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(54) Title: ANTI-PD-1 ANTIBODIES AND USES THEREOF

(57) Abstract: Anti-PD-1 antibodies and antigen-binding fragments thereof are described. Also described are nucleic acids encoding the antibodies, compositions comprising the antibodies, and methods of producing the antibodies and using the antibodies for treat-
ing or preventing diseases such as cancers and autoimmune diseases.



ANTI-PD-1 ANTIBODIES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is entitled to priority pursuant to Chinese Patent Application No.201510451773.6, filed July 28, 2015, the disclosure of which is incorporated by reference herein in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0002] This application contains a sequence listing, which is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file name "Sequence Listing File", creation date of July 6, 2016 and having a size of about 40 kB. The sequence listing submitted via EFS-Web is part of the specification and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The invention relates to monoclonal anti-PD-1 antibodies, nucleic acids and expression vectors encoding the antibodies, recombinant cells containing the vectors, and compositions comprising the antibodies. Methods of making the antibodies, and methods of using the antibodies to treat diseases including cancer and autoimmune diseases are also provided.

BACKGROUND OF THE INVENTION

[0004] Tumor cells are able to evade the immune system by "editing" host immunity in the tumor microenvironment in a variety of ways. One way in which tumors carry out this so-called "cancer immune escape" is by upregulating the expression of immune checkpoint proteins, which are key regulators of the immune system, thus suppressing the immune response. One such immunosuppressive co-signal is mediated by the PD-1 receptor and its ligand PD-L1.

[0005] PD-1 (Programmed Cell Death Protein 1) is a type I transmembrane protein that is 288 amino acids long and is one of the major immune checkpoint molecules (Blank et al., 2005, Cancer Immunotherapy, 54:307-314). PD-1 is primarily expressed on activated T cells, and it interacts with the ligands PD-L1 and PD-L2 to induce an inhibitory signal resulting in reduced T cell proliferation, cytokine production, and cytotoxic activity (Freeman et al., 2000, J. Exp. Med., 192:1027-34). PD-L1 is expressed on many cell types, including T, B, endothelial, epithelial, and antigen presenting cells, on cells of lung, liver and heart tissues, and on several types of tumor cells. In contrast, PD-L2 is narrowly expressed on professional antigen presenting cells, such as dendritic cells and macrophages.

[0006] The interaction between PD-1 and PD-L1 is critical for modulating the immune response, and it is the predominant mechanism by which PD-L1-expressing tumor cells escape from immune surveillance (Zippelius et al., 2015, Cancer Immunol Res., 3(3):236-44). Persistent expression of PD-1 by T cells is highly indicative of an exhausted phenotype, noted by a decrease in effector function. This phenotype has been observed in various types of tumor-infiltrating lymphocyte (TIL) and linked to poor prognosis and tumor recurrence (Wherry, 2011, Nat. Immunol., 12:492-99).

[0007] Blocking of the PD-1/PD-L1 interaction can activate the immune system and enhance anti-tumor immune responses, and it has been demonstrated that in multiple syngeneic mouse tumor models, blockade of PD-1 or its ligands promotes antitumor activity (Hirano et al., 2005, *Cancer Res.*, 65:1089-96). Thus, the interaction between PD-1 and PD-L1 is an attractive target for cancer immunotherapy.

5 [0008] Cancer immunotherapy, a recent breakthrough in cancer treatment, employs a patient's own immune system to attack tumor cells. Inhibitors of immune checkpoint proteins have the potential to treat a variety of tumor types, such as metastatic melanoma, lung cancer, breast cancer, renal cell carcinoma, etc. Recent studies using cancer immunotherapy approaches have shown promising results, especially in the case of metastatic carcinomas (Weinstock and McDermott, 2015, *TherAdv Urol.*, 7(6):365-77). In addition, cancer immunotherapy has shown great potential in the treatment of blood
10 cancers, including Hodgkin's lymphoma, multiple myeloma, bone marrow dysplasia syndrome, non-Hodgkin's lymphoma, etc. (Zou and Chen L, 2008, *Nat Rev Immunol.*, 8(6):467-77). Side effects caused by immune checkpoint inhibitors are negligible, reversible and manageable, and an effective immune checkpoint inhibitor may substantially improve the overall survival of cancer patients. Immune
15 checkpoint inhibitors can be used in combination with targeted therapy or conventional radiotherapy and chemotherapy, and such combinatorial therapy may be effective in the treatment of many types of cancer. Clinical trials of anti-PD-1 monoclonal antibodies have been initiated by BMS, Merck, MedImmune and CureTech, including, for example, three trials at the market stage; and clinical trials of anti-PD-L1 mAbs have also been initiated by Roche, MedImmune, Merck Serono and BMS, including,
20 for example, three trials that have been withdrawn or terminated (clinicaltrials.gov, 2015).

[0009] Phase I clinical trials of a fully human IgG4 mAb from BMS, Nivolumab (Opdivo®), have been carried out. The first of those trials, carried out in 2010, was conducted in 39 patients with advanced solid tumors (Brahmer et al., 2010, *J. Clin. Oncol.*, 28:3167-75). The response was 3/39, with mostly immune-related adverse effects (AEs) and one serious AE. The second trial, carried out in 2012,
25 was conducted in 294 patients (Topalian et al., 2014, *J. Clin. Oncol.*, 32:1020-30). There were objective responses in 16% of the non-small cell lung cancer patients, 28% of the metastatic melanoma patients, 29% of the renal cell cancer patients, and 0% of the castrate-resistant prostate cancer or colorectal cancer patients. Grade 3/4 treatment-related AEs occurred in 15% of patients, and there were three deaths.

[0010] Thus, despite the progress, there is a need in the art for more effective therapeutics
30 comprising anti-PD-1 antibodies that effectively inhibit the PD-1/PD-L1 signaling activity while causing minimal adverse side effects in humans.

BRIEF SUMMARY OF THE INVENTION

[0011] The invention satisfies this need by providing monoclonal antibodies that specifically bind
35 PD-1 with high affinity and induce the secretion of IFN- γ and IL-2 by immune cells, as measured in both a mixed lymphocyte reaction and a T lymphocyte stimulation assay. In particular, the fully human anti-PD-1 antibodies of the invention have a higher affinity to PD-1 than Nivolumab. Additionally, the antibodies demonstrate comparable characteristics to Pembrolizumab (Keytruda®, Merck), a humanized IgG4 anti-PD-1 mAb, but are predicted to have fewer immunogenic adverse side effects in humans than
40 Pembrolizumab due to the fact that they are fully human, rather than simply humanized mAbs.

[0012] In one general aspect, the invention relates to isolated monoclonal antibodies or antigen-binding fragments thereof that bind PD-1.

[0013] According to a particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of:

- (1) SEQ ID NOs: 30, 31, 32, 26, 27, and 28, respectively;
- (2) SEQ ID NOs: 6, 7, 8, 2, 3, and 4, respectively;
- (3) SEQ ID NOs: 14, 15, 16, 10, 11, and 12, respectively;
- (4) SEQ ID NOs: 22, 23, 24, 18, 19, and 20, respectively;
- (5) SEQ ID NOs: 38, 39, 40, 34, 35, and 36, respectively;
- (6) SEQ ID NOs: 46, 47, 48, 42, 43, and 44, respectively; or
- (7) SEQ ID NOs: 54, 55, 56, 50, 51, and 52, respectively;

wherein the antibody or antigen-binding fragment thereof binds PD-1, preferably binds specifically to human PD-1.

[0014] According to another particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, 1, 9, 17, 33, 41 or 49, or a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29, 5, 13, 21, 37, 45 or 53.

[0015] According to one embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof of the invention is human/rat chimeric.

[0016] According to another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof of the invention is human.

[0017] According to yet another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof of the invention further comprises a constant region, preferably a human heavy chain IgG4 constant region, more preferably a human heavy chain IgG4 constant region having one or more mutations, such as a S228P mutation, and a human antibody light chain kappa constant region.

[0018] In another general aspect, the invention relates to an isolated nucleic acid encoding a monoclonal antibody or antigen-binding fragment thereof of the invention.

[0019] In another general aspect, the invention relates to a vector comprising an isolated nucleic acid encoding a monoclonal antibody or antigen-binding fragment thereof of the invention.

[0020] In another general aspect, the invention relates to a host cell comprising an isolated nucleic acid encoding a monoclonal antibody or antigen-binding fragment thereof of the invention.

[0021] In another general aspect, the invention relates to a pharmaceutical composition, comprising an isolated monoclonal antibody or antigen-binding fragment thereof of the invention and a pharmaceutically acceptable carrier.

[0022] In another general aspect, the invention relates to a method of blocking the binding of PD-1 to PD-L1 or PD-L2, or a method of augmenting secretion of IFN- γ and IL-2, in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention.

[0023] In another general aspect, the invention relates to a method of treating a disease, disorder or condition, preferably an infectious disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention.

5 [0024] In another general aspect, the invention relates to a method of treating a hyperproliferative disease in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention. The hyperproliferative disease can be a non-malignant disease, including but not limited to, atherosclerosis, benign hyperplasia, benign prostatic hypertrophy. The hyperproliferative disease can also be a tumor, or a malignant disease. The tumor can be selected from
10 the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.

15 [0025] In another general aspect, the invention relates to a method of producing a monoclonal antibody or antigen-binding fragment thereof of the invention, comprising culturing a cell comprising a nucleic acid encoding the monoclonal antibody or antigen-binding fragment under conditions to produce the monoclonal antibody or antigen-binding fragment thereof, and recovering the antibody or antigen-binding fragment thereof from the cell or cell culture.

20 [0026] In another general aspect, the invention relates to a method of producing a pharmaceutical composition comprising a monoclonal antibody or antigen-binding fragment thereof of the invention, comprising combining the monoclonal antibody or antigen-binding fragment thereof with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition.

25 [0027] Other aspects, features and advantages of the invention will be apparent from the following disclosure, including the detailed description of the invention and its preferred embodiments and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

30 [0028] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. It should be understood that the invention is not limited to the precise embodiments shown in the drawings.

[0029] In the drawings:

[0030] Figure 1 shows the binding activity of PD-1-hFc protein to biotin-labeled PD-L1-hFc;

[0031] Figure 2 shows the flow cytometry profile of HEK293 cells stably transfected with PD-1 protein;

35 [0032] Figure 3 shows the ELISA measurement of serum antibody titers of transgenic mice after immunization with PD-1 protein, where 1671-1675 represent the mouse ID numbers;

[0033] Figure 4A and Figure 4B show the binding activity of chimeric anti-PD-1 antibodies according to embodiments of the invention to human PD-1-hFc protein, as measured by ELISA;

40 [0034] Figure 5A and Figure 5B show the binding activity of chimeric anti-PD-1 antibodies according to embodiments of the invention to cyno monkey PD-1-hFc protein, as measured by ELISA;

[0035] Figure 6A and Figure 6B show the binding activity of chimeric anti-PD-1 antibodies according to embodiments of the invention to other immune checkpoint proteins, as measured by ELISA;

[0036] Figures 7A-7C show the cell-based binding activity of chimeric anti-PD-1 antibodies according to embodiments of the invention to CHO-K1-hPD-1, as measured by flow cytometry;

[0037] Figures 8A-8C show the cell-based binding activity of chimeric anti-PD-1 antibodies according to embodiments of the invention to CHO-K1-cPD-1, as measured by flow cytometry;

[0038] Figures 9A-9C show the inhibition of binding of PD-1 protein to its ligand PD-L1 by chimeric anti-PD-1 antibodies according to embodiments of the invention, as measured by a protein-based receptor ligand blocking assay;

[0039] Figures 10A-10C show the inhibition of binding of PD-1 protein to its ligand PD-L2 by chimeric anti-PD-1 antibodies according to embodiments of the invention, as measured by a protein-based receptor ligand blocking assay;

[0040] Figures 11A&11B show the inhibition of binding of PD-1 protein to its ligand PD-L1 by chimeric anti-PD-1 antibodies according to embodiments of the invention, as measured by a cell-based receptor ligand blocking assay;

[0041] Figures 12A&12B show the inhibition of binding of PD-1 protein to its ligand PD-L2 by chimeric anti-PD-1 antibodies according to embodiments of the invention, as measured by a cell-based receptor ligand blocking assay;

[0042] Figure 13 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a T lymphocyte stimulation assay using PBMC's;

[0043] Figure 14 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IL-2 secretion in a mixed lymphocyte reaction using PBMC's from donor 1;

[0044] Figure 15 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IL-2 secretion in a mixed lymphocyte reaction using PBMC's from donor 2;

[0045] Figure 16 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte stimulation assay using PBMC's from donor 1;

[0046] Figure 17 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte stimulation assay using PBMC's from donor 2;

[0047] Figure 18 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte stimulation assay using PBMC's from donor 1;

[0048] Figure 19 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte stimulation assay using PBMC's from donor 2;

[0049] Figure 20 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte stimulation assay using PBMC's from donor 3;

[0050] Figure 21 shows the effect of chimeric anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte stimulation assay using PBMC's from donor 4;

[0051] Figures 22A-22C show the binding activity of fully human anti-PD-1 antibodies according to embodiments of the invention to human PD-1-hFc protein, as measured by ELISA;

[0052] Figures 23A-23C show the binding activity of fully human anti-PD-1 antibodies according to embodiments of the invention to cyno monkey PD-1-hFc protein, as measured by ELISA;

[0053] Figures 24A&24B show the cell-based binding activity of fully human anti-PD-1 antibodies according to embodiments of the invention to CHO-K1-hPD-1, as measured by flow cytometry;

[0054] Figures 25A&25B show the cell-based binding activity of fully human anti-PD-1 antibodies according to embodiments of the invention to CHO-K1-cPD-1, as measured by flow cytometry;

5 [0055] Figure 26 shows the binding activity of fully human anti-PD-1 antibodies according to embodiments of the invention to activated human PBMCs, as measured by flow cytometry;

[0056] Figure 27 shows the binding activity of fully human anti-PD-1 antibodies according to embodiments of the invention to activated cyno monkey PBMCs, as measured by flow cytometry;

[0057] Figures 28A-28C show the binding activity of fully human anti-PD-1 antibodies according to
10 embodiments of the invention to other immune checkpoint proteins, as measured by ELISA;

[0058] Figures 29A&29B show the inhibition of binding of PD-1 protein to its ligand PD-L1 by fully human anti-PD-1 antibodies according to embodiments of the invention, as measured by a protein-based receptor ligand blocking assay;

[0059] Figures 30A&30B show the inhibition of binding of PD-1 protein to its ligand PD-L2 by
15 fully human anti-PD-1 antibodies according to embodiments of the invention, as measured by a protein-based receptor ligand blocking assay;

[0060] Figures 31A&31B show the inhibition of binding of PD-1 protein to its ligand PD-L1 by fully human anti-PD-1 antibodies according to embodiments of the invention, as measured by a cell-based receptor ligand blocking assay;

20 [0061] Figures 32A&32B show the inhibition of binding of PD-1 protein to its ligand PD-L2 by fully human anti-PD-1 antibodies according to embodiments of the invention, as measured by a cell-based receptor ligand blocking assay;

[0062] Figure 33 shows the effect of fully human anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a T lymphocyte stimulation assay using PBMC's;

25 [0063] Figure 34 shows the effect of chimeric and fully human anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a mixed lymphocyte reaction;

[0064] Figure 35 shows the effect of fully human anti-PD-1 antibodies according to embodiments of the invention on IFN- γ secretion in a T lymphocyte stimulation assay using PBMC's;

[0065] Figures 36A&36B show the effect of fully human anti-PD-1 antibodies according to
30 embodiments of the invention on IFN- γ secretion in a mixed lymphocyte reaction;

[0066] Figure 37 shows the effect of fully human anti-PD-1 antibodies according to embodiments of the invention on Antibody-Dependent Cellular Cytotoxicity (ADCC);

[0067] Figure 38 shows the effect of fully human anti-PD-1 antibodies according to embodiments of the invention on Complement-Dependent Cytotoxicity;

35 [0068] Figure 39 shows the thermostability of fully human anti-PD-1 antibodies according to embodiments of the invention, as measured by Differential Scanning Calorimetry (DSC);

[0069] Figure 40 shows the freeze/thaw stability of fully human anti-PD-1 antibodies according to embodiments of the invention; and

[0070] Figure 41 shows the solubility of fully human anti-PD-1 antibodies according to

40 embodiments of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0071] Various publications, articles and patents are cited or described in the background and throughout the specification; each of these references is herein incorporated by reference in its entirety.

5 Discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is for the purpose of providing context for the invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

[0072] Unless defined otherwise, all technical and scientific terms used herein have the same
10 meaning commonly understood to one of ordinary skill in the art to which this invention pertains. Otherwise, certain terms used herein have the meanings as set in the specification. All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein. It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise.

15 [0073] Unless otherwise stated, any numerical value, such as a concentration or a concentration range described herein, are to be understood as being modified in all instances by the term “about.” Thus, a numerical value typically includes $\pm 10\%$ of the recited value. For example, a concentration of 1 mg/mL includes 0.9 mg/mL to 1.1 mg/mL. Likewise, a concentration range of 1% to 10% (w/v) includes 0.9% (w/v) to 11% (w/v). As used herein, the use of a numerical range expressly includes all
20 possible subranges, all individual numerical values within that range, including integers within such ranges and fractions of the values unless the context clearly indicates otherwise.

[0074] The invention generally relates to isolated anti-PD-1 antibodies, nucleic acids and expression vectors encoding the antibodies, recombinant cells containing the vectors, and compositions comprising the antibodies. Methods of making the antibodies, and methods of using the antibodies to
25 treat diseases including cancer and autoimmune diseases are also provided. The antibodies of the invention possess one or more desirable functional properties, including but not limited to high-affinity binding to PD-1, high specificity to PD-1, the ability to block the binding of PD-1 to its ligand PD-L1 and PD-L2, and the ability to stimulate secretion of the cytokines IFN- γ and IL-2.

[0075] In a general aspect, the invention relates to isolated monoclonal antibodies or antigen-
30 binding fragments thereof that bind PD-1.

[0076] As used herein, the term “antibody” is used in a broad sense and includes immunoglobulin or antibody molecules including human, humanized, composite and chimeric antibodies and antibody fragments that are monoclonal or polyclonal. In general, antibodies are proteins or peptide chains that exhibit binding specificity to a specific antigen. Antibody structures are well known. Immunoglobulins
35 can be assigned to five major classes (i.e., IgA, IgD, IgE, IgG and IgM), depending on the heavy chain constant domain amino acid sequence. IgA and IgG are further sub-classified as the isotypes IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4. Accordingly, the antibodies of the invention can be of any of the five major classes or corresponding sub-classes. Preferably, the antibodies of the invention are IgG1, IgG2, IgG3 or IgG4. Antibody light chains of vertebrate species can be assigned to one of two clearly
40 distinct types, namely kappa and lambda, based on the amino acid sequences of their constant domains.

Accordingly, the antibodies of the invention can contain a kappa or lambda light chain constant domain. According to particular embodiments, the antibodies of the invention include heavy and/or light chain constant regions from rat or human antibodies. In addition to the heavy and light constant domains, antibodies contain an antigen-binding region that is made up of a light chain variable region and a heavy chain variable region, each of which contains three domains (i.e., CDR1, CDR2, and CDR3). The light chain variable region domains are alternatively referred to as LCDR1, LCDR2 and LCDR3, and the heavy chain variable region domains are alternatively referred to as HCDR1, HCDR2 and HCDR3.

[0077] As used herein, the term an “isolated antibody” refers to an antibody which is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds to PD-1 is substantially free of antibodies that do not bind to PD-1). In addition, an isolated antibody is substantially free of other cellular material and/or chemicals.

[0078] As used herein, the term “monoclonal antibody” refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The monoclonal antibodies of the invention can be made by the hybridoma method, phage display technology, single lymphocyte gene cloning technology, or by recombinant DNA methods. For example, the monoclonal antibodies can be produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, such as a transgenic mouse or rat, having a genome comprising a human heavy chain transgene and a light chain transgene.

[0079] As used herein, the term “antigen-binding fragment” refers to an antibody fragment such as, for example, 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), a single domain antibody (sdab) an scFv dimer (bivalent diabody), a multispecific antibody formed from a portion of an antibody comprising one or more CDRs, a camelized single domain antibody, a nanobody, a domain antibody, a bivalent domain antibody, or any other antibody fragment that binds to an antigen but does not comprise a complete antibody structure. An antigen-binding fragment is capable of binding to the same antigen to which the parent antibody or a parent antibody fragment binds. According to particular embodiments, the antigen-binding fragment comprises a light chain variable region, a light chain constant region, and an Fd segment of the constant region of the heavy chain. According to other particular embodiments, the antigen-binding fragment comprises Fab and F(ab').

[0080] As used herein, the term “single-chain antibody” refers to a conventional single-chain antibody in the field, which comprises a heavy chain variable region and a light chain variable region connected by a short peptide of about 15 to about 20 amino acids. As used herein, the term “single domain antibody” refers to a conventional single domain antibody in the field, which comprises a heavy chain variable region and a heavy chain constant region or which comprises only a heavy chain variable region.

[0081] As used herein, the term “human antibody” refers to an antibody produced by a human or an antibody having an amino acid sequence corresponding to an antibody produced by a human made using any technique known in the art. This definition of a human antibody includes intact or full-length

antibodies, fragments thereof, and/or antibodies comprising at least one human heavy and/or light chain polypeptide.

[0082] As used herein, the term “humanized antibody” refers to a non-human antibody that is modified to increase the sequence homology to that of a human antibody, such that the antigen-binding properties of the antibody are retained, but its antigenicity in the human body is reduced.

[0083] As used herein, the term “chimeric antibody” refers to an antibody wherein the amino acid sequence of the immunoglobulin molecule is derived from two or more species. The variable region of both the light and heavy chains often corresponds to the variable region of an antibody derived from one species of mammal (e.g., mouse, rat, rabbit, etc.) having the desired specificity, affinity, and capability, while the constant regions correspond to the sequences of an antibody derived from another species of mammal (e.g., human) to avoid eliciting an immune response in that species.

[0084] As used herein, the term “PD-1” refers to the Programmed Cell Death 1 protein, a 50-55 kDa type I transmembrane receptor that is expressed on activated T, B, and myeloid lineage cells (Greenwald et al., 2005, *Annu. Rev. Immunol.* 23:515-48; Sharpe et al., 2007, *Nat. Immunol.* 8:239-45). The amino acid sequence of human PD-1 is represented in GenBank Accession No. NP_005009.2. Two ligands for PD-1 have been identified, PD-L1 and PD-L2.

[0085] As used herein, an antibody that “specifically binds to PD-1” refers to an antibody that binds to a PD-1, preferably a human PD-1, with a K_D of 1×10^{-7} M or less, preferably 1×10^{-8} M or less, more preferably 5×10^{-9} M or less, 1×10^{-9} M or less, 5×10^{-10} M or less, or 1×10^{-10} M or less. The term “ K_D ” refers to the dissociation constant, which is obtained from the ratio of K_d to K_a (i.e., K_d/K_a) and is expressed as a molar concentration (M). K_D values for antibodies can be determined using methods in the art in view of the present disclosure. For example, the K_D of an antibody can be determined by using surface plasmon resonance, such as by using a biosensor system, e.g., a Biacore® system, or by using bio-layer interferometry technology, such as a Octet RED96 system.

[0086] The smaller the value of the K_D of an antibody, the higher affinity that the antibody binds to a target antigen.

[0087] According to a particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of:

- (1) SEQ ID NOs: 30, 31, 32, 26, 27, and 28, respectively;
- (2) SEQ ID NOs: 6, 7, 8, 2, 3, and 4, respectively;
- (3) SEQ ID NOs: 14, 15, 16, 10, 11, and 12, respectively;
- (4) SEQ ID NOs: 22, 23, 24, 18, 19, and 20, respectively;
- (5) SEQ ID NOs: 38, 39, 40, 34, 35, and 36, respectively;
- (6) SEQ ID NOs: 46, 47, 48, 42, 43, and 44, respectively; or
- (7) SEQ ID NOs: 54, 55, 56, 50, 51, and 52, respectively;

wherein the antibody or antigen-binding fragment thereof binds PD-1, preferably specifically binds to human PD-1.

[0088] According to another particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof of the invention, comprising a heavy chain variable region

having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, 1, 9, 17, 33, 41 or 49, or a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29, 5, 13, 21, 37, 45 or 53. Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof of the invention comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, 1, 9, 17, 33, 41 or 49, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29, 5, 13, 21, 37, 45 or 53, respectively.

[0089] According to another particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof of the invention, comprising:

- a. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 25, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 29;
- b. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 1, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 5;
- c. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 9, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 13;
- d. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 17, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 21;
- e. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 33, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 37;
- f. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 41, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 45; or
- g. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 49, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 53.

[0090] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 6, 7, 8, 2, 3, and 4, respectively. In another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 1, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 5. Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 1; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 5.

[0091] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 14, 15, 16, 10, 11, and 12, respectively, respectively. In

another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 9, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 13.

Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 9; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 13.

[0092] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 22, 23, 24, 18, 19, and 20, respectively, respectively. In

another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 17, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 21.

Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 17; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 21.

[0093] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 30, 31, 32, 26, 27, and 28, respectively. In another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29.

Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 25; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 29.

[0094] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 38, 39, 40, 34, 35, and 36, respectively. In another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 33, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 37.

Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 33; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 37.

[0095] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 46, 47, 48, 42, 43, and 44, respectively. In another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 41, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 45.

Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy

chain variable region having the polypeptide sequence of SEQ ID NO: 41; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 45.

[0096] In one embodiment, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof, comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of SEQ ID NOs: 54, 55, 56, 50, 51, and 52, respectively. In another embodiment, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 49, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 53.

Preferably, the isolated monoclonal antibody or antigen-binding fragment thereof comprises a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 49; and a light chain variable region having the polypeptide sequence of SEQ ID NO: 53.

[0097] According to another particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof of the invention, wherein the antibody or antigen-binding fragment thereof is chimeric.

[0098] According to another particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof of the invention, wherein the antibody or antigen-binding fragment thereof is human.

[0099] According to another particular aspect, the invention relates to an isolated monoclonal antibody or antigen-binding fragment thereof of the invention, comprising a constant region, preferably a human heavy chain IgG4 constant region, more preferably a human heavy chain IgG4 constant region having a S228P mutation (SEQ ID NO: 75), and a human antibody light chain, preferably a human light chain kappa constant region (SEQ ID NO: 77).

[00100] In another general aspect, the invention relates to an isolated nucleic acid encoding a monoclonal antibody or antigen-binding fragment thereof of the invention. It will be appreciated by those skilled in the art that the coding sequence of a protein can be changed (e.g., replaced, deleted, inserted, etc.) without changing the amino acid sequence of the protein. Accordingly, it will be understood by those skilled in the art that nucleic acid sequences encoding monoclonal antibodies or antigen-binding fragments thereof of the invention can be altered without changing the amino acid sequences of the proteins.

[00101] In another general aspect, the invention relates to a vector comprising an isolated nucleic acid encoding a monoclonal antibody or antigen-binding fragment thereof of the invention. Any vector known to those skilled in the art in view of the present disclosure can be used, such as a plasmid, a cosmid, a phage vector or a viral vector. In some embodiments, the vector is a recombinant expression vector such as a plasmid. The vector can include any element to establish a conventional function of an expression vector, for example, a promoter, ribosome binding element, terminator, enhancer, selection marker, and origin of replication. The promoter can be a constitutive, inducible or repressible promoter. A number of expression vectors capable of delivering nucleic acids to a cell are known in the art and can be used herein for production of an antibody or antigen-binding fragment thereof in the cell.

Conventional cloning techniques or artificial gene synthesis can be used to generate a recombinant expression vector according to embodiments of the invention.

[00102] In another general aspect, the invention relates to a host cell comprising an isolated nucleic acid encoding a monoclonal antibody or antigen-binding fragment thereof of the invention. Any host cell known to those skilled in the art in view of the present disclosure can be used for recombinant expression of antibodies or antigen-binding fragments thereof of the invention. In some embodiments, the host cells are E. coli TG1 or BL21 cells (for expression of, e.g., an scFv or Fab antibody) or CHO-K1 cells (for expression of, e.g., a full-length IgG antibody). According to particular embodiments, the recombinant expression vector is transformed into host cells by conventional methods such as chemical transfection, heat shock, or electroporation, where it is stably integrated into the host cell genome such that the recombinant nucleic acid is effectively expressed.

[00103] In another general aspect, the invention relates to a method of producing a monoclonal antibody or antigen-binding fragment thereof of the invention, comprising culturing a cell comprising a nucleic acid encoding the monoclonal antibody or antigen-binding fragment thereof under conditions to produce a monoclonal antibody or antigen-binding fragment thereof of the invention, and recovering the antibody or antigen-binding fragment thereof from the cell or cell culture (e.g., from the supernatant). Expressed antibodies or antigen-binding fragments thereof can be harvested from the cells and purified according to conventional techniques known in the art and as described herein.

[00104] In another general aspect, the invention relates to a pharmaceutical composition, comprising an isolated monoclonal antibody or antigen-binding fragment thereof of the invention and a pharmaceutically acceptable carrier.

[00105] As used herein, the term “carrier” refers to any excipient, diluent, filler, salt, buffer, stabilizer, solubilizer, oil, lipid, lipid containing vesicle, microsphere, liposomal encapsulation, or other material well known in the art for use in pharmaceutical formulations. It will be understood that the characteristics of the carrier, excipient or diluent will depend on the route of administration for a particular application. As used herein, the term “pharmaceutically acceptable carrier” refers to a non-toxic material that does not interfere with the effectiveness of a composition according to the invention or the biological activity of a composition according to the invention. According to particular embodiments, in view of the present disclosure, any pharmaceutically acceptable carrier suitable for use in an antibody pharmaceutical composition can be used in the invention.

