "TCR V/C conjunction" refers to the boundary of TCR variable domain and constant domain, for example, the boundary between TCR Alpha variable domain and constant domain, or between TCRBeta variable domain and constant domain.

[000232] In certain embodiments, in the first and/or the second conjunction domain, the portion of the N terminal fragment of an antibody V/C conjunction and the portion of the C-terminal fragment of a TCR V/C conjunction are fused in such a way that a few residues at the C terminus of the antibody variable domain are substituted by the corresponding residues in the TCR counterpart. For example, as shown in the examples in Table 6, in the first conjunction domain having a sequence of SEQ ID NO: 35, the two C terminal residue "SS" of the antibody heavy variable region portion (i.e. SEQ ID NO: 23) are substituted by the TCR counterpart which are "LE" (see SEQ ID NO: 25).

[000233]

Table 6. First and second conjunction domain designs for VH-CBeta/VL-CAlpha

	Conjunction (heavy chain)	
	Variable	Constant
Antibody_VH (SEQ ID NO:23)	……LVTVSS	AS--TKGPS……
TCR_beta (SEQ ID NO:25)	……**RLTVLE**	**DLKNVFPPE**……

H_Conjunction_(SEQ ID NO: 35)	……LVTV**LE**	**DLKNVFPPE**……

[000234] In certain embodiments, the first polypeptide comprises a sequence comprising domains operably linked as in formula (I): VH-HCJ-C1, and the second polypeptide comprises a sequence comprising domains operably linked as in formula (II): VL-LCJ-C2, wherein:

VH is a heavy chain variable domain of an antibody;

HCJ is a first conjunction domain as defined supra;

C1 is a first TCR constant domain as defined supra;

VL is a light chain variable domain of an antibody;

LCJ is a second conjunction domain as defined supra;

C2 is a second TCR constant domain as defined supra.

[000235] Optionally, the bispecific antibody molecule may also have a spacer. In certain embodiments, the spacer comprises or is the amino acid sequence of GGGGSGGGGS (SEQ ID NO: 27).

[000236] Table 7 shows the sequences of two representative bispecific antibody molecules W3598-U6T1.G25-1.hIgG4.SP (also named as "W3598B") and W3598-U6T3.G25-1.uIgG4.SP (also named as "W3598C"), with the TCR constant domain highlighted in bold, and with the spacer underlined. The antibodies W3598B and W3598C are in the format of "G25," which is demonstrated in Figure 13.

Table 7.

Samples	Protein chain	Sequences
W3598B: W3598-U6T1.G25-1.hIgG4.SP	Light chain 1 (L1)	SYELTQPLSVSVALGQTARITCGGDNIGNKDVHWY QQKPGQAPVLVIYRDSNRPSGIPEGFSGSNSGNTAT LTISRAQAGDEADYYCQVWDSIWVFGGGTKLTVL**P DIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTQV SQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSQ KSDFACANAFQNSIIPEDTFFPSPESS** (SEQ ID NO: 28)
	Light chain 2 (L2)	DIVMTQTPLSLSVTPGQPASISCRSSQSLSDSAGITYL YWYLQKPGQSPQLLIYLASNLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQGIHVPLTFGQGTKL EIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR GEC (SEQ ID NO: 29)

	Heavy chain (H)	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSHAMS WVRQAPGKGLEWVSTITGGGGSIYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAKNRAGEG YFDYWGQGTLVTV**LEDLKNVFPPEVAVFEPSEAE ISHTQKATLVCLATGFYPDHVELSWWVNGKEVH SGVCTDPQPLKEQPALQDSRYALSSRLRVSATFW QNPRNHFRCQVQFYGLSENDEWTQDRAKPVTQI VSAEAWGR**GGGGSGGGGSQVQLQESGPGLVKPSE TLSLTCTVSGFSLTNYGVGWIRQPPGKGLEWIGIMT SGGSTYYNSALRARVTINRDTSKNQFSLKLSSVTAA DTAVYYCTRDGTTVETLFDYWGQGTMVTVSSAST KGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPA PEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSR LTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS LGK (SEQ ID NO: 30)
W3598C: W3598-U6T3.G25-1.uIgG4.SP	Light chain1 (L1)	SYELTQPLSVSVALGQTARITCGGDNIGNKDVHWY QQKPGQAPVLVIYRDSNRPSGIPEGFSGSNSGNTAT LTISRAQAGDEADYYCQVWDSIWVFGGGTKLTVL**P DIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTQV SQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSQ KSDFACANAFQNSIIPEDTFFPSPESS** (SEQ ID NO: 31)
	Light chain 2 (L2)	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSFKNK NYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGS GSGTDFTLTISSLQAEDVAVYYCQQYYSSPLTFGGG TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNN FYPREAKVQWKVDNALQSGNSQESVTEQDSKDST YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKS FNRGEC (SEQ ID NO: 32)
	Heavy chain (H)	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSHAMS WVRQAPGKGLEWVSTITGGGGSIYYADSVKGRFTI SRDNSKNTLYLQMNSLRAEDTAVYYCAKNRAGEG YFDYWGQGTLVTV**LEDLKNVFPPEVAVFEPSEAE ISHTQKATLVCLATGFYPDHVELSWWVNGKEVH SGVCTDPQPLKEQPALQDSRYALSSRLRVSATFW QNPRNHFRCQVQFYGLSENDEWTQDRAKPVTQI VSAEAWGR**GGGGSGGGGSEVQLLEAGGGPVQPG GSLRLSCAAAGFTFSNYAMSWVRQAPGKGLEWVS SISDQGGTAYHADSVQGRFTISRDNSKSTLYLQMNS LRAEDTAVYYCAKDFGDSPGYWGQGTLVTVSSAS TKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS SLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPC

		PAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKT ISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSL SLSLG (SEQ ID NO: 33)

Specifically, the heavy chain variable region in the heavy chain of the PD-1 binding moiety of the bispecific antibody WBP3598B or WBP3598C is EVQLLESGGGLVQPGGSLRLSCAASGFTFSSHAMSWVRQAPGKGLEWVSTITGGGGSIY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKNRAGEGYFDYWGQGTLVT V (SEQ ID NO: 34), which is derived from W3055 (SEQ ID NO: 23), with the two amino acids "SS" at the terminal deleted as compared to SEQ ID NO: 23.

[000237] Conjugates

[000238] 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.

[000239] 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.

[000240] 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.

[000241] 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.

[000242] 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.

[000243] 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.

[000244] In certain embodiments, the conjugate moiety can be a purification moiety such as a magnetic bead.

[000245] In certain embodiments, the bispecific antibody molecule provided herein is used for a base for a conjugate.

[000246] **Polynucleotides and Recombinant Methods**

[000247] The present disclosure provides isolated polynucleotides that encode the bispecific antibody molecules provided herein.

[000248] 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)).

[000249] 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.

[000250] 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.

[000251] 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.

[000252] 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*.

[000253] 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*.

[000254] 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.

[000255] 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.

[000256] 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.

[000257] 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.

[000258] 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.

[000259] 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.

[000260] 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.

[000261] 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).

[000262] Pharmaceutical Composition

[000263] The present disclosure further provides pharmaceutical compositions comprising the bispecific antibody molecule and one or more pharmaceutically acceptable carriers.

[000264] 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.

[000265] 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.

[000266] 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.

[000267] 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.

[000268] 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.

[000269] 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.

[000270] 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.

[000271] 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.

[000272] **Methods of Use**

[000273] 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.

[000274] 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 TIM-3-related condition or a disorder.

[000275] 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.

[000276] 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.

[000277] 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.

[000278] 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 qPCR, reverse transcriptase PCR, microarray, SAGE, 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).

[000279] 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).

[000280] 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 TIM-3-related condition or a disorder.

In some embodiment, the TIM-3-related condition or a disorder is cancer, autoimmune disease, inflammatory disease, or infectious disease.

[000281] 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..

[000282] 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.

[000283] 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.

[000284] 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.

[000285] 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.

[000286] 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.

[000287] 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.

[000288] 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.

[000289] 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.

[000290] 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.

[000291] The present disclosure further provides methods of using the bispecific antibody molecule thereof.

[000292] In some embodiments, the present disclosure provides methods of detecting presence or amount of TIM-3 and/or PD-1 in a sample, comprising contacting the sample with the bispecific antibody molecule, and determining the presence or the amount of TIM-3 and/or PD-1 in the sample.

