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(54) Title: Antibody Binding to PD-1

(57) Abstract: Disclosed herein are antibodies that bind to PD-1, comprising one or more CDRs selected from the amino acid sequences of SEQ ID NOs: 13, 14, 15, 16, 17, and 18. The antibodies have high affinity and low dissociation rate for PD-1, as well as the activity for neutralizing PD-1 in vitro. The antibodies disclosed herein can be full-length antibodies or antigen-binding fragments thereof. The antibodies can be used for detecting PD-1 and the like.

Antibody Binding to PD-1

TECHNICAL FIELD

The invention relates to the field of biotechnology, and more specifically to antibodies binding to PD-1.

5 BACKGROUND

Programmed cell death 1 (PD-1) is an immunoinhibitory receptor expressed on activated T cells, B cells, and myeloid cells, and is a member of the CD28 immunoglobulin superfamily. PD-1 is a 55kDa type I transmembrane glycoprotein comprising an Ig variable domain for binding to a ligand, and a cytoplasmic tail
10 region for binding to a signaling molecule. The PD-1 cytoplasmic tail region contains two tyrosine-based signaling motifs, immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM).

Two ligands for PD-1, PD-L1 and PD-L2, have been identified as constitutively
15 expressed or inducible on a variety of cells including non-hematopoietic tissues as well as various tumors. PD-L1 is expressed on B cells, T cells, myeloid cells, and dendritic cells as well as peripheral cells (such as microvascular endothelial cells) and non-lymphoid organs (such as heart, lung). In contrast, PD-L2 is present only in macrophages and dendritic cells.

20 The role of PD-1 in cancer has been reported in the literature. It was demonstrated that the interaction between PD-1 and its ligands down-regulates antigen receptor signaling, suppressing T cell response. The mechanism by which PD-1 attenuates T cell responses is similar to that of CTLA-4 in a manner that both molecules regulate an overlapping series of signal transduction proteins, but differs. PD-L1 is abundant
25 in a variety of human tumors (and can be induced by IFN γ in most PD-L1 negative tumor cells). There are numerous studies to date showing that the interaction between PD-1 and PD-L1 results in a decrease in tumor-infiltrating lymphocytes, a decrease in T cell receptor-mediated proliferation, and immune evasion of cancer cells. Blocking the interaction between PD-1 and PD-L1 was demonstrated to
30 increase T cell proliferation and cytokine production and to enhance capability of tumor-specific CD8⁺ T cells, thus contributing to the immune clearance of tumor

cells.

Immunohistochemistry has already found PD-1 (on tumor infiltrating lymphocytes) and/or PD-L1 (on tumor cells) in biopsies of many human primary tumors. Such biopsies include lesions of lung cancer, liver cancer, ovarian cancer, cervical
5 cancer, skin cancer, colon cancer, glioma, bladder cancer, breast cancer, kidney cancer, esophageal cancer, gastric cancer, oral squamous cell carcinoma, urothelial carcinoma, pancreatic cancer, and head and neck cancer. PD-1 ligand expression on cells of a variety of tumors is associated with poor prognosis.

In summary, the PD-1/PD-L1 pathway is an evident target for developing antibody
10 therapies for cancers. Thus, there is a need for agents that identify PD-1 and methods for using such agents.

SUMMARY

Provided herein are antibodies and antibody fragments that bind to PD-1.

In some embodiments, the PD-1 antibody or antibody fragment disclosed herein
15 comprises one, two, three, four, five or six CDRs selected from amino acid sequences of SEQ ID NOs: 13, 14, 15, 16, 17, and 18; or an amino acid sequence having one, two, or three conservatively substituted amino acid substitutions compared thereto.

In some embodiments, the antibody or antibody fragment binds to PD-1.

20 In some embodiments, the antibody or antibody fragment binds to human PD-1 (hPD-1).

In some embodiments, the antibody or antibody fragment blocks the binding of hPD-L1 (human PD-L1) to hPD-1 and/or disrupts hPD-1/hPD-L1 mediated downstream signaling pathways.

25 In some embodiments, a nucleotide sequence encoding SEQ ID NO: 13 of the above antibodies or antibody fragments is set forth in positions 73-114 of SEQ ID NO: 2.

In some embodiments, a nucleotide sequence encoding SEQ ID NO: 14 of any one of the above antibodies or antibody fragments is set forth in positions 160-177 of SEQ ID NO: 2.

5 In some embodiments, a nucleotide sequence encoding SEQ ID NO: 15 of any one of the above antibodies or antibody fragments is set forth in positions 271-303 of SEQ ID NO: 2.

In some embodiments, a nucleotide sequence encoding SEQ ID NO: 16 of any one of the above antibodies or antibody fragments is set forth in positions 91-108 of SEQ ID NO: 1.

10 In some embodiments, a nucleotide sequence encoding SEQ ID NO: 17 of any one of the above antibodies or antibody fragments is set forth in positions 148-192 of SEQ ID NO: 1.

In some embodiments, a nucleotide sequence encoding SEQ ID NO: 18 of any one of the above antibodies or antibody fragments is set forth in positions 292-327 of
15 SEQ ID NO: 1.

In some embodiments, the antibody or antibody fragment disclosed herein comprises one, two, or three heavy-chain CDRs selected from amino acid sequences of SEQ ID NOs: 16, 17, and 18; and/or

the antibody or antibody fragment comprises one, two, or three light-chain CDRs
20 selected from amino acid sequences of SEQ ID NOs: 13, 14, and 15.

In some embodiments, the above antibody or antibody fragment comprises light-chain CDRs having amino acid sequences of SEQ ID NOs: 13, 14, and 15, and heavy-chain CDRs having amino acid sequences of SEQ ID NOs: 16, 17, and 18.

25 In some embodiments, the antibody or antibody fragment is an isolated antibody or antibody fragment.

In some embodiments, the antibody or antibody fragment disclosed herein comprises a heavy-chain variable region and a light-chain variable region; the

heavy-chain variable region comprises an amino acid sequence of SEQ ID NO: 7, or an amino acid sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 7, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 7; and/or the light-chain variable region comprises an amino acid sequence of SEQ ID NO: 8, or an amino acid sequence having at least 90%, at least 95%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 8, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 8.

In some embodiments, the antibody or antibody fragment disclosed herein comprises a heavy-chain variable region and a light-chain variable region, the heavy-chain variable region comprising an amino acid sequence of SEQ ID NO: 7 and the light-chain variable region comprising an amino acid sequence of SEQ ID NO: 8.

In some embodiments, a nucleotide sequence encoding SEQ ID NO: 7 of any one of the above antibodies or antibody fragments is set forth in SEQ ID NO: 1, and a nucleotide sequence encoding SEQ ID NO: 8 is set forth in SEQ ID NO: 2.

In some embodiments, the antibody or antibody fragment disclosed herein further comprises a human $\gamma 4$ heavy-chain constant region or a variant thereof, and/or a human κ light-chain constant region or a variant thereof.

The present disclosure also provides an antibody or antibody fragment comprising a heavy chain and a light chain, the heavy chain comprising an amino acid sequence of SEQ ID NO: 71, an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 71, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 71; the light chain comprising an amino acid sequence of SEQ ID NO: 72, an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 72, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 72.

In some embodiments, the antibody or antibody fragment disclosed herein comprises a heavy chain set forth in SEQ ID NO: 71 and/or a light chain set forth in SEQ ID NO: 72.

5 In some embodiments, the antibody or antibody fragment disclosed herein comprises a heavy chain set forth in SEQ ID NO: 71 and a light chain set forth in SEQ ID NO: 72.

In some embodiments, a nucleotide sequence encoding SEQ ID NO: 71 of any one of the above antibodies or antibody fragments is set forth in SEQ ID NO: 73, and a nucleotide sequence encoding SEQ ID NO: 72 is set forth in SEQ ID NO: 74.

10 In some embodiments, the antibody or antibody fragment disclosed herein is a human antibody or human antibody fragment.

In some embodiments, the above antibody fragment disclosed herein is a Fab, Fv or scFv antibody.

15 In some embodiments, the antibody or antibody fragment disclosed herein is a monoclonal antibody (including a full-length monoclonal antibody), a polyclonal antibody, or a multispecific antibody (for example, bispecific antibody).

In another aspect, provided is a variant of any of the above antibodies or antibody fragments comprising up to 20, up to 10, up to 5, or up to 3 conservatively substituted amino acid substitutions.

20 In some embodiments, also provided is a variant of any one of the above antibodies or antibody fragments, comprising one, two, or three conservatively amino acid substitutions, while retaining the same function as the antibodies or antibody fragments.

In another aspect, also provided is a biological material comprising:

25 B1) a nucleic acid molecule encoding any one of the above antibodies or antibody fragments or the variants;

which can be a DNA, such as cDNA, genomic DNA, or recombinant DNA, or an

RNA, such as mRNA or hnRNA;

B2) an expression cassette, a recombinant vector, a recombinant cell, or a recombinant microorganism containing the nucleic acid molecule of B1).

The biological material is associated with the antibodies or antibody fragments or the variants disclosed herein, and can be used, for example, to produce the antibodies or antibody fragments or the variants disclosed herein.

In some embodiments, provided is a molecule comprising:

1) a nucleic acid fragment selected from:

- a nucleic acid fragment set forth in positions 91-108 of SEQ ID NO: 1;
- 10 a nucleic acid fragment set forth in positions 148-192 of SEQ ID NO: 1;
- a nucleic acid fragment set forth in positions 292-327 of SEQ ID NO: 1;
- a nucleic acid fragment set forth in positions 73-114 of SEQ ID NO: 2;
- a nucleic acid fragment set forth in positions 160-177 of SEQ ID NO: 2;
- a nucleic acid fragment set forth in positions 271-303 of SEQ ID NO: 2;
- 15 a nucleic acid fragment set forth in SEQ ID NO: 1;
- a nucleic acid fragment set forth in SEQ ID NO: 2;
- a nucleic acid fragment set forth as SEQ ID NO: 73; or
- a nucleic acid fragment set forth in SEQ ID NO: 74;

2) a nucleic acid fragment hybridizing with the nucleic acid fragment defined in 1) and encoding any one of the antibodies or antibody fragments or the variants disclosed herein;

3) a nucleic acid fragment having at least 90% sequence identity to the nucleic acid fragment defined in 1) or 2) and encoding any one of the antibodies or antibody fragments or the variants disclosed herein.

25 The nucleic acid molecule can be used in a part of the biological material.

The term "sequence identity" as used herein can be evaluated visually or using computer software, such as software programs described in Ausubel et al., Eds. (2007), Current Protocols in Molecular Biology. When a position of compared sequences is occupied by the same base or amino acid, then the molecules are

identical at that position. The sequence identity between two or more sequences can be represented by a percentage (%), which can be used to evaluate the identity of related sequences. A polynucleotide sequence or amino acid sequence having a certain percentage (e.g., 90%, 95%, 98%, or 99%) of "sequence identity" to another
5 sequence refers to that when the sequences are aligned, the percentage of bases or amino acids in the sequence are the same.

