PD-1 BINDING PROTEIN AND PHARMACEUTICAL USE

TECHNICAL FIELD

The present disclosure relates to a PD-1-binding protein and particularly to an anti-PD-1 antibody, a method for treating an inflammatory or autoimmune disease by using same, and pharmaceutical use thereof.

BACKGROUND

Programmed death-1 (PD-1), also known as programmed cell death-1, is a 268-amino acid type I transmembrane protein belonging to the CD28/CTLA-4 family of T cell regulators. It is a cell surface receptor protein mainly expressed on T cells and other immune cells. PD-1 has two ligands, PD-L1 and PD-L2, which interact with the cell surface receptor. Upon binding to its ligand PD-L1 or PD-L2, PD-1 down-regulates T cell function, including reducing T cell activation, differentiation, proliferation, cytokine secretion, etc.

The PD-1 pathway is a naturally occurring negative feedback regulation mechanism of the immune system. The mechanism of activating the PD-1 pathway to treat immune disorders is novel, and also has broad potential for treating diseases. The present disclosure provides a high-structure anti-PD-1 antibody that better activates the PD-1 signaling pathway and inhibits T cell activity, thereby improving the therapeutic effect on T cell-mediated inflammatory or autoimmune diseases.

SUMMARY

The present disclosure provides a PD-1-binding protein and a nucleic acid encoding same, a vector thereof, a host cell thereof, a pharmaceutical composition thereof, a method for treating an inflammatory or autoimmune disease by using same, and related pharmaceutical use thereof.

PD-1-Binding Protein

In one aspect, the present disclosure provides a PD-1-binding protein comprising at least one immunoglobulin single variable domain, wherein the immunoglobulin single variable domain comprises a CDR1, a CDR2, and a CDR3 from the amino acid sequence set forth in any one of SEQ ID NOs: 2 and 24-29, and the CDR1, the CDR2, and the CDR3 are defined according to the Kabat, IMGT, Chothia, AbM, or Contact numbering scheme. The immunoglobulin single variable domain specifically binds to a PD-1 protein or an epitope thereof.

In some embodiments, provided is the PD-1-binding protein, wherein the immunoglobulin single variable domain comprises a CDR1, a CDR2, and a CDR3, and the CDR1, the CDR2, and the CDR3 comprise the amino acid sequences set forth in SEQ ID NOs: 3-5, respectively. The CDRs are defined according to the Kabat numbering scheme.

In some embodiments, in the PD-1-binding protein, the immunoglobulin single variable domain comprises a combination of any 1, 2, or 3 of the aforementioned CDR1, CDR2, and CDR3.

In some embodiments, the immunoglobulin single variable domain is camelid-derived; in some specific embodiments, the immunoglobulin single variable domain comprises the amino acid sequence set forth in SEQ ID NO: 2 or an amino acid sequence having at least 90% sequence identity thereto.

In some embodiments, the immunoglobulin single variable domain is engineered by humanization and/or engineered by affinity maturation.

In some embodiments, in the aforementioned PD-1-binding protein, the immunoglobulin single variable domain comprises the amino acid sequence set forth in any one of SEQ ID NOs: 24-29 or an amino acid sequence having at least 90% sequence identity thereto. In some specific embodiments, the immunoglobulin single variable domain comprises the amino acid sequence set forth in SEQ ID NO: 27 or an amino acid sequence having at least 90% sequence identity thereto.

In some embodiments, in the aforementioned PD-1-binding protein, the immunoglobulin single variable domain comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid mutations compared to any one of SEQ ID NOs: 24-29.

In some specific embodiments, the amino acid mutations described above are amino acid replacements, substitutions, modifications, deletions, and/or additions (e.g., conservative amino acid replacements), and the mutations do not affect or substantially do not affect the function of specifically binding to PD-1.