[000293] 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 TIM-3 related disease or condition in a subject.

[0004] Advantages

[0005] 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 TIM-3 pathways, and they particularly resume CD8+ T cell function. The bispecific antibodies provided herein are superior to monospecific anti-PD1 antibodies, or monospecific anti-TIM-3 antibodies, or combination of monospecific anti-PD1 antibodies and monospecific anti-TIM-3 antibodies. The bispecific antibodies provided herein are also advantageous in that they are cross-reactive to human, monkey PD1 and TIM-3, and in some embodiments, cross-reactive to murine PD-1. 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.

[000294] 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

EXAMPLE 1: Generation of antigens and benchmark antibodies

[000295] 1. Research materials preparation

[000296] 1.1 Immunogen generation

[000297] DNAs encoding the ECD or full length of TIM-3 and PD-1 were synthesized in Sangon Biotech (Shanghai, China) and then subcloned into modified expression vectors

pcDNA3.3 with different tags (such as 6xhis, human Fc, or mouse Fc) in C-terminal. Then the expression vectors pcDNA3.3 was purified for use.

[000298] Expi293 cells (Invitrogen-A14527) were transfected with the purified expression vector pcDNA3.3. Cells were cultured for 5 days and supernatant was collected for protein purification using Ni-NTA column (GE Healthcare, 175248) or Protein A column (GE Healthcare, 175438) or Protein G column (GE Healthcare, 170618). The obtained human TIM-3, human PD-1, mouse PD-1 were quality controlled by SDS-PAGE and SEC, and then stored at -80 ^{o}C.

[000299] 1.2 Generation of benchmark antibodies

[000300] Six benchmark antibodies were applied in the examples as reference antibodies. Information for the benchmark antibodies are shown in Table 8 below.

Table 8.

Benchmark Antibodies	Reference	Name in the reference
305.BMK1	US9084776B2	5C4 (Opdivo)
305.BMK3	US8168757B2	MAB1B8
340.BMK5	WO2016/161270A1	lead antibody
340.BMK6	US20160200815A1	mAb15
340.BMK8	US9605070B2	ABTIM-3-hum11
359.BMK1	WO2017055404	PD1TIM3_0476

EXAMPLE 2: Stable cell lines establishment

[000296] Lipofectamine 2000 was used to transfect CHO-S or 293F cells with the expression vector containing the genes encoding full length human PD-1 or mouse PD-1. Cells were cultured in medium containing proper selection pressure. Human PD-1 high expression stable cell line (WBP305.CHO-S.hPro1.C6) and mouse PD-1 high expression stable cell line (WBP305.293F.mPro1.B4) were obtained by limiting dilution.

[000297] The genes of human TIM-3, human TIM-3vIII, and macaca fascicularis TIM-3 were respectively inserted into plasmids, the expression vector pcDNA 3.3. The plasmids were

then transfected to CHO-K1 cells respectively, as described below: 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°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 was mixed with 10 μg of Lipofectamine 2000 to make the final volume 200 μl in Opti-MEM medium. The solution in the tube pipette was added to the cells drop by drop. 6-8 hours after transfection, cells were washed with PBS and feed 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-TIM-3 antibody tagged with phycoerythrin (PE) and Anti-PE Microbeads (Miltenyi-013-048-801). Stable single cell clones were isolated by limited dilution and screened by FACS using anti-TIM-3 antibodies.

EXAMPLE 3: Bispecific antibody Generation

[000298] 3.1 Generation of an anti-PD-1 antibody "W3055"

[000299] An anti-PD-1 antibody, W3055, was prepared according to Chinese patent application CN106432494A, which was referred to as "1.153.7hAb" therein.

[000300] 3.2 Generation of an anti-TIM-3 antibody "W3402" or "W3405"

[000301] 3.2.1 Research materials preparation

[000302] Production of Antigens. DNA sequences encoding truncated (ECD and transmembrane) or full length of human TIM-3 (GenBank Accession No. NM_032782.3), mouse TIM-3 (GenBank Accession No. NM_134250.2) and cynomolgus monkey TIM-3 (GenBank Accession No. EHH54703.1) were synthesized in Sangon Biotech (Shanghai, China).

[000303] Stable cell lines: Cells expressing human (W340-CHO-K1.hPro1.G2, and W340-CHO-K1.hPro1.H1), mouse (W340-CHO-K1.mPro1.D3) or cynomolgus monkey TIM-3 (W340.293F.cynoPro1.17), as well as parental cell lines were provided by Biologics Discovery RM group.

[000304] 3.2.2 Hybridoma generation

[000305] 3.2.2.1 Hybridoma generation for W3402

[000306] Immunization and cell fusion: SD rats, 6-8 weeks of age, were immunized weekly by footpad and subcutaneous injections with 25 μg/animal of WBP340-hPro1.ECD.mFc or 25 μg/animal of WBP340-mPro1.ECD.hFc in adjuvant alternately. Post the 4^{th} immunization, serum samples were collected and examined every two weeks. Anti-hTIM-3 and anti-mTIM-3 antibody titers in the serum samples were determined by ELISA. Briefly, the plates coated with hTIM-3.ECD.His or mTIM-3.ECD.His were co-incubated with diluted rat serum (first 1:100, then 3-fold dilution in 2% BSA/PBS) for two hours. Goat anti rat-IgG-Fc-HRP was used as secondary antibody. The color was developed by dispensing 100 μL of TMB substrate, and then stopped by 100 μL of 2N HCl. The absorbance was read at 450 nM using a microplate spectrophotometer. When the serum antibody titer was sufficiently high, rats were given a final boost with both human and mouse TIM-3 ECD protein in D-PBS without adjuvant. On the day of fusion, lymph nodes and spleen were removed from immunized animal under sterile condition, and prepared into single cell suspension. The isolated cells were then mixed with myeloma cell SP2/0 at a ratio of 1:1. Electro cell fusion was performed using BTX 2001 Electro cell manipulator according to manufacturer's instruction. The cells were then seeded in 96-well plates at the density of 1×10^4 cells/well, and cultured at 37 °C, 5% CO_2, until ready for screening.

[000307] First and confirmation screen of hybridoma supernatants: human TIM-3-expressing cell binding assay by mirrorball was used as first screen method to test the binding of hybridoma supernatants to human TIM-3. Briefly, hybridoma supernatant samples, positive control and negative control were added into the 384-well plates, and co-incubated with human TIM-3 transfectant cells (WBP340.CHO-K1.hPro1.G2). Goat anti-rat IgG Fc PE antibody was used to determine the binding of anti-hTIM-3 antibody to the cells. Samples that had MFI$\geqslant$100 were considered positive hTIM-3 binders (NC: -15~10).

[000308] In order to confirm the initial binding results, the positive hybridoma line was further tested by FACS using WBP340.CHO-K1.hPro1.G2 as follow: Hybridoma supernatants were added to the cells, and the binding of rat antibodies onto the surface of the cells were detected by Alexa647-labeled goat anti-rat antibody. The MFI was evaluated by a flow cytometer and analyzed by FlowJo. Antibody binding to parental CHO-K1 cells was used as negative control.

[000309] 3.2.2.2 Hybridoma generation for W3405

[000310] Immunization: OMT rats (transgenic rats having recombinant immunoglobulin loci, as described and produced in US8,907,157 B2), 10~11 weeks of age, were immunized weekly by footpad and subcutaneous injections with 25 μg/animal of hTIM-3.ECD.mFc or 25 μg/animal of mTIM-3.ECD.hFc in adjuvant alternately.

[000311] Serum titer detection

[000312] Post the 4th immunization, serum samples from the immunized OMT rats were collected and examined every two weeks. Anti-hTIM-3 and anti-mTIM-3 antibody titers in the serum samples were determined by ELISA. Briefly, the plates coated with hTIM-3.ECD.His or mTIM-3.ECD.His were co-incubated with diluted rat serum (first 1:100 by volume, then 3-fold dilution in 2% BSA/PBS) for two hours. Goat anti rat-IgG-Fc-HRP was used as secondary antibody. The color was developed by dispensing 100 μL of TMB substrate, and then stopped by 100 μL of 2N HCl. The absorbance was read at 450 nM using a microplate spectrophotometer.