In some embodiments, treatment with an anti-PD-1 antibody or antibody fragment can be combined with radiotherapy. Radiotherapy induces cancer cell death and increases the availability of tumor antigens for presenting and activating immune
10 cells.

In some embodiments, treatment with the anti-PD-1 antibody or antibody fragment can be combined with surgery to remove cancer cells from a subject.

In other embodiments, the anti-PD-1 antibody or antibody fragment can be combined with a therapy capable of producing a synergistic effect on PD-1 blockade, the
15 therapy including a targeted medication for hormone clearance or inhibiting angiogenesis, or a targeted medication that targets proteins with activity in tumor cells. All of these lead to increased tumor cell death and increased availability of immunostimulatory tumor antigens. Increased T cell activation in combination with anti-PD-1 antibodies or antibody fragments can lead to persistent immune control
20 of cancers.

In some embodiments, the anti-PD-1 antibody or antibody fragment can be combined with another therapeutic antibody for treating cancer or infectious disease. Non-limiting solutions provided are combinations of anti-PD-1 antibody with antibodies targeting Her2/neu or EGF receptors. In another non-limiting
25 embodiment, the anti-PD-1 antibody or antibody fragment is combined with a therapy targeting VEGF or its receptor. In another embodiment, the anti-PD-1 antibody or antibody fragment is combined with anti-CTLA-4 antibody. In yet another non-limiting embodiment, the anti-PD-1 antibody is combined with an antibody targeting RSV.

30 In another aspect, also provided is a method for producing any one of the antibodies or antibody fragments or the variants, comprising:

a. under a condition for expressing the nucleic acid molecule encoding any one of the antibodies or antibody fragments or the variants, cultivating a recombinant cell containing the nucleic acid molecule to prepare the antibody or antibody fragment or the variant.

5 In some embodiments, the method further comprises:

b. isolating the antibody or antibody fragment or the variant from the recombinant cell or culture.

In another aspect, further provided is use of any one of the antibodies or antibody fragments or the variants in preparing any one of the products:

- 10 (1) a product for detecting PD-1;
(2) a product for stimulating or enhancing immune response;
(3) a product for treating cancer.

In some embodiments, the product for detecting PD-1 is a product for detecting hPD-1, such as a product detecting the expression of hPD-1 in specific cells,
15 tissues, or serum in vitro, or a product detecting hPD-1 in vivo.

In some embodiments, in the product for stimulating or enhancing immune response, the immune cells are in contact with an antigen and the antibody or antibody fragment or the variant, so as to increase the stimulation or to increase the immune response to the antigen.

20 In some embodiments, the immune response is a T cell-mediated immune response.

In some embodiments, in the product for treating cancer, the cancer is melanoma (e.g., metastatic malignant melanoma), renal carcinoma (e.g., clear cell carcinoma), prostate cancer (e.g., prostate cancer that is difficult to be controlled with hormones), pancreatic cancer, breast cancer, colon cancer, lung cancer (e.g.,
25 non-small cell lung cancer), esophageal cancer, head and neck squamous cell carcinoma, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, lymphoma, and other malignancies.

In another aspect, a pharmaceutical composition is provided, comprising any one of

the antibodies or antibody fragments or the variants disclosed herein as a therapeutic active ingredient and a pharmaceutically acceptable excipient, diluent, or carrier, or combinations thereof.

In another aspect, also provided is a method for treating cancer such as the
5 above-mentioned cancers, which comprises administering an effective amount of an antibody or antibody fragment or variant or a pharmaceutical composition disclosed herein to an individual in need thereof. The individual may be, for example, a rodent, canid, pig, primate, or human.

In some embodiments, the antibodies or antibody fragments provided herein bind to
10 hPD-1 with many excellent features including:

1. the antibodies or antibody fragments provided herein bind to hPD-1 with high affinity and can regulate the immune response. Specifically, the antibodies or antibody fragments provided herein can bind to hPD-1 with a K_D of about 20 pM or less.
- 15 2. the antibodies or antibody fragments provided herein can bind to hPD-1 with a k_{on} of about 1.98×10^6 1/Ms or faster.
3. the antibodies or antibody fragments provided herein can dissociate with hPD-1 with a k_{off} of about 4.11×10^{-5} 1/s or slower. The dissociation rate constant can be measured by biolayer interferometry (such as ForteBio Octet), or surface plasmon
20 resonance technology (such as Biacore).
4. the antibodies or antibody fragments provided herein may also block hPD-L1/hPD-1 binding with an IC_{50} of about 1 nM or less. By any method known in the art the blockade of ligand binding can be measured, and the IC_{50} , can be calculated, for example, by FACS method.
- 25 5. the antibodies or antibody fragments provided herein can disrupt the hPD-1/hPD-L1-mediated downstream signaling pathway by blocking the binding of hPD-1 and hPD-L1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates dose-dependent binding of anti-hPD-1 antibodies to purified hPD-1 in a protein ELISA.

Figure 2 illustrates k_{on} and k_{off} of antibody CL1 determined by a biolayer interferometry. Among them, each curve represents the dynamic process of antibody CL1 binding to the hPD-1 at different concentrations.

Figure 3 shows a flow cytometry detecting the blockade of the hPD-1/hPD-L1 binding by antibody CL1.

Figure 4 shows the functional activity of the anti-hPD-1 antibody measured by PD-1/PD-L1 Blockade Bioassay.

10 DETAILED DESCRIPTION

Unless otherwise stated, the methods used in the following examples are conventional methods.

The materials, reagents, and the like used in the following examples can be commercially available unless otherwise stated.

15 The present invention is further described below with reference to specific embodiments. It should be understood that the following examples are only used to illustrate the present invention, but not to limit the scope of the present invention.

Abbreviations and Definitions

20 Unless otherwise stated, the following terms shall have the meanings set forth below. Other terms or abbreviations have the meanings known in the art.

As used herein, unless otherwise stated, the singular forms “a” “an” and “the” include plural reference. Thus, for example, a reference to “an antibody” includes a plurality of antibodies.

25 "Antibody" refers to any form of an antibody that exhibits a desired biological activity, such as inhibiting the binding of a ligand to its receptor or inhibiting receptor-induced receptor signaling. Therefore, "antibody" is used in its broadest sense and includes, but is not limited to, a monoclonal antibody (including a

full-length monoclonal antibody), a polyclonal antibody and a multispecific antibody (such as bispecific antibody), a full human, humanized, primatized or chimeric antibody, a single chain antibody, etc.

"Antibody fragment" refers to a portion of an antibody, such as F(ab')₂, F(ab)₂, Fab', Fab, Fv, scFv, and the like. Regardless of its structure, the antibody fragment binds to the same antigen recognized by an intact antibody. The term "antibody fragment" includes aptamers, mirror isomers, and bivalent antibodies. The term "antibody fragment" also includes any synthetic or genetically engineered protein that functions as an antibody by binding to a particular antigen to form a complex.

10 "Fab fragment" consists of CH1 and variable regions of a light chain and a heavy chain. The heavy chain of a Fab molecule cannot form disulfide bonds with another heavy chain molecule.

"Fc region" contains two heavy-chain fragments comprising the CH2 and CH3 domains of an antibody. Two heavy-chain fragments are held together by two or
15 more disulfide bonds and by the hydrophobic interaction of the CH3 domains.

"Fv region" contains variable regions from both heavy and light chains, but lacks constant regions.

"Single-chain Fv antibody" (or "scFv antibody") refers to an antibody fragment comprising the VH and VL domains of an antibody, where these domains are
20 present in a single polypeptide chain. In general, Fv polypeptides additionally include a polypeptide linker between the VH and VL domains, which allows the scFv to form the desired structure for antigen binding. For a review of scFv, see US Pat. No. 6,423,538.

Those skilled in the art will understand that the classes of antibody heavy chains
25 include gamma, mu, alpha, delta, and epsilon (γ , μ , α , δ , and ϵ), and some subclasses (e.g., γ 1- γ 4). The nature of this chain determines the "type" of the antibody as IgG, IgM, IgA, IgD, or IgE. Immunoglobulin subclasses (isotypes), such as IgG1, IgG2, IgG3, IgG4, IgG5, etc., have been well characterized and the functional specificity imparted is also known. All types of immunoglobulins are
30 within the scope of the present invention. In some embodiments, the immunoglobulin molecule is an IgG species. IgG typically contains two identical

light chain polypeptides with a molecular weight of about 23,000 Daltons and two identical heavy chain polypeptides with a molecular weight of about 53,000-70,000. These four chains are connected in a "Y" configuration through disulfide bonds, wherein the light chain starts at the opening of "Y" configuration and extends through the variable region to surround the heavy chain. Antibodies in IgG4 form are a subclass of IgG, with their heavy chains being the $\gamma 4$ subtype. In some embodiments, the antibody disclosed herein is IgG4.

"Hypervariable region" refers to amino acid residues of an antibody responsible for antigen binding. Hypervariable regions include amino acid residues from "complementarity determining regions" or "CDRs" defined by sequence alignments. "Framework" residues or "FR" residues are variable domain residues other than hypervariable region residues as defined herein.

The antibodies disclosed herein may be derived from any animal, including birds and mammals. Preferably, the antibody is derived from a human, a mouse, a donkey, a rabbit, a goat, a camel, a llama, a horse, or a chicken source. In another embodiment, the variable region may come from a chondrichthoid source (e.g., from a shark). A "human antibody" is an antibody whose amino acid sequence corresponds to that of an antibody produced by a human, and/or an antibody that has been prepared using any of the techniques used to produce human antibodies as shown herein. This definition explicitly excludes humanized antibodies that comprise non-human antigen-binding residues.

"Isolated antibody" is an antibody that is separated from all or part of natural environment components. The contaminating component of its natural environment, including enzymes, hormones, and other proteinaceous or non-proteinaceous solutes, may interfere with the diagnostic or therapeutic application of the antibody. In some embodiments, the antibody is purified to the extent: (1) more than 95% by weight of the antibody, such as more than 99% by weight, as determined by the Lowry method; (2) sufficient to obtain at least 15 residues at the N-terminus or in internal amino acid sequence by use of a spinning cup sequencer; or (3) homogeneity by SDS-PAGE under reducing or non-reducing conditions with Coomassie blue or silver staining. Isolated antibodies include antibodies in situ within a recombinant cell, wherein at least one component of the

antibody's natural environment is not present. Isolated antibodies are usually prepared by at least one purification step. In some embodiments, the purity of the isolated antibody is at least about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 99%, or within the range between any two of these values (inclusive), or any values therein.