[00106] In another general aspect, the invention relates to a method of blocking the binding of PD-1 to PD-L1 or to PD-L2, or of augmenting secretion of IFN- γ and IL-2 in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention.

[00107] The functional activity of antibodies and antigen-binding fragments thereof that bind PD-1 can be characterized by methods known in the art and as described herein. Methods for characterizing antibodies and antigen-binding fragments thereof that bind PD-1 include, but are not limited to, affinity and specificity assays including Biacore, ELISA, and FACS analysis; receptor ligand binding assays to detect blocking of the binding of PD-1 to PD-L1 and PD-L2; assays to detect induction of lymphocytic cytokine production by the blocking of the binding of PD-1 to PD-L1 and PD-L2; cell cytotoxicity assays to detect the presence of antibody-dependent cell-mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC) activity of the antibodies; experiments to detect the inhibition of tumor growth in mouse tumor models; etc. According to particular embodiments, the

methods for characterizing antibodies and antigen-binding fragments thereof that bind PD-1 include those described in Examples 2-12 below.

[00108] In another general aspect, the invention relates to a method of treating an infectious disease or a graft versus host disease in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention. In another general aspect, the invention relates to a method of treating a tumor in a subject in need thereof, comprising administering to the subject a pharmaceutical composition of the invention.

[00109] As used herein, the term “subject” refers to an animal, and preferably a mammal.

According to particular embodiments, the subject is a mammal including a non-primate (e.g., a camel, donkey, zebra, cow, pig, horse, goat, sheep, cat, dog, rat, rabbit, guinea pig or mouse) or a primate (e.g., a monkey, chimpanzee, or human). In particular embodiments, the subject is a human.

[00110] According to embodiments of the invention, the pharmaceutical composition comprises a therapeutically effective amount of the anti-PD-1 antibody or antigen-binding fragment thereof. As used herein, the term “therapeutically effective amount” refers to an amount of an active ingredient or component that elicits the desired biological or medicinal response in a subject. A therapeutically effective amount can be determined empirically and in a routine manner, in relation to the stated purpose.

[00111] As used herein with reference to anti-PD-1 antibodies or antigen-binding fragments thereof, a therapeutically effective amount means an amount of the anti-PD-1 antibody or antigen-binding fragment thereof that stimulates an immune response in a subject in need thereof. Also as used herein with reference to anti-PD-1 antibodies or antigen-binding fragments thereof, a therapeutically effective amount means an amount of the anti-PD-1 antibody or antigen-binding fragment thereof that results in treatment of a disease, disorder, or condition; prevents or slows the progression of the disease, disorder, or condition; or reduces or completely alleviates symptoms associated with the immune disease, disorder, or condition.

[00112] According to particular embodiments, the disease, disorder or condition to be treated is a hyperproliferative disease, an infectious disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease. According to more particular embodiments, the disease, disorder or condition to be treated is an infectious disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease. According to more particular embodiments, the disease, disorder or condition to be treated is a non-malignant hyperproliferative disease, including but not limited to, atherosclerosis, benign hyperplasia, benign prostatic hypertrophy. According to other particular embodiments, the disease, disorder or condition to be treated is a tumor, or a malignant hyperproliferative disease, preferably a tumor selected from the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.

[00113] According to particular embodiments, a therapeutically effective amount refers to the amount of therapy which is sufficient to achieve one, two, three, four, or more of the following effects:

(i) reduce or ameliorate the severity of the disease, disorder or condition to be treated or a symptom

associated therewith; (ii) reduce the duration of the disease, disorder or condition to be treated, or a symptom associated therewith; (iii) prevent the progression of the disease, disorder or condition to be treated, or a symptom associated therewith; (iv) cause regression of the disease, disorder or condition to be treated, or a symptom associated therewith; (v) prevent the development or onset of the disease, disorder or condition to be treated, or a symptom associated therewith; (vi) prevent the recurrence of the disease, disorder or condition to be treated, or a symptom associated therewith; (vii) reduce hospitalization of a subject having the disease, disorder or condition to be treated, or a symptom associated therewith; (viii) reduce hospitalization length of a subject having the disease, disorder or condition to be treated, or a symptom associated therewith; (ix) increase the survival of a subject with the disease, disorder or condition to be treated, or a symptom associated therewith; (xi) inhibit or reduce the disease, disorder or condition to be treated, or a symptom associated therewith in a subject; and/or (xii) enhance or improve the prophylactic or therapeutic effect(s) of another therapy.

[00114] The therapeutically effective amount or dosage can vary according to various factors, such as the disease, disorder or condition to be treated, the means of administration, the target site, the physiological state of the subject (including, e.g., age, body weight, health), whether the subject is a human or an animal, other medications administered, and whether the treatment is prophylactic or therapeutic. Treatment dosages are optimally titrated to optimize safety and efficacy.

[00115] According to particular embodiments, the compositions described herein are formulated to be suitable for the intended route of administration to a subject. For example, the compositions described herein can be formulated to be suitable for intravenous, subcutaneous, or intramuscular administration.

[00116] As used herein, the terms “treat,” “treating,” and “treatment” are all intended to refer to an amelioration or reversal of at least one measurable physical parameter related to a cancer, an inflammatory disease, disorder or condition, an immune disease, disorder or condition, an autoimmune disease, disorder or condition, or an infectious disease, disorder or condition, which is not necessarily discernible in the subject, but can be discernible in the subject. The terms “treat,” “treating,” and “treatment,” can also refer to causing regression, preventing the progression, or at least slowing down the progression of the disease, disorder, or condition. In a particular embodiment, “treat,” “treating,” and “treatment” refer to an alleviation, prevention of the development or onset, or reduction in the duration of one or more symptoms associated with the disease, disorder, or condition, such as a tumor or more preferably a cancer. In a particular embodiment, “treat,” “treating,” and “treatment” refer to prevention of the recurrence of the disease, disorder, or condition. In a particular embodiment, “treat,” “treating,” and “treatment” refer to an increase in the survival of a subject having the disease, disorder, or condition. In a particular embodiment, “treat,” “treating,” and “treatment” refer to elimination of the disease, disorder, or condition in the subject.

[00117] According to particular embodiments, a composition used in the treatment of a cancer, an inflammatory disease, disorder or condition, an immune disease, disorder or condition, an autoimmune disease, disorder or condition, or an infectious disease, disorder or condition can be used in combination with another treatment including, but not limited to, a chemotherapy, an anti-CD20 mAb, an anti-CTLA-

4 antibody, an antiangiogenic agent, a radiation therapy, other immune-oncology drug, a targeted therapy, an anti-PD-L1 antibody or other anticancer drugs.

[00118] As used herein, the term “in combination,” in the context of the administration of two or more therapies to a subject, refers to the use of more than one therapy. The use of the term “in combination” does not restrict the order in which therapies are administered to a subject. For example, a first therapy (e.g., a composition described herein) can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy to a subject.

[00119] In another general aspect, the invention relates to a method of producing a pharmaceutical composition comprising a monoclonal antibody or antigen-binding fragment thereof of the invention, comprising combining a monoclonal antibody or antigen-binding fragment thereof with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition.

EMBODIMENTS

[00120] The invention provides also the following non-limiting embodiments.

[00121] Embodiment 1 is an isolated monoclonal antibody or antigen-binding fragment thereof comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of:

- (1) SEQ ID NOs: 30, 31, 32, 26, 27, and 28, respectively;
- (2) SEQ ID NOs: 6, 7, 8, 2, 3, and 4, respectively;
- (3) SEQ ID NOs: 14, 15, 16, 10, 11, and 12, respectively;
- (4) SEQ ID NOs: 22, 23, 24, 18, 19, and 20, respectively;
- (5) SEQ ID NOs: 38, 39, 40, 34, 35, and 36, respectively;
- (6) SEQ ID NOs: 46, 47, 48, 42, 43, and 44, respectively; or
- (7) SEQ ID NOs: 54, 55, 56, 50, 51, and 52, respectively;

wherein the antibody or antigen-binding fragment thereof binds PD-1.

[00122] Embodiment 2 is the isolated monoclonal antibody or antigen-binding fragment of Embodiment 1, comprising a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, 1, 9, 17, 33, 41 or 49, or a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29, 5, 13, 21, 37, 45 or 53.

[00123] Embodiment 3 is the isolated monoclonal antibody or antigen-binding fragment of Embodiment 2, comprising a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, 1, 9, 17, 33, 41 or 49, and a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29, 5, 13, 21, 37, 45 or 53, respectively.

[00124] Embodiment 4 is the isolated monoclonal antibody or antigen-binding fragment of Embodiment 3, comprising:

- (a) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 25, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 29;
- (b) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 1, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 5;
- (c) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 9, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 13;
- (d) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 17, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 21;
- (e) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 33, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 37;
- (f) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 41, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 45; or
- (g) a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 49, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 53.

[00125] Embodiment 5 is the isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 4, wherein the antibody or antigen-binding fragment thereof is chimeric.

[00126] Embodiment 6 is isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 5, wherein the antibody or antigen-binding fragment thereof is human.

[00127] Embodiment 7 is the isolated antibody or antigen-binding fragment of Embodiment 6, comprising a human heavy chain IgG4 constant region with a S228P mutation, and a human antibody light chain kappa constant region.

[00128] Embodiment 8 is the isolated antibody or antigen-binding fragment of any of Embodiments 1 to 7, wherein the antibody or antigen-binding fragment binds to a human PD-1 with a K_D of 5×10^{-9} M or less, preferably a K_D of 1×10^{-9} M or less, wherein the K_D is measured by surface plasmon resonance analysis, such as by using a Biacore system, or by a bio-layer interferometry technology, such as by using a Octet RED96 system.

[00129] Embodiment 9 is an isolated nucleic acid encoding the monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8.

[00130] Embodiment 10 is a vector comprising the isolated nucleic acid of Embodiment 9.

[00131] Embodiment 11 is a host cell comprising the nucleic acid of Embodiment 10.

[00132] Embodiment 12 is a pharmaceutical composition, comprising the isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8 and a pharmaceutically acceptable carrier.

[00133] Embodiment 13 is a method of blocking binding of PD-1 to PD-L1 or PD-L2, or augmenting secretion of IFN- γ and IL-2 in a subject in need thereof, comprising administering to the subject the pharmaceutical composition of Embodiment 12.

[00134] Embodiment 14 is a method of treating an infectious disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease in a subject in need thereof, comprising administering to the subject the pharmaceutical composition of Embodiment 12.

[00135] Embodiment 15 is the method of Embodiment 14, further comprising administering to the subject an additional agent for treating the infectious disease, inflammatory disease, immune disease, autoimmune disease, or graft versus host disease in the subject in need thereof.

[00136] Embodiment 16 is a method of treating a hyperproliferative disease in a subject in need thereof, comprising administering to the subject the pharmaceutical composition of Embodiment 12,

[00137] Embodiment 17 is the method of Embodiment 16, wherein the hyperproliferative disease is a non-malignant disease, preferably selected from the group consisting of atherosclerosis, benign hyperplasia and benign prostatic hypertrophy.

[00138] Embodiment 18 is the method of Embodiment 16, wherein the hyperproliferative disease is a tumor, or a malignant disease, preferably, the tumor is selected from the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.

[00139] Embodiment 19 is the method of any of Embodiments 16-18, further comprising administering to the subject an additional agent for treating the hyperproliferative disease in the subject in need thereof.

[00140] Embodiment 20 is a method of producing the monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8, comprising culturing a cell comprising a nucleic acid encoding the monoclonal antibody or antigen-binding fragment under conditions to produce the monoclonal antibody or antigen-binding fragment, and recovering the antibody or antigen-binding fragment from the cell or cell culture.

[00141] Embodiment 21 is a method of producing a pharmaceutical composition comprising the monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8, comprising combining the monoclonal antibody or antigen-binding fragment with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition.

[00142] Embodiment 22 is an isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8 for use in treating an infectious disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease in a subject in need thereof.

[00143] Embodiment 23 is an isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8 for use in treating a hyperproliferative disease, such as a non-malignant disease selected from the group consisting of atherosclerosis, benign hyperplasia and benign prostatic hypertrophy, or a tumor selected from the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.

[00144] Embodiment 24 is an use of an isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8 for manufacturing a pharmaceutical composition in treating an infectious

disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease in a subject in need thereof.

[00145] Embodiment 25 is an use of an isolated monoclonal antibody or antigen-binding fragment of any of Embodiments 1 to 8 for manufacturing a pharmaceutical composition in treating a

hyperproliferative disease, such as a non-malignant disease selected from the group consisting of atherosclerosis, benign hyperplasia and benign prostatic hypertrophy, or a tumor selected from the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.

EXAMPLES

[00146] The following examples of the invention are to further illustrate the nature of the invention. It should be understood that the following examples do not limit the invention and that the scope of the invention is to be determined by the appended claims.

[00147] Example 1 – Generation of anti-PD-1 antibodies

[00148] Human PD-1 protein was used as an immunogen to generate anti-PD-1 antibodies. The uses of human immunoglobulin transgenic mouse technology for the development and preparation of fully human antibodies was first described by Abgenix (xeno mouse and Medarex (HuMab "mouse"); Lonberg et al., 1994, Nature 368: 856-859; Lonberg and Huszar, 1995, Internal Rev. Immunol. 13:65-93; Harding and Lonberg, 1995, Ann. N.Y. Acad. Sci. 764:536-546).

[00149] Antibodies with high affinity ($K_D < 1 \times 10^{-9} M$) to PD-1 were obtained by carrying out pilot antibody production, purification and validation. The antibodies, which are specific for PD-1 and do not cross react with other immune checkpoints such as B7.1, CD28, CTLA-4, and ICOS, are able to block the binding of PD-1 to PD-L1 and PD-L2. The amino acid sequences of the heavy and light chain variable regions of the generated anti-PD-1 antibodies were determined using standard molecular biology methods and are summarized in Table 1.

[00150] Table 1. SEQ ID NOs of the amino acid sequences of the heavy chain variable regions, HCDRs, light chain variable regions and LCDRs of chimeric anti-PD-1 antibodies of the invention

Clone ID	SEQ ID Nos							
	Heavy Chain				Light Chain			
	variable region	CDR1	CDR2	CDR3	variable region	CDR1	CDR2	CDR3
9B2C6C9	1	2	3	4	5	6	7	8
76G5B3	9	10	11	12	13	14	15	16
32B5C7	17	18	19	20	21	22	23	24
223G6B5	25	26	27	28	29	30	31	32
21G8F11	33	34	35	36	37	38	39	40
226C3A7	41	42	43	44	45	46	47	48
204C5E12	49	50	51	52	53	54	55	56

[00151] The heavy chain and light chain variable regions of the anti-PD-1 antibodies listed in Table 1 are encoded by the nucleic acid sequences summarized in Table 2.

[00152] **Table 2.** SEQ ID NOs of the nucleic acid sequences of the heavy chain and light chain variable regions of chimeric anti-PD-1 antibodies of the invention

Clone ID	SEQ ID NOs (nucleotides of the sequence corresponding to CDRs)	
	Heavy Chain	Light Chain variable region
9B2C6C9	57 (CDR1: nt 76-108) (CDR2: nt 151-198) (CDR3: nt 295-336)	58 (CDR1: nt 70-117) (CDR2: nt 163-183) (CDR3: nt 280-306)
76G5B3	59 (CDR1: nt 76-105) (CDR2: nt 148-198) (CDR3: nt 295-309)	60 (CDR1: nt 70-102) (CDR2: nt 148-168) (CDR3: nt 265-291)
32B5C7	61 (CDR1: nt 76-105) (CDR2: nt 148-198) (CDR3: nt 295-312)	62 (CDR1: nt 70-102) (CDR2: nt 148-168) (CDR3: nt 265-291)
223G6B5	63 (CDR1: nt 76-108) (CDR2: nt 151-198) (CDR3: nt 295-324)	64 (CDR1: nt 70-102) (CDR2: nt 148-168) (CDR3: nt 265-291)
21G8F11	65 (CDR1: nt 76-105) (CDR2: nt 148-198) (CDR3: nt 295-327)	66 (CDR1: nt 70-102) (CDR2: nt 148-168) (CDR3: nt 265-291)
226C3A7	67 (CDR1: nt 76-111) (CDR2: nt 154-207) (CDR3: nt 304-342)	68 (CDR1: nt 70-102) (CDR2: nt 148-168) (CDR3: nt 265-291)
204C5E12	69 (CDR1: nt 76-105) (CDR2: nt 148-198) (CDR3: nt 295-348)	70 (CDR1: nt 70-117) (CDR2: nt 163-183) (CDR3: nt 280-306)

[00153] Fully human versions of the anti-PD-1 antibodies were generated. The fully human anti-PD-1 antibodies bound to the extracellular domain of human PD-1 with high affinity ($K_D < 1 \times 10^{-9} M$), and blocked the binding of PD-1 to its ligands, PD-L1 and PD-L2. The anti-PD-1 antibodies do not exhibit

non-specific binding to human B7.1, CD28, CTLA-4, ICOS or other similar protein antigens. The biological activities of the anti-PD-1 antibodies were evaluated by mixed lymphocyte and T cell stimulation assays, in which they increased secretion of the IFN- γ and IL-2 cytokines. While not wishing to be bound by theories, it is believed that the anti-PD-1 antibodies can be used to suppress PD-1-mediated signaling pathways that negatively-regulate immune responses, and to therefore enhance tumor-specific immune responses, either as a monotherapy or in combination with anti-PD-L1 monoclonal antibodies or other anticancer drugs as a cancer immunotherapy, particularly for patients having PD-L1 positive tumors. The anti-PD-1 antibodies can be used for the treatment of cancers and autoimmune diseases.

[00154] Example 2 – Preparation of anti-PD-1 antibodies

[00155] (step 1) Preparation of Immunogen A, PD-1^{ECD}-hFc protein (also referred to as PD-1-hFc herein)

[00156] The coding sequence of the human PD-1 extracellular domain (PD-1^{ECD}; SEQ ID NO: 71), corresponding to amino acids Leu25-Glu167 of SEQ ID No.72, was cloned along with the coding sequence of human IgG Fc fragment (hFc) into a pCpC vector (Invitrogen, #V044-50) using standard molecular biology cloning techniques (Sambrook and Russell, 1989, Molecular cloning: a laboratory manual, New York : Cold Spring Harbor Laboratory Press, 2nd ed.). HEK293 cells (Invitrogen) were transiently transfected using polyethylenimine (PEI, Polysciences) with the plasmid and expanded in FreeStyle 293 expression medium (Invitrogen) at 37°C. After 4 days of expansion, the culture medium was collected and centrifuged to remove cell components. The culture supernatant, which contained the recombinant PD-1^{ECD}-hFc, was subjected to Protein A chromatography (Mabselect, GE Healthcare). The ultraviolet (UV) absorption (A280nm) was monitored with a UV detector, and the samples were washed with PBS (pH 7.2) until the UV A280nm absorption returned to baseline levels, at which point the PD-1^{ECD}-hFc fusion protein was eluted from the Protein A affinity column with 0.1M glycine hydrochloride (pH2.5). The sample was dialyzed with PBS phosphate buffer (pH 7.2) at 4°C overnight. After dialysis, the purified PD-1 immunogen was passed through a 0.22 micron sterile filter, aliquoted and stored at -80°C.

[00157] Recombinant human PD-L1 extracellular domain (PD-L1^{ECD}) (corresponding to amino acids Phe19-Thr239 of Uniprot database protein Q9NZQ7.1) fused to hFc was prepared in the same way that the PD-1^{ECD}-hFc recombinant protein was prepared above. Purified PD-L1^{ECD}-hFc (also referred to as PD-L1-hFc herein) was biotinylated by mixing and incubating the protein with biotin (Sigma S3295).

[00158] To characterize the PD-1^{ECD}-hFc immunogen, the sample's protein concentration and purity were determined, and the immunogen's molecular weight and biological activity were determined.

[00159] The biological activity of the PD-1^{ECD}-hFc immunogen was determined by a ligand binding assay. Recombinant PD-1^{ECD}-hFc protein was diluted in PBS to a concentration of 1 μ g/mL, and 100 μ L of the diluted PD-1^{ECD}-hFc protein sample were added per well to microtiter plates, which were incubated overnight at 4°C to coat the plates with the recombinant protein. The plates were then blocked with a blocking solution (containing 1% BSA, pH7.4 PBS buffer, w/v) at 37°C for two hours and then incubated with serial dilutions of the biotinylated PD-L1^{ECD}-hFc protein or a control protein

(biotinylated non-PD-L1^{ECD}-hFc) for 1 hour at 37°C. Horseradish peroxidase (HRP) conjugated streptavidin (Sigma B2438) was added, and the plates were incubated at room temperature for 30 minutes. 100 μL of tetramethylbenzidine (TMB) were added, and the plates were incubated at room temperature for 15 minutes. 50 μL of 1N HCl were added to terminate the reaction, and the OD_{450nm} was determined by a microtiter plate reader. Figure 1 and Table 3 show the concentration-dependent binding of the biotinylated PD-L1^{ECD}-hFc protein or control protein to purified PD-1^{ECD}-hFc fusion protein. Binding to PD-1^{ECD}-hFc was observed with biotinylated PD-L1^{ECD}-hFc, but not with the control protein that does not contain the sequence of PD-L1^{ECD}.

Table 3. Binding activities of PD-1^{ECD}-hFc to biotinylated PD-L1^{ECD}-hFc

Biotinylated Sample	OD _{450nm}							
	PD-L1 ^{ECD} -hFc/Control Protein Concentration (μg/mL)							
	1	0.2	0.04	0.008	0.0016	0.0003	0.0001	0.00001
PD-L1 ^{ECD} -hFc Batch 1	3.23	3.205	2.97	0.705	0.205	0.11	0.145	0.09
PD-L1 ^{ECD} -hFc Batch 2	3.26	3.24	2.73	0.705	0.285	0.205	0.1	0.185
Control protein	0.13	0.095	0.09	0.14	0.195	0.195	0.32	0.205

(step 2) Preparation of Immunogen B, HEK293 cells overexpressing hPD-1

The nucleotide sequence encoding human PD-1 (SEQ ID NO: 73) was subcloned into a pIRES vector (Clontech), and the plasmid was prepared. HEK293 and CHO-K1 cells (Invitrogen) were transiently transfected with the plasmid using PEI, and transformants were selected in DMEM culture media containing 0.5 g/mL antibiotics and 10% (w/w) fetal bovine serum (FBS) for 2 weeks. A limited dilution into a 96-well culture plate was carried out, and the plate was incubated at 37°C with 5% (v/v) CO₂ for approximately 2 weeks. After selection, monoclones were expanded in 6-well plates, and the expanded clones were screened by flow cytometry using commercially available anti-PD-1 antibodies (R&D Systems). Clones exhibiting higher growth rates and higher fluorescence intensity as measured by FACS were further expanded and cryopreserved in liquid nitrogen.

Figure 2 shows the flow cytometry profile of the HEK293 cells stably transfected with the recombinant hPD-1 protein. The percentage of FACS-positive cells are shown in Table 4 as an indication of the expression levels of hPD-1 protein.

Table 4. hPD-1 expression levels of recombinant HEK293 clones, as determined by FACS

No.	Recombinant cell clone ID	PD mAb		IgG Control	
		Gated(%)	MFI	Gated(%)	MFI
1	293F-hPD1 1A2	43.38	17.8	0.83	3.11
2	293F-hPD1 1A6	96.23	275.9	0.45	2.9
3	293F-hPD1 1B4	95.9	266.52	0.54	3.13
4	293F-hPD1 1C1	95.5	261.6	0.92	3.09
5	293F-hPD1 1C10	96.43	297.88	0.44	3.13

6	293F-hPD1 1C6	97.47	196.04	2.8	3.7
7	293F-hPD1 1D2	96.95	226.39	0.57	3.08
8	293F-hPD1 1D4	96.71	202.08	1.45	3.31
9	293F-hPD1 1D6	94.41	255.87	0.74	3.11
10	293F-hPD1 1D7	95.13	246.53	0.81	3.03
11	293F-hPD1 1E1	95.77	174.74	0.53	3.17
12	293F-hPD1 1E2	95.67	222.33	1.74	3.66
13	293F-hPD1 1F1	96.68	303.23	0.72	3.37
14	293F-hPD1 1F4	97.05	248.22	1.38	2.93
15	293F-hPD1 1F5	96.01	148.82	0.64	2.86
16	293F-hPD1 1F8	96.16	234.31	0.7	3.21
17	293F-hPD1 1G1	96.45	312.87	0.71	3.08
18	293F-hPD1 1G10	98.67	341.57	0.44	2.9
19	293F-hPD1 1G11	95.53	300.49	0.59	3.4
20	293F-hPD1 1G12	95.47	339.92	0.43	3.13
21	293F-hPD1 1G4	97.87	366.88	0.71	3.27
22	293F-hPD1 1G6	97.38	293.46	0.67	2.94
23	293F-hPD1 1H5	96.99	223.54	1.14	3.46

[00165] (step 3) Preparation of Immunogen C, hPD-1-expressing construct

[00166] The coding sequence of full length human PD-1 protein (SEQ ID No.73) was subcloned into a pcDNA3.1 vector (Invitrogen), and the resulting plasmid was coated onto a 1.0um colloidal gold bullet (Bio-RAD) for subsequent immunization using a Helios gene gun (Bio-rad No. 165-2431), following the instructions of the Helios gene gun data sheet.

[00167] (step 4) Hybridoma cell fusion and antibody screening

[00168] Human Ig Fc does not interact with the mouse Fc receptor, so immune responses triggered in mice by hFc-containing immunogens are weak, resulting in low efficiency generation of monoclonal antibodies. Harbour H2L2 transgenic mice were generated by introducing the gene encoding the human immunoglobulin (Ig) variable region and the gene encoding the rat Ig constant region into the mouse genome, such that the mice contained a chimeric Ig comprising hV-rC, while expression of the mouse Ig was disabled (WO 2010/070263 A1). Harbour H2L2 transgenic mice are able to produce comparable immune responses and antibody titers to wild type mice (e.g., Balb/c).

[00169] (part 4A) 6-8 week old Harbour H2L2 transgenic mice (Beijing WeitongLihua) were immunized with Immunogen A, and the mice were kept under Specific Pathogen Free (SPF) conditions. In the first immunization, 50ug of Immunogen A was injected into the abdominal cavity of each mouse along with 0.25mL Complete Freund's Adjuvant (CFA). To enhance the immune response, 50ug of Immunogen A was injected into the abdominal cavity of each mouse along with 0.25 Incomplete Freund's Adjuvant (IFA) two weeks after the first immunization, and subsequent boosts were administered 3 weeks apart. Blood samples were collected one week after immunization. The antibody

titer and specificity in serum were determined by ELISA and FACS analysis, and the results are shown in Figure 3 and Table 5. Table 5 shows that serum from mice immunized with PD-1^{ECD}-hFc exhibited different levels of binding to Immunogen A. The highest serum dilution was about one million. The blank control was 1% (w/w) BSA. The OD450nm values shown in Table 5 are the serum titer values from 7 days after the third boost, as determined by ELISA.

[00170] Table 5. Serum titers of Harbour H2L2 transgenic mice immunized with hPD-1^{ECD}-hFc fusion protein, as determined by ELISA

Animal No.	OD _{450nm}						
	Serum dilution factors						Blank
	1:100	1:10 ³	1:10 ⁴	1:10 ⁵	1:10 ⁶	1:10 ⁷	
1671(TB3)	3.3651	3.3434	3.1001	1.3935	0.8103	0.7423	0.7921
1672(TB3)	3.2939	3.2384	2.1086	0.9142	0.7536	0.7248	0.7682
1673(TB3)	3.3487	3.3544	3.0128	1.3243	0.859	0.8475	0.7715
1674(TB3)	3.3584	3.325	2.5794	1.0904	0.788	0.7563	0.7558
1675(TB3)	3.3611	3.513	3.3012	2.2199	1.0601	0.9124	0.7528

[00171] (part4B) 6-8 week old Harbour H2L2 transgenic mice (Beijing WeitongLihua) were immunized with Immunogen B, and the mice were kept under SPF conditions. HEK293 cells were stably transfected with the pIRES plasmid encoding full length hPD-1 (see Example 2, step 2)) using Xtreme gene HP DNA Transfection reagent (Roche, #06 366 236 001). Cells were cultured in T-75 culture flasks. When the cells reached 90% confluence, the media was aspirated, and the cells were washed twice with DMEM medium (Invitrogen) and treated with non-enzymatic cell dissociation buffer (Invitrogen) at 37°C until the cells were detached from the culture flask. The cells were collected and washed twice with DMEM medium, and cell counts were determined and adjusted to 2 × 10⁷ cells/mL using PBS buffer (pH7.2). Mice were injected with 0.5mL of cell suspension for each immunization. Two weeks after the first immunization, a boost was administered, and subsequent boosts were administered 3 weeks apart. Blood samples were collected one week after immunization. The antibody titer and specificity in serum were determined by flow cytometry. After the second boost, the serum titer was over 1:1000, as determined by flow cytometry, and over 1:10,000, as determined by ELISA.

[00172] (part4C) 6-8 week old Harbour H2L2 transgenic mice (Beijing WeitongLihua) were immunized with Immunogen C, and the mice were kept under SPF conditions. All mice were immunized with the Helios gene gun 4 times, with 4 shots per immunization. Each shot contained 1μg cDNA. Two weeks after the first immunization, a boost was administered, and subsequent boosts were administered 3 weeks apart. Blood samples were collected one week after immunization, and the serum titer was determined by ELISA and FACS. The serum titer after the second boost was 1:1000, as determined by flow cytometry, and over 1:10,000, as determined by ELISA.