[000313] When the serum antibody titer was sufficiently high, the OMT rats were given a final boost with both human and mouse TIM-3 ECD proteins in D-PBS without adjuvant. On the day of fusion, lymph nodes were removed from the immunized OMT rats under sterile condition, and prepared into single cell suspension. The isolated cells were then mixed with myeloma cell SP2/0 at a ratio of 1:1. Electro cell fusion was performed using BTX 2001 Electro cell manipulator according to manufacturer's instruction. The cells were then seeded in 96-well plates at the density of 1×10^4 cells/well, and cultured at 37°C, 5% CO_2, until ready for screening.

[000314] 3.2.3 Hybridoma subcloning:

[000315] Once specific binding was verified through first and confirmation screening, the positive hybridoma cells were subcloned to get monoclonal anti-hTIM-3 antibodies by using semi-solid medium approach. The positive clones were confirmed by binding ELISA and FACS against human TIM-3 as described above.

[000316] 3.2.4 Hybridoma sequencing

[000317] Total RNA was isolated from hybridoma cells by using RNeasy Plus Mini Kit and first strand cDNA was prepared as follows:

[000318] cDNA amplification reaction (20 μL)

Component	Amount

Up to 5 μg total RNA	5 μL
Primer (50 μM oligo(dT)$_{20}$/50 ng/μL random hexamers)	1 μL/1μL
Annealing Buffer	1 μL
Bring the volume to 8 μL using RNase/DNase-free water	
65°C for 5min, then immediately place on ice for at least 1 minute	
2X First-Strand Reaction Mix	10 μL
SuperScript™ III/RNaseOUT™ Enzyme Mix	2 μL

[000319] cDNA amplification reaction condition

	Step 1	Step 2	Step 3	Step 4
Temperature (℃)	25	50	85	4
Time	10 min	50 min	5 min	∞

[000320] Antibody VH and VL genes were amplified from cDNA using 3'- constant region degenerated primer and 5'- degenerated primer sets, which are complementary to the upstream signal sequence-coding region of Ig variable sequences. The PCR reaction was done as follows:

[000321] PCR reaction system (50 μL)

Component	**Amount**
cDNA	2.0 μL
Premix Ex Taq	25 μL
5'- degenerated primer sets (10 pM)	2.5 μL
3'- constant region degenerated primer (10 pM)	1 μL
ddH_2O	19.5 μL

[000322] PCR reaction condition

	Step 1	Step 2	Step 3	Step 4	Step 5
Temperature (℃)	95	94	58	72	72
Time	4 min	45 sec	45 sec	1 min	10 min
Cycles	NA	30			NA

[000323] PCR product (10 μL) was ligated into pMD18-T vector and 10 μL of the ligation product was transformed into Top10 competent cells. Positive clones were randomly picked for sequencing by Shanghai Biosune Biotech Co., Ltd. Parental antibodies for W3402 and W3405 were obtained, named as "W3402-2.131.17" and "W3405-2.61.21", respectively.

[000324] 3.2.5 Antibody Optimization

[000325] 3.2.5.1 Humanization for W3402-2.131.17

[000326] "Best Fit" approach was used to humanize the light and heavy chains of parental W3402 antibody. The amino acid sequences of VH and VL were blasted against in-house human germline V-gene database. The first sequences of the humanized VH and VL were derived by replacing human CDR sequences in the top hit with the lead antibody's CDR sequences using Kabat CDR definition. Frameworks were defined using extended CDR definition, where Kabat CDR1 was extended by 5 amino acids at the N-terminus. For heavy chains, sixteen additional sequences were created by adding back mutations based on the first humanized VH sequence. Finally, the antibody "W3402" was obtained, and the sequence information for the antibody W3402 is provided in Table 1 and Table 2.

[000327] 3.2.5.2 Optimization for W3405-2.61.21

[000328] Fully human antibody construction

[000329] W3405-2.61.21 VH and VL genes were re-amplified with cloning primers containing appropriate restriction sites. DNA sequence encoding variable region of WBP3405-2.61.21 with the human IgG4 light chain on the C-terminal was cloned into a modified pcDNA3.3 expression vector. DNA sequence encoding variable region of WBP3405-2.61.21 with the constant region of human IgG4 (S228P) heavy chain on the C-terminal was cloned into a modified pcDNA3.3 expression vector, to express a fully human antibody named "W3405-2.61.21-uAb-hIgG4K."

[000330] Optimization and PTM removal

[000331] The fully human antibody "W3405-2.61.21-uAb-hIgG4K" was further optimized to improve expression level. Further, PTM site removing mutations were introduced by site-directed mutagenesis using QuickChange mutagenesis kit (Agilent Genomics) according to the manufacturer's protocol. Finally, the antibody "W3405" was obtained, and the sequence information for the antibody W3405 is provided in Table 1 and Table 2.

[000332] 3.3 Design and engineering of antibody and TCR chimeric proteins

[000333] TCR sequences

[000334] TCRs are heterodimeric proteins made up of two chains. About 95% human T cells have TCRs consisting of alpha and beta chains. Considering that more crystal structures are available for beta chain TRBC1, TRBC1 sequences were chosen as the major backbone to design the polypeptide complex disclosed herein ("WuXiBody"). A typical amino acid sequence of TRBC1 can be found in Protein Data Bgank (PDB) structure 4L4T.

[000335] Interchain disulphide-bond of TCR

[000336] TCR crystal structures were used to guide our WuXiBody design. Unlike native TCR anchored on the membrane of T cell surface, soluble TCR molecules are less stable, although its 3D structure is very similar to antibody Fab. As a matter of fact, the instability of TCR in soluble condition used to be a big obstacle that prevents the elucidation of its crystal structure (see Wang, *Protein Cell*, 5(9), pp.649–652 (2014)). We adopted a strategy of introducing a pair of Cys mutations in the TCR constant region and found it can significantly improve chain assembly and enhance expression.

[000337] The conjunctions connecting antibody variable and TCR constant domains, their relative fusion orientations, as well as the Fc-connecting conjunctions were all carefully fine-turned. As TCR structure is very similar to antibody Fab, we superimposed the antibody Fv homology model on TCR variable region (PDB 4L4T). The superimposed structure indicates that antibody Fv is structurally compatible with TCR constant domain. Based on this structural alignment and corresponding sequences, all the relevant engineering parameters were designed.

[000338] 3.4 Construction of expression vectors

[000339] Construction of bispecific antibodies: DNA sequence encoding VH and VL of an anti-PD1 antibody (e.g., W3055 as prepared in Section 3.1 above) was linked to the N- or C-terminus of an anti-TIM-3 antibody (e.g., W3402 or W3405 as prepared in Section 3.2 above). TCR constant domains were inserted to facilitate the correct pairing of VH and VL. The genes coding the above-mentioned bispecific antibodies were cloned into a modified pcDNA3.3 expression vector.

[000340] 3.5 Small scale Transfection, expression and purification

[000341] Heavy chain and light chain expression plasmids were co-transfected into ExpiCHO cells using expression kit (ThermoFisher- A29133) according to the manufacturer's instructions. 10 days after transfection, the supernatants were collected and used for protein purification using Protein A column (GE Healthcare-17543802) and further size exclusion chromatography (GE Healthcare-17104301). Antibody concentration was measured by Nano Drop. The purity of

proteins was evaluated by SDS-PAGE and SEC-HPLC. The bispecific antibodies were obtained after expression and purification.

[000342] The antibodies were purified using Protein A and analyzed using SEC-HPLC (Figure 1). One step protein A purification results in high purity of the protein >90%. The retention time around 6.7 min indicates the correct M.W of the samples.

[000343] Two bispecific antibodies, W3598-U6T1.G25-1.hIgG4.SP (also named as "W3598B") and W3598-U6T3.G25-1.uIgG4.SP (also named as "W3598C") were obtained, and used for in vitro characterization.

EXAMPLE 4: *In vitro* Characterization

[000344] 4.1 Differential scanning fluorimetry (DSF)

[000345] DSF was used to test the thermal stability of the bispecific antibodies. 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 and the resulting fluorescence data was collected. The data was analyzed automatically by its operation software and Th was calculated by taking the maximal value of negative derivative of the resulting fluorescence data with respect to temperature. Ton can be roughly determined as the temperature of negative derivative plot beginning to decrease from a pre-transition baseline.

[000346] As shown in Figure 2, T_h1 of the bispecific antibodies were approximately 63 °C.