"Nucleic acid" or "polynucleotide" refers to a polymer molecule consisting of single nucleotides: adenine (a), cytosine (c), guanine (g), thymine (t) (or uracil (u) in RNA), such as DNA, RNA, or modifications thereof. The nucleic acid molecule may be a natural nucleic acid molecule, a synthetic nucleic acid molecule, or a combination of one or more natural nucleic acid molecules and one or more synthetic nucleic acid molecules. Examples of nucleic acids include, but are not limited to: genes or gene fragments (such as probes, primers, EST or SAGE tags), exons, introns, messenger RNA (mRNA), transport RNA, ribosomal RNA, ribozymes, cDNA, dsRNA, siRNA, miRNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

"Isolated nucleic acid molecule" is a nucleic acid molecule that has been identified and separated from at least one contaminating nucleic acid molecule. An isolated nucleic acid molecule is different from its naturally occurring form or environment. Therefore, an isolated nucleic acid molecule is distinguished from a nucleic acid molecule that is present in its natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in a cell that normally expresses an antibody, for example, the chromosome position of the nucleic acid molecule is different from that in a natural cell.

"Monoclonal antibody" refers to an antibody obtained from a substantially homogeneous antibody population, and the individual antibodies that make up the population are identical. Monoclonal antibodies are highly specific and can be directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations, which typically include multiple different antibodies directed against multiple different determinants (epitopes), each monoclonal antibody is only directed against a single determinant of the antigen.

"Immune cells" include cells that are of hematopoietic origin and play a role in

immune response. Immune cells include: B lymphocytes and T lymphocytes; natural killer cells; and monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

As used herein, "variant" of a sequence refers to a sequence that differs from the sequence shown at one or more amino acid residues but retains the same biological activity for the resulting molecule as the sequence shown.

"Amino acid" refers to an organic compound containing both an amino group and a carboxyl group, such as an alpha-amino acid that can be encoded by a nucleic acid directly or in the form of a precursor. A single amino acid is encoded by a nucleic acid consisting of three nucleotides (so-called codon or base triplet). The same amino acid can be encoded by different codons, which is known as "codon degeneracy". Amino acids include natural amino acids and non-natural amino acids. Natural amino acids include alanine (three-letter code: ala, one-letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

"Conservatively-substituted variants" or "conservative amino acid substitutions" refer to amino acid substitutions known to those skilled in the art, and such substitutions generally do not alter the biological activity of the resulting molecule. In general, it is commonly recognized by those skilled in the art that a single amino acid substitution in a non-essential region of a polypeptide will not substantially alter the biological activity of the polypeptide. The conservative substitution can be a substitution by an amino acid with a side chain of similar chemical properties, such as: 1) an aliphatic side chain: glycine, alanine, valine, leucine, and isoleucine; 2) an aliphatic hydroxyl side chain: serine and threonine; 3) an amide-containing side chain: asparagine and glutamine; 4) an aromatic side chain: phenylalanine, tyrosine, and tryptophan; 5) a basic side chain: lysine, arginine, and histidine; and 6) an acidic side chain: aspartic acid and glutamine.

As used herein, the term "about" means that a value is within an acceptable error range of the particular value as determined by one of ordinary skill in the art, and

the value depends in part on how to measure or determine (i.e., the limits of the measurement system). Alternatively, "about" may mean a range of up to $\pm 20\%$, such as a range of $\pm 10\%$, $\pm 5\%$, or $\pm 1\%$. Unless otherwise stated, when a specific value appears in the application and the claims, "about" should be assumed to mean
5 that it is within an acceptable error range for that specific value.

When referring to a ligand/receptor binding pair, an antibody/antigen binding pair, or other binding pairs, "specific" binding refers to determining whether there is the binding reaction of a protein such as PD-1 in a heterogeneous population of the protein and/or other biological agents. Therefore, under the given conditions, a

10 specific ligand/antigen binds to a specific receptor/antibody and does not significantly bind to other proteins present in the sample. Specific binding can be described by the equilibrium dissociation constant (K_D). A smaller K_D means a tighter binding. Methods for determining whether two molecules specifically bind to each other are well known in the art, and include, for example, equilibrium
15 dialysis, surface plasmon resonance, biofilm layer interferometry, and the like.

Antibodies that "specifically bind" to PD-1 include antibodies that have an equilibrium dissociation constant K_D of less than or equal to about 100 nM, less than or equal to about 10 nM, less than or equal to about 5 nM, or less than or equal to about 1 nM with PD-1.

20 As used herein, the term "biological material" includes, but is not limited to, a nucleic acid molecule, an expression cassette, a recombinant vector, a recombinant cell, or a recombinant microorganism.

When "administer" and "treat" involves an animal, a human, a subject, a cell, a tissue, an organ, or a biological fluid, it refers to contacting an exogenous

25 medicament, a therapeutic agent, a diagnostic agent or a composition with the animal, the human, the subject, the cell, the tissue, the organ, or the biological fluid.

"Administer" and "treat" may refer to, for example, a treatment method, a pharmacokinetic method, a diagnostic method, a research method, and an experimental method. Treating cells includes contacting an agent with cells and
30 contacting the agent with a fluid, wherein the fluid is in contact with cells.

"Administer" and "treat" also mean in-vitro and ex-vivo treatment of cells, for example, by an agent, a diagnostic agent, a binding composition, or other cells.

As used herein, "inhibit" or "treat" or "treatment" involves delaying the development of symptoms associated with a disease and/or alleviating the severity of these symptoms that the disease develops or is expected to develop. The term also involves alleviating existing symptoms, preventing additional symptoms, and
5 alleviating or preventing underlying causes of these existing symptoms. Thus, the term indicates that a beneficial result has been imparted to a vertebrate subject with a disease, such as a human.

As used herein, the term "therapeutically effective amount" or "effective amount" refers to an amount of the anti-PD-1 antibody that can effectively prevent or
10 alleviate the disease or disorder to be treated when the anti-PD-1 antibody or the fragment thereof is administered alone or in combination with another therapeutic agent to a cell, a tissue, or a subject in that amount. A therapeutically effective amount further refers to an amount of the antibody or the fragment thereof that is sufficient to cause a reduction in symptoms, for example, treating, curing,
15 preventing, or alleviating a related medical state, or increasing the treatment rate, cure rate, prevention rate, or alleviation rate for the symptom. The effective amount for a particular subject can vary depending on a variety of factors, such as the disease to be treated, the overall health of a patient, the route and dose of administration, and the severity of a side effect. An effective amount can be the
20 maximum dose or administration regimen that avoids significant side effects or toxic effects. When an active ingredient is administered to an individual alone, a therapeutically effective amount refers to the amount of that individual ingredient. When a combination is administered, a therapeutically effective amount refers to the combined amount of active ingredients that produces a therapeutic effect,
25 regardless of whether these active ingredients are administered in combination, continuously, or simultaneously. A therapeutically effective amount may reduce symptoms by at least 10%, generally at least 20%, preferably at least about 30%, more preferably at least 40%, and most preferably at least 50%. In some embodiments, the effective amount of the anti-PD-1 antibody or a fragment thereof
30 is 0.01 mg to 1 g. In some embodiments, the effective amount of the anti-PD-1 antibody or a fragment thereof is 10 mg to 750 mg. The antibodies described herein can be administered by conventional methods, such as intravenous or subcutaneous injection.

Monoclonal antibody

Monoclonal antibodies for PD-1 can be prepared according to knowledge and skills in the art, and antibodies or antibody fragments can be isolated from scFv yeast display libraries.

- 5 DNA encoding the monoclonal antibody can be easily isolated and sequenced by conventional methods. Once the DNA is isolated, it can be placed in an expression vector, and the vector is then transfected into a host cell to achieve the synthesis of monoclonal antibodies in a recombinant host cell. The host cell is, for example, an E. coli cell, a simian COS cell, a Chinese Hamster Ovary (CHO) cell, or a myeloma
10 cell that does not otherwise produce an immunoglobulin. Recombinant production of the antibody will be described in greater detail below.

Chimeric antibody

- DNA of the antibody can also be modified, for example, by using coding sequences for human heavy and light chain constant domains to replace the homologous
15 mouse sequences, or by combining a sequence encoding an immunoglobulin with all or part of a sequence encoding a non-immunoglobulin substance (such as a protein domain) with covalent linkages. The non-immunoglobulin substance is usually used to replace the constant domain of an antibody or the variable domain of an antigen-binding site of the antibody to generate a chimeric bivalent antibody,
20 and the chimeric bivalent antibody contains one antigen-binding site having specificity for an antigen and another antigen-binding site having specificity for a different antigen.

Antibody purification

- With a recombinant technology, the antibody can be produced intracellularly or in
25 the periplasmic space, or secreted directly into the culture. If the antibody is produced intracellularly or in the periplasmic space, the cell slurry is frozen and thawed repeatedly in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonyl fluoride (PMSF) in about 30 minutes, and then cell debris is removed by centrifugation. If the antibody is secreted into the culture, the
30 supernatant from the expression system can be first concentrated using a

commercially-available protein concentration filter (such as an Amicon or Millipore Pellicon ultrafiltration unit). In any of the aforementioned steps, a protease inhibitor (such as PMSF) can be used to inhibit the proteolysis, and an antibiotic can be used to prevent the growth of foreign contaminants.

- 5 An antibody composition can be prepared from cells using, for example, hydroxyapatite, gel electrophoresis, dialysis, and affinity chromatography, wherein the affinity chromatography is a preferred purification technique. Whether protein A is suitable as an affinity ligand depends on the type and the isotype of the immunoglobulin Fc region present in the antibody. Protein A can be used for the
- 10 purification of antibodies based on human $\gamma 1$, $\gamma 2$ or $\gamma 4$ heavy chains (Lindmark et al., 1983, J.Immunol.Meth.62: 1-13). The matrix to which the affinity ligand is attached is usually agarose, but other matrices can also be used. Mechanically stable matrices, such as controlled porosity glass or poly(styrene-divinylbenzene), can achieve a higher flow rate and a shorter processing time than agarose can
- 15 achieve. If the antibody contains a CH3 domain, it can be purified using Bakerbond ABXTM resin. Depending on the antibody to be recovered, other protein purification techniques can be used, such as fractionation on an ion exchange column, ethanol precipitation, reverse-phase HPLC, anion or cation exchange resin (e.g., polyaspartic acid column) chromatography, chromatofocusing, and
- 20 SDS-PAGE.