[00173] Prior to the completion of steps 4A-C above, mice with specific immune response against hPD-1 were selected for fusion and were given a final boost by intraperitoneal injection of 100μg of purified PD-1^{ECD}-hFc (for mice immunized with Immunogen A or Immunogen C) or HEK293 cells

stably transfected with hPD-1 (for mice immunized with Immunogen B). Five days later, the mice were sacrificed, and their splenocytes were collected. NH_4OH was added to the splenocyte samples to a final concentration of 1% (w/w) to lyse red blood cells in the sample. The samples were centrifuged at 1000 rpm and washed three times with DMEM culture media. The viability of the splenocytes was

determined, and viable splenocytes were then fused with mouse myeloma cells SP2/0 (ATCC) at a ratio of 5:1 by the high efficiency electric fusion method (see Methods in Enzymology, Vol. 220).

[00174] Fused cells were re-suspended in DMEM media containing 20% FBS and 1x hypoxanthine-aminopterin-thymidine (HAT) medium (w/w), and the concentration was adjusted to 10^5 cells/200uL. 200uL of fused cells were added to each well of 96-well plate, which was incubated at 37°C , 5% CO_2 .

14 days after cell fusion, hybridoma supernatants were collected and screened by ELISA and Acumen (microplate cell assay). Clones with an OD450nm greater than 1.0 by ELISA and an MFI value greater than 100 by Acumen were expanded in a 24-well plate containing DMEM with 10% (w/w) heat-inactivated FBS at 37°C , 5% (v/v) CO_2 . Supernatants were collected after 3 days of culturing. The antibody isotypes were determined, and their ability to bind to recombinant PD-1^{ECD} protein and PD-1-expressing cells was determined by ELISA and flow cytometry (see Example 3). A receptor ligand binding assay was performed to determine the blocking activity of the hybridoma supernatant (see Examples 4-5).

[00175] Based on the 24-well plate screening results, clones with OD450nm values greater than 1.0 by the ELISA binding assay, MFI values greater than 50 by the FACS binding assay, and inhibition rates greater than 60% by the receptor ligand binding assay were selected and subcloned. Subcloning was carried out by limited dilution in a 96-well plate with DMEM media containing 10% (v/v) FBS at 37°C and 5% (v/v) CO_2 . After 10 days of culturing, the supernatants were collected and preliminarily screened by ELISA and Acumen. Positive clones were expanded in a 24-well plate and cultured for 3 days. Supernatants were collected. ELISA and FACS binding assays were performed to determine the binding activity, and receptor ligand binding assays were performed to assess the biological activity. The selection criteria was as follows: OD450 nm > 1.0 by ELISA, MFI value > 50 by FACS, and inhibition rate > 60% by receptor ligand binding assay. Clones that met the selection criteria were expanded in DMEM media containing 10% (w/w) FBS at 37°C , 5% (v/v) CO_2 and frozen in liquid nitrogen so that the hybridoma cells could be used for subsequent antibody production and purification.

[00176] (step 5) Production and purification of lead candidate antibodies

[00177] The antibody concentrations from the hybridoma cells were low, about 1-10ug/mL, and there was a large variation in antibody concentrations. In addition, the FBS and the components of the culture medium can interfere with the analysis. Therefore, it was necessary to perform small scale antibody production and purification (1-5 mg).

[00178] Hybridoma cells from Example 2 (part 4) were cultured in T-75 culture flasks using Hybridoma serum free medium (Invitrogen), and passaged for 3 generations. Cells were transferred to 2L culture flasks when the hybridoma cells were in good condition. 500mL of production media was added to each culture flask, and the cell density was adjusted to 10^5 cells/mL. The culture flasks were placed onto a rotary incubator at 37°C with a rotating speed of 3 cycles per minute. Hybridoma cells were cultured for 14 days, after which the supernatant was collected and the cells were removed. The

supernatants were then filtered through 0.45 micron filtration. The culture supernatants were then ready for purification or storage at -30°C.

[00179] Monoclonal antibodies were purified by passing the hybridoma culture supernatants through 2mL Protein G columns (GE Healthcare). Protein G columns were first equilibrated with PBS buffer (pH7.2), and the hybridoma culture supernatants were then applied to the equilibrated Protein G columns with a constant flow rate of 3mL/minute. The columns were then washed with 4 volumes of PBS buffer. The anti-PD-1 antibodies were then eluted with elution buffer (0.1M acetate buffer, pH2.5), and the UV absorbance of the eluates were monitored using a UV detector (A280 UV absorption peak). 10% of 1.0M Tris-HCL buffer was added to the eluates to neutralize the pH, and the samples were sterile-filtered by passing them through 0.22 micron filters. Sterile-filtered purified anti-PD-1 antibodies were obtained.

[00180] The concentrations of purified anti-PD-1 antibodies was determined by UV absorbance (A280/1.4), and the purity and endotoxin level (Lonza kit) were measured. The results of the analyses are shown in Table 6. The purified anti-PD-1 antibodies had endotoxin concentrations less than 1.0 EU/mg.

[00181] **Table 6. Quality Control analysis of Purified Chimeric PD-1 mAbs from Hybridoma**

Clone ID	Purity	Protein Concentration ($\mu\text{g/mL}$)	Endotoxin (EU/ μg)
9B2C6C9	> 90%	0.44	4.76
76G5B3	> 90%	0.52	0.56
32B5C7	> 90%	1.65	0.99
223G6B5	> 90%	11.9	0.097
21G8F11	ND	3.5	ND

[00182] **Example 3 – Characterization of lead candidate antibodies**

[00183] (part A) Detection of the binding of anti-PD-1 antibodies to recombinant PD-1^{ECD}-hFc protein by ELISA

[00184] The binding of purified anti-PD-1 antibodies from Example 2 to recombinant human PD-1^{ECD}-hFc protein, cyno PD-1^{ECD}-hFc protein and other PD-1 family-related immune checkpoint proteins was analyzed by ELISA to determine the specificity of the antibodies.

[00185] Purified Immunogen A (hPD-1^{ECD}-hFc protein) from Example 2, cyno PD-1^{ECD}-hFc protein (prepared using the method for preparing Immunogen A (see Example 2, step 1), comprising the amino acid sequence of the extracellular domain of cyno PD-1, which corresponds to amino acids Leu25-Gln167 of Uniprot database protein B0LAJ3), and other immune checkpoint proteins (CD28, B7-1, ICOS, CTLA4 and NC-Fc) (R&D Systems) were diluted to 1 $\mu\text{g/mL}$ in PBS. 100 μL of the diluted recombinant proteins were added to each well of 96-well plate. The plates were sealed with plastic film and incubated at 4°C overnight. The plates were washed twice with wash buffer (PBS + 0.01% (v/v) Tween20), and incubated with blocking buffer (PBS + 0.01% (v/v) Tween20 + 1% (w/w) BSA) at room temperature for 2 hours. The blocking buffer was aspirated away, and 100 μL of purified anti-PD-1

antibodies were added to each well and incubated for 2 hours at 37°C. The plates were washed three times with wash buffer (PBS + 0.01% (v/v) Tween20). HRP-conjugated secondary antibody (Sigma) was added to each well and incubated for 2 hours at 37°C. After the plates were washed three times with wash buffer, 100uL TMB substrate was added to each well and incubated for 30 minutes at room temperature. 100uL stop solution (0.1N HCl) was added to each well to stop the reaction. The absorbance at 450nm was measured with an ELISA plate reader (384plus SpectraMax, Molecular Devices). The results are shown in Figures 4-6 and in Tables 7-9. The IgG control was human IgG.

[00186] Table 7. Binding activities of chimeric anti-PD-1 mAbs to human PD-1^{ECD}-hFc, as measured by ELISA

Clone ID	OD _{450nm}									
	Antibody concentration(nM)									
	100	31.6	10	3.16	1	0.316	0.1	0.0316	0.01	0.00316
9B2C6C9	3.07	3.26	3.25	3.14	3.10	2.95	1.93	0.93	0.50	0.36
76G5B3	3.05	3.13	3.12	3.07	3.07	3.04	2.43	1.31	0.65	0.38
32B5C7	3.05	3.14	3.14	3.07	3.02	2.81	2.11	1.09	0.55	0.36
IgG control	0.10	0.08	0.08	0.09	0.12	0.10	0.09	0.09	0.09	0.08

Clone ID	OD _{450nm}							
	Antibody concentration(nM)							
	100	10	1	0.1	0.01	0.001	0.0001	0.00001
223G6B5	2.86	2.87	2.89	2.47	0.771	0.451	0.385	0.375
21G8F11	2.73	2.87	2.83	1.77	0.669	0.468	0.433	0.405
226C3A7	2.83	2.86	2.86	2.54	0.90	0.45	0.40	0.39
204C5E12	2.77	2.84	2.87	2.13	0.68	0.48	0.43	0.43
IgG Control	0.395	0.287	0.288	0.297	0.298	0.311	0.298	0.287

[00187] Table 8. Binding activities of chimeric anti-PD-1 mAbs to Cyno Monkey PD-1^{ECD}-hFc, as measured by ELISA

Clone ID	OD _{450nm}									
	Antibody concentration(nM)									
	100	31.6	10	3.16	1	0.316	0.1	0.0316	0.01	0.00316
9B2C6C9	1.96	1.75	1.61	1.41	1.05	0.65	0.33	0.17	0.11	0.07
76G5B3	1.89	1.78	1.62	1.56	1.40	1.01	0.56	0.25	0.14	0.07
32B5C7	1.66	1.90	1.51	1.35	1.03	0.57	0.29	0.15	0.10	0.08
IgG control	0.09	0.07	0.07	0.07	0.07	0.07	0.08	0.07	0.07	0.07

Clone ID	OD _{450nm}							
	Antibody concentration(nM)							
	100	10	1	0.1	0.01	0.001	0.0001	0.00001
223G6B5	2.70	2.74	2.61	1.28	0.342	0.193	0.170	0.174
21G8F11	2.61	2.63	2.45	0.972	0.270	0.172	0.182	0.171
IgG Control	0.423	0.146	0.105	0.097	0.105	0.106	0.110	0.116

[00188] Table 9. Binding activities of chimeric anti-PD-1 mAbs to other immune checkpoint proteins, as measured by ELISA

Clone ID	OD _{450nm}					
	Immune check point proteins					
	hPD 1	CD28	B7.1	ICOS	CTLA4	NC-Fc
9B2C6C9	2.732	0.066	0.27	0.161	0.281	0.264
76G5B3	2.761	0.061	0.271	0.173	0.135	0.268
32B5C7	2.694	0.062	0.36	0.483	0.172	0.327
223G6B5	2.43	0.166	0.174	0.140	0.106	0.207
21G8F11	2.25	0.144	0.154	0.128	0.091	0.161
IgG control	0.082	0.064	0.07	0.142	0.062	0.068

5

[00189] (part B) Detection of the binding of anti-PD-1 antibodies to cells expressing PD-1 by flow cytometry

[00190] CHO-K1 cells were stably transfected with pIRES plasmid containing the nucleic acid sequence encoding full length human PD-1 (see Example 2, step 2) to generate CHO-K1 cells stably expressing human PD-1 (herein referred to as CHO-K1-hPD-1 cells). Other CHO-K1 were stably transfected with pIRES plasmid containing the nucleic acid sequence encoding full length cyno PD-1 to generate CHO-K1 cells stably expressing cyno PD-1 (herein referred to as CHO-K1-cPD-1 cells). CHO-K1-hPD-1 and CHO-K1-cPD-1 cells were cultured and expanded in T-75 culture flasks to 90% confluence. The culture media was aspirated, and the cells were washed twice with HBSS (Hanks Balanced Salt Solution, Invitrogen). The cells were treated with enzyme-free cell dissociation solution (Versene solution, Invitrogen) and collected. The cells were then washed twice with HBSS, cell counts were determined, and cells were resuspended with HBSS at 2×10^6 cells/mL. Goat serum was added to the cell suspension to a final concentration of 1%, and the cells were blocked for 30 minutes on ice and then washed twice with HBSS. The cells were collected after centrifugation and resuspended in FACS buffer (HBSS + 1%BSA, v/v) at 2×10^6 cells/mL. 100uL of the cell suspension were then added to each well of 96-well plate. 100uL of purified anti-PD-1 antibodies from Example 2 were added to each well of the 96-well plate and incubated for 2 hours on ice. Cells were washed twice with FACS buffer, and 100uL of Alexa 488-labeled secondary antibody (Invitrogen) were added to the 96-well plate and incubated for 1 hour on ice. The samples were washed three times with FACS buffer, and 100uL

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fixation buffer (4% paraformaldehyde v/v) were added to each well and incubated for 10 minutes. The cells were then washed twice with FACS buffer and resuspended in 100uL FACS buffer. The mean fluorescence intensity (MFI) was determined using FACS Calibur (BD), and the results are shown in Figures 7-8 and in Tables 10-11. The IgG control was human IgG, and the values in the tables are the mean fluorescence intensity of the cell population.

[00191] Table 10. Binding activities of chimeric anti-PD-1 mAbs to CHO-K1-hPD-1, as determined by FACS

Clone ID	Mean fluorescence intensity							
	Antibody concentration(nM)							
	100	31.6	10	3.16	1	0.316	0.1	0.0316
9B2C6C9	410.6	407.6	340.7	206.1	105.05	49	23.4	2.15
76G5B3	443.2	466.25	434.55	279.9	165.95	76.8	36.25	2.2
32B5C7	252.55	241.2	155.9	97.5	51.1	26.1	13.2	2.1
226C3A7	824.7	825.5	203.2	22.9	5.2	3.4	3.2	4.1
204C5E12	1492.9	985.5	160.2	21.4	5.0	3.0	2.9	3.0
IgG control	2.75	2.5	2.25	2.2	2.15	2.15	2.15	2.25

Clone ID	Mean fluorescence intensity							
	Antibody concentration(nM)							
	100	10	1	0.1	0.01	0.001	0.0001	0.00001
223G6B5	1587	1623	369.0	45.0	7.9	4.2	3.3	3.3
21G8F11	661.1	696.2	165.6	22.8	5.4	3.1	2.9	2.9
IgG Control	4.3	8.3	3.6	3.1	2.9	2.9	3.1	2.7

[00192] Table 11. Binding activities of chimeric anti-PD-1 mAbs to CHO-K1-cPD-1, as determined by FACS

Clone ID	Mean fluorescence intensity							
	Antibody concentration(nM)							
	100	31.6	10	3.16	1	0.316	0.1	0.0316
9B2C6C9	340.4	313.8	262.2	180.8	94.6	43.3	21.1	2.2
76G5B3	296.7	314.5	326.2	217.3	131.7	59.3	28.4	2.3
32B5C7	150.2	151.4	173.9	89.0	46.3	25.8	13.7	2.2
226C3A7	932.6	848.4	220.8	28	5.8	3.6	3.3	3.3
204C5E12	944.5	772.9	156.3	23.4	5.1	3.1	3.1	2.9
IgG control	2.9	3.0	2.4	2.3	2.2	2.2	2.2	2.2

Clone ID	Mean fluorescence intensity							
	Antibody concentration(nM)							
	100	10	1	0.1	0.01	0.001	0.0001	0.00001
223G6B5	2339	2125	474.6	50.6	8.9	4.0	3.3	3.3
21G8F11	944.5	772.9	156.3	23.4	5.1	3.1	3.1	2.9
IgG Control	5.0	4.5	3.2	3.6	3.2	3.0	3.0	2.9

[00193] (part C) Binding affinity and Dissociation constant of anti-PD-1 antibodies

[00194] Dissociation constants were determined by Octet red 96 (Fortiebio). The detailed operation and methods were followed according to the specifications of the instrument provided by the manufacturer. Briefly, a streptavidin sensor (SA sensor, Fortiebio) was used for the affinity determination. Biotinylated PD-1^{ECD}-hFc (Immunogen A) was diluted to 10ug/mL in PBS buffer (pH7.4) containing 0.1% (w/w) BSA and 0.02% (v/v) Tween20 and incubated with the streptavidin sensor. Five different concentrations of anti-PD-1 antibody were incubated with the Immunogen A-loaded streptavidin sensor at 30°C for 3 minutes. The reaction mixture was further incubated in PBS buffer (pH7.4) containing 0.1% (v/w) BSA and 0.02% (v/v) Tween20 at 30°C for 5 minutes. The association and dissociation signals of anti-PD-1 antibodies to Immunogen A were recorded in real time using Octet Red 96. The affinity, association and dissociation constants were determined using Octet User software, and the results are shown in Table 12.

[00195] **Table 12.** Binding kinetics and affinities of anti-PD-1 chimeric mAbs to human PD-1^{ECD}-hFc protein, as determined by Octet Red 96

Clone ID	K _D (nM)	k _a (1/Ms)	k _d (1/s)
9B2C6C9	0.269	9.01x10 ⁴	2.43x10 ⁻⁵
76G5B3	0.032	4.08x10 ⁵	1.30x10 ⁻⁵
32B5C7	0.045	2.12x10 ⁵	9.64x10 ⁻⁶

[00196] **Example 4 – Determination of the ability of the anti-PD-1 antibodies to block the binding of PD-1 to its ligands PD-L1 and PD-L2**

[00197] Protein- and cell-based receptor ligand binding assays were performed to determine the ability of the anti-PD-1 antibodies to block the binding of PD-1 to its ligands PD-L1 and PD-L2.

[00198] Biotinylated recombinant PD-L1^{ECD} and PD-L2^{ECD} proteins were prepared as described for biotinylated recombinant PD-1^{ECD}-hFc in Example 2. The extracellular domain of PD-L1 corresponds to amino acids Phe19-THr239 of Uniprot database protein Q9NZQ7.1, and the extracellular domain of PD-L2 corresponds to amino acids Leu20-Pro219 of Uniprot database protein Q9BQ51.

[00199] Purified PD-1^{ECD}-hFc (Example 2) was diluted with PBS to a final concentration of 1.0ug/mL, and 100uL diluted PD-1^{ECD}-hFc were added to each well of a 96-well plate that was then

sealed with plastic film and incubated at 4°C overnight. The plate was washed twice with wash buffer (PBS + 0.01% (v/v) Tween 20) and incubated with blocking buffer (PBS + 0.01% (v/v) BSA + 1% Tween 20 (w/w)) at room temperature for 2 hours. The blocking buffer was aspirated, and 50uL purified anti-PD-1 antibodies from Example 2 were added to each well of 96-well plate. 100uL of biotinylated recombinant PD-L1^{ECD} or PD-L2^{ECD} protein were added to each well, mixed, and incubated at 37°C for 2 hours. The plate was washed three times with wash buffer (PBS + 0.01% (v/v) Tween 20). 100uL HRP-conjugated streptavidin (Sigma) were then added to each well and incubated at 37°C for 2 hours. The plate was then washed three times with wash buffer, and 100uL TMB substrate were added to each well. After 30 minutes of incubation at room temperature, the reaction was stopped by adding 100uL stop solution (0.1N HCl). The absorbance at OD450nm was measured with an ELISA plate reader (384plus SpectraMax, Molecular Devices). The results, shown in Figures 9-10, demonstrate that the anti-PD-1 antibodies can block the binding of PD-1 to its ligands, PD-L1 and PD-L2.

[00200] CHO-K1-hPD-1 cells were cultured and expanded in T-75 culture flasks to 60-80% confluence. The culture media was aspirated, and the cells were washed twice with PBS. The cells were treated with TrypLE TM Express (Invitrogen) and collected. Trypsin was neutralized by the addition of 8mL culture medium, and cell counts were determined. Cells were centrifuged at 300g for 5 minutes and resuspended in blocking buffer at 1×10^6 cells/mL. The cells were blocked for 15 minutes at 37°C. Meanwhile, the wells of 96-well round-bottom plates were blocked with 200uL blocking buffer for 1 hour at 37°C. The blocking buffer was discarded, and 200uL cells were dispensed to each well of the 96-well plates (2×10^5 cells/well). The plates were centrifuged at 500g for 5 minutes, and the supernatants were discarded. The cells were resuspended in 100uL antibody prepared in blocking buffer. 100uL of biotinylated PD-L1-Fc or biotinylated PD-L2-Fc (60ug/mL in blocking buffer) were added to each well of 96-well plate and mixed by shaking gently. The plates were incubated at 4°C for 90 minutes and washed twice with 200uL blocking buffer. The blocking buffer was discarded, and the cells were resuspended in 100uL streptavidin-Alexa 488 solution (1:500 in blocking buffer, Invitrogen) and incubated at 4°C for 1 hour. The plates were washed three times with blocking buffer and resuspended in 200uL blocking buffer. The mean fluorescence intensity (MFI) was determined using FACS Calibur (BD). The results, shown in Figures 11-12, demonstrate that the anti-PD-1 antibodies can block the binding of cell-expressed PD-1 to its ligands, PD-L1 and PD-L2.

[00201] **Example 5 – Lymphocyte stimulation assay to examine the ability of the anti-PD-1 antibodies to block the binding of PD-1 to its ligands PD-L1 and PD-L2**

[00202] (A) A T cell stimulation assay was performed to examine the effect of the anti-PD-1 antibodies on T cell stimulation by their blocking of the binding of PD-1 to its ligands PD-L1 and PD-L2.

[00203] (step 1) Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood by a Ficoll gradient.

[00204] Whole blood was diluted with PBS at a ratio of 1:1 (v/v) and gently added on top of Ficoll solution (GE Healthcare) using a sterile pipette. The volume ratio of Ficoll to diluted whole blood was 3:4. The samples were centrifuged at 400g at 20°C for 30 minutes. Three layers of solution were formed

after centrifugation, with the upper layer being plasma, and the middle milk white layer being mononuclear cells. A sterile pipette was used to collect the mononuclear cells from the middle layer and transfer them to a new centrifugal tube. Three times the sample volume of PBS was added, and the samples were centrifuged at 100g at room temperature for 10 minutes. The supernatant was discarded, and the lymphocytes were resuspended with 10mL PBS buffer. The lymphocytes were washed with PBS three times to remove blood platelets. The lymphocyte suspension was then resuspended with 10mL RPMI1640 culture medium (Invitrogen) containing 10% FBS.

[00205] (step 2) PBMC stimulation test

[00206] Constructs encoding full-length PD-L1 protein were generated by subcloning PD-L1 nucleotide sequence into pIRES plasmid (pIRES-puro-PD-L1). To anchor anti-CD3 (OKT3) (see Kipriyanov et al., 1997, Peds. 10:445-453) into the cell membrane, OKT3 scFv was fused to the C-terminus of mouse CD8a (amino acids 113-220 of NCBI accession No: NP - 1074579.1) and subcloned into pIRES-OS8 (see Sambrook and Russell, *Id.*). pIRES-puro-PD-L1 and pIRES-OS8 were co-transfected to CHO-K1 and 293F cells following the preparation method in Example 2 to generate the stable cell lines CHO-K1-PD-L1/OS8 and 293F-PD-L1/OS8. The cells were used to stimulate T lymphocytes. Before the experiments, CHO-K1-PD-L1/OS8 and 293F-PD-L1/OS8 cells were treated with 10ug/mL mitomycin at 37°C for 3 hours.

[00207] 100uL PBMC (containing 5×10^4 cells) were added to the wells of a 96-well plate, and the test antibody solution was then added to the 96-well plate and incubated for 15 minutes at room temperature. 50uL of 5×10^3 CHO-K1-PD-L1/OS8 or 293F-PD-L1/OS8 were added to each well and cultured at 37°C, 5% CO₂ for 72 hours. The supernatants were collected and stored at -20°C until analysis.

[00208] (step 3) Detection of interferon gamma (IFN- γ) or interleukin IL-2 secretion by ELISA

[00209] Quantification of the levels of IFN- γ or IL-2 in culture supernatant was carried out using Human IFN-gamma Quantikine ELISA Kit (R&D Systems SIF50) and Human IL-2 Quantikine ELISA Kit D2050 (R&D Systems S2050), respectively, following the manufacturer-provided operating instructions and kit reagents. Briefly, the IFN- γ and IL-2 polyclonal antibodies were coated onto the ELISA microplates, and 400uL of culture supernatant as well as the standard were added to each well and incubated at room temperature for 2 hours. The plates were washed 4 times with wash buffer, followed by the addition of HRP-conjugated anti-human IFN- γ and IL-2 antibodies, and incubated at room temperature for 2 hours. After washes, a chromogenic substrate was added and incubated in the dark at room temperature for 30 minutes, and the reaction was terminated by the addition of a stop solution. The absorbance at 450nm was determined using an ELISA plate reader, and the results, shown in Figure 13 and in Table 13, demonstrate that the anti-PD-1 antibodies analyzed by the PBMC lymphocyte stimulation test can increase IFN- γ secretion. The IgG control is human IgG, and the values listed in the table are the IFN- γ concentrations in the culture supernatant (pg/mL).

[00210] Table13. Chimeric anti-PD-1 mAbs induce IFN- γ release from stimulated PBMCs

Clone ID	IFN- γ (pg/mL)		
	Antibody Concentration(ug/mL)		
	10	1	0.1
9B2C6C9	20596.6	14651.4	5805.1
76G5B3	17419.6	18640.1	13643.3
IgG Control	4036.6	4544.7	5193.8

[00211] (B) A mixed lymphocyte reaction was performed to examine the impact of the anti-PD-1 antibodies on T cell stimulation by their blocking of the binding of PD-1 to its ligands PD-L1 and PD-L2.

[00212] (step 1) Isolation and culture of dendritic cells from human CD14⁺ cells

[00213] FicollPaque Plus (GE Healthcare) was used to isolate PBMCs from whole blood, following the manufacturer-provided instructions. The protocol was the same as that described in Example 5A, step 1.

[00214] PBMCs were resuspended in RPMI 1640 complete medium containing 10% FBS, and the cell concentration was adjusted to 1×10^5 cells/mL. The cells were cultured in T-75 culture flasks at 37°C, 5% (v/v) CO₂ for 2 hours. The culture supernatant and non-adhered cells were transferred to a new T-75 culture flask, and the original T-75 culture flask was replenished with new RPMI 1640 complete culture medium supplemented with 10% FBS and incubated at 37°C and 5% (v/v) CO₂ for 2 hours. The culture supernatant and non-adhered cells were removed, and the adherent cells were replenished with RPMI1640 culture media containing 10% FBS and incubated at 37°C, 5% (v/v) CO₂ for 18 hours. The culture supernatant and non-adhered cells were removed, and the adherent cells were replenished with RPMI1640 complete culture media supplemented with 500U/mL recombinant human GM-CSF (PEPROTECH) and 500U/mL recombinant human interleukin IL-4 (PEPROTECH) and cultured for 4 days. After 4 days, the culture media was replenished with complete RPMI 1640 media supplemented with GM-CSF and IL-4, and the cells were cultured for an additional 2 days. The culture medium was then replaced with RPMI 1640 complete media with 1ug/mL LPS and incubated for 18 hours. Dendritic cells were then collected by adding PBS containing EDTA and centrifuging at 300g for 5 minutes. The supernatant was aspirated, and the cells were washed once more with PBS. The collected human CD14⁺ dendritic cells were resuspended in RPMI 1640 complete media, and the cell counts were determined.

[00215] (step 2) Isolation and purification of human CD4⁺ T cells.

[00216] Human CD4⁺ T cells were isolated and purified from PBMCs using a MagCollect™ human CD4 + T Cell Isolation Kit (R&D Systems), following the manufacturer-provided instructions.

[00217] (step 3) Mixed lymphocyte reaction

[00218] Purified CD4⁺ cells from different healthy volunteers were co-cultured with dendritic cells in 96-well plates. The cell densities were adjusted to 10^5 cells per 80uL. 10^5 purified CD4⁺ T cells and 2×10^4 dendritic cells were added to each well of a 96-cell plate, purified anti-PD-1 antibodies from

Example 2 were added to the appropriate wells in the plate, and the plates were incubated at 37°C in a 5% CO₂ incubator for 6 days. The supernatants were collected, and the cytokine levels were determined.

[00219] (step 4) Measurement of IFN- γ and IL-2 levels in supernatant by ELISA

[00220] Quantification of the levels of IFN- γ or IL-2 in the supernatants collected in the previous step was carried out using Human IFN-gamma Quantikine ELISA Kit (R&D Systems SIF50) and Human IL-2 Quantikine ELISA Kit D2050 (R&D Systems S2050), respectively, following the manufacturer-provided operating instructions and kit reagents.

[00221] The results for IL-2, shown in Figures 14-15 and in Tables 14-15, show that the IL-2 level increased with an increase in anti-PD-1 antibody concentrations. The IgG control is human IgG, and the values listed in Tables 14-15 are the IL-2 concentration (pg/mL) in the culture supernatants. PBMC donor -1 and PBMC donor -2 refer to blood donor ID.

[00222] **Table 14.** Chimeric anti-PD-1 mAbs induce IL-2 release, as determined by a mixed lymphocyte reaction assay (PBMC donor-1)

Clone ID	IL-2 (pg/mL)				
	Antibody Concentration(μ g/mL)				
	0.001	0.01	0.1	1	10
9B2C6C9	82.3	96.0	144.4	142.1	151.4
76G5B3	88.8	130.7	147.7	139.7	150.1
223G6B5	75.8	116.0	148.8	209.0	ND
21G8F11	33.1	48.9	106.4	65.6	ND
IgG Control	-	-	76.7	76.0	83.0

[00223] **Table 15.** Chimeric anti-PD-1 mAbs induce IL-2 release, as determined by a mixed lymphocyte reaction assay (PBMC donor-2)

Clone ID	IL-2 (pg/mL)				
	Antibody Concentration(μ g/mL)				
	0.001	0.01	0.1	1	10
9B2C6C9	107.7	141.5	148.9	194.3	169.0
76G5B3	93.3	145.2	182.2	193.6	188.2
223G6B5	28.7	43.7	71.1	107.0	ND
21G8F11	30.0	43.4	74.2	102.7	ND
IgG Control	-	-	115.0	89.4	93.6

[00224] The results for IFN- γ , shown in Figures 16-21 and in Tables 16-19, show that the IFN- γ level increased with an increase in anti-PD-1 antibody concentrations. The IgG control is human IgG, and the values listed in Tables 16-19 are the IFN- γ concentration (pg/mL) in the culture supernatants. PBMC donor -1 and PBMC donor -2 refer to blood donor ID.