[000347] 4.2 TIM-3- binding ELISA and FACS

[000348] A 96-well ELISA plate (Nunc MaxiSorp, ThermoFisher) was coated overnight at 4℃ with 0.5 μg/ml antigen (TIM-3-ECD) in Carbonate-bicarbonate buffer. After a 1 hour blocking step with 2% (w/v) bovine serum albumin (Pierce) dissolved in PBS, serial dilutions of the different TIM-3×PD-1 bispecific antibodies in PBS containing 2% bovine serum albumin were incubated on the plates for 2 hours at room temperature. Following the incubation, the plates were washed three times with 300 μL per well of PBS containing 0.5% (v/v) Tween 20. 100 ng/ml Goat-anti-human IgG Fc-HRP (Bethyl, #A80-304P) is added and incubated on the plates for 1 hour at room 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) is added for the detection. The reaction is stopped after approximate 8 minutes through the addition of 100

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

[000349] Binding of TIM-3×PD-1 bispecific antibodies to TIM-3 expressing cells was determined by flow cytometry. Briefly, 1×10^5 TIM-3 over-expressed stable cells were incubated for 60 minutes at 4℃ with serial dilutions of TIM-3×PD-1 bispecific or hIgG4 isotype control antibodies. After washing twice with cold PBS supplemented with 1% bovine serum albumin (wash buffer), cell surface bound antibody was detected by incubating the cells with fluorescence-labeled anti-human IgG antibody for 30 minutes at 4℃. The cells were washed twice in the same buffer and the mean fluorescence (MFI) of stained cells was measured using a FACS Canto II cytometer (BD Biosciences). The wells containing no antibody or secondary antibody only were used to establish background fluorescence. Four-parameter non-linear regression analysis was used to obtain EC_{50} values for cell binding using GraphPad Prism software.

[000350] The two antibodies bound to human TIM-3 with EC_{50} of 0.28 and 0.33 nM (Figure 5) and to cyno TIM-3 with EC_{50} of 0.61 and 0.89 nM (Figure 6), more potent than a benchmark bispecific antibody (W359.BMK1.uIgG1).

[000351] 4.3 Human PD-1- binding ELISA and FACS

[000352] For ELISA binding, non-tissue culture treated flat-bottom 96-well plates were pre-coated with 0.5ug/ml in house human PD-1 protein WBP305-hPro1.ECD.mFc overnight at 4°C. After 2% BSA blocking, 100 μL 3-fold titrated Abs from 25 nM to 0.0001 nM Abs were pipetted into each well and incubated for 1 hour at ambient temperature. Following removal of the unbound substances, HRP-labeled goat anti-human IgG were added to the wells and incubated for 1 hour. The color was developed by dispensing 100 μL TMB substrate, and then stopped by 100 μL 2N HCl. The absorbance was read at 450 nm using a Microplate Spectrophotometer.

[000353] For FACS binding, engineered human PD-1 expressing cells WBP305.CHO-S.hPro1.C6 were seeded at 1×10^5 cells/well in U-bottom 96-well plates. 3-Fold titrated Abs from 83.3 nM to 0.001 nM were added to the 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 and the mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000354] Results show that the bispecific antibodies can bind to human PD1 with EC_{50} of 1.78 nM and 2.38 nM (Figure 3).

[000355] 4.4 Mouse and cynomolgus monkey PD-1-binding

[000356] Antibodies were detected on their binding to mouse PD-1 in a FACS assay. Briefly, engineered mouse PD-1 expressing cells WBP305.293F.mPro1.B4 were seeded at 1×10^5 cells/well in U-bottom 96-well plates. 3-Fold titrated Abs from 133.3 nM to 0.06 nM were added to the 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 and the mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000357] Cynomolgus monkey PD-1-binding ELISA was used to test the antibodies. Briefly, flat-bottom 96-well plates were pre-coated with 0.5ug/ml in-house made cynomolgus PD-1 protein WBP305-cPro1.ECD.his overnight at 4°C. After 2% BSA blocking, 100 μL 3-fold titrated Abs from 25 nM to 0.0001 nM Abs were pipetted into each well and incubated for 1 hour at ambient temperature. Following removal of the unbound substances, HRP-labeled goat anti-human IgG was added to the wells and incubated for 1 hour. The color was developed by dispensing 100 μL TMB substrate, and then stopped by 100 μL 2N HCl. The absorbance was read at 450 nm using a Microplate Spectrophotometer.

[000358] Results show that the antibodies also can bind to cyno PD1 with EC_{50} of 1.64 and 2.01 nM (Figure 4).

[000359] 4.5 PD-1 and TIM-3 dual binding (ELISA and FACS)

[000360] In order to test whether the bispecific antibodies could bind to both PD-1 and TIM-3, an ELISA assay was developed as below. A 96-well ELISA plate (Nunc MaxiSorp, ThermoFisher) was coated overnight at 4℃ with 0.5 μg/ml TIM-3-ECD in carbonate-bicarbonate buffer. After a 1 hour blocking step with 2% (w/v) bovine serum albumin (Pierce) dissolved in PBS, serial dilutions of the different TIM-3×PD-1 bispecific antibodies in PBS containing 2% bovine serum albumin are incubated on the plates for 1 hour at room temperature. Following the incubation, plates are washed three times with 300 μL per well of PBS containing 0.5% (v/v) Tween 20. 0.1 μg/ml PD1-ECD-hFc.Biotin) was added to plates and incubation 1 hour. After washing the plates three times, Streptavidin-HRP (Invitrogen, #SNN1004)(1:25000 diluted) is added and incubated on the plates for 1 hour at room 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) is added for the detection. The reaction is 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^e).

[000361] As shown in Figure 7, the two antibodies bound to both targets with EC_{50} of 0.19 and 1.66 nM, respectively, whereas the bispecific benchmark antibody showed minimal signal.

[000362] 4.6 TIM-3 and PD-1 Affinity (SPR)

[000363] SPR technology was used to measure the on-rate constant (ka) and off-rate constant (kd) of the antibodies to ECD of TIM-3 or PD-1. The affinity constant (KD) was consequently determined.

[000364] 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 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 s with the activation buffer. 30 μg/mL of goat anti-human IgG Fcγ antibody in 10 mM NaAc (pH 4.5) was then injected to Fc1-Fc4 channels for 200s at a flow rate of 5 μL/min. The chip was deactivated by 1 M ethanolamine-HCl (GE). Then the antibodies were captured on the chip. Briefly, 4 μg/mL antibodies in running buffer (HBS-EP+) was injected individually to Fc3 channel for 30 s 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 analyte ECD of TIM-3 or PD-1 and blank running buffer were injected orderly to Fc1-Fc4 channels at a flow rate of 30 μL/min for an association phase of 120 s, followed by 2400 s dissociation phase. Regeneration buffer (10 mM Glycine pH 1.5) was injected at 10 μL/ min for 30 s following every dissociation phase.

[000365] The sensorgrams of blank surface and buffer channel were subtracted from the test sensorgrams. The experimental data was fitted by 1:1 model using Langmiur analysis.

[000366] As shown in Figure 8, the two bispecific antibodies bind to human PD1 with K_D of 1.09 nM and 1.72 nM, comparable to their parental antibody. The two bispecific antibodies bind to cyno PD1 with KD of 6.67 nM and 1.16 nM. Their binding to human TIM-3 with K_D of 0.344 nM and 0.201 nM, respectively.

[000367] 4.7 Antibody competition with PD-1/PDL1 assays

[000368] Both ELISA and FACS were used to measure antibody blocking PD-1 and PD-L1 interaction.

[000369] In an ELISA, flat-bottom 96-well plates were pre-coated with 0.5ug/ml WBP305-hPro1.ECD.hFc overnight at 4 °C. After 2% BSA blocking, 100 μL 3-fold titrated Abs from 25

nM to 0.0001 nM Abs coupled with 2ug/ml in house human PD-L1 protein W315-hPro1.ECD.mFc were pipetted into each well and incubated for 1 hour at ambient temperature. The binding of W315-hPro1.ECD.mFc to WBP305-hPro1.ECD.hFc was detected by HRP-labeled goat anti-mouse IgG. The color was developed by dispensing 100 μL of TMB substrate, and then stopped by 100 μL of 2N HCl. The absorbance was read at 450 nM using a Microplate Spectrophotometer.