Therapeutic use of antibodies and antibody fragments disclosed herein

- The antibodies or antigen-binding fragments disclosed herein that specifically bind to PD-1 can be used for stimulating or enhancing an immune response. The antibodies and antibody fragments disclosed herein are particularly suitable for
- 25 treating a subject suffering from a disease that can be treated by increasing a T cell-mediated immune response. In some embodiments, the subject includes a human patient in need of an increased immune response.

- In some embodiments, provided is a method for treating disease that can be treated by increasing a T cell-mediated immune response, the method comprising
- 30 administered to a patient in need thereof a therapeutically effective amount of an antibody or antigen-binding fragment disclosed herein. In some embodiments, the disease that can be treated by increasing a T cell-mediated immune response is a

cancer.

Cancer

The antibodies or antigen-binding fragments disclosed herein can be used for treating a cancer (i.e., inhibiting the growth or survival of tumor cells). In some
5 embodiments, cancers whose growth can be inhibited with the antibodies disclosed herein include cancers that generally respond to an immunotherapy, but also include cancers that have not yet been associated with an immunotherapy so far. In some embodiments, non-limiting examples of cancers include melanoma (e.g., metastatic malignant melanoma), renal carcinoma (e.g., clear cell carcinoma),
10 prostate cancer (e.g., prostate cancer that is difficult to be controlled with hormones), pancreatic cancer, breast cancer, colon cancer, lung cancer (e.g., non-small cell lung cancer), esophageal cancer, head and neck squamous cell carcinoma, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, lymphoma, and other malignancies. In some embodiments, the cancer is a
15 refractory or relapsed cancer that can be inhibited by the antibodies or antigen-binding fragments.

Non-therapeutic use of the antibodies and antibody fragments

Anti-PD-1 antibody products for non-therapeutic use already exist, such as the anti-hPD-1 monoclonal antibody Mab1086 commercially available from R&D
20 Systems of Minneapolis, MN, USA, which can be used for flow cytometry, Western Blot, and ELISA. The antibodies disclosed herein can be used for any non-therapeutic purpose provided by Mab1086.

The antibodies disclosed herein can be used for diagnostic assay, for example, for detecting the expression of PD-1 in specific cells, tissues, or serum. For diagnostic
25 use, the antibody is usually labeled (directly or indirectly) with a detectable moiety. Numerous labels are available, generally comprising the following categories: biotin, fluorochrome, radionucleotide, enzyme, iodine, and a biosynthetic label.

The antibodies of the invention can also be used for in-vivo diagnostic assays. The antibodies are usually labeled with a radionuclide (e.g., ^{111}In , ^{99}Tc , ^4C , ^{125}I , ^3H , ^{32}P ,
30 ^{35}S , or ^{18}F) so that antigens or cells that express the antibodies can be localized by

immunoscintigraphy or positron emission tomography.

Pharmaceutical compositions

Also provided are pharmaceutical compositions. Such compositions comprise an effective dose of an antibody or antibody fragment and a pharmaceutically acceptable carrier.

In some embodiments, the term "pharmaceutically acceptable carrier" refers to a substance approved by a government regulatory agency or listed in other commonly-recognized pharmacopeias for use in animals, and particularly in humans. In addition, the "pharmaceutically acceptable carrier" may be any type of non-toxic solid, semi-solid or liquid filler, diluent, an encapsulating material, or a formulation additive.

The term "carrier" refers to a diluent, adjuvant, excipient, or carrier that is administered with an active ingredient for treatment. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including oils originated from petroleum, animal, plant or synthesis, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. In some embodiments, when the pharmaceutical composition is administered intravenously, the carrier can be water. A saline aqueous solution, a glucose aqueous solution, and a glycerol solution can also be used as a liquid carrier, especially for injection. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin, which is hereby incorporated herein by reference. Such compositions will contain a clinically effective dose of an antibody or antibody fragment and a suitable carrier, to provide a form suitable for administration to a patient. The formulation should be suitable for the administration mode. The formulation can be encapsulated in an ampoule bottle, a disposable syringe, or a multi-dose vial made of glass or plastic.

In some embodiments, the composition is formulated into a pharmaceutical composition suitable for intravenous injection into a human body according to conventional steps. A composition for intravenous administration is usually a solution in sterile isotonic aqueous buffer. The pharmaceutical composition may also include a solubilizer and a local anesthetic, such as lidocaine, to alleviate the

pain at the injection site. In general, the active ingredients are provided in a unit dosage form individually or as a mixture, for example, the active ingredients are encapsulated in sealed containers (such as ampoule bottles or sachets) that can indicate the amount of the active agent, in the form of lyophilized powder or
5 anhydrous concentrate. Where the composition is administered by infusion, the composition can be dispensed in infusion bottles containing sterile, pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule bottle containing sterile water or saline for injection can be used, so that the active ingredients can be mixed before administration.

- 10 The antibodies or antibody fragments provided herein include salt forms thereof. Pharmaceutically acceptable salts include salts of anions derived from acids such as hydrochloric acid, phosphoric acid, acetic acid, oxalic acid, tartaric acid, and the like; and salts of cations derived from sodium, potassium, ammonium, calcium, iron, isopropylamine, triethylamine, 2-ethylaminoethanol, histidine,
15 procaine cations, and the like.

Reagents or materials

hPD-1 was obtained from Sino Biological Inc., Catalog No.: 10377-H03H-B.

hIgG was obtained from Nanjing Genscript Biotechnology Co., Ltd., Catalog No.: A01006.

- 20 hPD-1-expressing Jurkat cells were obtained from Promega, Catalog No.: J1252.

Unlabeled hPD-L1 was obtained from Sino Biological Inc., Catalog No.: 10084-HNAH.

PE-labeled goat anti-human Fc was obtained from Thermo Fisher Scientific, Catalog No.: 12-4998-82.

- 25 CHO-K1 cells were obtained from Promega, Catalog No.: J1252.

F-12 was obtained from Thermo Fisher Scientific, Catalog No.: 11765062.

Bio-Glo Luciferase Assay System was obtained from Promega, Catalog No.:

G7940.

Example 1: Preparation and sequencing of the anti-hPD-1 antibodies

First, human VL and VH cDNAs were prepared based on human lymphocyte mRNAs, and then a scFv yeast display library was established. The CL1 and

- 5 CL1-related antibodies disclosed herein were isolated by screening the library. The methodologies for establishing and screening such libraries are known in the art. Methods and reagents particularly suitable for the establishment and screening of antibody display libraries can be found in the following document: such as U.S. Pat. No. 6423538 of Wittrup et al.

- 10 The DNA sequences encoding the scFv variable regions expressed by yeast were determined by gene sequencing. The results of each sequencing reaction for each clone were analyzed with Seqman. The sequences are disclosed in the Sequence Listing and listed in Table 1.

Table 1 DNA or amino acid sequences of the anti-hPD-1 antibodies

SEQ ID NO:	Description	Sequence
1	CL1 heavy-chain variable region (DNA)	caggtgcagctggtgcagtcggcgctggaggtgaagaagcctggcgccagcgtgaaggtgtcctgtaaggccagcggctacaccttcaccaattactatatgtattgggtgcggcaggcccggccagggactggagtggtggagggcatcaatcccagcaacggcgccaccaacttc aatgagaagttaagaaccgggtgaccctgaccaccgatagcagcaccaccaccgcttac atggagctgaagagcctgcagtttgacgataccgctgtgtactattgcgctgcccgggatca ggctggacatgggcttcgagttctggggccagggcaccaccgtgaccgtgtccagc
2	CL1 light-chain variable region (DNA)	gagatcgtgctgaccagtcctcccgctaccctgagcctgtccccggagagcgggctacc ctgtctgtcgggcctccaagggcgtgagcaccagcggatactcctatctgactggtacca gcagaagcccggccagggtcccagggtgctgatctacctggcttcctacctggagagcgg cgtgcccgtaggtttagcggcagcggcagcgggaaccgatttcaccctgaccatcagctcc ctggagcccaggattttgccgtgtactactgccagcacgcttacgacctgcccctgaccttt ggccggcgccaccaaggtggagatcaag
3	CL5 heavy-chain variable region (DNA)	caagttcaattagtcctaaagtgtgtcgaagtaagaagccaggtgcctcagtcctcaagtcag ttgtaaagcctcaggttatacatcttacaactactacatgtactgggttagacaagccccaggt caaggtttggaatggatgggtggtatcaatccttcaaacgggtggcaccacttcaacgaaaa gtcaagaacagagtaactttgactacagattcttcaaccactacagcatacatggaattgaa atctttacaattcgatgacacagctgtttactactgtgcagcgcgtgattacagattcgacatg ggtttcgaatactgggggtcaaggtaccactgttactgtctcctct

4	CL5 light-chain variable region (DNA)	gaaattgtcttgactcaatctccagctacattgtccttaagtcctggtgaaagagccacattgtc atgcagagcttccaaaggtgtatctacctcaggtactcttacttgcatggtatcaacaaaag ccaggtcaagcacctagattgttgatctacttgccagttacttggaatctggtgtccagcta gattttccggtagtgggtctggtactgacttcacattgaccatatcttcattagaacctgaagatt tcgcagtctattactgccaacattcttatgacctccacttacattcggaggtggtacaaaggta gaaataaaa
5	CL9 heavy-chain variable region (DNA)	caagttcaattagtcctaaagtggtgtcgaagtaaagaagccaggtgcctcagtcctcaagtcag ttgtaaagcctcaggttatacatattacaaactactacatgtactgggttagacaagccccaggt caaggtttggaatggatgggtggtatcaatccttcaaacgggtggcaccacttcaacgaaaa gttcaagaacagagtaactttgactacagattcttcaaccactacagcatacatggaattgaa atctttacaattcgatgacacagctgtttactactgtgcagcacgtgattacaaattcgacatgg gtttcgaattctgggtgcaaggtaccactgttactgtctcctct
6	CL9 light-chain variable region (DNA)	gaaattgtcttgactcaatctccagctacattgtccttaagtcctggtgaaagagccacattgtc atgcagagcttccaaaggtgtatctacctcaggtactcttacttgcatggtatcaacaaaag ccaggtcaagcacctagattgttgatctacttgccagttacttggaatctggtgtccagcta gattttccggtagtgggtctggtactgacttcacattgaccatatcttcattagaacctgaagatt tcgcagtctattattgccaacattcctatggccttcagttacattcgggtggtggtacaaaggta gaaataaaa
7	CL1 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYRLDMGFEFWGQGT TVTVSS
8	CL1 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTIS LEPEDFAVYYCQHAYDLPLTFGGGGTKVEIK
9	CL5 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYRFDMDGFEYWGQG TTTVTVSS
10	CL5 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTIS LEPEDFAVYYCQHSYDLPLTFGGGGTKVEIK
11	CL9 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYKFDMDGFEFWGQGT TVTVSS
12	CL9 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTIS LEPEDFAVYYCQHSYGLPVTTFGGGGTKVEIK
13	CL1 light-chain CDR1 (AA)	ASKGVSTSGYSYLH
14	CL1 light-chain CDR2 (AA)	LASYLE
15	CL1 light-chain CDR3 (AA)	YCQHAYDLPLT
16	CL1 heavy-chain CDR1 (AA)	NYYMYW