[00225] **Table16.** Chimeric PD-1 mAbs induce IFN- γ release, as determined by a mixed lymphocyte reaction assay (PBMC donor-1)

Clone ID	IFN- γ (pg/mL)				
	Antibody Concentration(μ g/mL)				
	0.001	0.01	0.1	1	10
9B2C6C9	2229.6	4687.1	17891.3	11404.4	7591.3
76G5B3	2895.3	7526.0	12139.7	8108.7	14490.4
223G6B5	4893.5	9376.9	27300.3	33964.3	ND
21G8F11	3005.3	5910.4	15299.6	15248.1	ND
IgG Control	-	-	2076.4	1720.7	2384.1

[00226] **Table17.** Chimeric PD-1 mAbs induce IFN- γ release, as determined by a mixed lymphocyte reaction assay (PBMC donor-2)

Clone ID	IFN- γ (pg/mL)				
	Antibody Concentration(μ g/mL)				
	0.001	0.01	0.1	1	10
9B2C6C9	997.0	3496.1	7352.7	15169.3	17565.9
76G5B3	1805.6	5518.5	12716.3	15900.6	13348.2
223G6B5	1744.6	2573.7	4300.4	5428.3	ND
21G8F11	2089.3	2720.7	2626.4	2827.1	ND
IgG Control	-	-	1183.6	1871.4	2588.0

[00227] **Table 18.** Chimeric PD-1 mAbs induce IFN- γ release, as determined by a mixed lymphocyte reaction assay (PBMC donor-1)

Clone ID	IFN- γ (pg/mL)				
	Antibody Concentration(μ g/mL)				
	0.001	0.01	0.1	1	10
32B5C7	339.95	4719.75	6197.8	10864.95	21250.1
IgG Control	-	1458.32	1446.08	1251.255	-

[00228] **Table 19.** Chimeric PD-1 mAbs induce IFN- γ release, as determined by a mixed lymphocyte reaction assay (PBMC donor-2)

Clone ID	IFN- γ (pg/mL)			
	Antibody Concentration(μ g/mL)			
	0.01	0.1	1	10
32B5C7	11865.3	26675.9	32325.9	46764.8
IgG Control	352.8	281.3	127.1	-

[00229] Example 6 – Determination of the amino acid sequences in the variable regions of the anti-PD-1 antibodies

[00230] Total RNA isolation: After the supernatants from the hybridoma subclones obtained from Example 2 were characterized (i.e., validation and determination of bioactivity, Examples 3-5),

5 5×10^7 hybridoma cells were collected by centrifugation. 1mL Trizol was added to the cell pellets, mixed and transferred to 1.5mL centrifuge tubes, and incubated at room temperature for 5 minutes. 0.2mL chloroform were added to the samples and vortexed for 15 seconds. After standing for 2 minutes, the mixtures were centrifuged at 12000g at 4°C for 5 minutes. The supernatants were collected and transferred to new 1.5mL centrifuge tubes, 0.5mL isopropyl alcohol was added and mixed gently, and
10 the samples were incubated at room temperature for 10 minutes. The samples were centrifuged at 12000g at 4°C for 15 minutes. The supernatants were aspirated, and the precipitates were washed with 1mL 75% (v/v) ethanol. The mixtures were centrifuged at 12000g at 4°C for 5 minutes, the supernatants were decanted, and the precipitates were air-dried. Total RNA was obtained by adding DEPC-treated water to the precipitates (55°C water bath for 10 minutes).

15 **[00231]** Reverse transcription and PCR: 1 μ g of RNA and reverse transcriptase were added to a reaction mixture of a final volume of 20 μ L, and the mixture was incubated at 42°C for 60 minutes and then at 70°C for 10 minutes to terminate the reaction. A 50 μ L PCR reaction mixture was prepared, containing 1 μ L cDNA, 25 pmol of each primer, 1 μ L DNA polymerase, 250 μ mol dNTPs, and the buffer system. The PCR program settings were as follows: denaturation at 95°C for 3 minutes, 35 cycles of
20 denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds) and elongation (72°C for 35 seconds), followed by a final extension at 72°C for 5 minutes to obtain the PCR product. The commercially available reverse transcription kit used was PrimeScript RT Master Mix (Takara, RR036), and the commercially available Q5 ultra-fidelity polymerase PCR kit was from NEB (M0492).

[00232] Cloning and sequencing: 5 μ L PCR products were examined by agarose gel electrophoresis,
25 and the samples were recovered from the agarose gel using NucleoSpin Gel & PCR Clean-up kit (MACHEREY-NAGEL, 740609). Ligation reaction: To a 50ng sample, 50ng T vector, 0.5 μ L ligase, and 1 μ L buffer were added and brought to a final volume of 10 μ L with water. The reaction mixture was incubated at 16°C for 30 minutes using the T4 DNA Ligase (NEB, M0402). 5 μ L ligation product was added to 100 μ L competent cells (Ecos 101 competent cells, Yeastern, FYE607), which were incubated
30 on ice for 5 minutes, heat shocked at 42°C for 1 minute and incubated on ice again for 1 minute. Cells were recovered by adding 650 μ L SOC medium without antibiotics and incubating at 37°C in a shaking incubator for 30 minutes at 200 rpm. 200 μ L of each bacterial culture was spread onto an LB agar plate containing antibiotics at 37°C overnight. The next day, PCR reactions were set up using the T vector primers M13F and M13R. Pipette tips were used to pick bacterial colonies and were dipped into PCR
35 reaction mixture and pipetted up and down. Half of the reaction mixture was transferred to an LB agar plate containing 100nM ampicillin. After the PCR reactions, 5 μ L of the PCR products were removed and examined by agarose gel electrophoresis, and positive samples were sent for sequencing and analysis (Kabat, 1991, "Sequences of proteins of objective interest," the NIH, Bethesda, MD). The sequencing results are shown in Tables 1-2.

[00233] Example 7 – Conversion, expression and purification of fully human anti-PD-1 antibody

[00234] (step 1) Plasmid construction and preparation: Sequences of the anti-PD-1 antibody heavy and light chain variable regions were obtained according to Example 6. The anti-PD-1 antibodies' heavy chain variable region sequences were subcloned into expression vectors containing a signal peptide and a human heavy chain IgG4 constant region with a S228P mutation. The anti-PD-1 antibodies' light chain variable region sequences were subcloned into expression vectors containing a signal peptide and a human antibody light chain kappa constant region. The recombinant plasmids were verified and confirmed by sequencing (the sequencing method was the same as in Example 6). Alkaline lysis was performed using a reagent kit (MACHEREY-NAGEL) to improve the purity and quality of the recombinant plasmids, and the plasmids were filtered through 0.22uM filters (Millipore). The purified plasmids were used for transfection.

[00235] (step 2) Transfection: HEK293E cells (Invitrogen) were cultured in FreeStyle 293 medium (Invitrogen) at 37°C, 130RPM, 8% CO₂ (v/v). HEK293E cells were adjusted to 1-1.5x10⁶/mL cell density for transfection. 10% (v/v) F68 (Invitrogen) was added to the FreeStyle 293 medium to a final concentration of 0.1% (v/v), as Medium A. 5mL of Medium A and 200ug/mL PEI (Sigma) were mixed to generate Medium B. 5mL of Medium A and 100ug/mL recombinant plasmid from step 1 were mixed to generate Medium C. After 5 minutes of incubation, Medium B and Medium C were mixed and incubated for 15 minutes to generate Mixture D. 10mL of Mixture D were slowly added to 100mL HEK293E cells with continuous stirring to avoid local accumulation of PEI. HEK293E cells were incubated overnight while shaking. The next day, peptone was added to a final concentration of 0.5% (w/v). On about day 5-7, the antibodies' titers were determined. On about day 6-7, the HEK293E cultures were centrifuged (30 minutes, 3500RPM), and the supernatants were collected and filtered through 0.22uM filters for purification.

[00236] (step 3) Antibody purification: Protein A columns (GE) were washed with 0.1M NaOH for 30 minutes or with 5 bed volumes of 0.5M NaOH to get rid of endotoxin. Columns that had not been used in a long time were soaked in 1M NaOH for at least 1 hour, washed with endotoxin-free water to a neutral pH, and washed with 10 bed volumes of 1% Triton X100. The columns were then equilibrated with 5 bed volumes of PBS (PBS phosphate buffer, pH7.2). The filtered supernatants from step 2 were loaded onto the columns, and the flow through was collected, if necessary. The columns were washed with 5 bed volume of PBS and then eluted with 5 bed volumes of 0.1M Glycine-HCl pH3.0. The eluates containing anti-PD-1 antibodies were neutralized with 0.5 bed volumes of 1M Tris-HCl (NaCl 1.5M) pH8.5. The human anti-PD-1 antibodies were dialyzed in 1X PBS for 4 hours, to avoid endotoxin contamination. After dialysis, the anti-PD-1 antibody concentrations were determined by spectrophotometry or a reagent kit, the purities of the antibodies were determined by HPLC-SEC, and the contents of endotoxin were determined by an endotoxin test kit (Lonza). The fully human anti-PD-1 antibodies were characterized, and the results are shown in Figures 22-41, and in Tables 20-25.

[00237] Table 20. Binding activities of fully human anti-PD-1 mAbs to human PD-1^{ECD}-hFc, as measured by ELISA

Clone ID	OD _{450nm}								
	Antibody concentration(nM)								
	100	10	1	0.1	0.01	0.001	0.0001	0.00001	0
9B2C6C9	2.60	2.55	2.51	1.56	0.34	0.12	0.10	0.10	0.09
76G5B3	2.58	2.44	2.47	1.34	0.28	0.12	0.10	0.10	0.09
32B5C7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
223G6B5	2.78	2.80	2.86	2.38	0.62	0.32	0.29	N/A	0.30
21G8F11	3.03	3.02	3.03	2.62	0.58	0.26	0.16	N/A	0.18
IgG control	0.34	0.26	0.25	0.24	0.23	0.24	0.25	N/A	0.28

[00238] Table 21. Binding activities of fully human anti-PD-1 mAbs to Cyno Monkey PD-1^{ECD}-hFc, as measured by ELISA

Clone ID	OD _{450nm}								
	Antibody concentration(nM)								
	100	10	1	0.1	0.01	0.001	0.0001	0.00001	0
9B2C6C9	2.43	2.38	2.11	0.73	0.14	0.07	0.07	0.07	0.06
76G5B3	2.45	2.41	2.32	1.06	0.20	0.08	0.06	0.06	0.06
32B5C7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
223G6B5	2.85	2.87	2.88	2.50	0.83	0.33	0.29	N/A	0.31
21G8F11	3.00	3.02	2.99	2.39	0.51	0.22	0.15	N/A	0.19
IgG control	0.33	0.28	0.27	0.26	0.27	0.26	0.27	N/A	0.31

5 [00239] Table 22. Binding activities of fully human anti-PD-1 mAbs to other immune checkpoint proteins, as measured by ELISA

Clone ID	OD _{450nm}					
	Immunocheck point proteins					
	hPD 1	CD28	B7.1	ICOS	CTLA4	NC-Fc
9B2C6C9	3.10	0.09	0.33	0.34	0.28	0.35
76G5B3	3.01	0.10	0.37	0.38	0.32	0.41
32B5C7	N/A	N/A	N/A	N/A	N/A	N/A
223G6B5	1.85	0.05	0.25	0.12	0.13	0.20
21G8F11	2.70	0.27	0.25	0.16	0.09	0.21
IgG control	0.28	0.10	0.29	0.28	0.23	0.31

[00240] Table 23. Binding activities of fully human anti-PD-1 mAbs to CHO-K1-hPD-1, as determined by FACS

Clone ID	Mean fluorescence intensity									
	Antibody concentration(nM)									
	100.0	31.6	10.0	3.2	1.0	0.32	0.10	0.03	0.01	0
9B2C6C9	1574	1646	1668	1519	830	324.0	108.0	38.7	16.1	4.2
76G5B3	1884	1943	1920	1881	1200	504.9	167.5	58.9	22.9	4.1
223G6B5	1404	1413	1434	1421	1114	501.3	168.1	58.4	22.3	4.0
IgG control	6.0	4.8	4.4	4.2	4.0	4.0	3.9	4.0	4.0	3.9

Clone ID	Mean fluorescence intensity								
	Antibody concentration(nM)								
	100	10	1	0.1	0.01	0.001	0.0001	0	
21G8F11	1729	1794	1794	1700	1015	414.5	137.7	48.8	
IgG control	705.2	708.7	719.0	712.6	559.0	252.6	86.0	31.2	

Clone ID	Mean fluorescence intensity							
	Antibody concentration(nM)							
	66.7	6.7	0.7	0.07	0.01	0.0007	0.00	
32B5C7	479.9	252.1	53.6	9.0	2.8	2.6	2.4	

5

[00241] Table 24. Binding activities of fully human anti-PD-1 mAbs to CHO-K1-cPD-1, as determined by FACS

Clone ID	Mean fluorescence intensity									
	Antibody concentration(nM)									
	100.0	31.6	10.0	3.2	1.0	0.3	0.1	0.03	0.01	0.0
9B2C6C9	5.0	4.4	4.2	3.9	3.9	3.9	3.9	3.9	3.9	4.0
76G5B3	100.0	31.6	10.0	3.2	1.0	0.3	0.1	0.0	0.0	0.0
223G6B5	859.0	879.8	864.6	752.9	416.8	181.3	63.5	24.4	11.2	3.8
IgG control	5.0	4.4	4.2	4.0	4.0	4.0	3.9	3.9	3.9	3.9

Clone ID	Mean fluorescence intensity								
	Antibody concentration(nM)								
	100	10	1	0.1	0.01	0.001	0.0001	0	
21G8F11	1744.7	1593.2	566.6	104.2	24.0	10.9	5.5	3.7	
IgG control	33.1	8.7	19.6	13.8	5.3	3.9	3.8	3.7	

10

[00242] Table 25. Binding kinetics and affinities of fully human anti-PD-1 mAbs to His-tagged human PD-1 ECD protein, as determined by Biacore

Clone ID	K _D (M)	k _a (1/Ms)	k _d (1/s)
9B2C6C9	7.209E-9	1.674E+4	1.207E-4
76G5B3	9.357E-10	9.996E+4	9.354E-5
223G6B5	5.496E-9	8.650E+4	4.754E-4
pembrolizumab	8.02E-9	3.35E+5	2.69E-3
nivolumab	1.08E-8	1.30E+5	1.40E-3

[00243] Example 8 – Determination of binding and dissociation constants by Biacore

[00244] Anti-human Fc IgG was immobilized on flow cells 1 and 2: HBS-EP+ (10mM HEPES, 150mM NaCl, 3mM EDTA, 0.05% P20, pH 7.4) was used as running buffer, and the immobilization of anti-human Fc IgG was carried out using the immobilization wizard template. Flow cells 1 and 2 of a Series S CM5 sensor chip were activated with freshly-mixed 50mmol/L NHS and 200mmol/L EDC. 20ug/mL of anti-human Fc IgG diluted in 10mM NaAC (pH 4.5) was injected into the activated flow cells 1 and 2. The remaining active coupling sites were blocked with 1M ethanolamine.

[00245] Recombinant His-tagged hPD-1 ECD protein was diluted to 50nM, followed by four 2-fold serial dilutions with HBS-EP+ buffer. The His-tagged hPD-1 ECD protein concentrations were 0nM, 3.125nM, 6.25nM, 12.5nM, 25nM and 50nM. K_D measurements were carried out with HBS-EP+ as the running buffer. Each antibody was injected over the CM5 sensor flow cell 2 with a flow rate of 10 uL/min to reach response 230 RU. Prepared His-tagged hPD-1 ECD protein was then injected over flow cells 1 and 2, at a flow rate of 30uL/min for 180sec. Buffer flow was maintained for 400 seconds for dissociation measurements (30uL/min). To remove the tested antibody from the surface, 10mM glycine-HCl pH 1.5 was injected for 20 seconds (30uL/min). Flow cell 1 was used as reference flow cell. The above steps were repeated for each concentration of serially-diluted His-tagged PD-1^{ECD} protein. The K_D value for each antibody was evaluated using Biacore T200 evaluation software 1.0, and the data was fit with a 1:1 binding model. The results are shown in Table 25.

[00246] Example 9 – ADCC and CDC Effector Function Analysis

[00247] To confirm the presumed absence of effector function of fully human anti-PD-1 antibodies, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays were performed.

[00248] For the ADCC assay, samples of PD-1-expressing Jurkat T cells were adjusted to a concentration of 12.5×10^4 cells/mL with ADCC medium (without phenol red). 40uL of cell suspensions (1×10^4 viable cells) were added to each well of a v-bottom 96-well plate. 20uL of antibody were serially diluted in ADCC medium (without phenol red) and added to each well in triplicate. The final antibody concentrations were: 1.4ng/mL, 4.1ng/mL, 12.3ng/mL, 0.037ug/mL, 0.111ug/mL, 0.333ug/mL, and 1ug/mL. The plate was incubated at 22-25°C for 30 minutes. NK92 cells stably transfected with FcγRIII 158V were adjusted with ADCC medium (without phenol red) so that by adding 40uL of NK92 cells stably transfected with FcγRIII 158V to the target cells, the ratio of effector to target cells was 1:1. The plate was then incubated at 37°C for 4 hours. After 4 hours incubation, 100uL substrate from CytoTox 96 kit (Promega) were added to each well. For maximum cell lysis control, 2uL

of lysis solution (CytoTox-ONETM kit, Promega) was added 10 minutes before the addition of 100uL substrate from CytoTox 96 kit. The plate was incubated at room temperature for 10 minutes, and 50uL of stop solution were then added to each well and mixed for 30 seconds. The absorbance at 560/590nm was measured, and the 1% lysis values were calculated using GraphPad Prism 5.0. The results, shown in Figure 37, show that the anti-HLA antibody induced ADCC in PD-1-expressing Jurkat T cell cells, but the fully human anti-PD-1 antibodies had no ADCC effect on PD-1-expressing Jurkat T cells.

[00249] For the CDC assay, PBMCs were harvested and CD4+ T cells were isolated using a CD4+ T cell isolation kit (Stemcell, No.19052). CD4+ cells were incubated with anti-CD3 antibody (Clone: OKT3) for 48 hours to induce expression of PD-1. Cells were harvested, and the cell concentrations were adjusted to 1.25×10^6 cells/mL with cell culture medium. To a flat-bottom 96-well white plate, 40uL/well of cells were added. 40uL/well of fully human anti-PD-1 antibody, serially diluted in CDC medium, were added to each well in duplicate. The final antibody concentrations were: 0ng/mL, 16ng/mL, 80ng/mL, 0.4ug/mL, 2ug/mL, and 10ug/mL. The plates were incubated in the hood for 30 minutes. Commercially available purified human complement (Quidel, cat # 042637) was added to the plates containing cells at 20uL/well to a final concentration of 20%. The plates were incubated at 37°C for 20 hours. The Celltiter-glo luminescent cell viability kit (Promega, No. G7573) was used to test cell viability according to the manufacture-provided protocol. The plates were shaken on a microplate shaker for 2 minutes at a speed of 200 and then incubated at room temperature for 10 minutes. The luminescence signal was read with Envision. For data analysis, the % cytotoxicity was calculated using GraphPad Prism 5.0. The results, shown in Figure 38, show that the anti-HLA antibody induced CDC in activated CD4+ T cells, but the fully human anti-PD-1 antibodies had no CDC effect on activated CD4+ T cells.

[00250] Example 10 – Antibody thermostability, measured by differential scanning calorimetry (DSC)

[00251] Fully human anti-PD-1 antibodies were adjusted to 1mg/mL and a final volume of about 700uL with sample buffer. The parameters were set up as follows (VP-DSC): Starting Temperature 30°C; Final Temperature 100°C; Scan rate 50°C/hour; Number of Rescans 0; PreScan Thermostat 3 min; PostScan Thermostat 0 min; Post Cycle Thermostat 25°C; Filtering Period 25 seconds; Feedback Mode/Gain None; Cell Refill Parameters 35°C. The resulting protein-buffer thermograms were processed by subtracting a corresponding buffer–buffer scan and subsequently fitting a baseline to the trace. The T_m s were recorded at each peak maxima observed in the thermograms using Origin™ 7.0 software. The results are shown in Figure 39.

[00252] Example 11 – Antibody freeze/thaw stability

[00253] The freeze/thaw stabilities of the fully human anti-PD-1 antibodies were characterized as follows. A 100uL aliquot from the frozen stocks of each anti-PD-1 antibody was thawed at room temperature. Once fully thawed, the samples were then rapidly frozen in the -80°C freezer and kept at -80°C for at least two hours before being thawed again at room temperature. The samples went through three identical freeze/thaw cycles. Visual inspection was used to check for precipitation. 20uL aliquots

were removed from the samples for size-exclusion chromatography (SEC) analysis after three freeze/thaw cycles. The stability of the fully human anti-PD-1 antibodies before and after the freeze/thaw cycles were analyzed by HPLC-SEC characterization. The results, shown in Figure 40, demonstrated that after three freeze/thaw cycles, monomer IgGs accounted for more than 95% of each of the anti-PD-1 antibodies tested.

[00254] Example 12 – Antibody solubility

[00255] The solubility of the fully human anti-PD-1 antibodies was characterized by concentrating 10mg IgG using centrifugal filters (Amicon Ultra-0.5mL 30K) at 14000g at 4°C down to >100mg/mL. 2mL or more of IgG was added into the centrifugal filters and concentrated at 14000g at 4°C. The setting time of centrifuging was 2 min, 3 min, 5 min, 8 min, 15 min, and 20 min, and each time 20uL were aliquoted to a collection tube to measure the concentration with a nanodrop at A280. The centrifugation was finished when the concentration reached 100mg/mL. For HPLC-SEC characterization, 6uL of concentrated samples were injected into an HPLC-SEC column, and the percentages of monomers and aggregates were determined based on the peak area. The results, shown in Figure 41, demonstrated that all of the fully human anti-PD-1 antibodies tested had a solubility over 100mg/mL and that monomer IgGs were higher than 95% for all of the anti-PD-1 antibodies tested.

[00256] While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

REFERENCES

- Blank et al., 2005, Cancer Immunotherapy, 54:307-314
- Brahmer et al., 2010, J. Clin. Oncol., 28:3167-75
- Freeman et al., 2000, J. Exp. Med., 192:1027-34
- Greenwald et al., 2005, Annu. Rev. Immunol. 23:515-48
- Harding and Lonberg, 1995, Ann. N.Y. Acad. Sci. 764:536-546
- Hirano et al., 2005, Cancer Res., 65:1089-96
- Kabat, 1991, "Sequences of proteins of objective interest," the NIH, Bethesda, MD
- Kipriyanov et al., 1997, Peds. 10:445-453
- Lonberg and Huszar, 1995, Internal Rev. Immunol. 13:65-93
- Lonberg et al., 1994, Nature 368: 856-859
- Sambrook and Russell, 1989, Molecular cloning: a laboratory manual, New York : Cold Spring Harbor Laboratory Press, 2nd ed.
- Sharpe et al., 2007, Nat. Immunol. 8:239-45
- Topalian et al., 2014, J. Clin. Oncol., 32:1020-30
- Weinstock and McDermott, 2015, TherAdv Urol., 7(6):365-77
- Wherry, 2011, Nat. Immunol., 12:492-99
- Zippelius et al., 2015, Cancer Immunol Res.; 3(3):236-44
- Zou and Chen L, 2008, Nat Rev Immunol., 8(6):467-77

CLAIMS

We claim:

1. An isolated monoclonal antibody or antigen-binding fragment thereof comprising LCDR1, LCDR2, LCDR3, HCDR1, HCDR2 and HCDR3, having the polypeptide sequences of:
 - (1) SEQ ID NOs: 30, 31, 32, 26, 27, and 28, respectively
 - (2) SEQ ID NOs: 6, 7, 8, 2, 3, and 4, respectively;
 - (3) SEQ ID NOs: 14, 15, 16, 10, 11, and 12, respectively;
 - (4) SEQ ID NOs: 22, 23, 24, 18, 19, and 20, respectively;
 - (5) SEQ ID NOs: 38, 39, 40, 34, 35, and 36, respectively;
 - (6) SEQ ID NOs: 46, 47, 48, 42, 43, and 44, respectively; or
 - (7) SEQ ID NOs: 54, 55, 56, 50, 51, and 52, respectively;wherein the antibody or antigen-binding fragment thereof binds PD-1.
2. The isolated monoclonal antibody or antigen-binding fragment of claim 1, comprising a heavy chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 25, 1, 9, 17, 33, 41 or 49, or a light chain variable region having a polypeptide sequence at least 95% identical to SEQ ID NO: 29, 5, 13, 21, 37, 45 or 53.
3. The isolated monoclonal antibody or antigen-binding fragment of claim 1 or 2, comprising:
 - a. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 25, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 29;
 - b. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 1, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 5;
 - c. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 9, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 13;
 - d. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 17, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 21;
 - e. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 33, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 37;
 - f. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 41, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 45; or
 - g. a heavy chain variable region having the polypeptide sequence of SEQ ID NO: 49, and a light chain variable region having the polypeptide sequence of SEQ ID NO: 53.

4. The isolated monoclonal antibody or antigen-binding fragment of any of claims 1 to 3, wherein the antibody or antigen-binding fragment thereof is chimeric.
5. The isolated monoclonal antibody or antigen-binding fragment of any of claims 1 to 3, wherein the antibody or antigen-binding fragment thereof is human.
6. The isolated monoclonal antibody or antigen-binding fragment of claim 5, comprising a human heavy chain IgG4 constant region having a S228P mutation, and a human antibody light chain kappa constant region constant region.
7. An isolated nucleic acid encoding the monoclonal antibody or antigen-binding fragment of any of claims 1 to 6.
8. A vector comprising the isolated nucleic acid of claim 7.
9. A host cell comprising the nucleic acid of claim 8.
10. A pharmaceutical composition, comprising the isolated monoclonal antibody or antigen-binding fragment of any of claims 1 to 6 and a pharmaceutically acceptable carrier.
11. A method of blocking binding of PD-1 to PD-L1 or PD-L2, or augmenting secretion of IFN- γ and IL-2 in a subject in need thereof, comprising administering to the subject the pharmaceutical composition of claim 10.
12. A method of treating an infectious disease, an inflammatory disease, an immune disease, an autoimmune disease, or a graft versus host disease in a subject in need thereof, comprising administering to the subject the pharmaceutical composition of claim 10.
13. A method of treating a tumor in a subject in need thereof, comprising administering to the subject the pharmaceutical composition of claim 10, wherein the tumor is selected from the group consisting of a solid tumor, a hematologic cancer, bladder cancer, biliary cancer, brain cancer, breast cancer, colon cancer, esophageal cancer, gastric cancer, glioma, head cancer, leukemia, liver cancer, lung cancer, lymphoma, myeloma, neck cancer, ovarian cancer, melanoma, pancreatic cancer, renal cancer, salivary cancer, stomach cancer, thymic epithelial cancer, and thyroid cancer.
14. A method of producing the monoclonal antibody or antigen-binding fragment of any of claims 1 to 6, comprising culturing a cell comprising a nucleic acid encoding the monoclonal antibody or antigen-binding fragment under conditions to produce the monoclonal antibody or antigen-binding fragment, and recovering the antibody or antigen-binding fragment from the cell or cell culture.
15. A method of producing a pharmaceutical composition comprising the monoclonal antibody or antigen-binding fragment of any of claims 1 to 6, comprising combining the monoclonal antibody or antigen-binding fragment with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition.