[000370] For blocking human PD-L1 binding to human PD-1 by FACS, engineered human PD-1 expressing cells WBP305.CHO-S.hPro1.C6 were seeded at 1×10^5 cells/well in U-bottom 96-well plates. 3-Fold titrated Abs from 250 nM to 0.001 nM coupled with 5ug/ml in house human PD-L1 protein W315-hPro1.ECD.mFc were added to the cells. Plates were incubated at 4 ^{o}C for 1 hour. After wash, the binding of W315-hPro1.ECD.mFc to cell expressive human PD-1 was detected by PE-labeled goat anti-mouse antibody. The competition binding of antibodies to the cells was tested by flow cytometry and the mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000371] For blocking mouse PD-L1 binding to mouse PD-1 by FACS, engineered mouse PD-1 expressing cells WBP305.293F.mPro1.B4 were seeded at 1×10^5 cells/well in U-bottom 96-well plates. 3-Fold titrated Abs from 400 nM to 0.183 nM coupled with 5ug/ml in house mouse PD-L1 protein W315-mPro1.ECD.mFc were added to the cells. Plates were incubated at 4 ^{o}C for 1 hour. After wash, the binding of W315-mPro1.ECD.mFc to cell expressive mouse PD-1 was detected by PE-labeled goat anti-mouse antibody. The competition binding of antibodies to the cells was tested by flow cytometry and the mean fluorescence intensity (MFI) was analyzed by FlowJo.

[000372] Results show that the two bispecific antibodies can block PDL1 binding to PD1+ cells with IC_{50} of 2.00 and 2.59 nM (Figure 9), and they can also block the interaction of PS-TIM-3, with IC_{50} of 21.61 nM and 19.87 nM (Figure 10).

[000373] 4.8 Mixed lymphocyte reaction (MLR)

[000374] Mixed lymphocyte reaction (MLR) was used to test the agonistic effect of antibodies on cytokine secretion and proliferation of activated $CD4^+$ T cells.

[000375] 4.8.1 Cell isolation, cell culture and induction

[000376] Human peripheral blood mononuclear cells (PBMCs) were freshly isolated from healthy donors using Ficoll-Paque PLUS gradient centrifugation. Isolated PBMCs were cultured in complete RPMI-1640 (containing 10% FBS and 1% PS) supplemented with 100 U recombinant human IL-2.

[000377] 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 recombinant human GM-CSF and 50 ng/mL rhIL-4. Cell suspension was seeded at a concentration of 2×10^6 cells/mL, 2.5 mL/well in 6-well plate. Cells were cultured for 5 to 7 days to differentiate into DCs. Cytokines were replenished every 2-3 days by replacing half of the media with fresh media supplemented with cytokines. Eighteen to twenty-four hours before MLR, 1 μg/mL LPS was added to the culture to induce DCs maturation.

[000378] Human $CD4^+$ T cells were isolated using Human $CD4^+$ T cell Enrichment kit according to the manufacturer's protocol.

[000379] 4.8.2 Mix lymphocyte reaction

[000380] For human allogeneic MLR, purified $CD4^+$ T cells were co-cultured with immature or mature allogeneic DCs (iDCs or mDCs). MLR was set up in 96-well round bottom plates using complete RPMI-1640 medium. $CD4^+$ T cells, various concentrations of antibodies, and iDC or mDC were added to the plates. The plates were incubated at 37°C, 5% CO_2. IL-2 and IFN-γ production was determined at day 3 and day 5, respectively. The cells were harvest at day 5 to measure $CD4^+$ T cell proliferation by ^{3}H-TDR.

[000381] 4.8.3 Cytokine detection

[000382] Human IL-2 and IFN-γ release were measured by ELISA using matched antibody pairs. Recombinant human IFN-γ and IL-2 were used as standards, respectively. The plates were pre-coated with human IL-2 or IFN-γ capture antibody overnight at 4 °C. After blocking, 100 μL of standards or samples were pipetted into each well and incubated for 2 hours at ambient temperature. Following removal of the unbound substances, the biotin-conjugated detecting antibody for corresponding cytokine were added to the wells and incubated for 1 hour. HRP-streptavidin was then added to the wells for 30 minutes incubation at ambient temperature. The color was developed by dispensing 100 μL of TMB substrate, and then stopped by 100 μL of 2N HCl. The absorbance was read at 450 nM using a Microplate Spectrophotometer.

[000383] Results show that the two bispecific antibodies can enhance cytokine release in MLR assay (Figure 11).

[000384] 4.9 Reporter gene assay

[000385] Jurkat E6.1 cells, which were stably integrated IL-2 luciferase reporter gene, were transfected to express human TIM-3. The TIM-3+ Jurkat cells were activated by anti-CD28 antibody and anti-CD3 antibody in the presence of various concentrations of testing antibodies

overnight at 37°C, 5% CO2. After incubation, reconstituted luciferase substrate was added and the luciferase intensity was measured by a microplate spectrophotometer.

[000386] Results show that the two antibodies can inhibit signaling in TIM-3 report gene assay (Figure 12).

Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments that have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

WHAT IS CLAIMED IS

1. A bispecific polypeptide complex comprising a first antigen-binding moiety associated with a second antigen-binding moiety, wherein:

the first antigen-binding moiety comprises:

a first polypeptide comprising, from N-terminus to C-terminus, a first heavy chain variable domain (VH1) of a first antibody operably linked to a first T cell receptor (TCR) constant region (C1), and

a second polypeptide comprising, from N-terminus to C-terminus, a first light chain variable domain (VL1) of the first antibody operably linked to a second TCR constant region (C2),

wherein:

C1 and C2 are capable of forming a dimer comprising at least one non-native interchain bond between C1 and C2, and the non-native interchain bond is capable of stabilizing the dimer;

and

the second antigen-binding moiety comprises:

a second heavy chain variable domain (VH2) of a second antibody operably linked to an antibody heavy chain CH1 domain, and

a second light chain variable domain (VL2) of the second antibody operably linked to an antibody light chain constant (CL) domain,

wherein:

one of the first and the second antigen-binding moiety is an PD-1 binding moiety, and the other one is a TIM-3 binding moiety,

the PD-1 binding moiety is derived from an anti-PD-1 antibody comprising:

a) a heavy chain CDR1 comprising an amino acid sequence of SEQ ID NO: 17,

b) a heavy chain CDR2 comprising an amino acid sequence of SEQ ID NO: 19,

c) a heavy chain CDR3 comprising an amino acid sequence of SEQ ID NO: 21,

d) a light chain CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 18,

e) a light chain CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20, and

f) a light chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 22; and

the TIM-3 binding moiety is derived from an anti-TIM-3 antibody comprising:

a) a heavy chain CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and 7,

b) a heavy chain CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and 9,

c) a heavy chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 5 and 11,

d) a light chain CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 2 and 8,

e) a light chain CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4 and 10, and

f) a light chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 6 and 12.

2. The bispecific polypeptide complex of claim 1, wherein the first antigen-binding moiety is the PD-1 binding moiety, and the second antigen-binding moiety is the TIM-3 binding moiety.

3. The bispecific polypeptide complex of claim 1 or 2, wherein the PD-1 binding moiety comprises a heavy chain variable region comprising or consisting of SEQ ID NO: 34, and a light chain variable region comprising or consisting of SEQ ID NO: 24.

4. The bispecific polypeptide complex of claim 1 or 2, wherein the TIM-3-binding moiety comprises a heavy chain variable region comprising or consisting of SEQ ID NO: 13 or 15, and a light chain variable region comprising or consisting of SEQ ID NO: 14 or 16.

5. The bispecific polypeptide complex of any of the preceding claims, wherein C1 has the amino acid sequence as set forth in SEQ ID NO: 25.

6. The bispecific polypeptide complex of any of the preceding claims, wherein C2 has the

amino acid sequence as set forth in SEQ ID NO: 26.

7. The bispecific polypeptide complex of any of the preceding claims, wherein the first polypeptide of the PD-1 binding moiety is linked to the VH2 of the TIM-3 binding moiety via a spacer.

8. The bispecific polypeptide complex of claim 7, wherein the spacer comprises the amino acid of SEQ ID NO: 27.

9. The bispecific polypeptide complex of any of claims 1-8, wherein the bispecific polypeptide complex comprises a combination of three polypeptide sequences: SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

10. The bispecific polypeptide complex of any of claims 1-8, wherein the bispecific polypeptide comprises a combination of three polypeptide sequences: SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33.

11. The bispecific polypeptide complex of any of the preceding claims, wherein the TIM-3-binding moiety further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to TIM-3, and/or the PD-1 binding moiety further comprises one or more amino acid residue substitutions or modifications yet retains specific binding affinity to PD-1.