17	CL1 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKF
18	CL1 heavy-chain CDR3 (AA)	ARDYRLDMGFEF
19	CL5 light-chain CDR1 (AA)	ASKGVSTSGYSYLH
20	CL5 light-chain CDR2 (AA)	LASYLE
21	CL5 light-chain CDR3 (AA)	YCQHSYDLPLT
22	CL5 heavy-chain CDR1 (AA)	NYYMYW
23	CL5 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKF
24	CL5 heavy-chain CDR3 (AA)	ARDYRFDMGFEY
25	CL9 light-chain CDR1 (AA)	ASKGVSTSGYSYLH
26	CL9 light-chain CDR2 (AA)	LASYLE
27	CL9 light-chain CDR3 (AA)	YCQHSYGLPVT
28	CL9 heavy-chain CDR1 (AA)	NYYMYW
29	CL9 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKF
30	CL9 heavy-chain CDR3 (AA)	ARDYKFDMGFEF
31	CL2 heavy-chain variable region (DNA)	caagttcaattagtccaaagtggtgtcgaagtaaagaagccaggtgcctcagtc aaagtcag ttgtaaagcctcaggttatacatttacaactactacatgtactgggttagacaagccccaggt caaggtttggaatggatgggtggtatcaatccttcaaacggtggcaccacactcaacgaaaa gtccaagaacagagtaactttgactacagattctcaaccactacagcatacatggaattgaa atctttacaattcgatgacacagctgtttactactgtgcagcccgtgattacaaattcgacatgg gtttcgagtactggggtaaggtaccactgttactgtctcctct
32	CL2 light-chain variable region (DNA)	gaaattgtcttgactcaatctccagctacattgtccttaagtctctggtgaaagagccacattgtc atgcagagcttcaaagggtgtatctacctcaggttactcttacttgcatgggtatcaacaaaag ccaggtcaagcacctagattgttgatctacttggccaggtacttggaaatctgggtgtccagcta gattttccggtagtggttctggtactgacttcacattgaccatatcttcattagaacctgaagatt tcgcagctctattattgccaacattcctatgggtcttccacttacattcgggtgggtggtacaaaggta gaaataaaa
33	CL7 heavy-chain variable region (DNA)	caagttcaattagtccaaagtggtgtcgaagtaaagaagccaggtgcctcagtc aaagtcag ttgtaaagcctcaggttatacatttacaactactacatgtactgggttagacaagccccaggt caaggtttggaatggatgggtggtatcaatccttcaaacggtggcaccacactcaacgaaaa gtccaagaacagagtaactttgactacagattctcaaccactacagcatacatggaattgaa atctttacaattcgatgacacagctgtttactactgtgcagcccgtgattaccgattcgacatgg gtttcgagttttggggtaaggtaccactgttactgtctcctct

34	CL7 light-chain variable region (DNA)	gaaattgtcttgactcaatctccagctacattgtccttaagtcctggtgaaagagccacattgtc atgcagagcttccaaaggtgtatctacctcaggtactcttacttgcatgggtatcaacaaaag ccaggtcaagcacctagattgttgatctacttgccaggtacttggaatctggtgtccagcta gattttccggtagtgggtctggtactgacttcacattgaccatatcttcattagaacctgaagatt tcgcagctctattattgccaacattcctatagttttccactcacattcggtggtggtacaaaggta gaaataaaa
35	CL6 heavy-chain variable region (DNA)	caagttcaattagtcctaaagtggtgtcgaagtaaagaagccaggtgcctcagtcctcaagtcag ttgtaaagcctcaggttatacatattacaaactactacatgtactgggttacacaagccccaggt caaggtttggaatggatgggtgtatcaatccttcaaacgggtggcaccactcaacgaaaa gttcaagaacagagtaactttgactacagattcttcaaccactacagcatacatggaattgaa atctttacaattcgatgacacagctgtttactactgtgcagcccggtgattacagatttgacatgg gtttcgagttctggggtcaaggtaccactgttactgtctcctct
36	CL6 light-chain variable region (DNA)	gaaattgtcttgactcaatctccagctacattgtccttaagtcctggtgaaagagccacattgtc atgcagagcttccaaaggtgtatctacctcaggtactcttacttgcatgggtatcaacaaaag ccaggtcaagcacctagattgttgatctacttgccaggtacttggaatctggtgtccagcta gatgttccggtagtgggtctggtactgacttcacattgaccatatcttcattagaacctgaagat gtcgcagctctattattgccaacattcctatgacctccactacattcggtggtggtacaaaggta agagataaaa
37	CL2 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYKFDMGFEYWGQG TTVTVSS
38	CL2 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTIS LEPEDFAVYYCQHSYGLPLTFGGGKVEIK
39	CL7 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYRFDMGFEFWGQGT TTVTVSS
40	CL7 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTIS LEPEDFAVYYCQHSYSFPLTFGGGKVEIK
41	CL6 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYRFDMGFEFWGQGT TTVTVSS
42	CL6 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTIS LEPEDFAVYYCQHSYDLPLTFGGGKVEIK
43	CL2 light-chain CDR1 (AA)	ASKGVSTSGYSYLH
44	CL2 light-chain CDR2 (AA)	LASYLE
45	CL2 light-chain CDR3 (AA)	YCQHSYGLPLT
46	CL2 heavy-chain CDR1 (AA)	NYYMYW

47	CL2 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKF
48	CL2 heavy-chain CDR3 (AA)	ARDYKFDMGFEY
49	CL7 light-chain CDR1 (AA)	ASKGVSTSGYSYLH
50	CL7 light-chain CDR2 (AA)	LASYLE
51	CL7 light-chain CDR3 (AA)	YCQHSYSFPLT
52	CL7 heavy-chain CDR1 (AA)	NYMYW
53	CL7 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKF
54	CL7 heavy-chain CDR3 (AA)	ARDYRFDMGFEF
55	CL6 light-chain CDR1 (AA)	ASKGVSTSGYSYLH
56	CL6 light-chain CDR2 (AA)	LASYLE
57	CL6 light-chain CDR3 (AA)	YCQHSYDLPLT
58	CL6 heavy-chain CDR1 (AA)	NYMYW
59	CL6 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKF
60	CL6 heavy-chain CDR3 (AA)	ARDYRFDMGFEF
61	CL20 heavy-chain variable region (DNA)	caggtgcagctggtgcagtcaggggtggaggtcaaaaagcccgtgcctcagtc aaagtgtctgtaaagcctctggatatacttactaactactatatgtactgggtgaggcaggcaccaggacagggtctggagtgatggcggaatcaaccctctaattgggggtaccaacttcaacgaaaagtttaaaaaccgggtgacactgaccacagattccagcactaccacagcctatatggagctgaagtcctgcagtcgacgatacagcagtgactattgcgccaggcgggactacagattcgatatgggctttgactattggggccagggaactaccgtcacctgtctagt
62	CL20 light-chain variable region (DNA)	gaaattgtgctgaccagagccccgccactctgtctctgagtcaggagagagggccaccctgtcatgccgagctccaaggcggtgtccacctccggctacagtattctgcactggtaccagcagaaacccggacaggcacctagactgctgatctacctggccagctatctggaatctggcgtgccagctaggtcagcggtctggaagtgggaccgactttaccctgacaatttcagcctggagcccgaagatttcgcagctctactattgccagcattctcgtgacctgcccctgacattcggcgggtggaacaaaagtggaaatcaag
63	CL20 heavy-chain variable region (AA)	QVQLVQSGVEVKKPGASVKVSKASGYTFTNYYMYWV RQAPGQGLEWMGGINPSNGGTNFNEKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCARRDYRFDMGFDYWGGQ TTVTVSS

64	CL20 light-chain variable region (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTSS LEPEDFAVYYCQHSRDLPLTFGGGTKVEIK
65	CL20 light-chain CDR1 (AA)	RASKGVSTSGYSYLH
66	CL20 light-chain CDR2 (AA)	LASYLEs
67	CL20 light-chain CDR3 (AA)	QHSRDLPLT
68	CL20 heavy-chain CDR1 (AA)	NYMY
69	CL20 heavy-chain CDR2 (AA)	GINPSNGGTNFNEKFKN
70	CL20 heavy-chain CDR3 (AA)	RDYRFDMGFDY
71	CL1 heavy chain full length (AA)	QVQLVQSGVEVKKPGASVKVSCKASGYTFTNYYMYWV RQAPGGLEWMGGINPSNGGTNFNEKFKNRVTLTDSST TTAYMELKSLQFDDTAVYYCAARDYRLDMGFEFWGQGT TVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPE PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQF NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPS QEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT TPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEAL HNHYTQKSLSLGK
72	CL1 light chain full length (AA)	EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWY QQKPGQAPRLLIYLAstyleSGVPARFSGSGSGTDFTLTSS LEPEDFAVYYCQHAYDLPLTFGGGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC