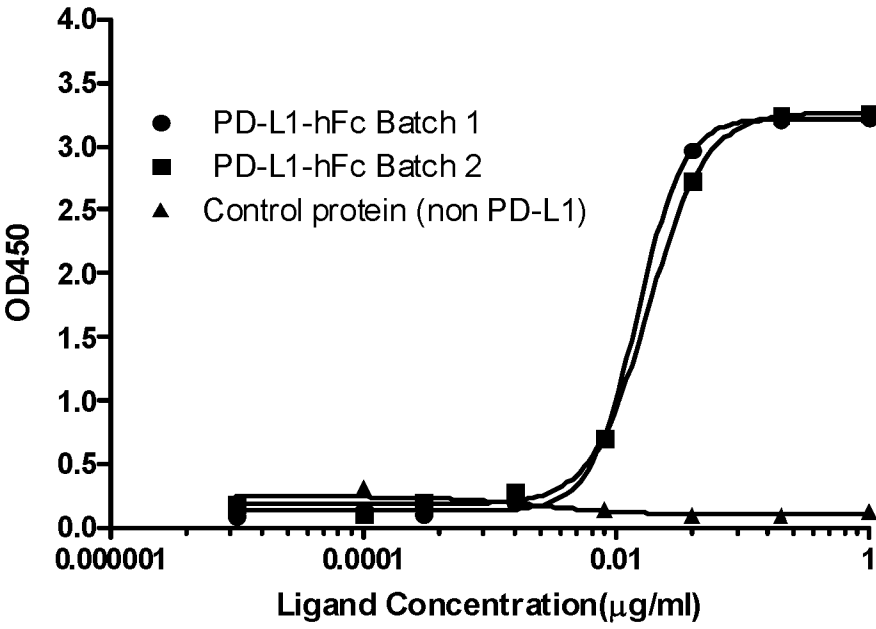


Figure 1

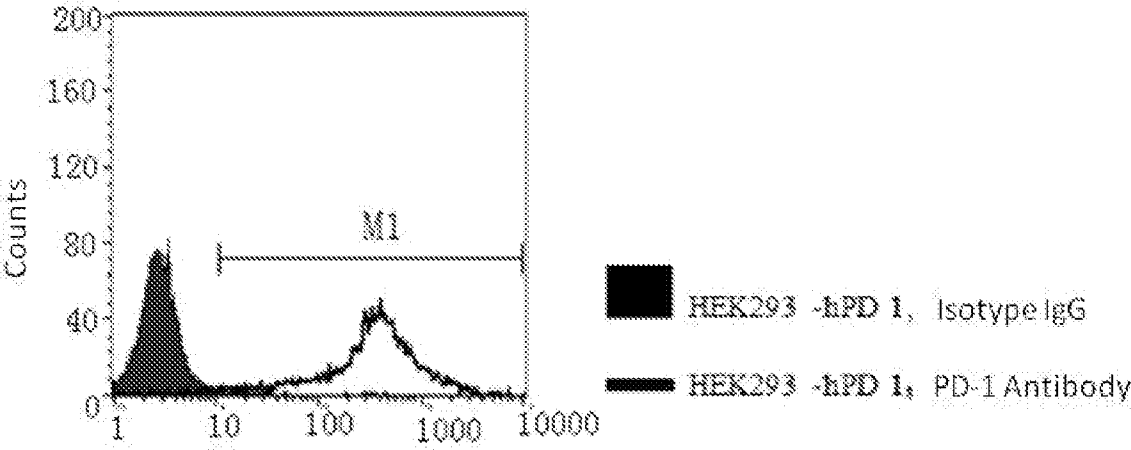


Figure 2

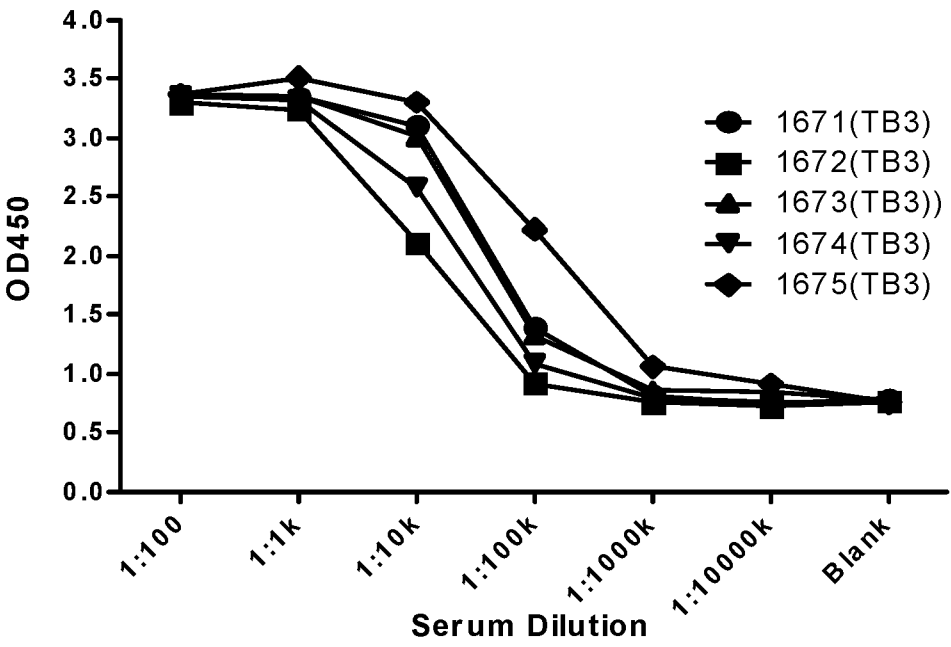


Figure 3

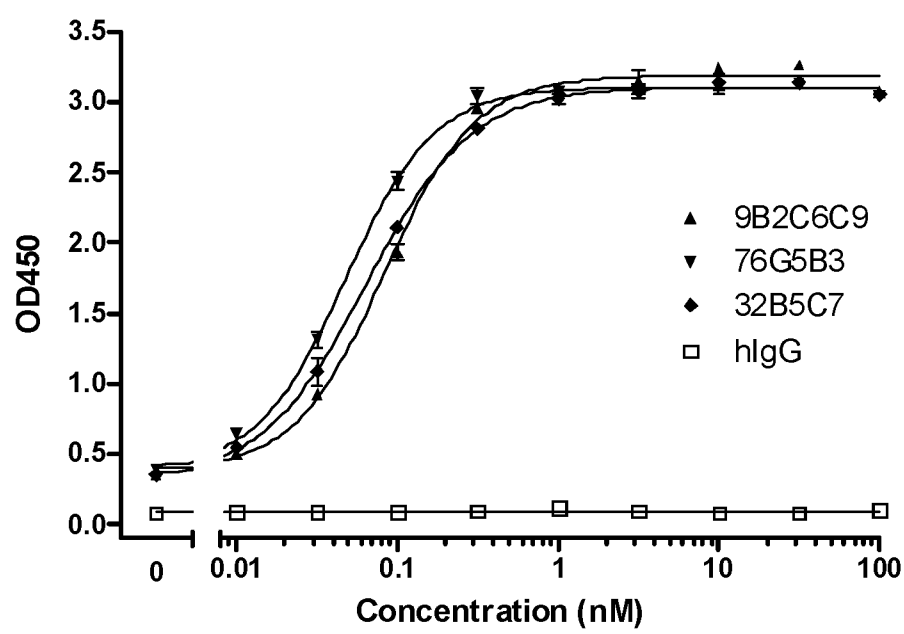


Figure 4A

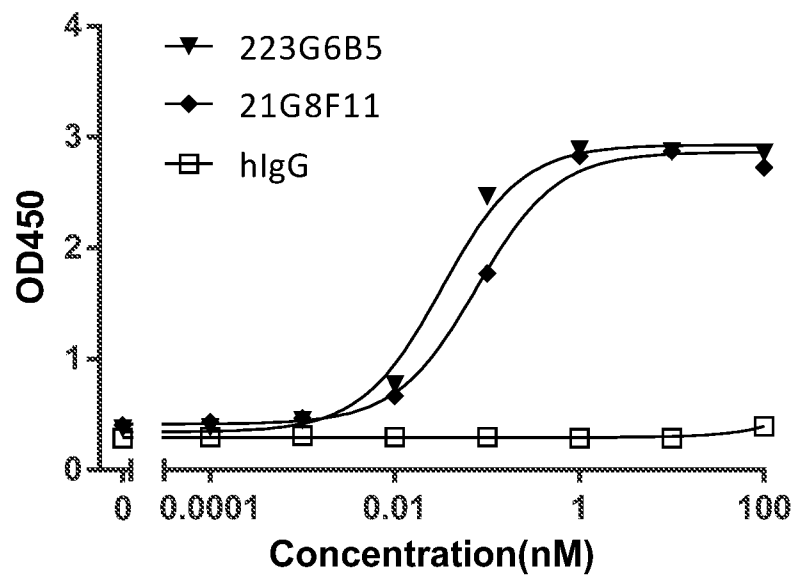


Figure 4B

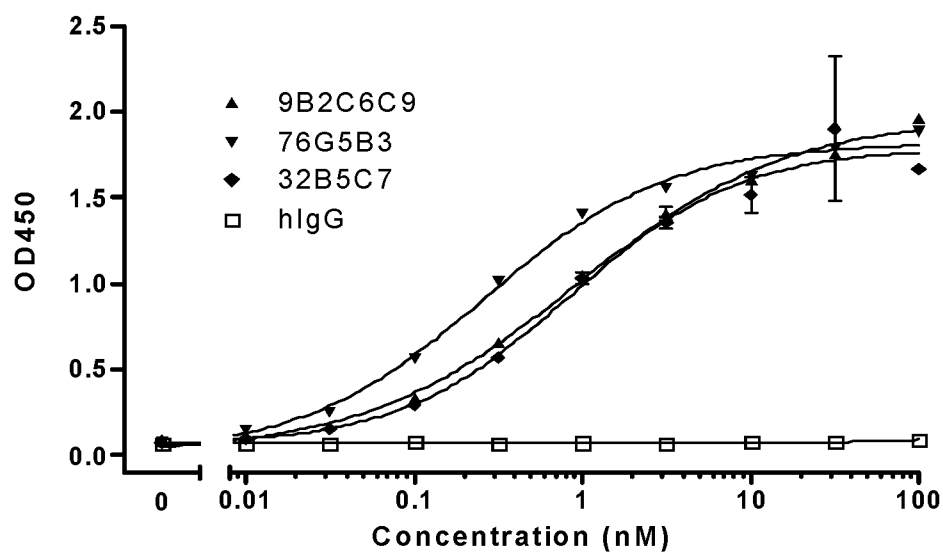


Figure 5A

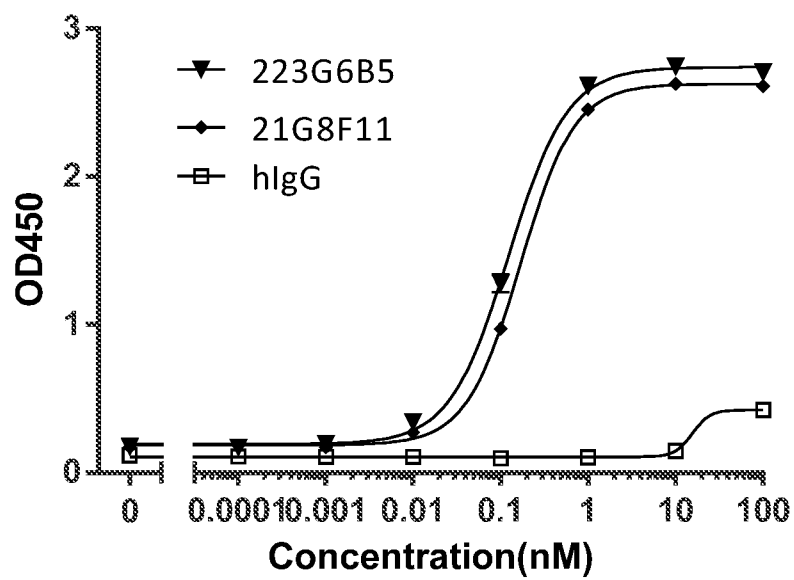


Figure 5B

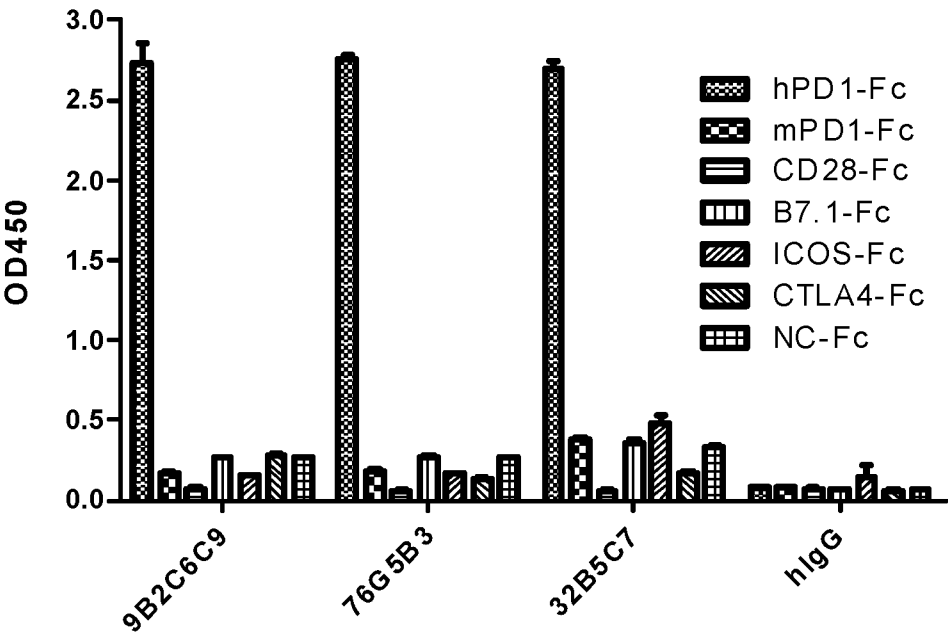


Figure 6A

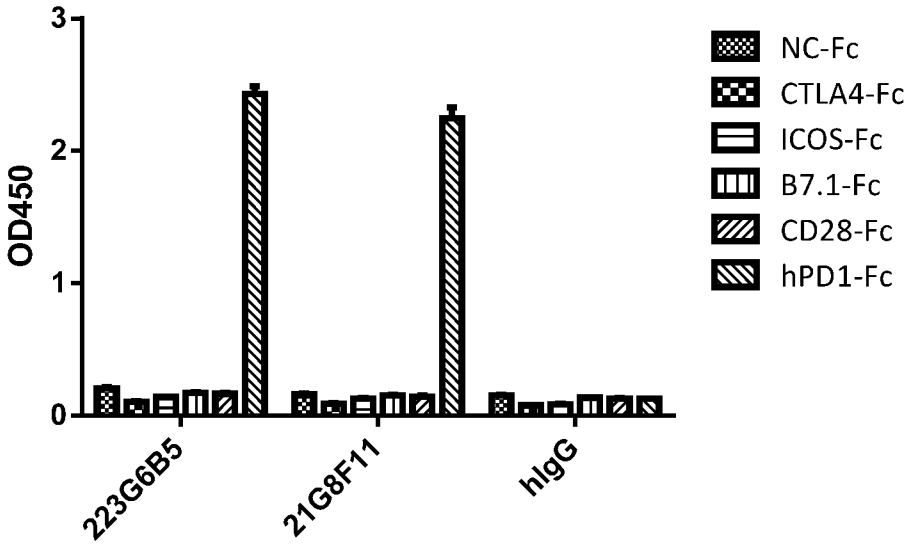


Figure 6B

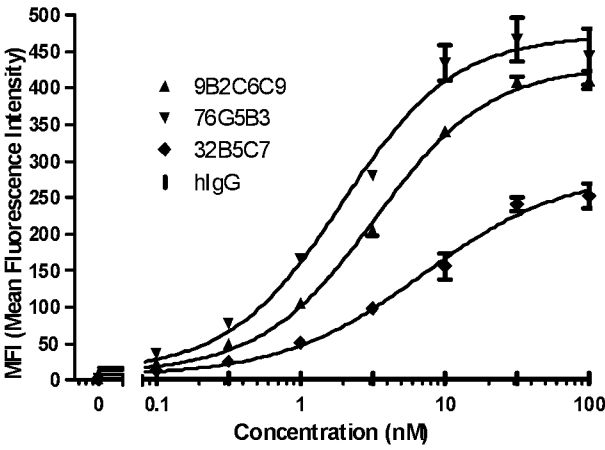


Figure 7A

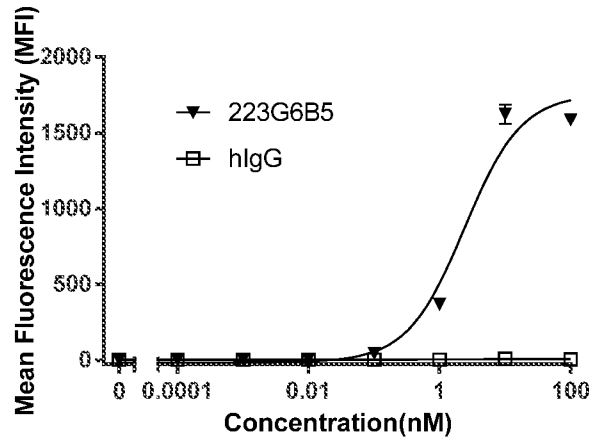


Figure 7B

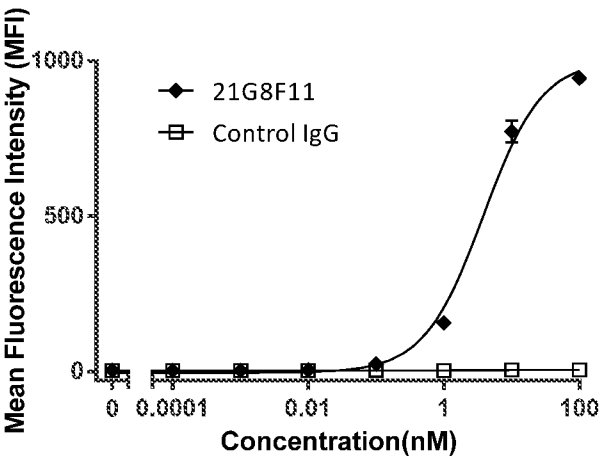


Figure 7C

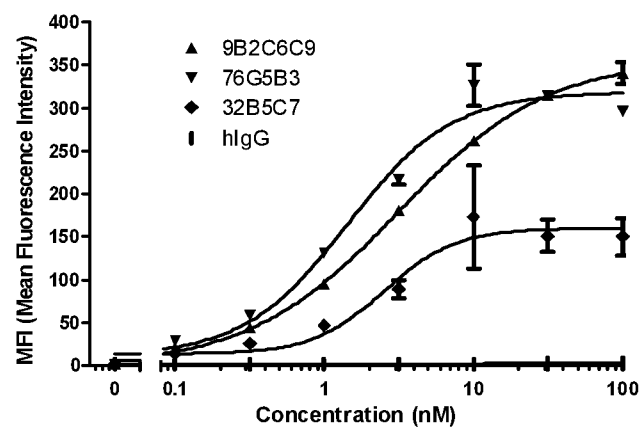


Figure 8A

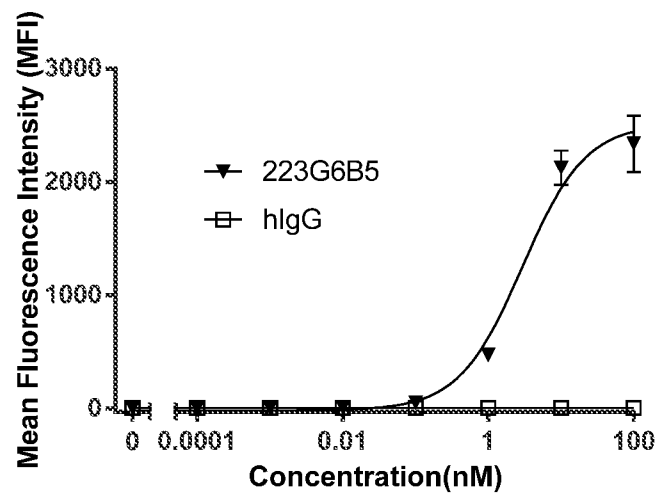


Figure 8B

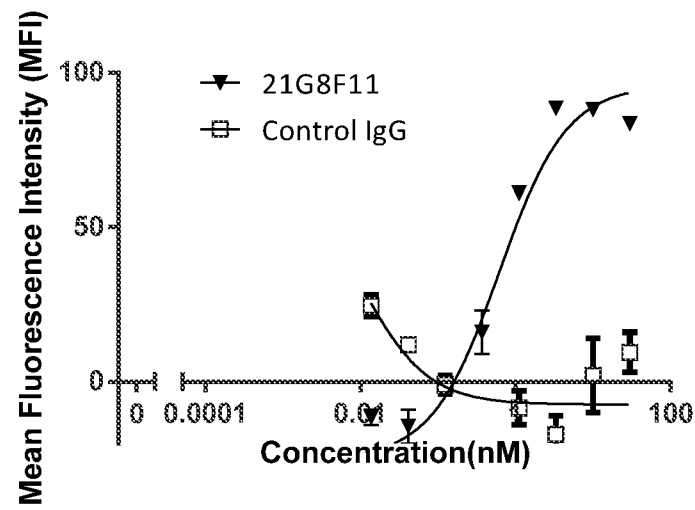


Figure 8C

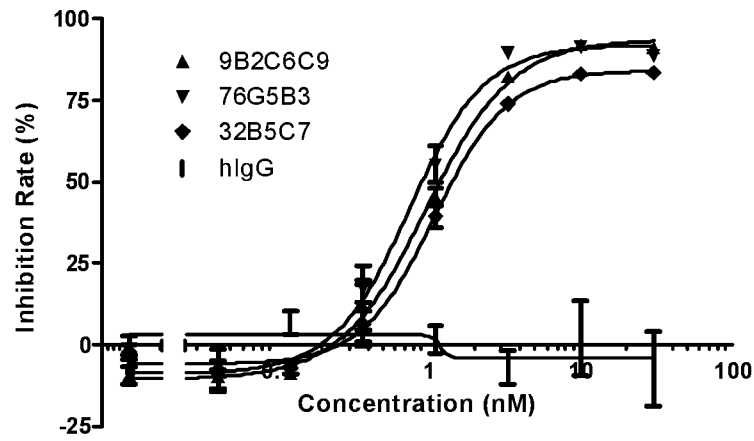


Figure 9A

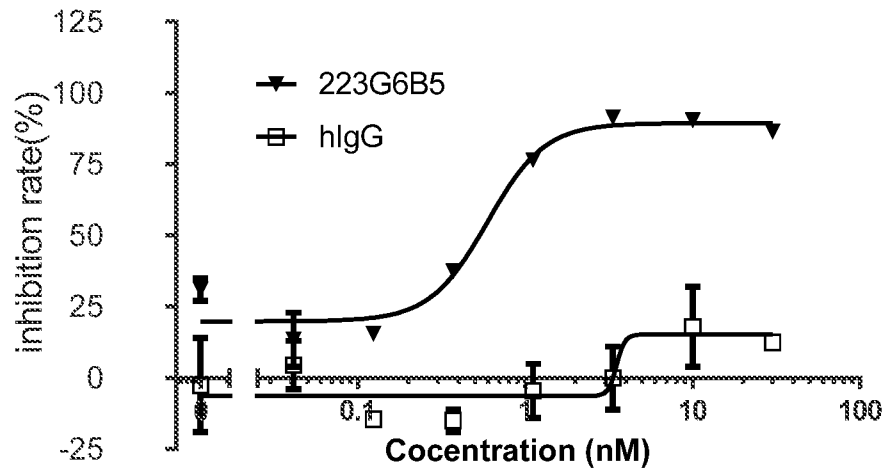


Figure 9B

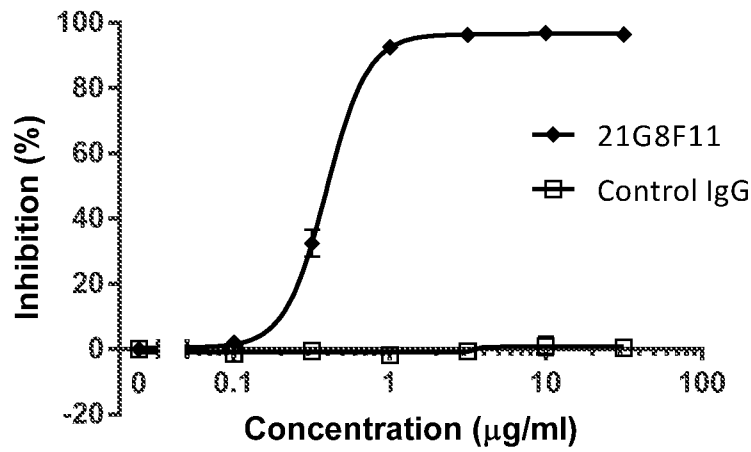


Figure 9C

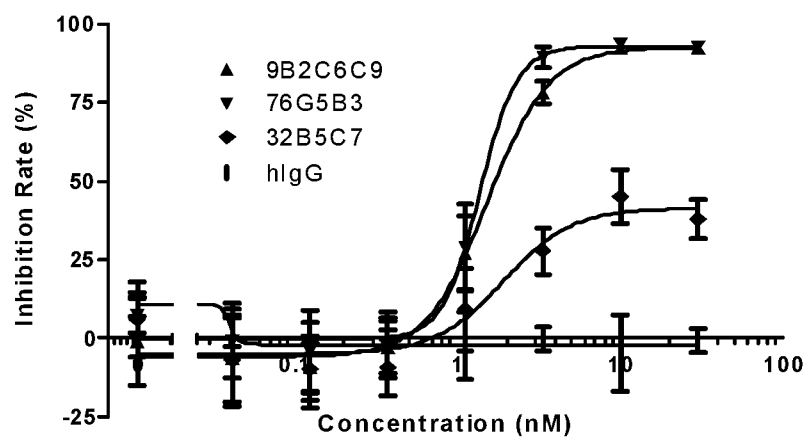


Figure 10A

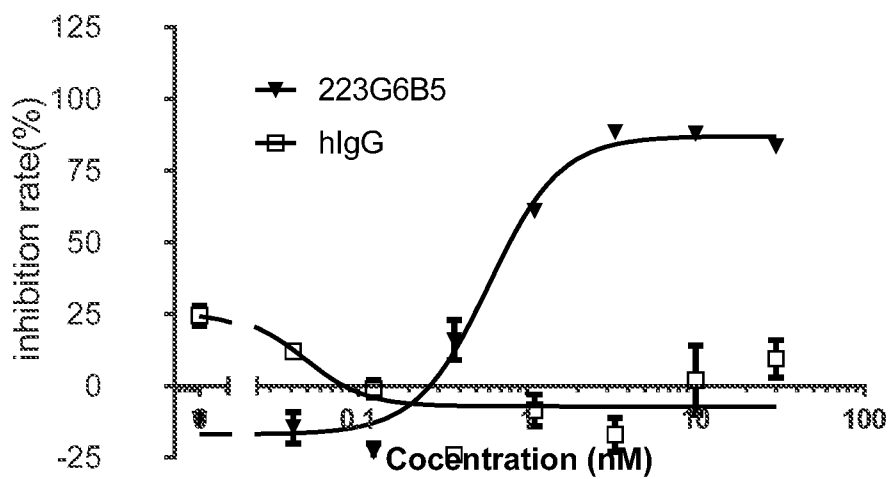


Figure 10B

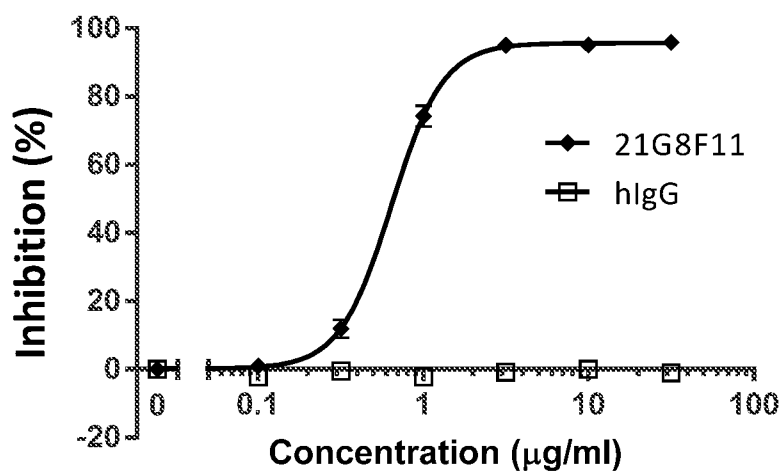


Figure 10C

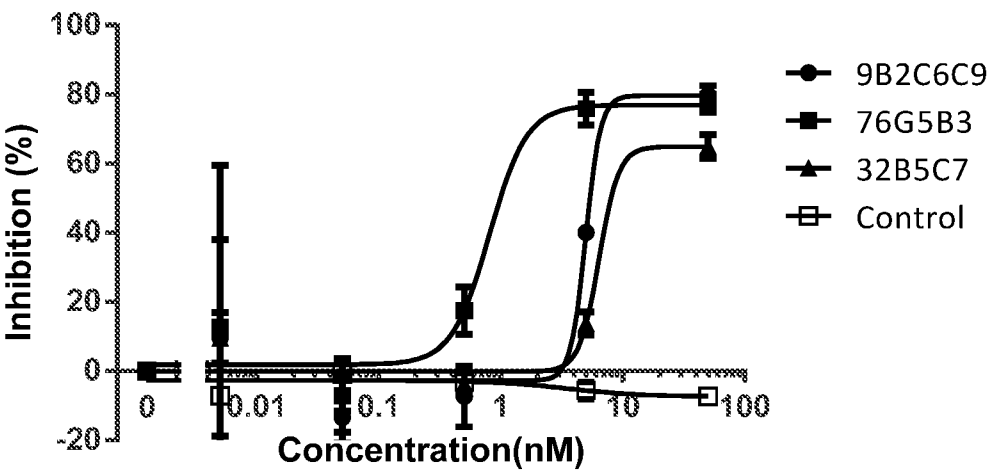


Figure 11A

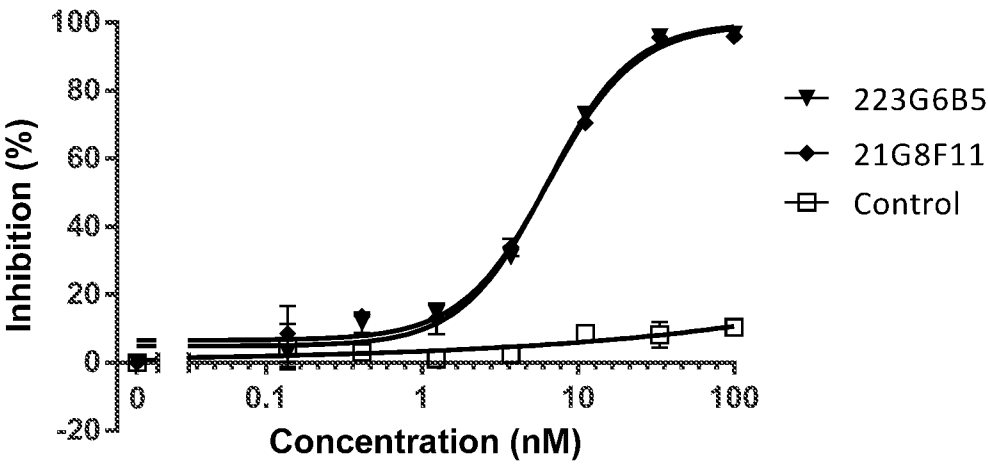


Figure 11B

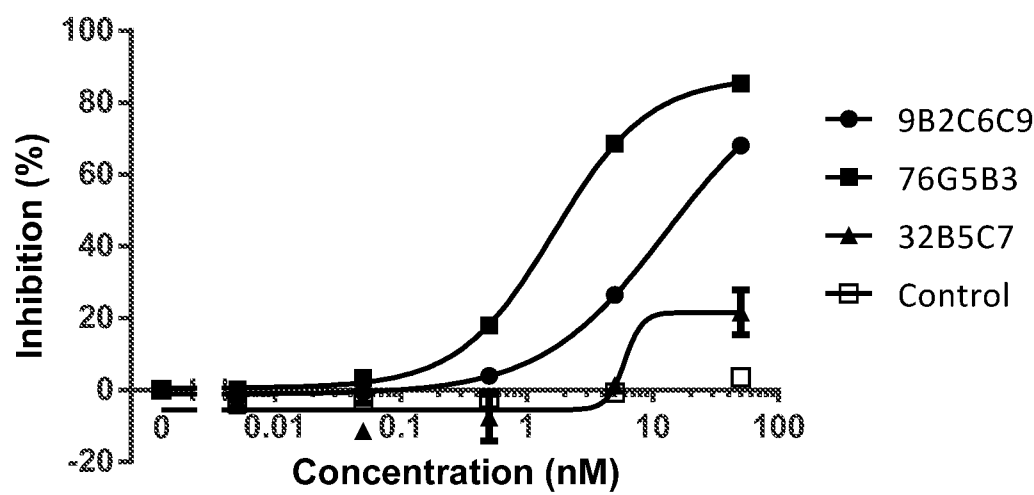


Figure 12A

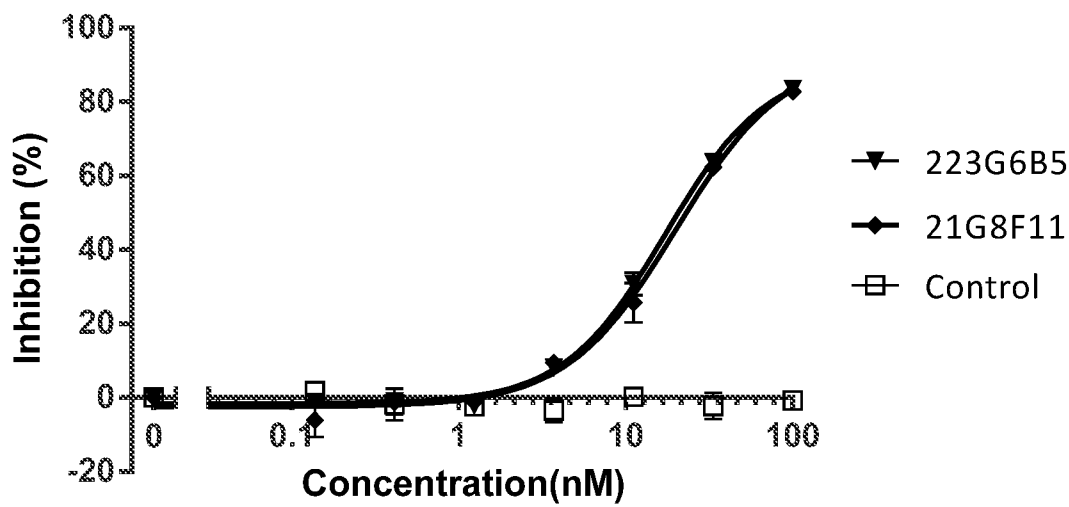


Figure 12B

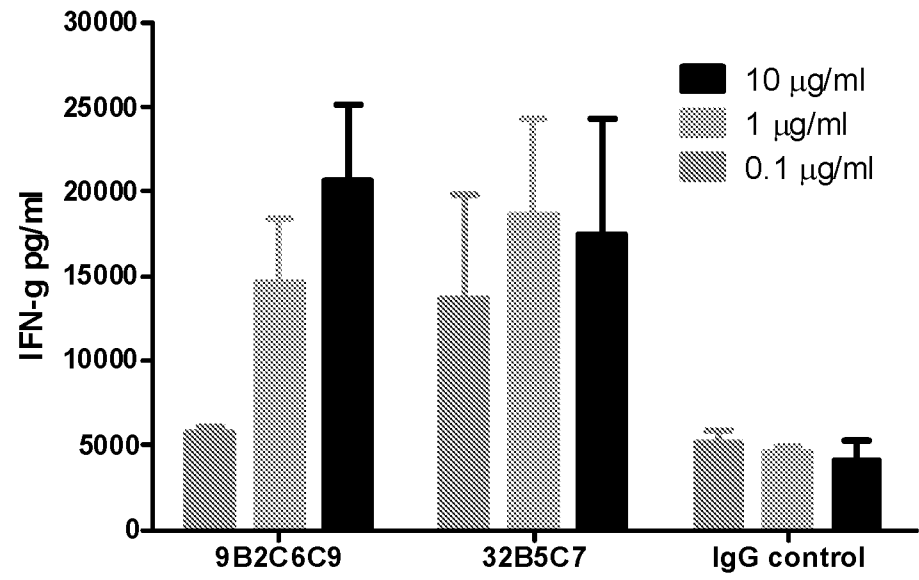


Figure 13

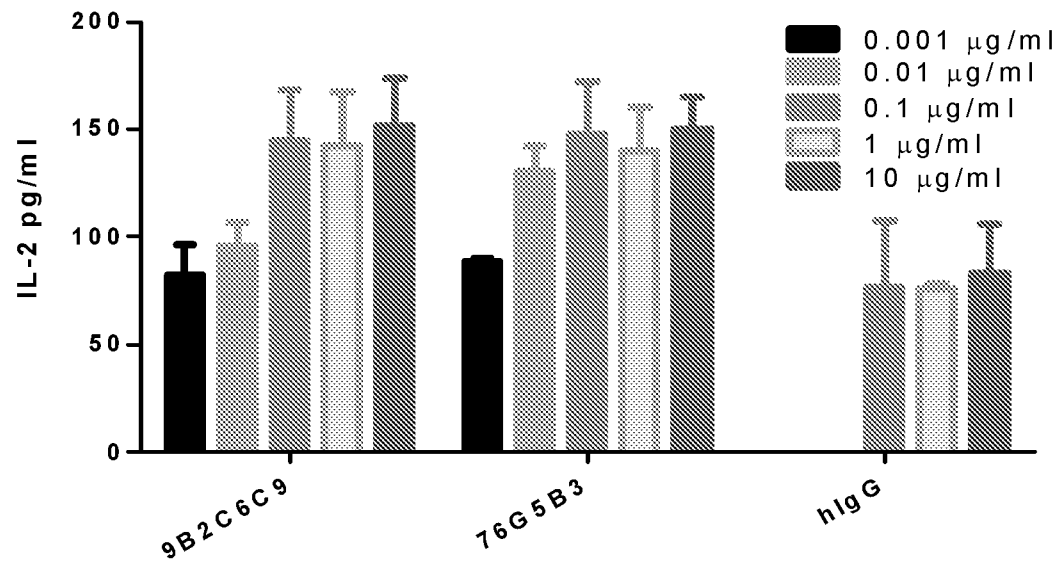


Figure 14

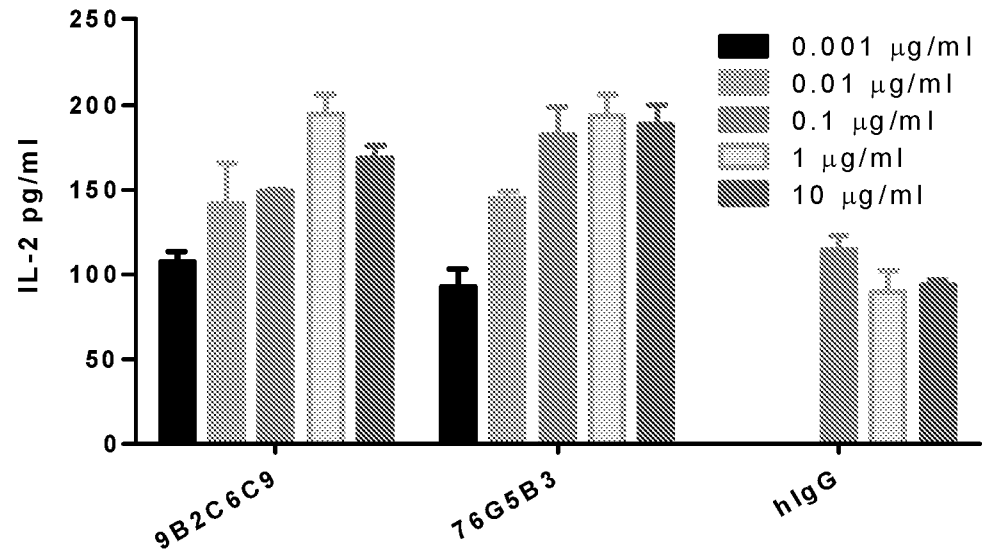


Figure 15

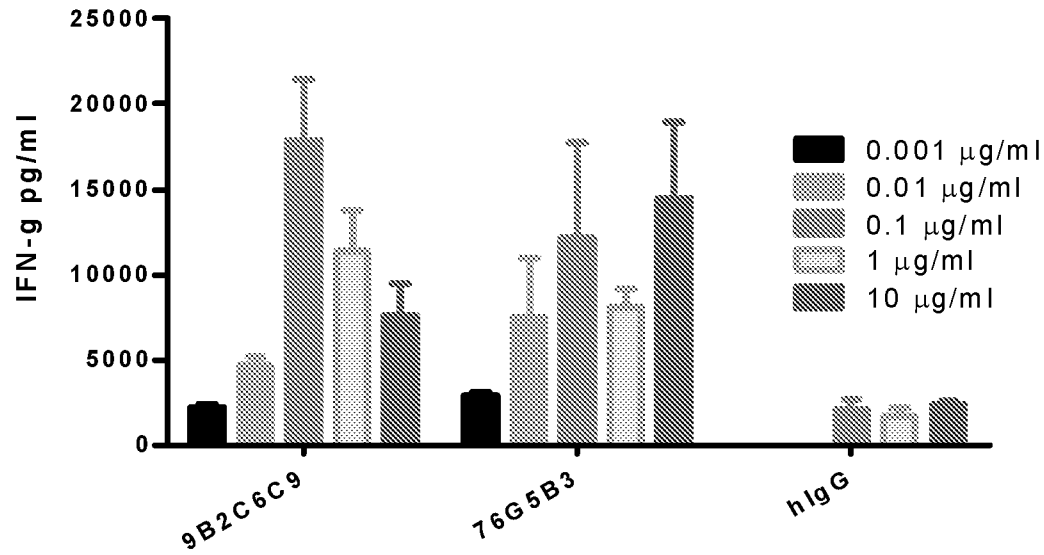


Figure 16

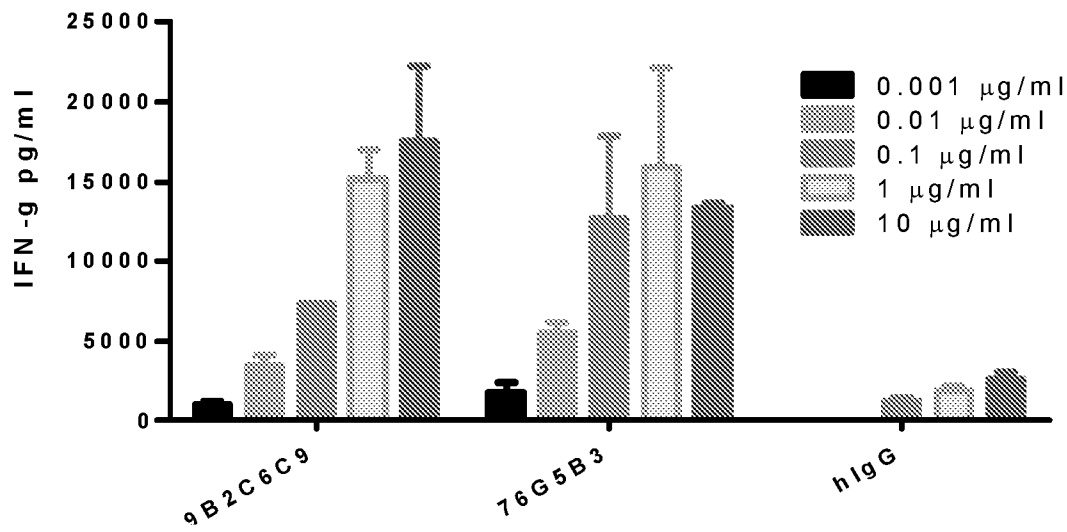


Figure 17

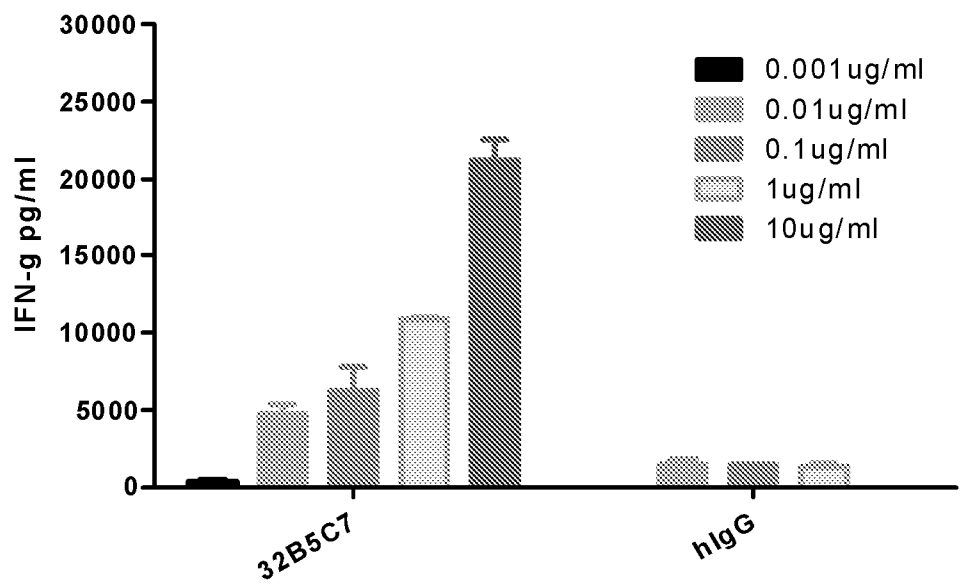


Figure 18

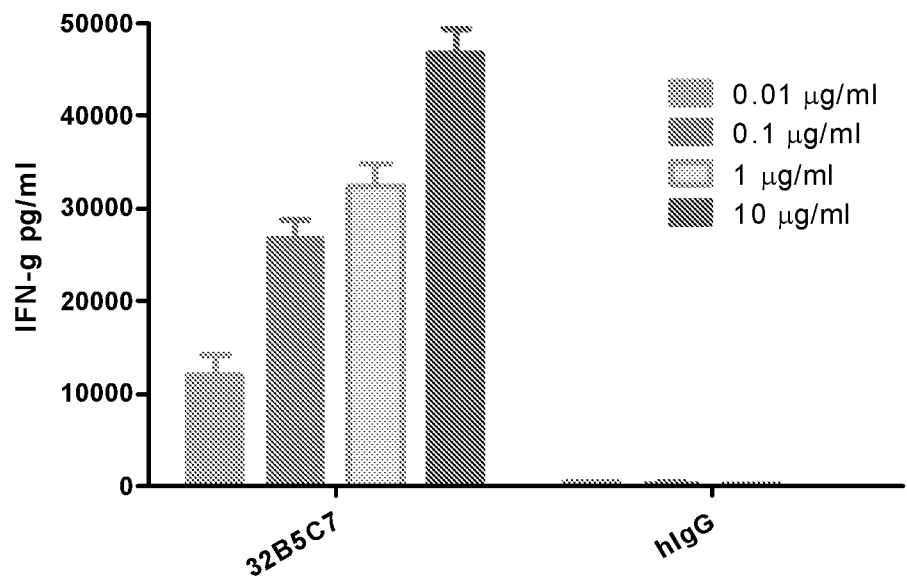


Figure 19

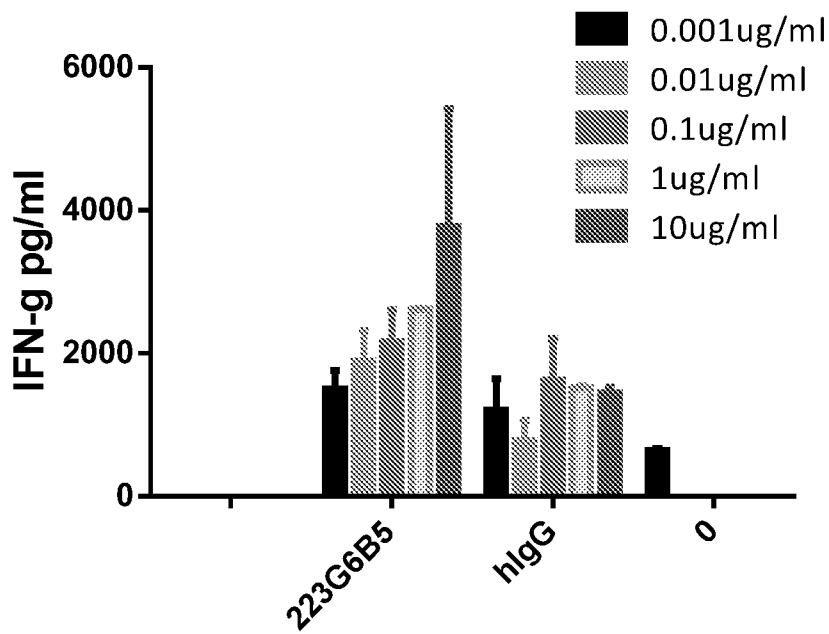


Figure 20

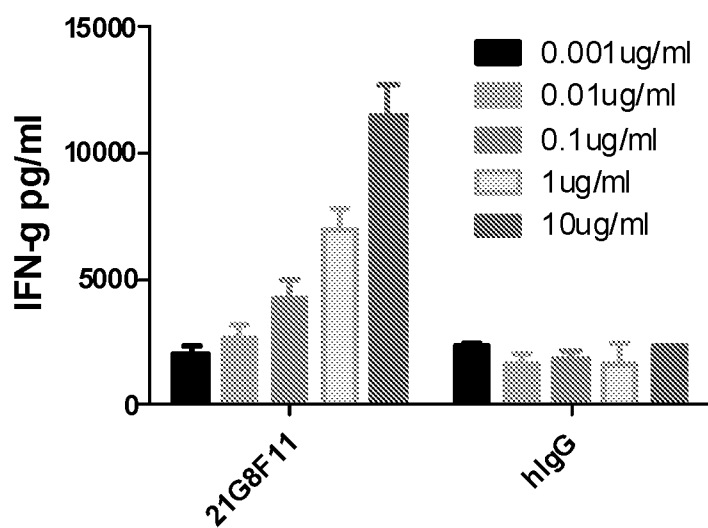


Figure 21

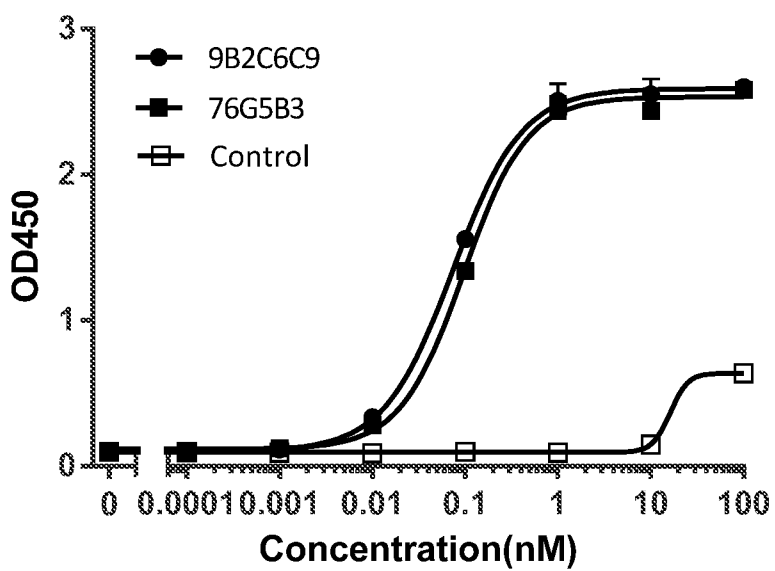


Figure 22A

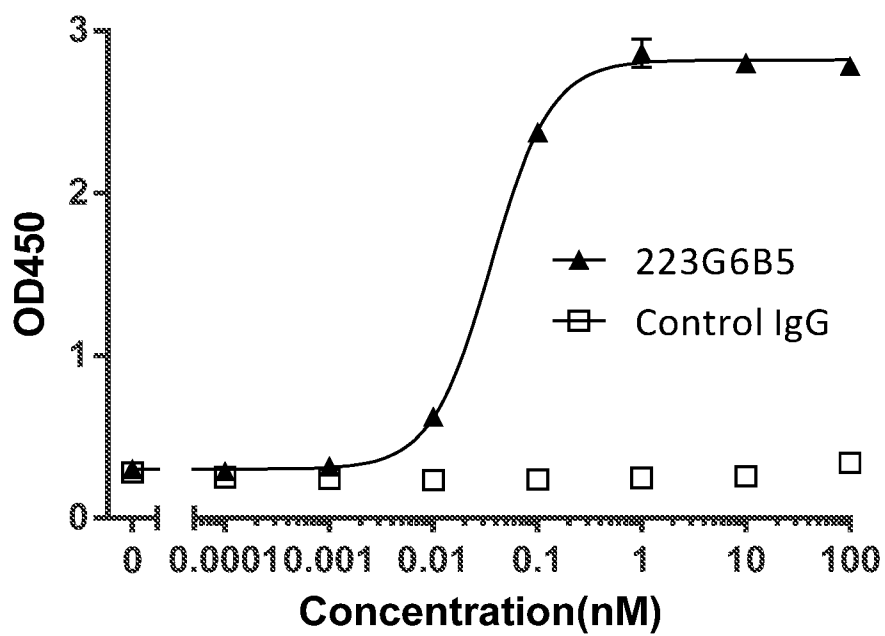


Figure 22B

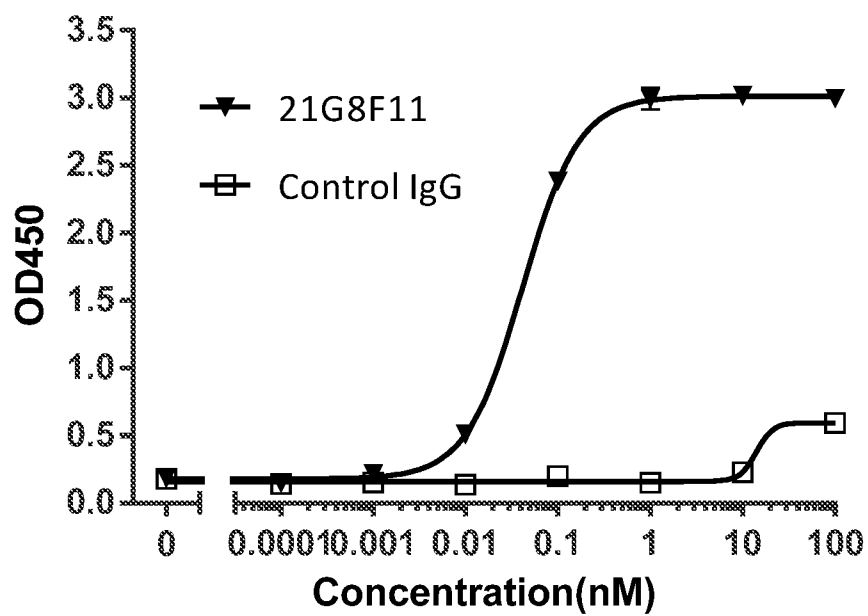


Figure 22C

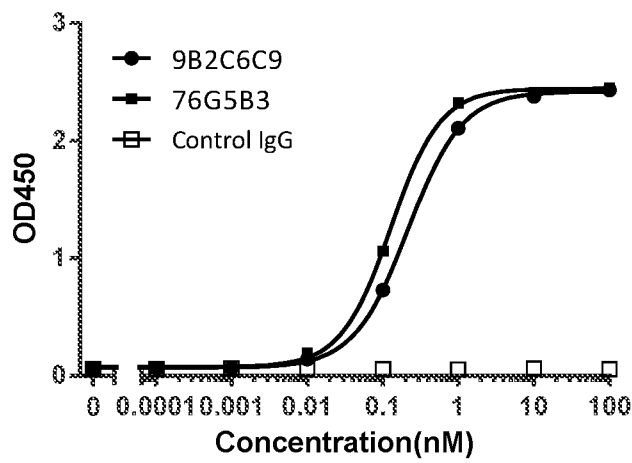


Figure 23A

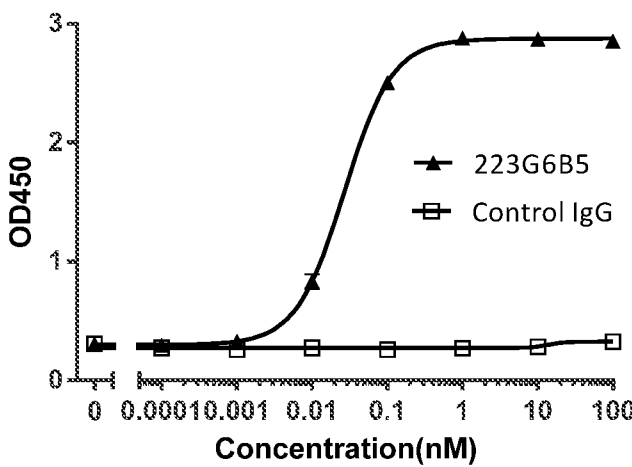


Figure 23B

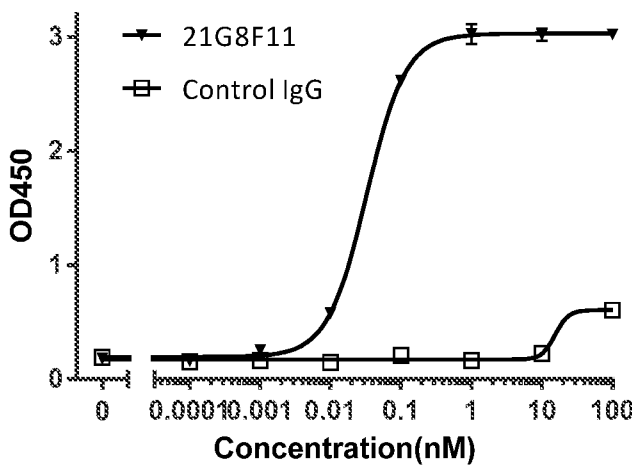


Figure 23C

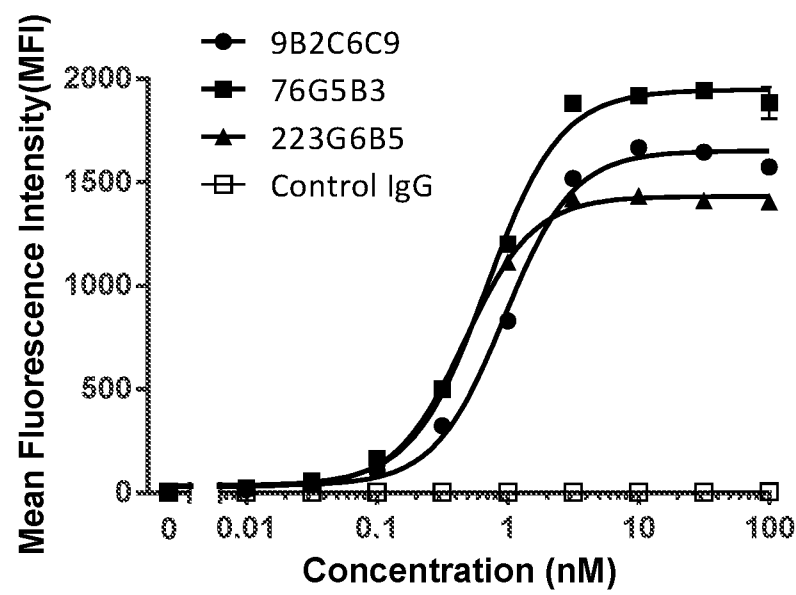


Figure 24A

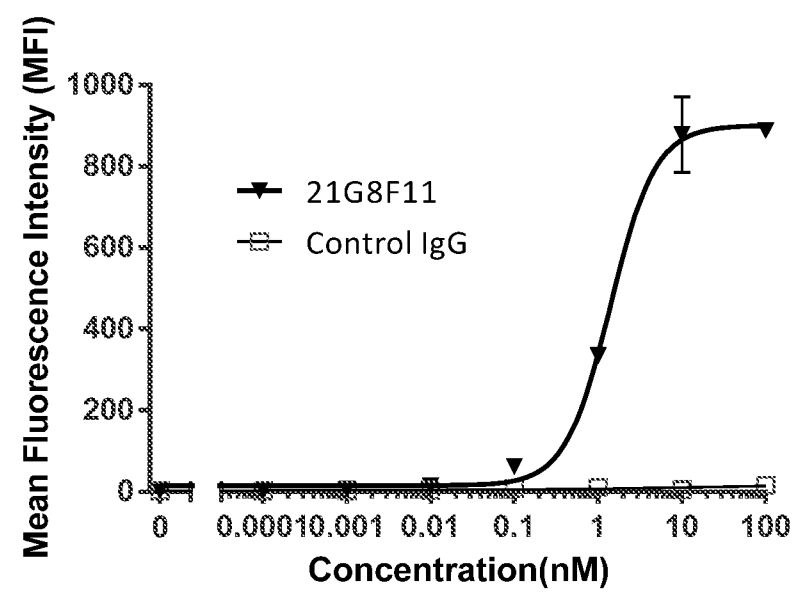


Figure 24B

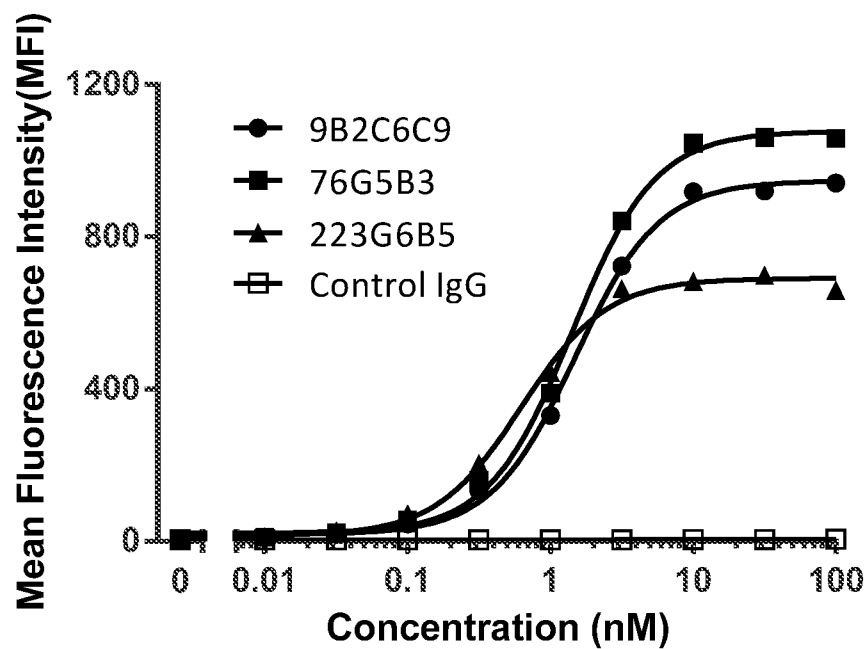


Figure 25A

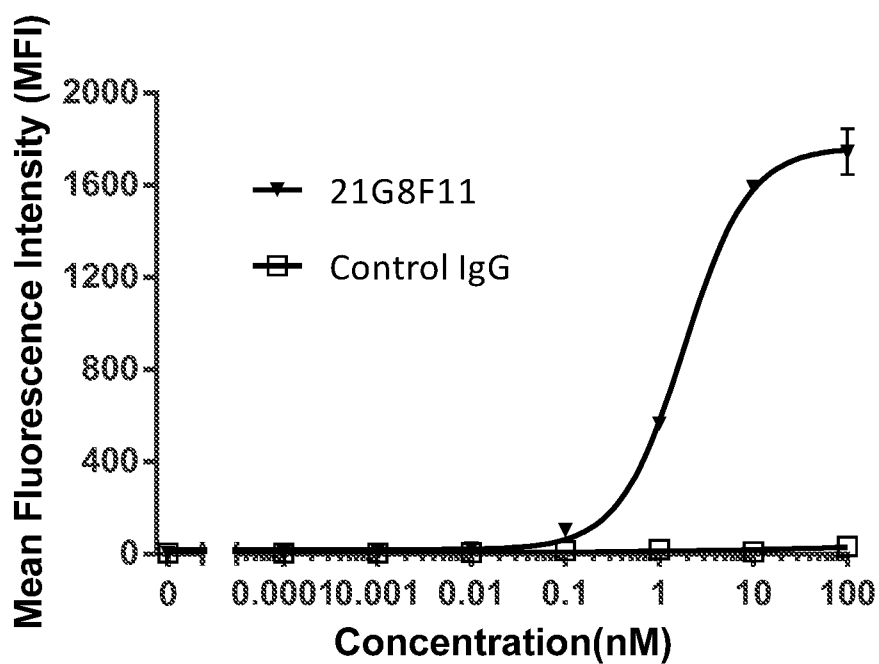


Figure 25B

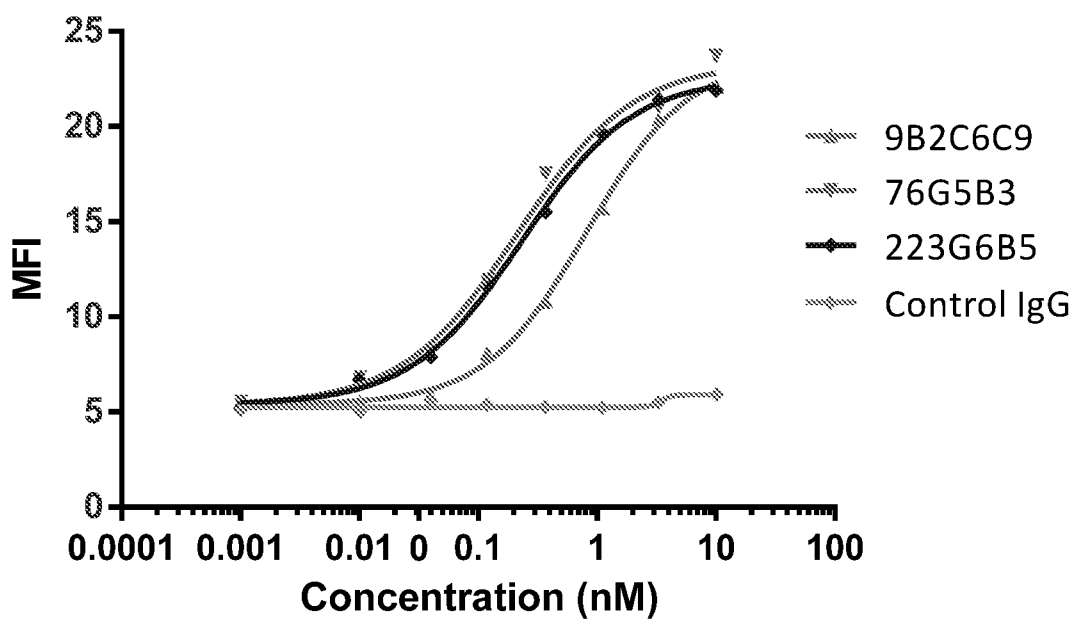


Figure 26

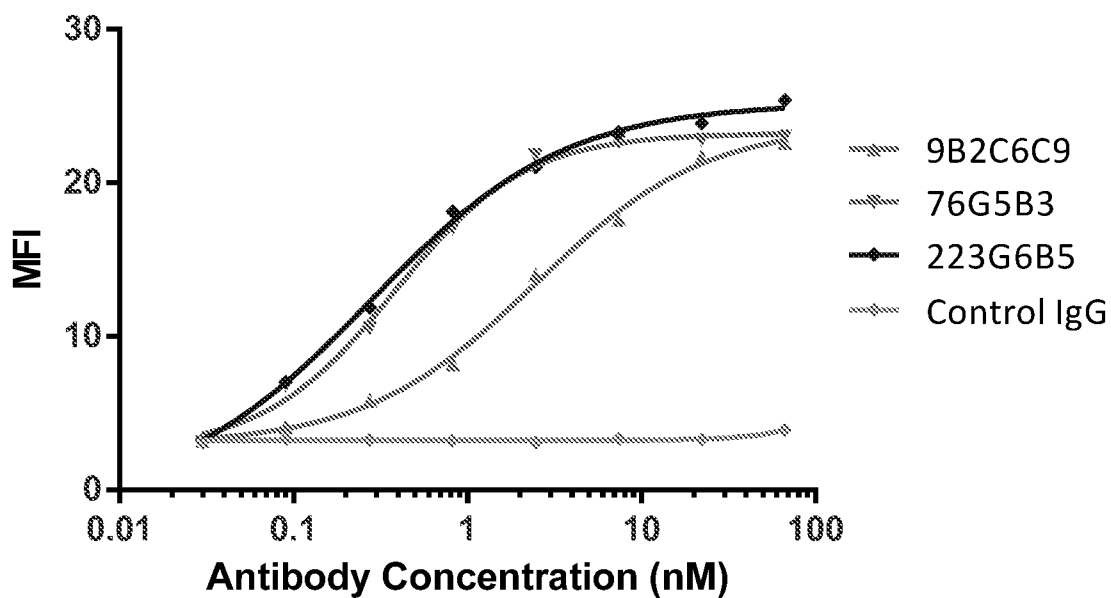


Figure 27

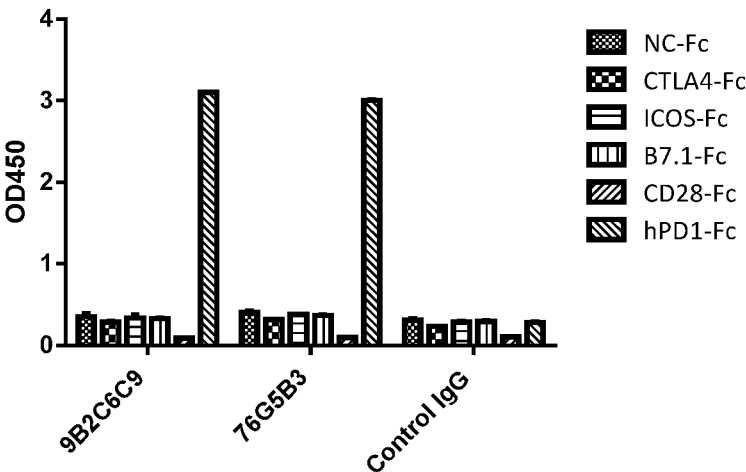


Figure 28A

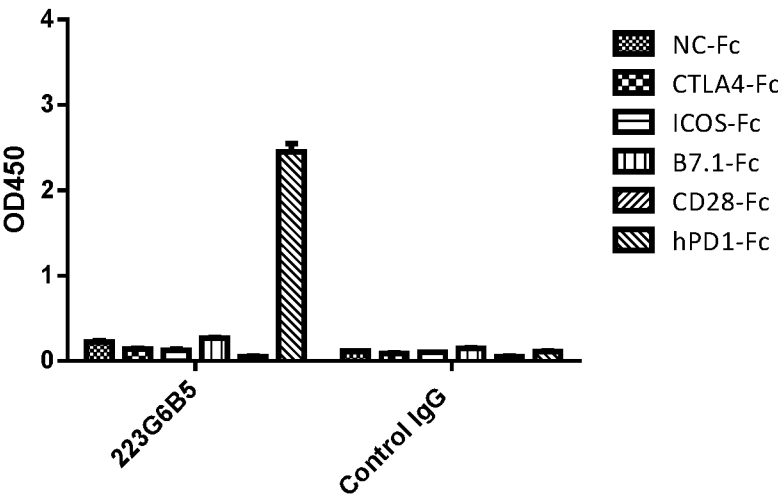