In some embodiments, the aforementioned PD-1-binding protein comprises or is an antibody that specifically binds to a PD-1 protein or an epitope thereof. In some specific embodiments, the antibody is a camelid antibody, a chimeric antibody, a humanized antibody, a fully human antibody, or an antigen-binding fragment thereof.

In some embodiments, the aforementioned PD-1-binding protein is a linear antibody, a single-chain antibody, a nanobody, a peptibody, a domain antibody, a multispecific antibody (a bispecific antibody, a diabody, a triabody, a tetrabody, a tandem di-scFv, or a tandem tri-scFv), or an antigen-binding fragment or conjugate of any one of the foregoing.

In some embodiments, the immunoglobulin single variable domain in the aforementioned PD-1-binding protein is a single-domain antibody or VHH.

In some embodiments, the present disclosure provides a PD-1-binding protein comprising one or more (e.g., 2, 3, 4, 5, 6, 7, or 8) of the aforementioned immunoglobulin single variable domains, wherein any two of the immunoglobulin single variable domains may be identical or different and may form a dimeric or multimeric molecule.

In some embodiments, the aforementioned PD-1-binding protein further comprises an immunoglobulin Fc region. In some embodiments, the PD-1-binding protein further comprises a human immunoglobulin Fc region. For example, the Fc region is an Fc region of human IgG1, IgG2, or IgG4. The PD-1-binding protein in the present disclosure may

have an Fc region of a wild-type IgG or a variant thereof.

In some embodiments, the Fc comprises a C220 mutation, e.g., C220A.

In some embodiments, the Fc region is an Fc region that increases an effector function, for example, an Fc region that increases antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and/or complement-dependent cytotoxicity (CDC).

Exemplary IgG1 Fc regions comprise the following substitutions: 239D; 239E; 239K, 241A; 262A; 264D; 264L; 264A; 264S; 265A; 265S; 265V; 296A; 296A; 301A; 332E; 239D/332E; 239D/330S/332E; 239D/330L/332E; 298A/333A/334A; 247I/339D; 247I/339Q; 280H/290S; 280H/290S/298D; 280H/290S/298V; 243L/292P/300L; 243L/292P/300L/396L; 243L/292P/300L/305I/396L; 236A/239D/332E; 326A/333A; 326W/333S; 290E/298G/299A; 290N/298G/299A; 290E/298G/299A/326E; or 290N/298G/299A/326E; or any combination of any of the positions described above. The mutations are defined according to the EU numbering system.

Exemplary IgG1 Fc regions comprise the following substitutions: S239D; S239E; S239K, F241A; V262A; V264D; V264L; V264A; V264S; D265A; D265S; D265V; F296A; Y296A; R301A; I332E; S239D/I332E; S239D/A330S/I332E; S239D/A330L/I332E; S298A/D333A/K334A; P247I/A339D; P247I/A339Q; D280H/K290S; D280H/K290S/S298D; D280H/K290S/S298V; F243L/R292P/Y300L; F243L/R292P/Y300L/P396L; F243L/R292P/Y300L/V305I/P396L; G236A/S239D/I332E; K326A/E333A; K326W/E333S; K290E/S298G/T299A; K290N/S298G/T299A; K290E/S298G/T299A/K326E; or K290N/S298G/T299A/K326E, or any combination of any of the positions described above.

In some embodiments, the Fc region is an Fc region that weakens an effector function, for example, an Fc region that weakens ADCC, ADCP, and/or CDC. Exemplary Fc regions with a reduced effector function include those that comprise the following substitutions: N297A or N297Q (IgG1); L234A/L235A (IgG1); V234A/G237A (IgG2); L235A/G237A/E318A (IgG4); H268Q/V309L/A330S/A331S (IgG2); C220S/C226S/C229S/P238S (IgG1); C226S/C229S/E233P/L234V/L235A (IgG1); L234F/L235E/P331S (IgG1); or S267E/L328F (IgG1).

In some embodiments, the Fc region is a human IgG1-Fc region with mutation C220A.