12. The bispecific polypeptide complex of claim 11, 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.

13. The bispecific polypeptide complex of any of the preceding claims, wherein the TIM-3-binding moiety is operably linked to the N terminus or the C terminus of PD-1 binding moiety.

14. The bispecific polypeptide complex of any of the preceding claims, wherein the PD-1 binding moiety is operably linked to the N terminus or the C terminus of the TIM-3-binding moiety.

15. The bispecific polypeptide complex of any of the preceding claims, wherein the bispecific

polypeptide complex further comprises an immunoglobulin constant region, optionally a constant region of IgG, or optionally a constant region of human IgG.

16. The bispecific polypeptide complex of claim 15, wherein the PD-1 moiety or the TIM-3-binding moiety is operably linked to the C terminus of the constant region.

17. The bispecific polypeptide complex of any of the preceding claims, wherein the TIM-3-binding moiety and/or the antigen-binding moiety is humanized.

18. The bispecific polypeptide complex of any of the preceding claims, wherein the TIM-3-binding moiety is capable of specifically binding to human TIM-3 at a K_D value of no more than 5×10^{-9}M, 5×10^{-10}M, or 5×10^{-11}M as measured by surface plasmon resonance (SPR), and/or the PD-1 binding moiety is capable of specifically binding to human PD-1 at a K_D value of no more than 10^{-8} or 5×10^{-8} M as measured by surface plasmon resonance.

19. The bispecific polypeptide complex of any of the preceding claims, wherein the TIM-3-binding moiety is capable of specifically binding to human TIM-3 expressed on a cell surface at an EC_{50} of no more than 5nM, 1nM, or 0.2 nM as measured by flow cytometry, and/or the antigen-binding moiety is capable of specifically binding to human PD-1 expressed on a cell surface at a K_D value of no more than 10^{-8} M or 5×10^{-10} M as measured by flow cytometry.

20. The bispecific polypeptide complex of any of the preceding claims, wherein the TIM-3-binding moiety is capable of specifically binding to Cynomolgus monkey TIM-3, rat TIM-3, and/or mouse TIM-3.

21. The bispecific polypeptide complex of any of the preceding claims, wherein the PD-1 moiety is capable of specifically binding to Cynomolgus monkey PD-1, and/or mouse PD-1.

22. The bispecific polypeptide complex of any of the preceding claims linked to one or more conjugate moieties.

23. The bispecific polypeptide complex of claim 22, 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.

24. A pharmaceutical composition comprising the bispecific polypeptide complex of any of claims 1-23, and a pharmaceutically acceptable carrier.

25. An isolated polynucleotide encoding the bispecific polypeptide complex of any of claims 1-23.

26. A vector comprising the isolated polynucleotide of claim 25.

27. A host cell comprising the vector of claim 26.

28. A method of expressing the bispecific polypeptide complex of any of claims 1-23, comprising culturing the host cell of claim 27 under the condition at which the vector of claim 26 is expressed.

29. 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 polypeptide complex of any of claims 1-23 or the pharmaceutical composition of claim 24.

30. The method of claim 29, wherein 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.

31. The method of claim 29 or 30, wherein the disease or condition is PD-1-related and/or TIM-3-related.

32. The method of any of claims 29-31, wherein the disease or condition is cancer, autoimmune disease, inflammatory disease, or infectious disease.

33. The method of any of claims 29-32, wherein the cancer is lymphoma, basal cell carcinoma, biliary tract cancer, bladder cancer, bone cancer, brain and central nervous system cancer,

breast cancer, peritoneal cancer, cervical cancer, uterine or endometrial cancer, choriocarcinoma, colon cancer, colorectal cancer, rectal cancer, connective tissue cancer, esophageal cancer, mesothelioma, nasopharyngeal cancer, eye cancer, head and neck cancer, anal cancer, gastrointestinal cancer, glioblastoma, intra-epithelial neoplasm, kidney or renal cancer, larynx cancer, leukemia, liver cancer, lung cancer, melanoma, myeloma, neuroblastoma, oral cavity cancer, germ cell cancer, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland carcinoma, sarcoma, skin cancer, squamous cell cancer, stomach cancer, testicular cancer, thyroid cancer, or vulval cancer.

34. The method of any of claims 29-33, wherein the disease or condition is B cell lymphoma, optionally Hodgkin lymphoma or non-Hodgkin lymphoma (NHL), wherein the NHL comprises: diffuse large B-cell lymphoma (DLBCL), small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, mantle cell lymphoma, AIDS-related lymphoma, Waldenstrom's Macroglobulinemia, chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), hairy cell leukemia, chronic myeloblastic leukemia, and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema, and Meigs' syndrome.

35. The method of any of claims 29-34, wherein the subject is human.

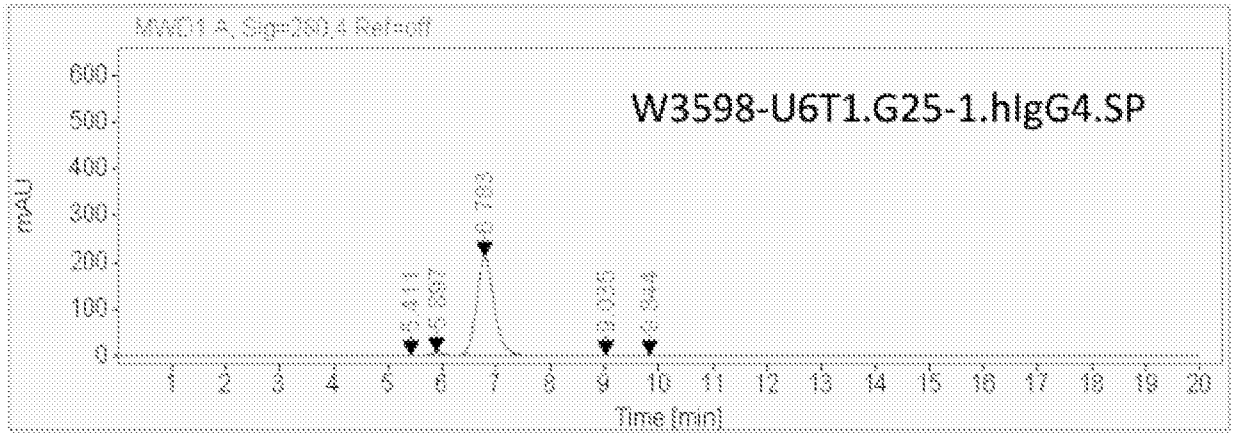
36. The method of any of claims 29-35, wherein the administration is via oral, nasal, intravenous, subcutaneous, sublingual, or intramuscular administration.

37. A method of modulating TIM-3 activity in a TIM-3-expressing cell, comprising exposing the TIM-3-expressing cell to the bispecific polypeptide complex of any of claims 1-23.

38. Use of the bispecific polypeptide complex of any of claims 1-23 in the manufacture of a medicament for treating a disease or condition that would benefit from upregulation of an immune response.

39. Use of the bispecific polypeptide complex of any of claims 1-23 in the manufacture of a medicament for treating a disease or condition that is PD-1 and/or TIM-3-related.