Figure 28B

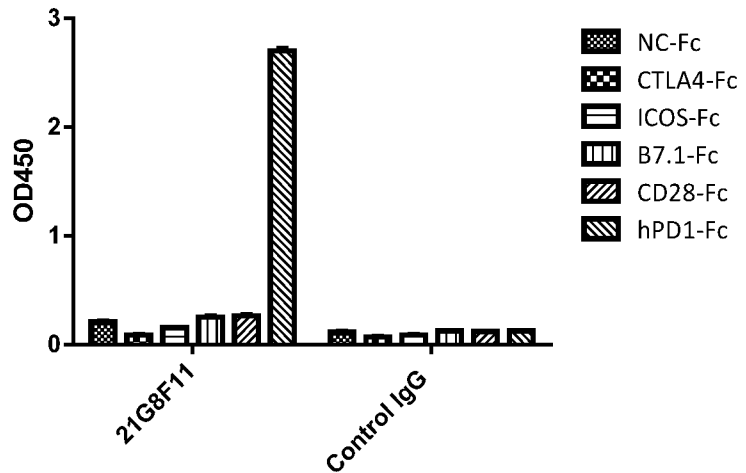


Figure 28C

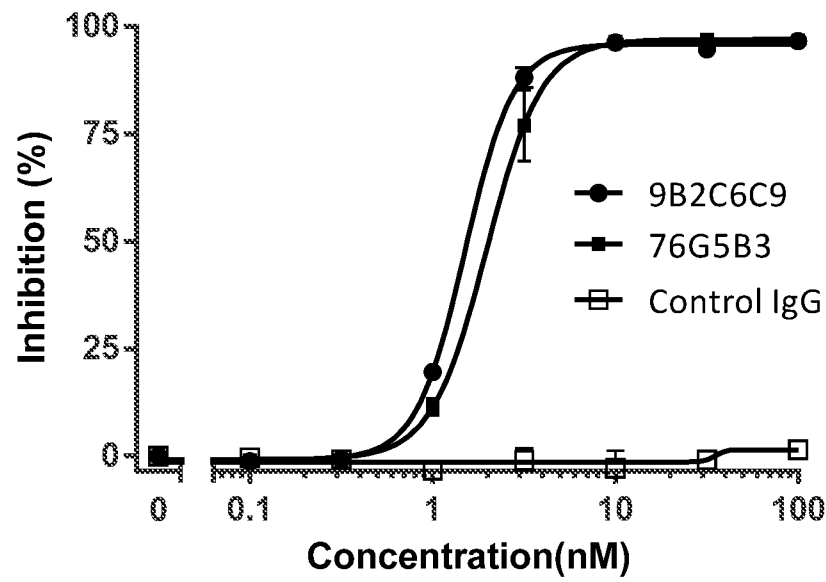


Figure 29A

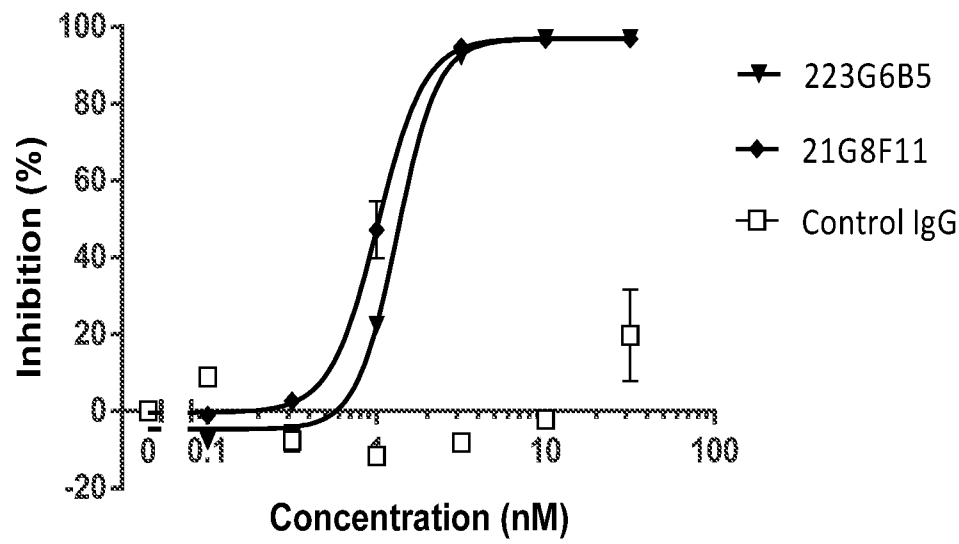


Figure 29B

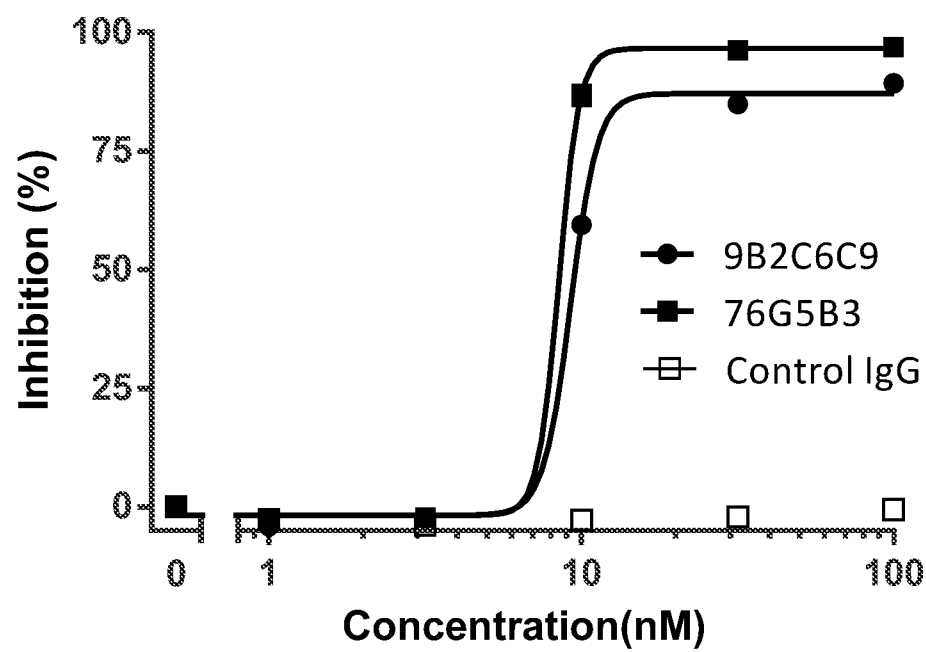


Figure 30A

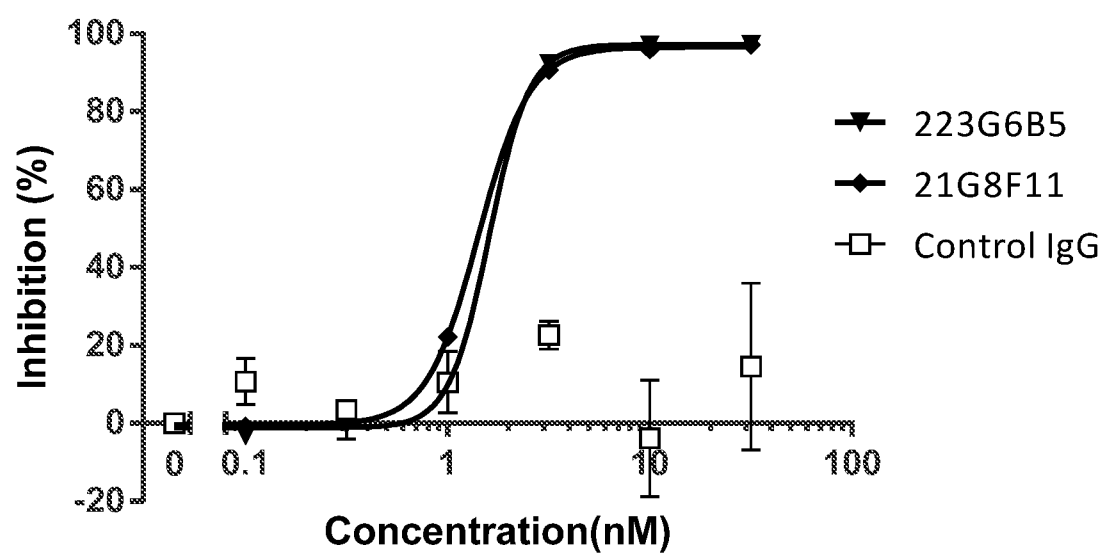


Figure 30B

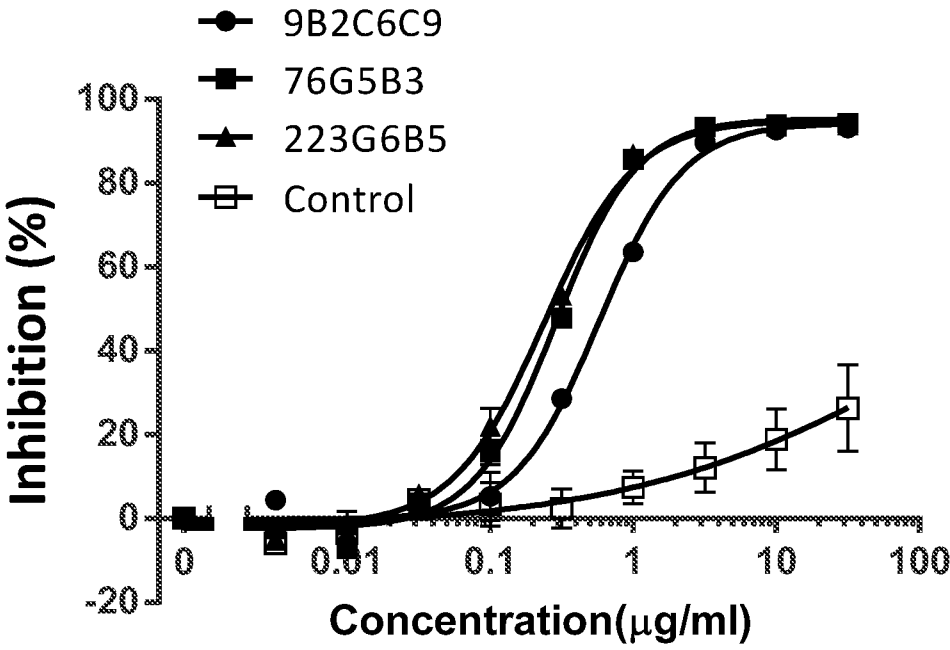


Figure 31A

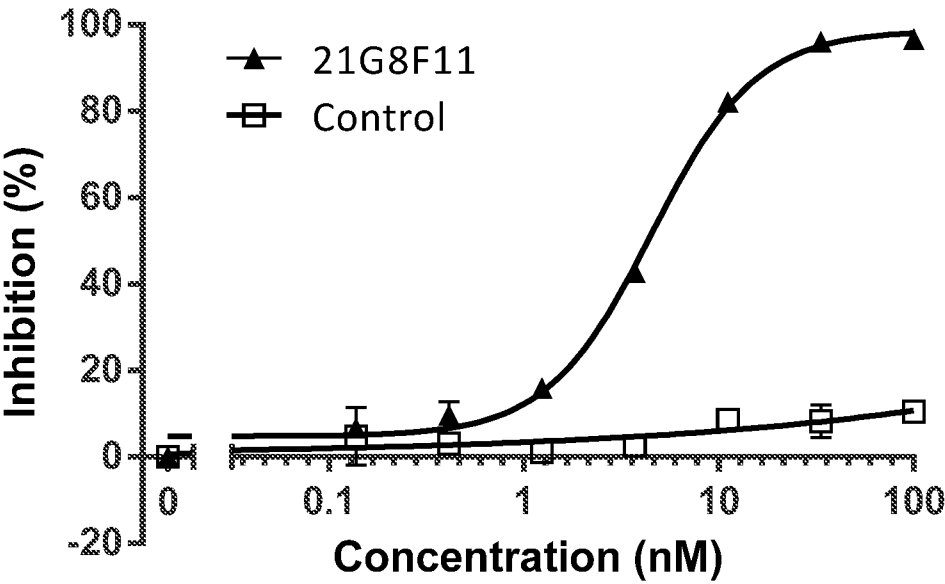


Figure 31B

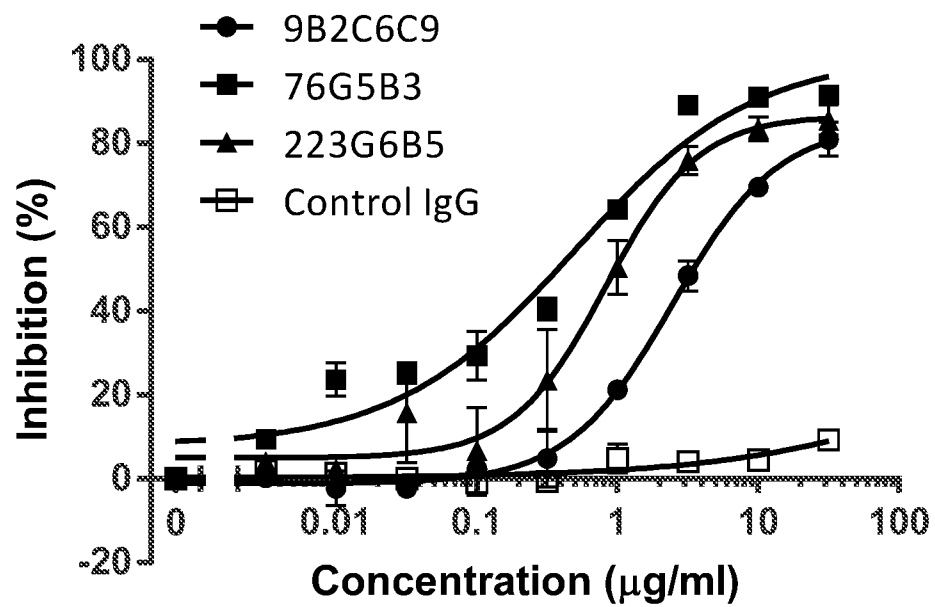


Figure 32A

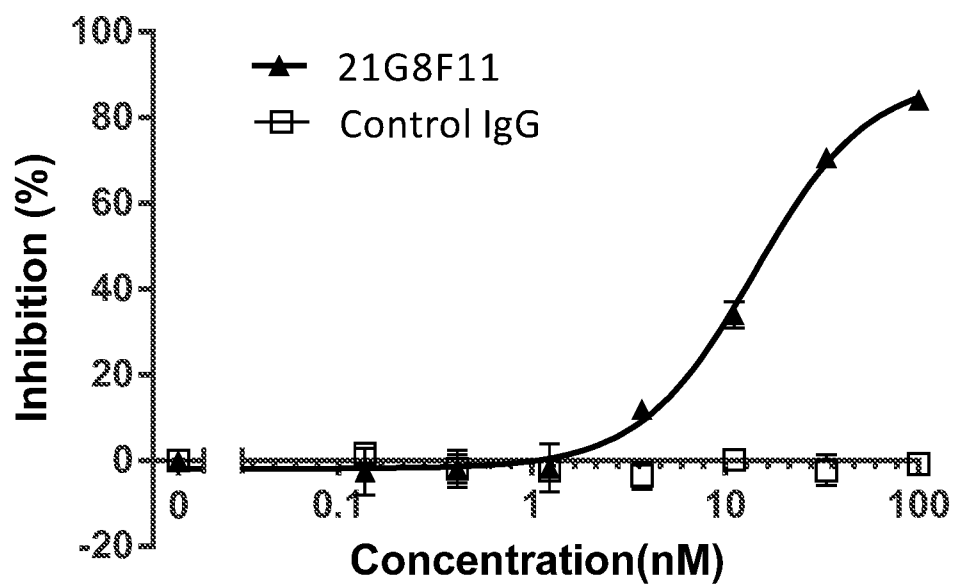


Figure 32B

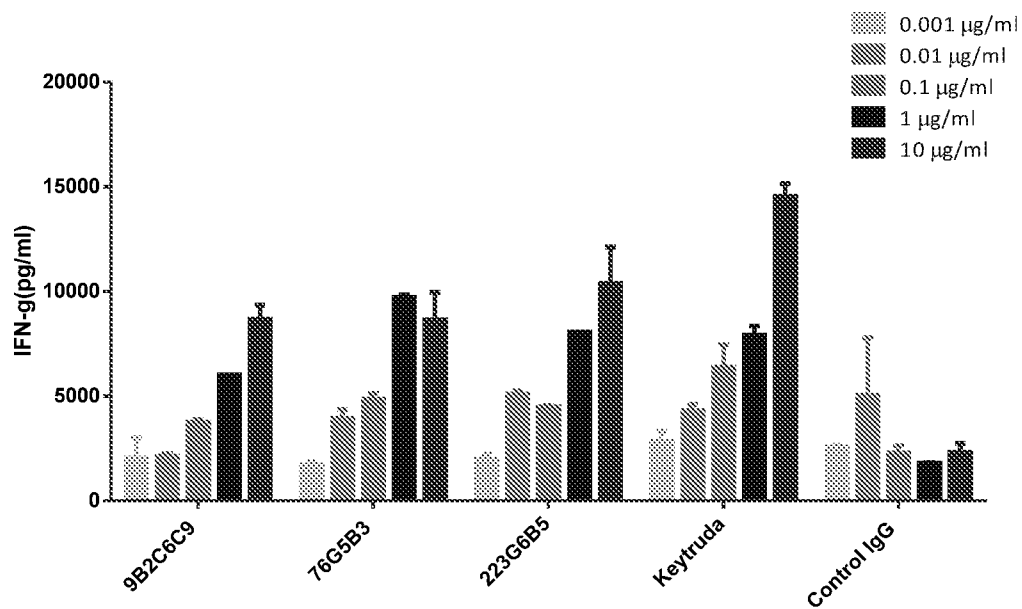


Figure 33

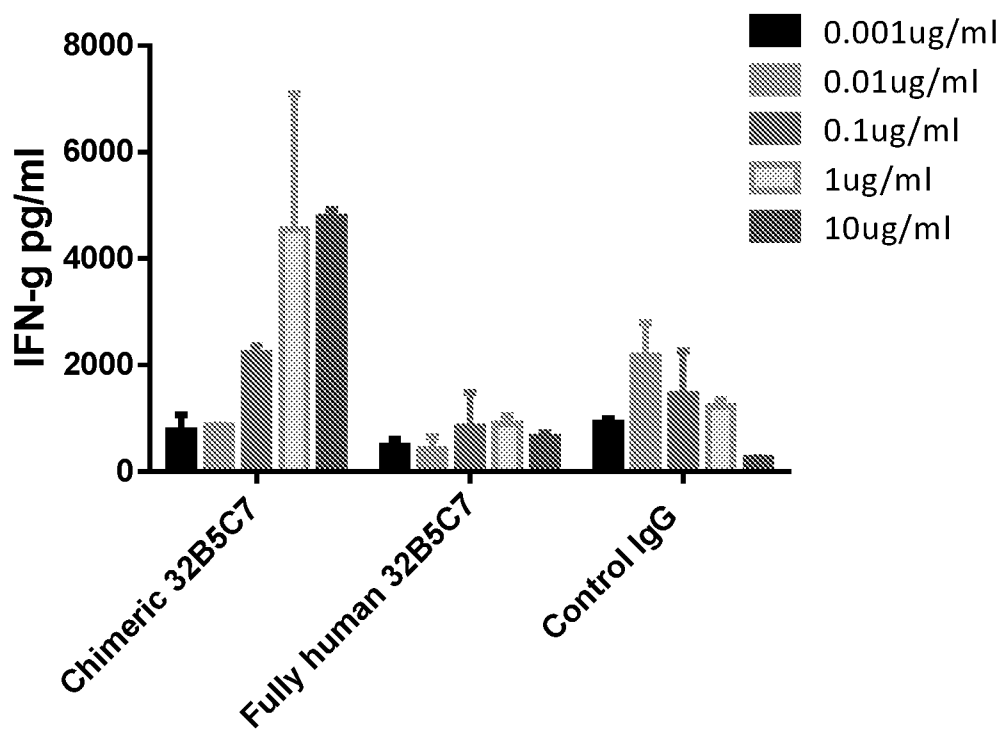


Figure 34

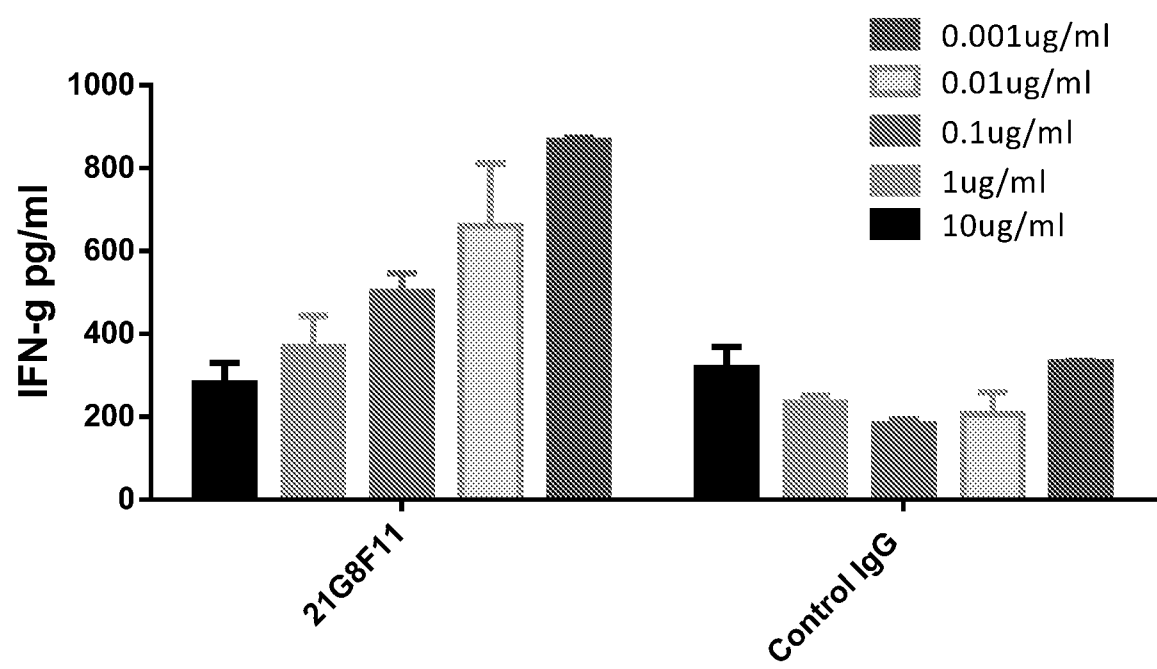


Figure 35

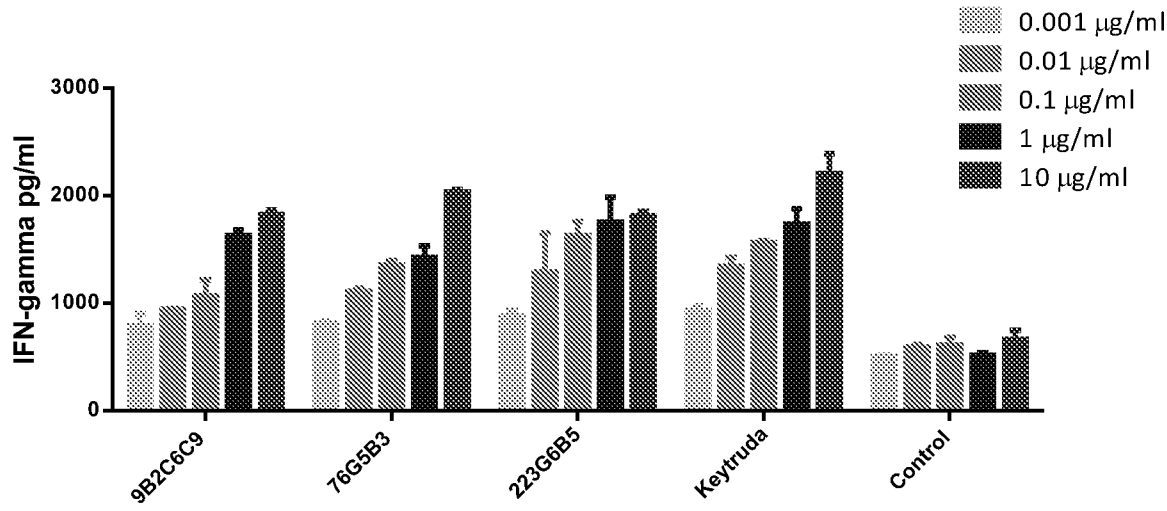


Figure 36A

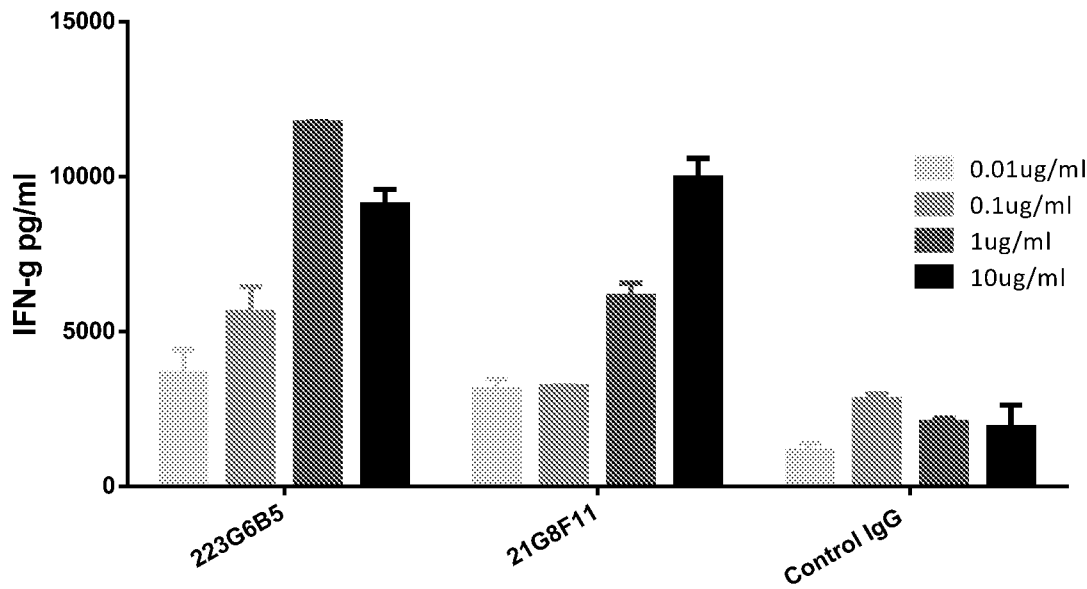


Figure 36B

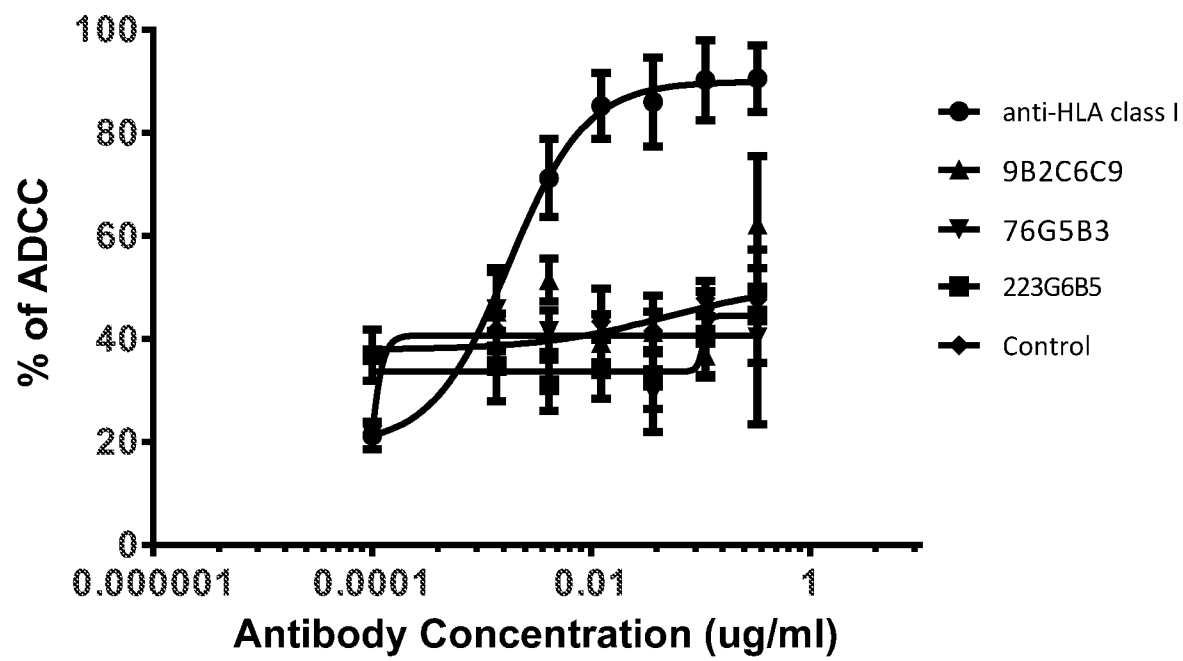


Figure 37

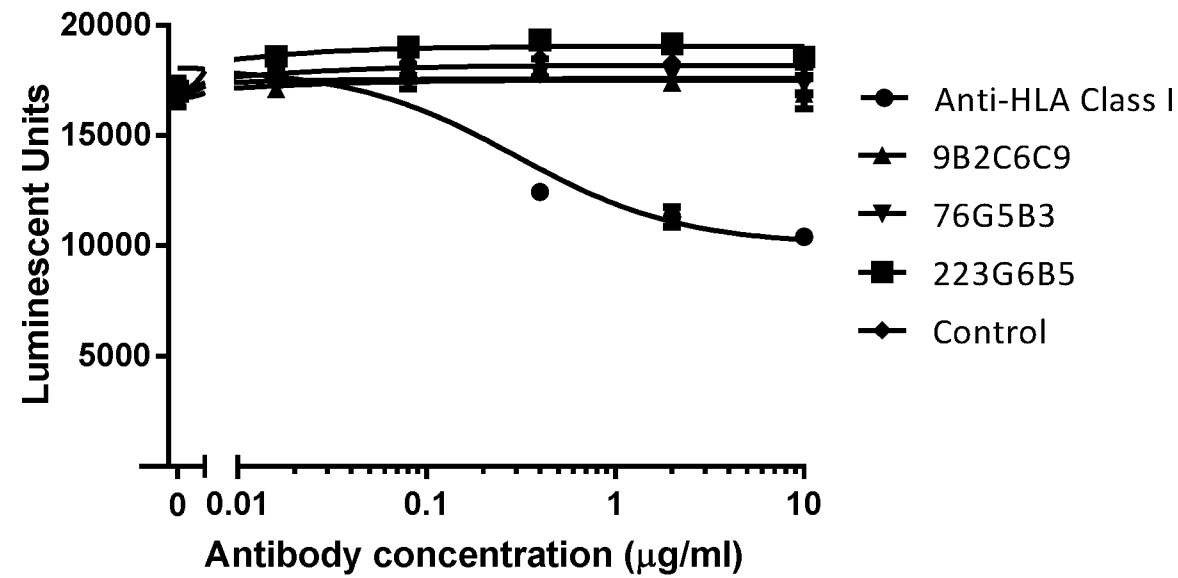


Figure 38

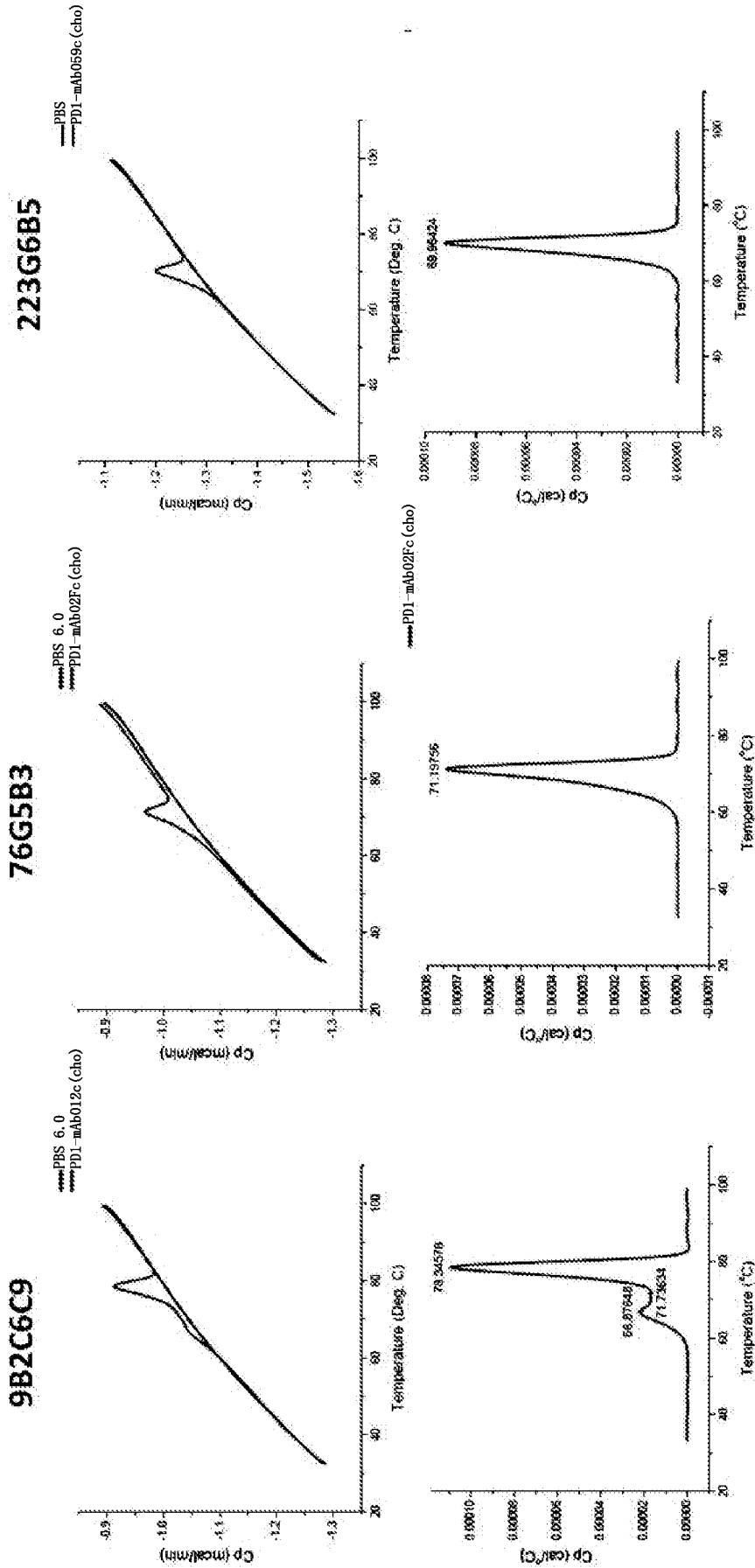
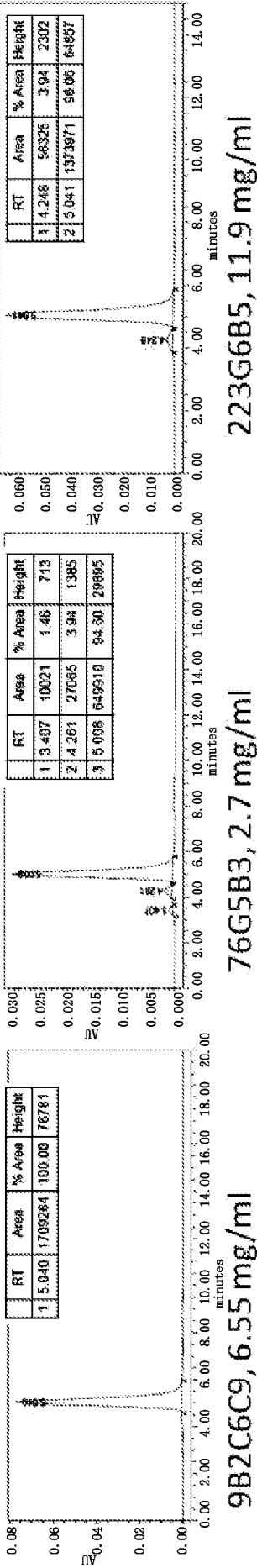


Figure 39

Before freeze/thaw



After 3 cycles of freeze/thaw

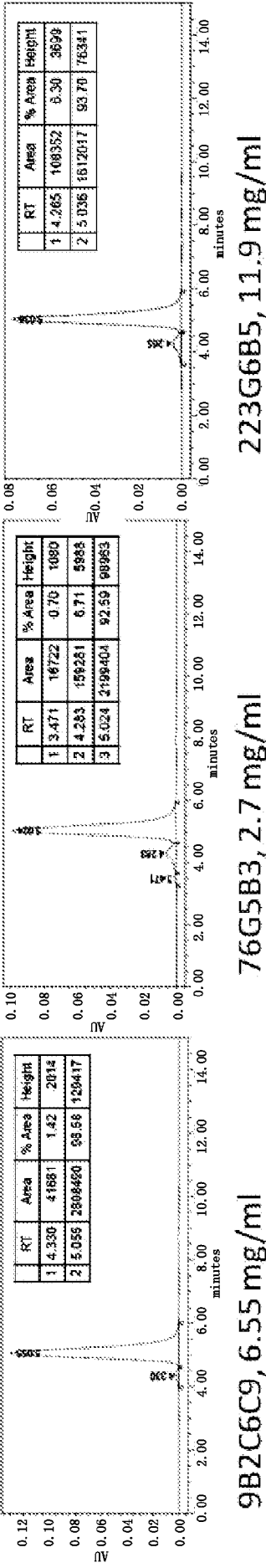
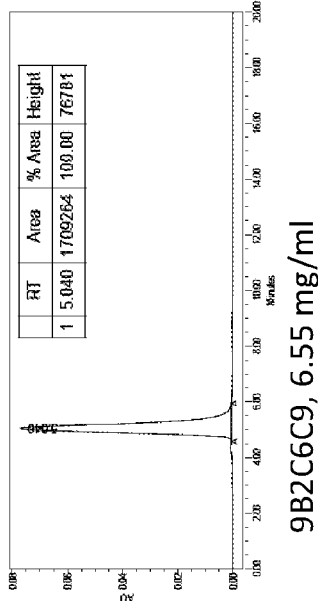


Figure 40

Before concentration



After concentration

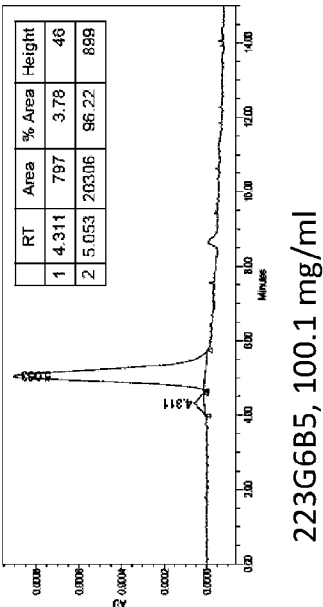
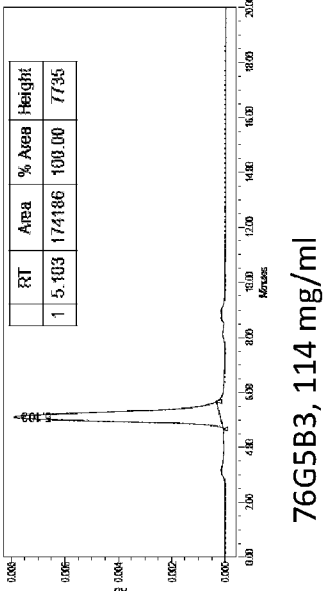
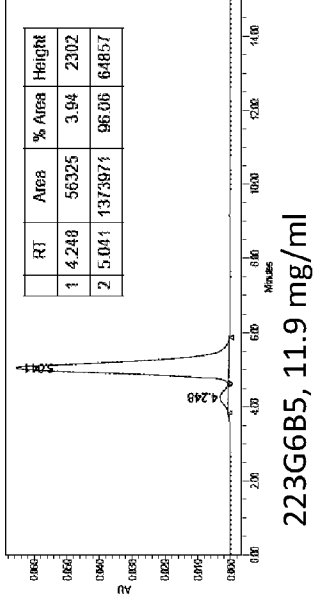
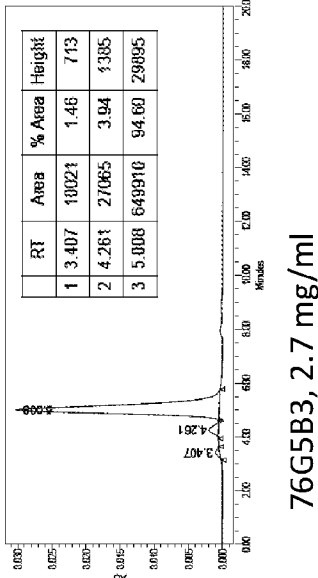
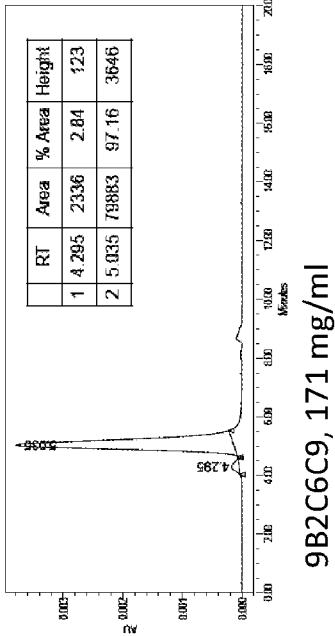


Figure 41

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/091962**A. CLASSIFICATION OF SUBJECT MATTER**

C07K 16/28(2006.01)i; C12N 15/13(2006.01)i; C12N 15/63(2006.01)i; C12N 5/10(2006.01)i; A61K 39/395(2006.01)i; A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K; C12N; A61K; A61P

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

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

DWPI; SIPOABS; CPRSABS; CNKI; NCBI; ISI Web of knowledge; ELSEVIER and keywords: antibody, programmed death-1, PD-1, receptor, etc. GenBank; EMBL; Retrieving System for Biological Sequence of Chinese Patent and searched sequences: SEQ ID NOS: 25-32

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2004056875 A1 (WYETH CORP) 08 July 2004 (2004-07-08) the abstract, claims 1-34	1-10, 14, 15 (all partially)
A	WO 2006121168 A1 (ONO PHARMACEUTICAL CO) 16 November 2006 (2006-11-16) the abstract, claims 1-105	1-10, 14, 15 (all partially)

☐ 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

27 October 2016

Date of mailing of the international search report

02 November 2016

Name and mailing address of the ISA/CN

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YANG, Zhenyu