In some embodiments, the Fc region comprises the amino acid sequence set forth in SEQ ID NO: 6 or 45. In some specific embodiments, the PD-1-binding protein comprises an Fc region set forth in SEQ ID NO: 6.

In some embodiments, provided is a PD-1-binding protein comprising the amino acid sequence set forth in any one of SEQ ID NOs: 7, 30, and 48 or an amino acid sequence having at least 90% sequence identity thereto. In some specific embodiments, the PD-1-binding protein comprises the amino acid sequence set forth in SEQ ID NO: 30.

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof that binds to or competes for binding to the same epitope with the immunoglobulin

single variable domain in the aforementioned PD-1-binding protein of the present disclosure.

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof that does not bind to or compete for binding to the same epitope with the immunoglobulin single variable domain in the aforementioned PD-1-binding protein of the present disclosure.

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof that blocks the binding of the immunoglobulin single variable domain in the aforementioned PD-1-binding protein of the present disclosure to PD-1 (e.g., human PD-1).

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof whose binding to PD-1 (e.g., human PD-1) is blocked by the immunoglobulin single variable domain in the aforementioned PD-1-binding protein of the present disclosure.

In some embodiments, the aforementioned PD-1-binding protein has an activity selected from the group consisting of at least one of the following:

(a) binding to human PD-1 or an epitope thereof with a K_D value of $\leq 10^{-7}$;

(b) not blocking or hardly blocking the signaling pathway of PD-L1 and PD-1;

(c) activating the PD-1 signaling pathway;

(d) inhibiting cytokine-releasing activity of T cells, e.g., inhibiting IFNγ-secreting activity of T cells;

(e) inhibiting T cell proliferation, e.g., inhibiting memory CD8+ T cell proliferation;

(f) reducing PD-1-expressing T cells; and

(g) maintaining a low level of PD-1-expressing T cells.

In some embodiments, the aforementioned PD-1-binding protein of the present disclosure may bind to PD-1 with a K_D value of $\leq 1 \times 10^{-7}$ M, e.g., $\leq 1 \times 10^{-8}$ M, or $\leq 1 \times 10^{-9}$ M, or $\leq 1 \times 10^{-10}$ M.

In another aspect, the present disclosure provides a PD-1-binding protein comprising a heavy chain variable region (VH) and a light chain variable region (VL), wherein:

the VH comprises a HCDR1, a HCDR2, and a HCDR3 from the amino acid sequence set forth in any one of SEQ ID NOs: 8 and 31-36; and the VL comprises a LCDR1, a LCDR2, and a LCDR3 from the amino acid sequences set forth in SEQ ID NOs: 9 and 37-42, and the CDR1, the CDR2, and the CDR3 are defined according to the Kabat, IMGT, Chothia, AbM, or Contact numbering scheme. The VH and the VL specifically bind to a PD-1 antigen or an epitope thereof.

In some embodiments, provided is a PD-1-binding protein comprising a heavy chain variable region (VH) and a light chain variable region (VL);

the VH comprises a HCDR1, a HCDR2, and a HCDR3 that comprise the amino acid sequences set forth in SEQ ID NOs: 10-12, respectively; and

the VL comprises a LCDR1, a LCDR2, and a LCDR3 that comprise the amino acid sequences set forth in SEQ ID NOs: 13-15, respectively. The CDRs are CDRs defined

according to the Kabat numbering scheme.

In some embodiments, the VH of the PD-1-binding protein comprises any 1, 2, or 3 of the HCDR1, the HCDR2, and the HCDR3 set forth in SEQ ID NOs: 10-12, and/or the VL comprises any 1, 2, or 3 of the LCDR1, the LCDR2, and the LCDR3 set forth in SEQ ID NOs: 13-15.

In some embodiments, the PD-1-binding protein is an anti-PD-1 antibody.

In some embodiments, the heavy chain variable region (VH) and the light chain variable region (VL) of the anti-PD-1 antibody are murine-derived; in some specific embodiments, the VH comprises the amino acid sequence set forth in SEQ ID NO: 8 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 9 or an amino acid sequence having at least 90% sequence identity thereto.