73	CL1 heavy chain full length (DNA)	cagggtgcagctgggtgcagtccggcgtggaggtgaagaagcctggcgccagcgtgaaggt gtcctgtaaggccagcgggtacaccttcaccaattactatgtattgggtgcggcaggccc ccggccagggactggagtggatgggaggcatcaatccagcaacggcgccaccaacttc aatgagaagttaagaaccgggtgacctgaccaccgatagcagcaccaccaccgcttac atggagctgaagagcctgcagtttgacgataccgctgtgtactattgcgctgccgggatta caggctggacatgggcttcgagttctggggccaggggcaccaccgtgaccgtgtccagcgc tagcaccaaggggcccttcggtgtccccctggccccctgtagccgggtccacctctgagagc accgctgctctgggctgtctggtgaaggatttcccgaaccgggtgaccgtgtcatggaa ctccggggctctgacatccgggtgtccacacttttctgcagtgtgcatccggcctgta cagcctgagctctgtggtcacagtcccaagttcatccctgggaaccaagacataacttgca acgtggatcataaaccagcaataactaaggtcgacaaacgagtggagtctaagtacggacc acctgcccaccatgtccagcacctgagttcctgggaggaccaagcgtgttctgtttcctcc aaagcctaaagataccctgatgatcagtcggactcccagggtcacctgcgtggctgtggac gtgtcccaggaggaccctgaagtccagtcaactggtagcgtggacggcgtcgaagtgcac aatgctaagacaaaacctcgagaggaacagtttaactccacataccgtgtcgtgagcgtcct gactgtgctgcatcaggattggctgaacggcaaggagtataagtgcaaagtgagcaataag ggactgccaagctctatcgagaaaactatttctaaggctaaaggacagcctagggaaccac aggtgtacacctgccccctagtccaggaggaaatgactaagaaccaggtctcactgacctg tctggtgaaagggttctatccttcagatattgcagtggagtgggaatccaatggtcagccaga gaacaattacaagacaactccaccctgctggacagcgtgggtctttcttctgtattctaga ctgaccgtggacaaaagtcgctggcaggagggtaatgtctttctgtagtgtgatgcacgaa gcctgcacaaccactacactcagaaaagcctgtcactgtccctgggtaaa
74	CL1 light chain full length (DNA)	gagatcgtgctgaccagtcccccgctaccctgagcctgtccccggagagcgggctacc ctgtcttgcgggctccaaggcggtgagcaccagcggatactctatctgactggtacca gcagaagcccggccaggctcccaggctgctgatctacctggcttctacctggagagcgg cgtgcccgtaggtttagcggcagcggcagcggaaaccgattcacctgacctcagctcc ctggagcccaggattttgccgtgtactactgccagcagccttacgacctgcccctgaccttt ggcggcgccaccaaggtggagatcaagcgtacgggtggctgcaccatctgtcttcatctcc cgccatctgatgagcagttgaaatctggaactgcctctgttgtgtcctgctgaataactctat cccagagaggccaaagtacagtggaaaggtggataacgcctccaatcgggtaactccag gagagtgtcacagagcaggacagcaaggacagcacctacagcctcagcagcacctgac gctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacccatcagg gcctgagctgcccgtcacaagagcttcaacaggggagagtg

Notes: DNA within brackets in the table refers to nucleotides and AA refers to amino acids.

The nucleotide sequence encoding SEQ ID NO: 13 is shown in positions 73-114 of SEQ ID NO: 2;

- 5 The nucleotide sequence encoding SEQ ID NO: 14 is shown in positions 160-177 of SEQ ID NO: 2;

The nucleotide sequence encoding SEQ ID NO: 15 is shown in positions 271-303 of SEQ ID NO: 2;

- 10 The nucleotide sequence encoding SEQ ID NO: 16 is shown in positions 91-108 of SEQ ID NO: 1;

The nucleotide sequence encoding SEQ ID NO: 17 is shown in positions 148-192 of SEQ ID NO: 1;

The nucleotide sequence encoding SEQ ID NO: 18 is shown in positions 292 - 327 of SEQ ID NO: 1.

5 **Example 2: Construction and expression of antibody CL1 and related antibodies**

The DNA sequences of the VL and VH regions of antibody CL1 cloned by PCR were linked to the human κ light-chain constant region and the IgG4 constant region (γ 4 heavy-chain constant region) respectively to construct the light-chain
10 and heavy-chain DNA sequences. The 5' and 3' terminuses of the sequences were modified with PCR primers designed to add an appropriate leader sequence to each strand, and then the resulting sequences were cloned to the existing recombinant antibody expression vector. It was determined, by sequencing assay, that the vector was correctly constructed. 40 μ g of expression plasmid was thoroughly mixed with
15 700 μ L of CHO cell suspension that contained 10^7 cells in total, and the mixture was added to a 0.4cm electroporation cuvette and placed in an electroporation apparatus. The electroporation was completed under electroporation parameters set as: voltage 300 V, electric pulse duration 17 ms, and square wave. After the electroporation was completed, these cells were cultivated in 8 mL of growth
20 medium for 3 days. The antibody concentration in the supernatant after transfection was measured by ELISA, and finally, the engineered cell line having high expression and good growth was determined.

Cells were cultivated in CD-CHO (Gibco) in shake flasks for 12 days. The antibody in the cell supernatant was purified by protein A affinity chromatography, washed,
25 eluted with 1 M acetic acid, and neutralized with 3 M Tris. Finally, the buffer was replaced with 100 mM acetic acid which had been adjusted to pH 5.5 with 1 M Tris to obtain antibody CL1.

Antibodies CL5, CL9, CL2, CL7, CL6, and CL20 were constructed and expressed in a similar manner as described above.

30 The amino acid sequences of antibodies CL1, CL5, CL9, CL2, CL7, CL6 and CL20 obtained after purification were as expected.

Example 3: Assay for affinity of the anti-hPD-1 antibodies to hPD-1

The binding of the antibodies to hPD-1 was determined by enzyme-linked immunosorbent assay (ELISA). hPD-1 was coated on a 96-well plate by incubation overnight at 4 °C. After coating, the plate was washed 5 times with PBST.

- 5 Dilutions of antibodies CL1, CL5, CL9, CL2, CL7, CL6, CL20, and hIgG at different concentrations were prepared with PBST, and incubated with coated hPD-1 for 1 hour at room temperature. After the binding, the plate was washed 5 times with PBST. The resulting solution was incubated at room temperature in PBST that contained peroxidase (HRP)-labeled goat anti-mouse IgG solution
10 diluted to 1/10000 for 1 hour, washed again, and developed with TMB.

ELISA results are shown in Fig. 1. With a half maximal effective concentration (EC_{50}) representing the binding affinity, results are shown in Table 2.

- The experimental results indicate that anti-hPD-1 antibodies CL1, CL5, CL9, CL2, CL7, CL6, and CL20 all bind to hPD-1, and CL1 demonstrates a strongest affinity,
15 with an EC_{50} of about 0.208 nM for the binding to hPD-1, and is significantly superior to CL5, CL9, CL2, CL7, CL6, and CL20.

Example 4: Kinetics analysis for the binding of antibody CL1 to hPD-1

- To further characterize the binding properties of the antibody, antibody CL1 was profiled by bio-layer interferometry on the Octet system (ForteBio, Menlo Park,
20 California) to illustrate the binding kinetics and calculate the equilibrium binding constant. The assay was performed by coupling a hPD-1 fusion protein to a streptavidin biosensor using biotin.

- hPD-1 needs to be biotinylated to facilitate the fixing of hPD-1 on a streptavidin biosensor matrix. 1.0 mg of biotin was first dissolved in 100 μ l of
25 dimethyl sulfoxide to obtain a biotin solution at 10mg/ml, then each milliliter of hPD-1 (1.8 mg/ml) solution was added with 10 μ l of the biotin solution, and the reaction solution was gently stirred and incubated in the dark at room temperature for 2 hours to obtain biotinylated hPD-1. A PD-10 column packed with Sephadex G-25M (Pharmacia, Catalog No.: 17-0851-01) was equilibrated with 25 ml of cold
30 PBS, and 2 ml of biotinylated hPD-1 was loaded per column. The column was eluted with 10×1 ml cold PBS. The eluent was collected and read at OD280 to obtain purified biotinylated hPD-1, and the obtained biotinylated hPD-1 was stored

at -80 °C until use.

A streptavidin biosensor was pre-wetted by immersing in PBS in a well for 10 minutes, and then the biosensor was equilibrated with PBS for 2 minutes. The biosensor was immersed in the solution of 20 µg/mL biotinylated hPD-1 in PBS for 5 minutes to couple with biotinylated hPD-1, and then was equilibrated with PBS for 2 minutes. The biosensors were placed in wells containing antibody solutions of various concentrations (0.125 to 2 nM, SDS-PAGE purity > 99%) to observe their binding to the anti-PD-1 antibody, and the binding was monitored and measured for 30 minutes. After the biosensor was transferred to PBS, dissociation was monitored and measured for 90 minutes. The observed binding and dissociation rates (k_{binding} and $k_{\text{dissociation}}$) were fitted with a 1:1 binding global fit model containing all test concentrations, and the equilibrium binding constant K_D was calculated.

The results of kinetics assays are shown in Table 2 and Fig. 2.

The experimental results show that K_D of the binding of antibody CL1 to hPD-1 is about 0.0207 nM. Such a strong affinity is resulting from a much higher binding rate and a slower dissociation rate than ordinary antibodies.

Example 5: Assay for the blocking of hPD-1/hPD-L1 binding by antibody CL1

The blocking of ligand binding was assayed by flow cytometry. Jurkat cells expressing hPD-1 were plated in a 96-well plate, and then antibody solutions (CL1 or hIgG (as a negative control)) of various concentrations and unlabeled hPD-L1 at a concentration of 1 µg/mL were added. The mixture was placed on ice for 30 minutes, washed 3 times with FACS buffer (PBS containing 0.3% BSA), and incubated with PE-labeled goat anti-human Fc on ice for another 30 minutes. Cells were washed three more times with FACS buffer and analyzed by flow cytometry. Data were analyzed using nonlinear regression of Prism software, and IC_{50} values were calculated. The results are shown in Table 2 and Fig. 3.

The experimental results show that the anti-PD-1 antibody CL1 can completely inhibit the binding of hPD-L1 to hPD-1 of Jurkat cells in a dose-dependent manner, with an IC_{50} value of about 0.77 nM.

Table 2 Affinity, kinetics, ligand binding blocking, and functional activity of the anti-hPD-1 antibody

	CL1	CL5	CL9	CL2	CL7	CL6	CL20
ELISA affinity assay EC ₅₀ (nM)	0.208	0.399	0.594	0.451	0.346	1.336	0.242
ForteBio kinetics analysis k _{binding} (1/Ms) k _{dissociation} (1/s) KD (nM)	1.98×10 ⁶ 4.11×10 ⁻⁵ 0.0207	NA	NA	NA	NA	NA	NA
Assay for the blocking of ligand binding IC ₅₀ (nM)	0.77	NA	NA	NA	NA	NA	NA
Assay for the functional activity EC ₅₀ (μg/mL)	0.269	0.932	NA	NA	NA	NA	NA

NA: No relevant experiments have been conducted and data are available.

Example 6: Kinetics analysis for antibodies related to antibody CL1

- A series of antibodies related to antibody CL1 were analyzed by bio-layer interferometry as described in Example 4. The amino acid sequences of VL and VH regions of the related antibodies are shown in the sequence listing. The equilibrium binding constant KD of each relevant antibody is shown in Table 3.