Telephone No. (86-10)62089435

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/091962**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **11-13**
because they relate to subject matter not required to be searched by this Authority, namely:
[1] PCT Rule 39.1(iv) - Method for treatment of the human or animal body by surgery or therapy.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- [1] Invention 1: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 223G6B5 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof;
- [2] invention 2: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 9B2C6C9 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof;
- [3] invention 3: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 76G5B3 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof;
- [4] invention 4: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 32B5C7 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof;
- [5] invention 5: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 21G8F11 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof;
- [6] invention 6: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 226C3A7 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof;
- [7] invention 7: claims 1-10 and 14-15 (all partially), directed to the anti-PD-1 antibody 204C5E12 or antigen-binding fragment thereof, and an isolated nucleic acid, a vector, a host cell, a pharmaceutical composition and methods related thereof.
- [8] The same or corresponding technical feature of inventions 1-7 is an isolated monoclonal antibody binding PD-1. Such a technical feature has been disclosed by the prior art (e.g. CN103059138 A). It follows that the same or corresponding technical feature of inventions 1-7 does not make a contribution over the prior art and can not be considered as a special technical feature within the meaning of PCT Rule 13.2. The application, hence does not meet the requirements of unity of invention as defined in PCT Rules 13.1.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/091962

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **claims 1-10 and 14-15 (all partially) directed the antibody 223G6B5**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2016/091962

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2004056875	A1	08 July 2004	MX	PA05006828	A	08 September 2005
				US	2008311117	A1	18 December 2008
				AT	514713	T	15 July 2011
				US	2010028330	A1	04 February 2010
				US	7488802	B2	10 February 2009
				JP	2006521783	A	28 September 2006
				IL	169152	D0	04 July 2007