In some embodiments, the anti-PD-1 antibody is engineered by humanization. In some specific embodiments, during humanization engineering, IGHV1-3 is used as a human germline heavy chain template, and/or IGkV1-16 is used as a human germline light chain template.

In some embodiments, the anti-PD-1 antibody is a murine-derived antibody, a chimeric antibody, or a humanized antibody or an antigen-binding fragment thereof. In some specific embodiments, the humanized antibody uses IGHV1-3 as a human germline heavy chain template and/or IGkV1-16 as a human germline light chain template.

In some embodiments, the VH of the aforementioned PD-1-binding protein comprises the amino acid sequence set forth in any one of SEQ ID NOs: 8 and 31-36 or an amino acid sequence having at least 90% sequence identity thereto, and/or the VL comprises the amino acid sequence set forth in any one of SEQ ID NOs: 9 and 37-42 or an amino acid sequence having at least 90% sequence identity thereto.

In some embodiments, the aforementioned PD-1-binding protein comprises a VH and a VL, wherein:

the VH comprises the amino acid sequence set forth in SEQ ID NO: 35 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 41 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid

sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 35 or an amino acid

sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 35 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 42 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 36 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto; or

the VH comprises the amino acid sequence set forth in SEQ ID NO: 36 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto.

In some specific embodiments, the aforementioned PD-1-binding protein comprises a VH and a VL, wherein:

the VH comprises the amino acid sequence set forth in SEQ ID NO: 35, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 41;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 31, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33, and the VL

comprises the amino acid sequence set forth in SEQ ID NO: 39;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 33, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 35, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 35, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 42;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 36, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39; or
the VH comprises the amino acid sequence set forth in SEQ ID NO: 36, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40.

In some embodiments, in the aforementioned PD-1-binding protein, the VH comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid mutations compared to any one of SEQ ID NOs: 31-36, and/or the VL comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid mutations compared to any one of SEQ ID NOs: 37-42.

In some specific embodiments, the amino acid mutations described above are amino acid replacements, substitutions, modifications, deletions, and/or additions (e.g., conservative amino acid replacements), and the mutations do not affect or substantially do not affect the function of specifically binding to PD-1.

In some embodiments, the aforementioned PD-1-binding protein comprises or is an antibody that specifically binds to a PD-1 protein or an epitope thereof. In some specific embodiments, the antibody is a murine-derived antibody, a chimeric antibody, a humanized antibody, a fully human antibody, or an antigen-binding fragment thereof.

In some embodiments, the aforementioned PD-1-binding protein comprises a Fab, Fab', $F(ab')_2$, or single-chain antibody (scFv) that specifically binds to PD-1.

In some embodiments, the aforementioned PD-1-binding protein further comprises an immunoglobulin Fc region. In some embodiments, the PD-1-binding protein further comprises a human immunoglobulin Fc region. For example, the Fc region is an Fc region of human IgG1, IgG2, or IgG4. The PD-1-binding protein in the present disclosure may have an Fc region of a wild-type IgG or a variant thereof.

In some embodiments, the Fc region is an Fc region that increases an effector function, for example, an Fc region that increases antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and/or complement-dependent cytotoxicity (CDC).

Exemplary IgG1 Fc regions comprise the following substitutions: 239D; 239E; 239K, 241A; 262A; 264D; 264L; 264A; 264S; 265A; 265S; 265V; 296A; 296A; 301A; 332E; 239D/332E; 239D/330S/332E; 239D/330L/332E; 298A/333A/334A; 247I/339D; 247I/339Q; 280H/290S; 280H/290S/298D; 280H/290S/298V; 243L/292P/300L; 243L/292P/300L/396L; 243L/292P/300L/305I/396L; 236A/239D/332E; 326A/333A; 326W/333S; 290E/298G/299A; 290N/298G/299A; 290E/298G/299A/326E; or

290N/298G/299A/326E; or any combination of any of the positions described above. The mutations are defined according to the EU numbering system.