Table 3 The binding of CL1 antibody-related antibodies to hPD-1

VH	VL	Form	KD (nM)
CL1 VH (SEQ ID NO: 7)	CL1 VL (SEQ ID NO: 8)	IgG4	0.0207
CL1 VH (SEQ ID NO: 7)	CL1 VL (SEQ ID NO: 8)	scFv	0.082
CL1 VH (SEQ ID NO: 7)	CL5 VL (SEQ ID NO: 10)	IgG4	0.076
CL1 VH (SEQ ID NO: 7)	CL5 VL (SEQ ID NO: 10)	scFv	0.143
CL1 VH (SEQ ID NO: 7)	CL9 VL (SEQ ID NO: 12)	IgG4	0.229
CL1 VH (SEQ ID NO: 7)	CL9 VL (SEQ ID NO: 12)	scFv	0.518
CL1 VH (SEQ ID NO: 7)	CL2 VL (SEQ ID NO: 38)	IgG4	0.189
CL1 VH (SEQ ID NO: 7)	CL2 VL (SEQ ID NO: 38)	scFv	0.447
CL1 VH (SEQ ID NO: 7)	CL7 VL (SEQ ID NO: 40)	IgG4	0.061
CL1 VH (SEQ ID NO: 7)	CL7 VL (SEQ ID NO: 40)	scFv	0.149

The results in Table 3 demonstrate that, with the same VH and VL, the affinity of an antibody in the form of scFv to hPD-1 is lower than that of an antibody in the form of IgG4. With the same form, namely, all in the form of IgG4 or scFv, antibodies, obtained by maintaining the VH amino acid sequence of antibody CL1 unchanged and replacing the VL of antibody CL1 with the VL of antibody CL5, the VL of antibody CL9, the VL of antibody CL2, or the VL of antibody CL7, had a decreased affinity, and the one obtained by replacing the VL of antibody CL1 with the VL of antibody CL9 had the lowest affinity. It is shown that the VL of antibody CL1 plays an important role in the binding of CL1 to hPD-1, and the change of an amino acid sequence may lead to a change in conformation and thus the binding to hPD-1 is affected.

Example 7: Assay for the functional activity of the anti-hPD-1 antibody

The ability of an anti-hPD-1 antibody to inhibit the hPD-1 activity was detected in vitro to determine the functional activity of the anti-hPD-1 antibody.

The anti-hPD-1 antibody blocked the interaction between hPD-1 and hPD-L1 and activated the TCR downstream immune signaling pathways in Jurkat cells.

A reporter gene is a gene whose expression product is easily identified. The coding sequence of the reporter gene was fused with the regulatory sequence of the target gene to form a chimeric gene, and expressed under the control of the regulatory sequence, and then upstream signaling was indirectly detected by detecting the expression product of the reporter gene. The PD-1/PD-L1 Blockade Bioassay system is suitable for the biological activity assay of the anti-PD-1 monoclonal antibody. The primary principle thereof is as follows: a CHO-K1 cell line stably expressing PD-L1 and T-cell receptor activator (TCR activator) on the cell membrane is constructed, mimicking antigen presenting cells (APCs); and Jurkat cell line, a human T lymphocyte line expressing PD-1 on the cell membrane, is transfected with a Luciferase reporter vector having NFAT as a promoter. When the two cell lines are co-incubated, the TCR activator of a CHO-K1 cell binds to TCR on the surface of a Jurkat cell to induce the downstream signaling pathway and eventually activate the expression of a reporter gene, but at the same time the PD-1/PD-L1 interaction will block this activation. After the anti-PD-1 monoclonal antibody is added, the PD-1/PD-L1 interaction is blocked, and the luciferase

reporter gene restarts to express. A fluorescence signal is generated, and the signal value is positively correlated to the amount of the anti-PD-1 monoclonal antibody added.

The anti-hPD-1 antibody was assayed and evaluated by co-incubating the antibody with the cells as described below. CHO-K1 cells were resuspended in F-12 + 10% FBS medium (containing 200 $\mu\text{g/ml}$ Hygromycin B and 250 $\mu\text{g/ml}$ G418), with a cell density being adjusted to 4.0×10^5 cells/ml, and were inoculated to a 96-well plate at 100 $\mu\text{l/well}$. The 96-well plate was placed in an incubator of 37 °C and 5% CO₂, and incubated overnight. 125 μl of the antibody solution (CL1, CL5, and hIgG) in the 96-well microplate was serially 2.5-fold diluted in triplicate along the plate with an assay diluent (RPMI medium containing 1% fetal bovine serum (FBS)). Jurkat cells were resuspended with the assay diluent, and the cell density was adjusted to 1.25×10^6 cells/ml. The 96-well plate was taken out, and the medium was removed. 40 μl of the prepared reference substance (hIgG) and the test samples (CL1 and CL5) were added to the 96-well plate, and then 40 μl of Jurkat cell suspension was added to the 96-well plate. The 96-well plate was placed in an incubator of 37 °C and 5% CO₂ for 6 hours, and then placed at room temperature for 10 minutes. Then 80 μl of Bio-Glo Luciferase Assay System reagent was added to each well, and the plate was placed in the dark at room temperature for 30 minutes. The fluorescence value for each well of the 96-well plate was read by the autofluorescence detection function of a multifunctional microplate reader. Curve fitting was performed on the data of reference and test samples with a 4-parameter model by using SoftMax Pro 6.5.1 software, and the EC₅₀ was determined by a standard method. The results are shown in Table 2 and Fig. 4.

The results in Table 2 and Fig. 4 show that in the presence of the anti-hPD-1 antibody, the PD-1/PD-L1 interaction is blocked, the inhibited TCR downstream signaling pathways of Jurkat cells are reactivated, the Luciferase reporter gene expression resumes, and a fluorescence signal is generated. The anti-hPD-1 antibody blocks the PD-1/PD-L1 interaction in a dose-dependent manner. Further, EC₅₀, representing the ability to inhibit the hPD-1 activity, is about 0.269 $\mu\text{g/ml}$ and 0.932 $\mu\text{g/ml}$ respectively for antibodies CL1 and CL5, indicating that CL1 has a stronger ability to inhibit the hPD-1 activity. It has been proved once again at a cellular level that the antibody CL1 is superior to CL5 in terms of the functional activity, which is also consistent with the results of Example 3.

Example 8: Comparison of kinetics analyses for the binding of antibodies CL1 and CL20 to hPD-1

Kinetics of the binding of antibodies CL1 and CL20 to hPD-1 was analyzed and compared on the Biacore system (GE, Boston, MA) using the surface plasmon resonance technology.

A protein A chip was used for detection. An antibody CL1 or CL20 solution with a concentration of 5 $\mu\text{g/ml}$ passed through the experimental flow path at a flow rate of 10 $\mu\text{l/min}$, and the capture amount was about 200 RU for 10 s. Then the flow rate was adjusted to 30 $\mu\text{l/min}$, and PD-1 solutions of different concentrations (0.625 nM, 1.25 nM, 2.5 nM, 5 nM, and 10 nM) were fed in sequence, with a binding time of 180 s and a dissociation time of 1,200 s. The binding and dissociation curves were obtained by fitting with a 1:1 Langmuir binding model, and the kinetic parameters of the binding of antibodies CL1 and CL20 to hPD-1 were obtained (k_{binding} : binding rate; $k_{\text{dissociation}}$: dissociation rate; K_D : equilibrium dissociation constant). The results of kinetics assays are shown in Table 4. K_D of the binding of antibody CL1 to hPD-1 is about 0.606 nM, K_D of the binding of antibody CL20 to hPD-1 is about 4.434 nM, and the affinity of antibody CL1 to hPD-1 is about 8 times that of antibody CL20 to hPD-1.

Table 4 Kinetics data of the binding of antibodies CL1 and CL20 to hPD-1

	CL1	CL20
k binding (1/Ms)	8.252×10^5	1.025×10^6
k dissociation (1/s)	4.999×10^{-4}	4.544×10^{-3}
K_D (nM)	0.606	4.434

CLAIMS

1. An antibody or antibody fragment comprising one, two, three, four, five or six CDRs selected from amino acid sequences of SEQ ID NOs: 13, 14, 15, 16, 17, and 18.
2. The antibody or antibody fragment according to claim 1, characterized in that the antibody or antibody fragment comprises one, two, or three heavy-chain CDRs selected from amino acid sequences of SEQ ID NOs: 16, 17, and 18; and/or the antibody or antibody fragment comprises one, two, or three light-chain CDRs selected from amino acid sequences of SEQ ID NOs: 13, 14, and 15.
3. The antibody or antibody fragment according to claim 1 or 2, characterized in that the antibody or antibody fragment comprises light-chain CDRs having amino acid sequences of SEQ ID NOs: 13, 14, and 15, and heavy-chain CDRs having amino acid sequences of SEQ ID NOs: 16, 17, and 18.
4. The antibody or antibody fragment according to any one of claims 1-3, characterized in that the antibody or antibody fragment is an isolated antibody or antibody fragment.
5. The antibody or antibody fragment according to any one of claims 1-4 characterized in that the antibody or antibody fragment comprises a heavy-chain variable region and a light-chain variable region; wherein the heavy-chain variable region comprises an amino acid sequence of SEQ ID NO: 7, or an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 7, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 7; and/or the light-chain variable region comprises an amino acid sequence of SEQ ID NO: 8, or an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 8, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 8.

6. The antibody or antibody fragment according to any one of claims 1-5, characterized in that the antibody or antibody fragment further comprises a human $\gamma 4$ heavy-chain constant region or a variant thereof, and/or a human κ light-chain constant region or a variant thereof.

5 7. An antibody or antibody fragment comprising a heavy chain and a light chain, characterized in that the heavy chain comprises an amino acid sequence of SEQ ID NO: 71, an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 71, or an amino acid sequence an amino acid sequence having one or more conservative amino acid substitutions compared to
10 the amino acid sequence of SEQ ID NO: 71; the light chain comprises an amino acid sequence of SEQ ID NO: 72, an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 72, or an amino acid sequence having one or more conservative amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 72.

15 8. A variant of the antibody or antibody fragment according to any one of claims 1-7, comprising one, two, or three conservative amino acid substitutions.

9. A biological material related to the antibody or antibody fragment according to any one of claims 1-7 or the variant according to claim 8, characterized in that the biological material is:

20 B1) a nucleic acid molecule encoding the antibody or antibody fragment according to any one of claims 1-7 or the variant according to claim 8; or

B2) an expression cassette, a recombinant vector, a recombinant cell, or a recombinant microorganism comprising the nucleic acid molecule of B1).

10. The biological material according to claim 9, characterized in that the nucleic acid molecule of B1) comprises:
25

1) a DNA molecule selected from:

a nucleic acid fragment set forth in positions 91-108 of SEQ ID NO: 1;

a nucleic acid fragment set forth in positions 148-192 of SEQ ID NO: 1;

a nucleic acid fragment set forth in positions 292-327 of SEQ ID NO: 1;

a nucleic acid fragment set forth in positions 73-114 of SEQ ID NO: 2;
a nucleic acid fragment set forth in positions 160-177 of SEQ ID NO: 2;
a nucleic acid fragment set forth in positions 271-303 of SEQ ID NO: 2;
a nucleic acid fragment of SEQ ID NO: 1;
5 a nucleic acid fragment of SEQ ID NO: 2;
a nucleic acid fragment of SEQ ID NO: 73; or
a nucleic acid fragment of SEQ ID NO: 74;

2) a nucleic acid fragment hybridizing with the nucleic acid fragment defined in 1) and encoding the antibody or antibody fragment according to any one of claims
10 1-7 or the variant according to claim 8;

3) a nucleic acid fragment having at least 90% sequence identity to the DNA molecule defined in 1) or 2) and encoding the antibody or antibody fragment according to any one of claims 1-7 or the variant according to claim 8.