				AU	2003288675	B2	22 July 2010
				AU	2003288675	A8	14 July 2004
				US	2004213795	A1	28 October 2004
				EP	1576014	B1	29 June 2011
				CA	2508660	C	20 August 2013
				CA	2508660	A1	08 July 2004
				JP	4511943	B2	28 July 2010
				CN	101899114	A	01 December 2010
				HK	1083510	A1	16 December 2011
				BR	0316880	A	25 October 2005
				NO	20053389	D0	12 July 2005
				US	7521051	B2	21 April 2009
				NO	336442	B1	17 August 2015
				US	8088905	B2	03 January 2012
				NO	20053389	A	12 July 2005
				IL	169152	A	30 November 2010
				ES	2367430	T3	03 November 2011
				EP	1576014	A1	21 September 2005
				CN	1753912	B	02 November 2011
				US	2006210567	A1	21 September 2006
				AU	2003288675	A1	14 July 2004
				AU	2010235966	A1	11 November 2010
				CN	1753912	A	29 March 2006
				JP	2010189395	A	02 September 2010
WO	2006121168	A1	16 November 2006	US	9358289	B2	07 June 2016
				JP	2006340714	A	21 December 2006
				EP	2161336	B1	31 July 2013
				NL	300782	I2	18 May 2016
				RU	2010135087	A	27 February 2012
				RU	2013133714	A	27 January 2015
				US	9084776	B2	21 July 2015
				US	9387247	B2	12 July 2016
				US	2014348743	A1	27 November 2014
				US	2009217401	A1	27 August 2009
				ES	2427646	T3	31 October 2013
				JP	2016033135	A	10 March 2016
				KR	101339628	B1	09 December 2013
				AU	2006244885	B2	31 March 2011
				JP	5028700	B2	19 September 2012
				KR	101498834	B1	05 March 2015
				IL	208642	A	30 August 2012
				EP	2418278	A2	15 February 2012
				DK	2161336	T3	28 October 2013

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2016/091962

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
		JP 2012158605 A	23 August 2012
		CN 103059138 B	28 October 2015
		JP 5872377 B2	01 March 2016
		US 2015165025 A1	18 June 2015
		EP 1896582 A4	08 April 2009
		US 8008449 B2	30 August 2011
		EP 2418278 A3	04 July 2012
		CN 105315373 A	10 February 2016
		KR 20130114226 A	16 October 2013
		US 2014328833 A1	06 November 2014
		KR 20130032908 A	02 April 2013
		TW I379898 B	21 December 2012
		CN 103059138 A	24 April 2013
		JP 2009155338 A	16 July 2009
		US 8779105 B2	15 July 2014
		US 2013133091 A1	23 May 2013
		EP 2161336 A1	10 March 2010
		EP 2439272 A2	11 April 2012
		PT 2161336 E	03 October 2013
		IL 187108 A	30 June 2011
		AU 2006244885 A1	16 November 2006
		CA 2607147 A1	16 November 2006
		KR 101318469 B1	23 October 2013
		EP 2439273 A2	11 April 2012
		LU 92904 I2	10 February 2016
		EP 1896582 A1	12 March 2008
		EP 2439272 A3	31 July 2013
		KR 20080011428 A	04 February 2008
		US 2014212422 A1	31 July 2014
		NZ 563193 A	28 May 2010
		NO 20075697 A	11 February 2008