Exemplary IgG1 Fc regions comprise the following substitutions: S239D; S239E; S239K, F241A; V262A; V264D; V264L; V264A; V264S; D265A; D265S; D265V; F296A; Y296A; R301A; I332E; S239D/I332E; S239D/A330S/I332E; S239D/A330L/I332E; S298A/D333A/K334A; P247I/A339D; P247I/A339Q; D280H/K290S; D280H/K290S/S298D; D280H/K290S/S298V; F243L/R292P/Y300L; F243L/R292P/Y300L/P396L; F243L/R292P/Y300L/V305I/P396L; G236A/S239D/I332E; K326A/E333A; K326W/E333S; K290E/S298G/T299A; K290N/S298G/T299A; K290E/S298G/T299A/K326E; or K290N/S298G/T299A/K326E, or any combination of any of the positions described above.

In some embodiments, the Fc region is an Fc region that weakens an effector function, for example, an Fc region that weakens ADCC, ADCP, and/or CDC. Exemplary Fc regions with a reduced effector function include those that comprise the following substitutions: N297A or N297Q (IgG1); L234A/L235A (IgG1); V234A/G237A (IgG2); L235A/G237A/E318A (IgG4); H268Q/V309L/A330S/A331S (IgG2); C220S/C226S/C229S/P238S (IgG1); C226S/C229S/E233P/L234V/L235A (IgG1); L234F/L235E/P331S (IgG1); or S267E/L328F (IgG1).

In some embodiments, the aforementioned PD-1-binding protein further comprises a human immunoglobulin Fc region, and the Fc region is set forth in SEQ ID NO: 6 or 45 or has at least 80% or at least 90% sequence identity to any one of SEQ ID NO: 6 or 45.

In some embodiments, the aforementioned PD-1-binding protein further comprises a human immunoglobulin Fc region, and the Fc region is set forth in SEQ ID NO: 45.

In some embodiments, the aforementioned PD-1-binding protein further comprises a heavy chain constant region and a light chain constant region.

In some embodiments, the aforementioned PD-1-binding protein further comprises a human IgG1 heavy chain constant region and a human κ light chain constant region, preferably.

In some embodiments, the human IgG1 heavy chain constant region comprises the amino acid sequence set forth in SEQ ID NO: 16, and the human κ light chain constant region comprises the amino acid sequence set forth in SEQ ID NO: 17.

In some embodiments, provided is a PD-1-binding protein comprising a heavy chain and a light chain, wherein the heavy chain comprises a constant region sequence that is set forth in SEQ ID NO: 16 or has at least 90% sequence identity thereto, and the light chain comprises a constant region sequence that is set forth in SEQ ID NO: 17 or has at least 90% sequence identity thereto.

In some embodiments, provided is a PD-1-binding protein comprising a heavy chain and a light chain, wherein the heavy chain comprises the amino acid sequence set forth in any one of SEQ ID NO: 18 or 43 or an amino acid sequence having at least 90% sequence identity thereto, and/or the light chain comprises the amino acid sequence set forth in any

one of SEQ ID NO: 19 or 44 or an amino acid sequence having at least 90% sequence identity thereto.

In some embodiments, provided is a PD-1-binding protein comprising a heavy chain and a light chain, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO: 43 or an amino acid sequence having at least 90% sequence identity thereto, and the light chain comprises the amino acid sequence set forth in SEQ ID NO: 44 or an amino acid sequence having at least 90% sequence identity thereto.

In some specific embodiments, the heavy chain comprises the amino acid sequence set forth in SEQ ID NO: 43, and/or the light chain comprises the amino acid sequence set forth in SEQ ID NO: 44.