11. A method for preparing the antibody or antibody fragment according to any one
15 of claims 1-7 or the variant according to claim 8, the method comprising:

under a condition for expressing the nucleic acid molecule according to B1) of claim 9, cultivating a recombinant cell comprising the nucleic acid molecule to prepare the antibody or antibody fragment or the variant.

12. A pharmaceutical composition comprising the antibody or antibody fragment
20 according to any one of claims 1-7 or the variant according to claim 8, and a pharmaceutically acceptable excipient, diluent, or carrier, or a combination thereof.

13. Use of the antibody or antibody fragment according to any one of claims 1-7 or the variant according to claim 8 in the preparation of any one of the following products:

25 (1) a product for detecting PD-1;
(2) a product for stimulating or enhancing immune response;
(3) a product for treating cancer.

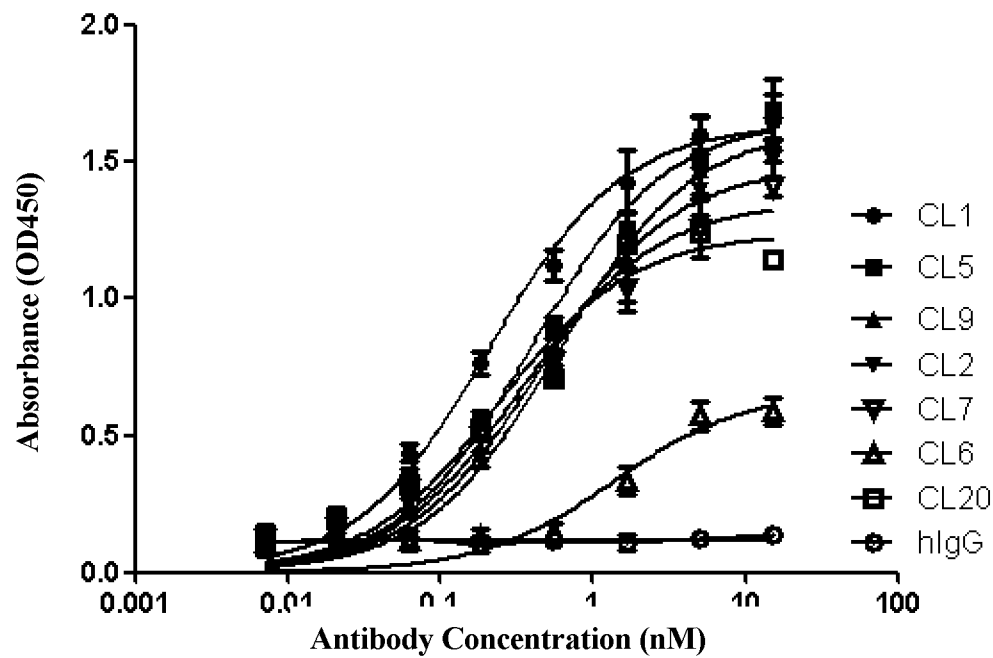


Fig. 1

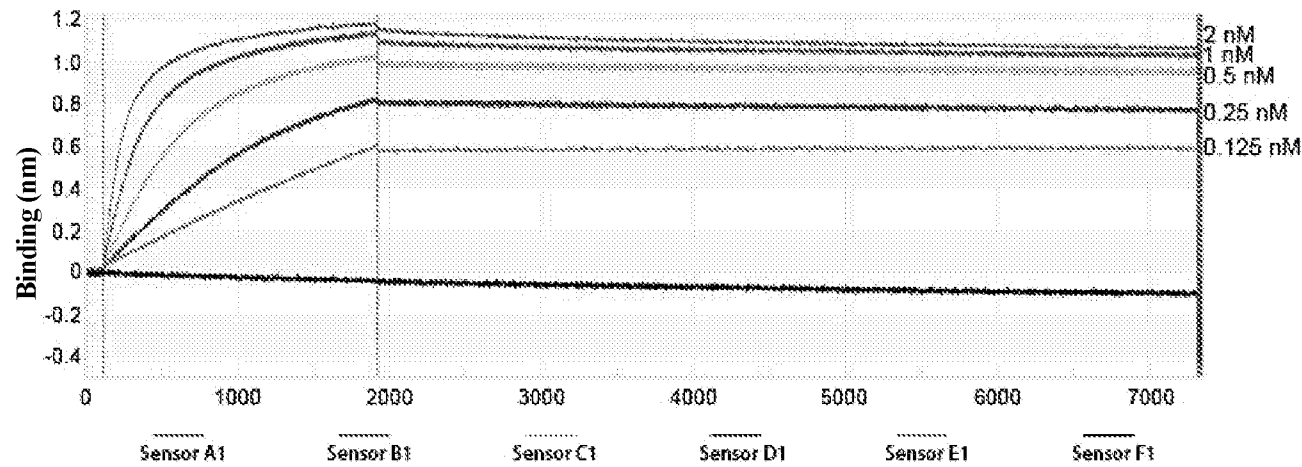


Fig. 2

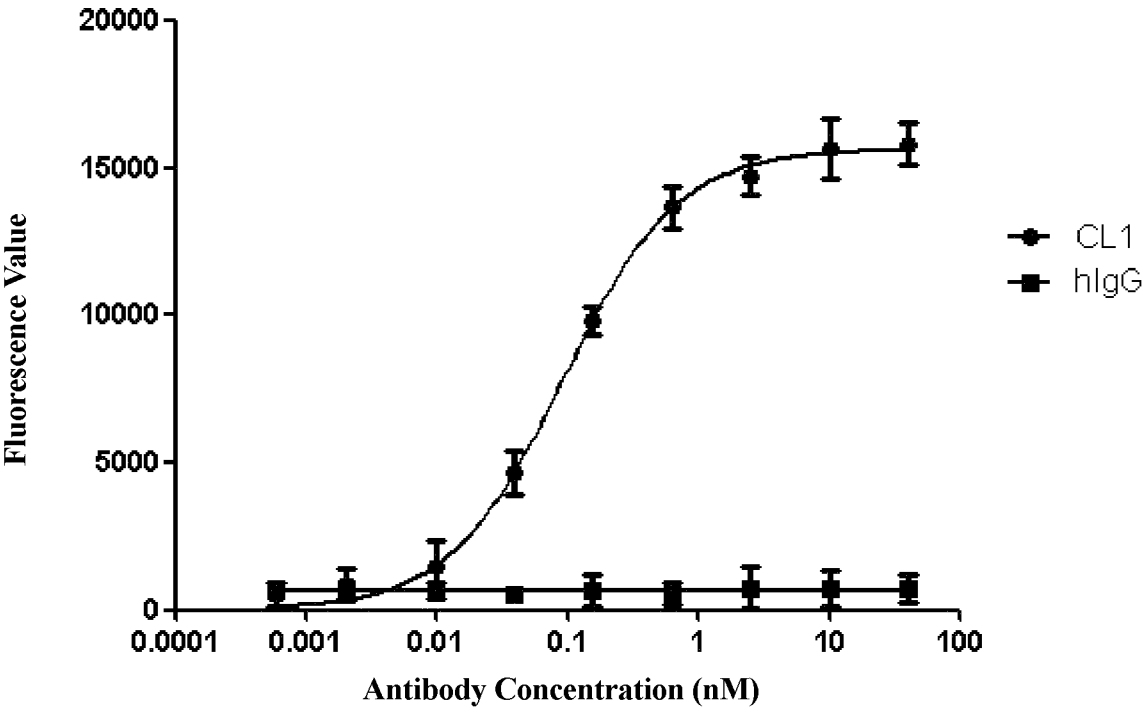


Fig. 3

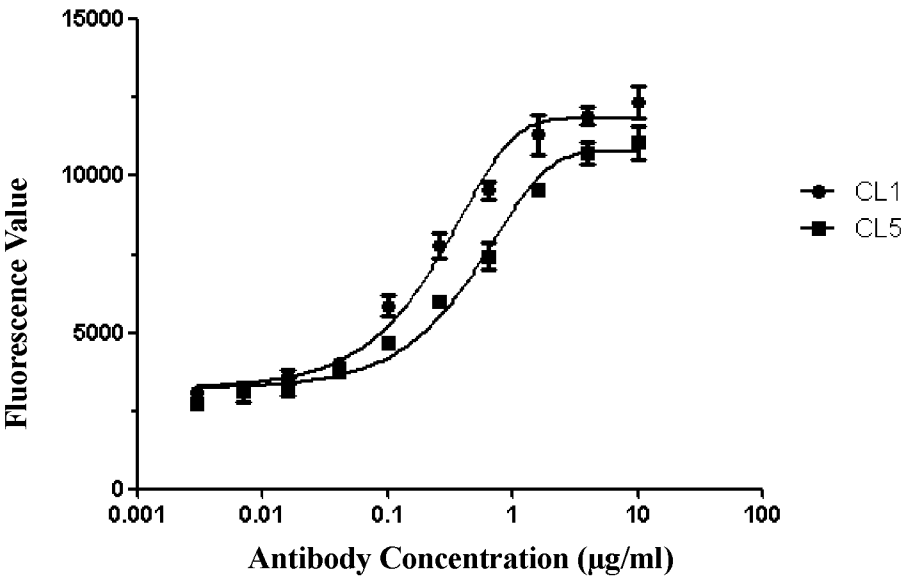


Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2020/083954**A. CLASSIFICATION OF SUBJECT MATTER**

C07K 16/28(2006.01)i; C12N 15/13(2006.01)i; C12N 15/63(2006.01)i; A61K 39/395(2006.01)i; A61P 37/04(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)

CNKI, CNABS, CNTXT, DWPI, SIPOABS, EPTXT, WOTXT, USTXT, JPTXT, ELSEVIER, EMBASE, ISI web of science and searched terms: antibody, PD-1, etc.; STN, GENBANK, EMBL, Retrieving System for Biological Sequence of Chinese Patent and searched sequences: SEQ ID NOs: 1-18, and 71-74.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008156712 A1 (ORGANON NV et al.) 24 December 2008 (2008-12-24) claims 1-23 and embodiments 1-4	1-13



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

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“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

28 May 2020

Date of mailing of the international search report

24 June 2020

Name and mailing address of the ISA/CN

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2020/083954

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