SEC-HPLC of purified antibodies



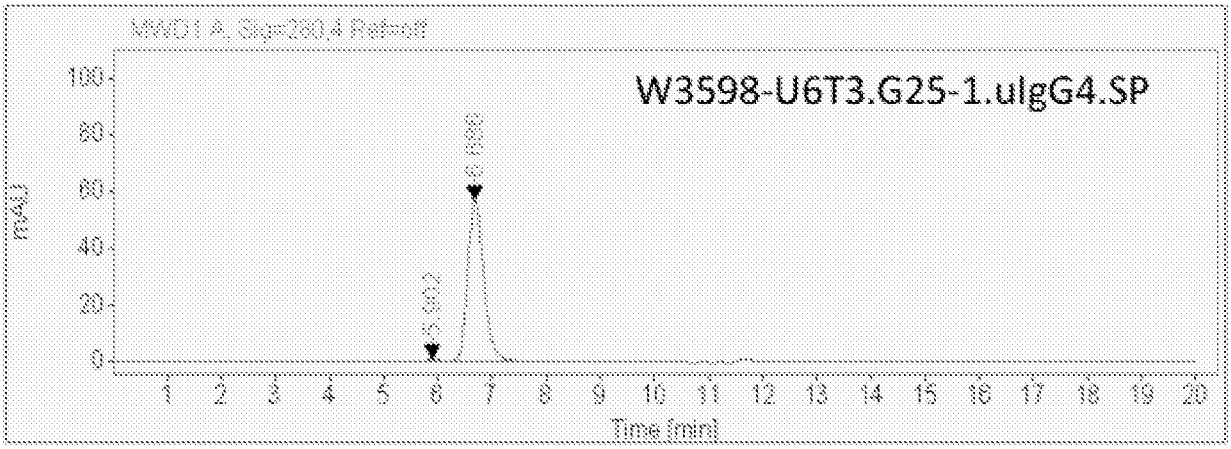


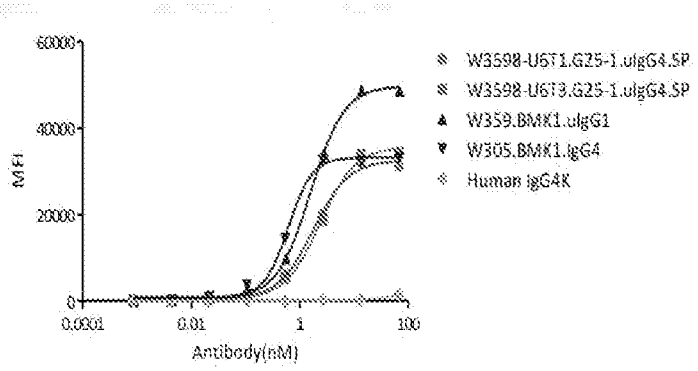
Figure 1

DSF

Ab Name	Isotype	pI	Buffer	T_m1 [°C]
W3598-U6T1.G25-1. hIgG4.SP	hIgG4, kappa, lambda	5.74	PBS	63.7
W3598-U6T3.G25-1.uIgG4.SP	hIgG4, kappa, lambda	5.63	50 mM NaAC +7% Sucrose pH 5.6	63.1

Figure 2

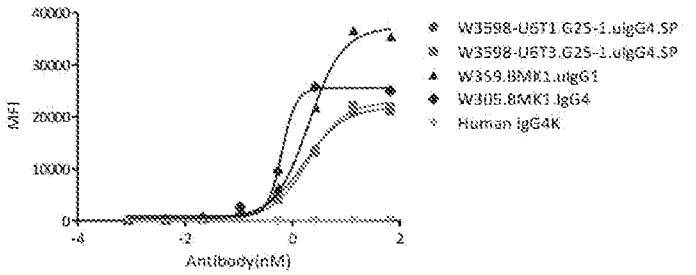
hPD-1-Binding as measured by FACS



Antibody	Top (MFI)	EC_{50} (nM)
W3598-U6T1.G25-1.uIgG4.SP	32540	1.72
W3598-U6T3.G25-1.uIgG4.SP	35639	2.38
W359.BMK1.uIgG1	49731	1.58
W305.BMK1.IgG4	33197	0.61
Human IgG4K	N.A.	N.A.

Figure 3

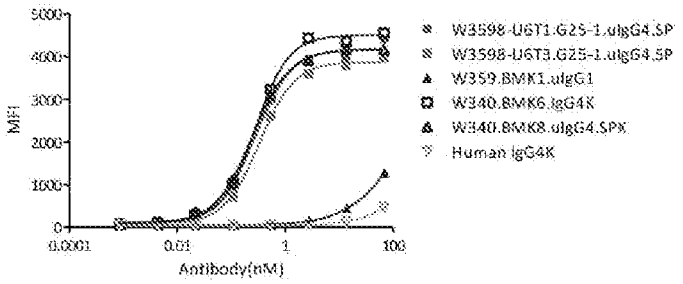
Cyno PD-1-Binding as measured by FACS



Antibody	Top (MFI)	EC_{50} (nM)
W3598-U6T1.G25-1.uIgG4.SP	21833	1.64
W3598-U6T3.G25-1.uIgG4.SP	22863	2.01
W359.BMK1.uIgG1	37097	2.01
W305.BMK1.IgG4	27163	0.74
Human IgG4K	N.A.	N.A.

Figure 4

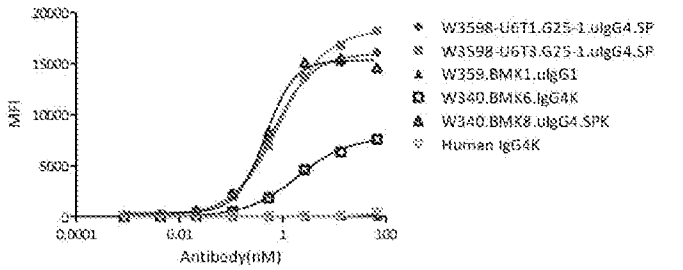
Human TIM3-Binding as measured by FACS



Antibody	Top (MFI)	EC50 (nM)
W3598-U6T1.G25-1.ulgG4.SP	4184	0.28
W3598-U6T3.G25-1.ulgG4.SP	3874	0.33
W359.8MK1.ulgG1	N.A.	N.A.
W340.8MK6.IgG4K	4506	0.28
W340.8MK8.ulgG4.SPK	4156	0.24
Human IgG4K	N.A.	N.A.

Figure 5

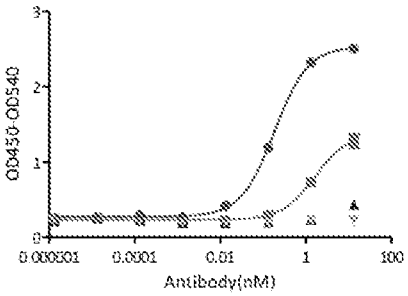
Cyno TIM3-Binding as measured by FACS



Antibody	Top (MFI)	EC_{50} (nM)
W3598-U6T1.G25-1.ulgG4.SP	15981	0.61
W3598-U6T3.G25-1.ulgG4.SP	18290	0.89
W359.8MK1.ulgG1	N.A.	N.A.
W340.8MK6.IgG4K	7832	1.95
W340.8MK8.ulgG4.SPK	15353	0.46
Human IgG4K	N.A.	N.A.

Figure 6

Dual Binding as measured by ELISA



Antibody	EC_{50} (nM)	Top (Mifi)
W3598-U6T1.G2S-1.uIgG4.SP	0.19	2.531
W3598-U6T3.G2S-1.uIgG4.SP	1.66	1.378
W359.8MK1.uIgG1	N.A.	N.A.
Human IgG4K	N.A.	N.A.

Figure 7

SPR Results

Antigens	Antibodies	Ka (1/Ms)	Kd (1/s)	KD (M)
Human PD1.ECD.His	W3598-U6T1.G2S-1.hIgG4.SP	6.00E+05	6.54E-04	1.09E-09
	W3598-U6T3.G2S-1.hIgG4.SP	4.95E+05	8.50E-04	1.72E-09
Cyno PD1.ECD.His	W3598-U6T1.G2S-1.hIgG4.SP	2.09E+05	1.39E-02	6.67E-08
	W3598-U6T3.G2S-1.hIgG4.SP	1.33E+06	1.55E-02	1.17E-08
Human TIM3.ECD.MBPHis	W3598-U6T1.G2S-1.hIgG4.SP	4.54E+05	1.56E-04	3.44E-10
	W3598-U6T3.G2S-1.hIgG4.SP	1.46E+05	2.93E-05	2.01E-10

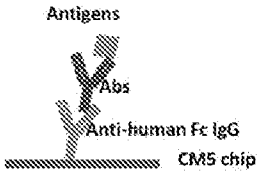
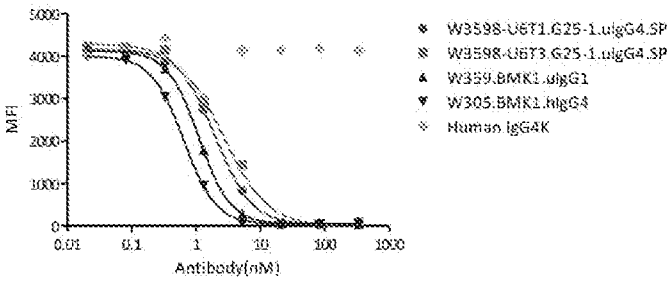


Figure 8

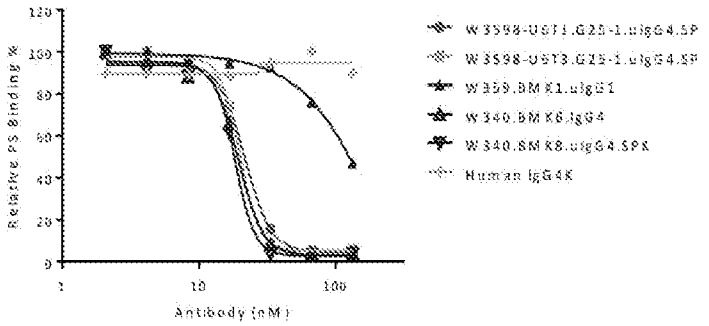
Competitive Binding to human PD1 as measured by FACS



Antibody	IC_{50} (nM)	Bottom (MFI)
W3598-U6T1.G25-1.ulgG4.SP	2.00	23.01
W3598-U6T3.G25-1.ulgG4.SP	2.59	-26.65
W359.BMK1.ulgG1	1.11	40.64
W305.BMK1.hIgG4	0.64	32.26
Human IgG4K	N.A.	N.A.