In some embodiments, provided is a PD-1-binding protein comprising a heavy chain and a light chain, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO: 49 or an amino acid sequence having at least 90% sequence identity thereto, and the light chain comprises the amino acid sequence set forth in SEQ ID NO: 50 or an amino acid sequence having at least 90% sequence identity thereto.

In some embodiments, the Fc region contained in the aforementioned PD-1-binding protein may cause the binding protein to form a dimeric molecule.

In some embodiments, the Fc region contained in the aforementioned PD-1-binding protein may extend the *in vivo* half-life of the binding protein.

In some embodiments, the PD-1-binding protein of the present disclosure is an anti-PD-1 antibody or a conjugate or fusion protein comprising the antibody.

In some embodiments, the aforementioned PD-1-binding protein has an activity selected from the group consisting of at least one of the following:

(a) binding to human PD-1 or an epitope thereof with a K_D value of $\leq 10^{-7}$;

(b) not blocking or hardly blocking the signaling pathway of PD-L1 and PD-1;

(c) activating the PD-1 signaling pathway;

(d) inhibiting cytokine-releasing activity of T cells, e.g., inhibiting IFNγ-secreting activity of T cells;

(e) inhibiting T cell proliferation, e.g., inhibiting memory CD8+ T cell proliferation;

(f) reducing PD-1-expressing T cells; and

(g) maintaining a low level of PD-1-expressing T cells.

In some embodiments, the aforementioned PD-1-binding protein of the present disclosure may bind to PD-1 with a K_D value of $\leq 1 \times 10^{-7}$ M, e.g., $\leq 1 \times 10^{-8}$ M, or $\leq 1 \times 10^{-9}$ M, or $\leq 1 \times 10^{-10}$ M.

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof that binds to or competes for binding to the same epitope with the aforementioned PD-1-binding protein of the present disclosure.

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof that does not bind to or compete for binding to the same epitope with the immunoglobulin single variable domain in the aforementioned PD-1-binding protein of

the present disclosure.

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof that blocks the binding of the immunoglobulin single variable domain in the aforementioned PD-1-binding protein of the present disclosure to PD-1 (e.g., human PD-1).

In some embodiments, provided is an anti-PD-1 antibody or an antigen-binding fragment thereof whose binding to PD-1 (e.g., human PD-1) is blocked by the immunoglobulin single variable domain in the aforementioned PD-1-binding protein of the present disclosure.

In the present disclosure, "at least 80% (sequence) identity" encompasses at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% (sequence) identity; "at least 90% (sequence) identity" encompasses at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% (sequence) identity.

Polynucleotide and Vector

The present disclosure provides a polynucleotide encoding the PD-1-binding protein of the present disclosure. The polynucleotide of the present disclosure may be an RNA, a DNA, or a cDNA. According to some embodiments of the present disclosure, the polynucleotide of the present disclosure is a substantially isolated polynucleotide.

The polynucleotide of the present disclosure may also be in the form of a vector, may be present in a vector, and/or may be part of a vector, and the vector is, for example, a plasmid, cosmid, YAC, or viral vector. The vector may especially be an expression vector, i.e., a vector that provides the *in vitro* and/or *in vivo* (i.e., in suitable host cells, host organisms, and/or expression systems) expression of the PD-1-binding protein. The expression vector generally comprises at least one of the polynucleotides of the present disclosure, which is operably linked to one or more suitable expression regulatory elements (e.g., promoters, enhancers, and terminators). The selection of the elements and sequences thereof for expression in a particular host is common knowledge of those skilled in the art. Regulatory elements and other elements useful or necessary for expression of the PD-1-binding protein of the present disclosure are, for example, promoters, enhancers, terminators, integration factors, selection labels, leader sequences, and reporter genes.

The polynucleotide of the present disclosure may be prepared or obtained by known means (e.g., by automatic DNA synthesis and/or recombinant DNA technology) based on information on the amino acid sequence of the polypeptide of the present disclosure, and/or may be isolated from a suitable natural source.