Figure 9

Competitive Binding to PS-TIM3 as measured by FACS



Antibody	IC_{50} (nM)	Bottom
W3598-U6T1.G25-1.ulgG4.SP	21.61	4.557
W3598-U6T3.G25-1.ulgG4.SP	19.87	5.248
W359.BMK1.ulgG1	N.A.	N.A.
W340.BMK8.IgG4	19.34	3.169
W340.BMK8.ulgG4.SPK	18.39	2.892
Human IgG4K	N.A.	N.A.

Figure 10

MLR Assay

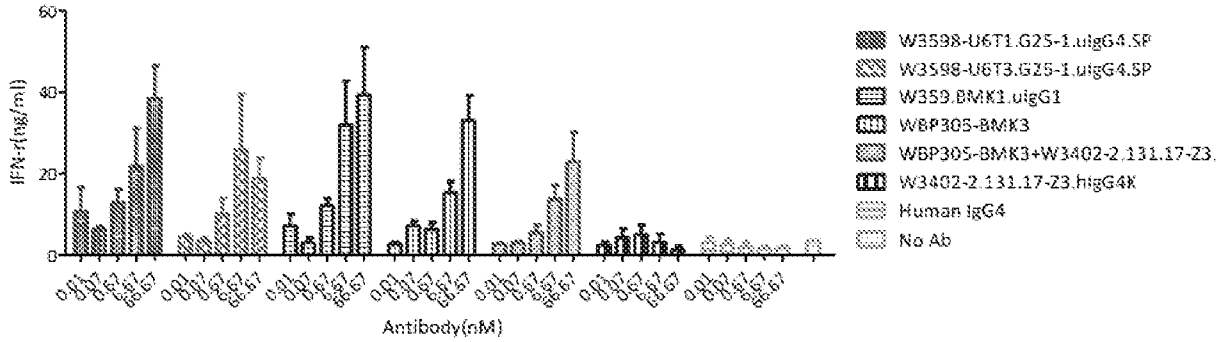
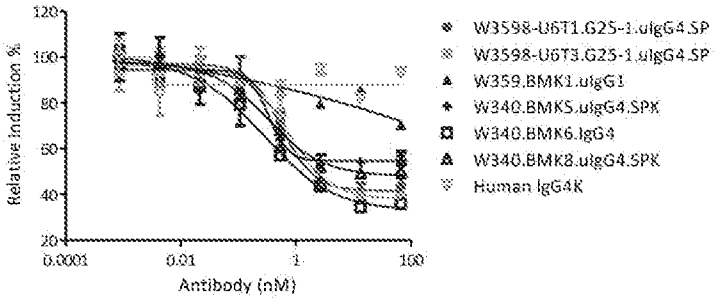


Figure 11

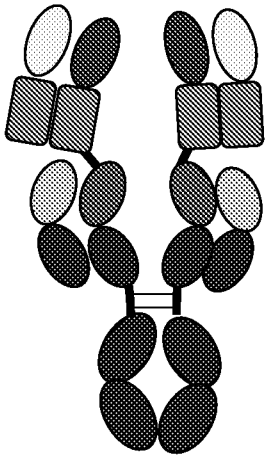
TIM3 Reporter Gene Assay



Ab	EC50 (nM)	Emax%
W3598-U6T1.G25-1.uIgG4.SP	0.56	41.58
W3598-U6T3.G25-1.uIgG4.SP	0.73	37.98
W359.BMK1.uIgG1	N.A.	N.A.
W340.BMK5.uIgG4.SPK	0.33	54.59
W340.BMK6.IgG4	0.27	32.74
W340.BMK8.uIgG4.SPK	0.32	48.12
Human IgG4K	N.A.	N.A.

Figure 12

Format G25



First antibody variable region

TCR constant region

Second antibody variable region
antibody constant region CH1/CL

antibody constant region CH2-CH3

Figure 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2020/081258

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i; C07K 16/28(2006.01)i; C07K 14/46(2006.01)i; C07K 16/46(2006.01)i; A61K 39/00(2006.01)i; C07K 16/00(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)

A61K, C07K

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,CNKI,CNTXT,WOTXT,USTXT,EPTXT,JPTXT,PubMed,ISI web of knowledge,google scholar:bispecific polypeptide,antigen,antibody,heavy chain, light chain, PD-1, TIM-3, CDR1-3, SEQ ID NO:13-16,23-27, immune response

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 106432494 A (HARBIN GLORIA PHARMACEUTICALS CO. LTD.) 22 February 2017 (2017-02-22) the whole document	1-39
A	CN 107847589 A (ANAPTYSBIO INC.) 27 March 2018 (2018-03-27) the whole document	1-39
A	CN 108350082 A (I-MAB BIOPHARMA CO. LTD.) 31 July 2018 (2018-07-31) the whole document	1-39
A	CN 108659112 A (SHANGHAI TONGJI HOSPITAL) 16 October 2018 (2018-10-16) the whole document	1-39
A	WO 2017019896 A1 (NOVARTIS AG et al.) 02 February 2017 (2017-02-02) the whole document	1-39
A	CN 107987169 A (LI, H.S.) 04 May 2018 (2018-05-04) the whole document	1-39

☐ 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
01 June 2020	**30 June 2020**
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 **LV,Jian** Telephone No. **86-(10)-53962098**

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2020/081258

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	106432494	A	22 February 2017	None			
CN	107847589	A	27 March 2018	SG	10201909173P	A	28 November 2019
				SG	11201707705U	A	30 October 2017
				MX	2017012429	A	26 January 2018
				EA	201792184	A1	30 April 2018
				EP	3277321	A1	07 February 2018
				KR	20170133458	A	05 December 2017
				WO	2016161270	A1	06 October 2016
				US	10508149	B2	17 December 2019
				EP	3277321	A4	12 December 2018
				IL	254559	D0	10 February 2019
				BR	112017021040	A2	24 July 2018
				AU	2016242964	A1	09 November 2017
				JP	2018512138	A	17 May 2018
				US	2018127500	A1	10 May 2018
				CA	2981520	A1	06 October 2016
CN	108350082	A	31 July 2018	US	2018346574	A1	06 December 2018
				MX	2018015584	A	18 September 2019
				KR	20180039182	A	17 April 2018
				KR	101996019	B1	03 July 2019
				US	2018208659	A1	26 July 2018
				US	2018346573	A1	06 December 2018
				IL	263509	D0	31 January 2019
				CL	2018003583	A1	17 May 2019
				SG	11201811003P	A	30 January 2019
				BR	112018075737	A2	26 March 2019
				JP	2019531256	A	31 October 2019
				AU	2017284632	A1	03 January 2019
				CA	3027209	A1	21 December 2017
				KR	20190079713	A	05 July 2019
				EP	3325513	A1	30 May 2018
				PH	12018502623	A1	07 October 2019
				WO	2017215590	A1	21 December 2017
				US	10059769	B2	28 August 2018
				EP	3325513	A4	19 December 2018
				US	10208119	B2	19 February 2019
				CO	2018013500	A2	19 February 2019
				PE	20190510	A1	10 April 2019
CN	108659112	A	16 October 2018	None			
WO	2017019896	A1	02 February 2017	US	2018222982	A1	09 August 2018
				EP	3328418	A1	06 June 2018
				JP	2018522027	A	09 August 2018
				CN	108025051	A	11 May 2018
CN	107987169	A	04 May 2018	None			