Host Cell

The present disclosure provides a recombinant host cell that expresses or is capable of expressing one or more of the PD-1-binding proteins of the present disclosure and/or

comprises the nucleic acid or vector of the present disclosure. In some embodiments, the host cell is a bacterial cell, a fungal cell, or a mammalian cell.

Bacterial cells include, e.g., cells of gram-negative bacterial strains (e.g., *Escherichia coli* strains, *Proteus* strains, and *Pseudomonas* strains) and gram-positive bacterial strains (e.g., *Bacillus* strains, *Streptomyces* strains, *Staphylococcus* strains, and *Lactococcus* strains).

Fungal cells include, e.g., cells of the species of *Trichoderma*, *Neurospora*, and *Aspergillus*; or include cells of the species of *Saccharomyces* (e.g., *Saccharomyces cerevisiae*), *Schizosaccharomyces* (e.g., *Schizosaccharomyces pombe*), *Pichia* (e.g., *Pichia pastoris* and *Pichia methanolica*), and *Hansenula*.

Mammalian cells include, for example, HEK293 cells, CHO cells, BHK cells, HeLa cells, and COS cells.

However, amphibian cells, insect cells, plant cells, and any other cells used in the art for expressing heterologous proteins may also be used in the present disclosure.

The cell of the present disclosure is unable to develop into a complete plant or animal individual.

Production or Preparation Method

The present disclosure provides a method for preparing the PD-1-binding protein of the present disclosure, and the method generally comprises the following steps:

- culturing the host cell of the present disclosure under conditions that allow expression of the binding protein of the present disclosure; and
- collecting the binding protein expressed by the host cell from the culture; and
- optionally, further purifying and/or modifying the binding protein of the present disclosure.

The PD-1-binding protein of the present disclosure may be produced intracellularly (e.g., in the cytoplasm, in the periplasm, or in inclusion bodies) in a cell as described above, and then the protein is isolated from the host cell and is optionally further purified; or the protein may be produced extracellularly (e.g., in the culture medium in which the host cell is cultured), and then the protein is isolated from the culture medium and is optionally further purified.

Methods and reagents for recombinant production of polypeptides, e.g., specific suitable expression vectors, transformation or transfection methods, selection labels, methods for inducing protein expression, and culture conditions, are known in the art. Similarly, isolation and purification techniques suitable for use in the manufacture of the binding protein or antibody of the present disclosure are well known to those skilled in the art. Methods for producing and purifying antibodies are well known in the prior art and can be found in, for example, “Antibodies: A Laboratory Manual”, Cold Spring Harbor Press (chapters 5-8 and 15). The engineered antibodies of the present disclosure may also be prepared and purified by conventional methods. For example, cDNA sequences encoding heavy and light chains can be cloned and recombined into an expression vector. The

expression vector can stably transfect cells. Mammalian expression systems may result in glycosylation of antibodies, particularly at the highly conserved N-terminus of the Fc region. Stable clones are obtained by expression of antibodies specifically binding to human antigens. Positive clones are expanded in a serum-free culture medium in a bioreactor to produce antibodies. The culture solution with the secreted antibodies can be purified and collected by conventional techniques. Antibodies can be filtered and concentrated by conventional methods. Soluble mixtures and multimers can also be removed by conventional methods, such as molecular sieves and ion exchange. The resulting products need to be immediately frozen, such as at -70 °C, or lyophilized. However, the PD-1-binding protein of the present disclosure may also be obtained by other methods for producing proteins known in the art, such as chemical synthesis, including solid-phase or liquid-phase synthesis.

Composition/Pharmaceutical Composition

The present disclosure provides a composition comprising the aforementioned PD-1-binding protein.

For example, provided is a pharmaceutical composition comprising a therapeutically, palliatively, or prophylactically effective amount of the PD-1-binding protein described above and at least one pharmaceutically acceptable excipient, diluent, or carrier.

In some specific embodiments, a unit dose of the pharmaceutical composition may comprise 0.01 to 99 wt% of the PD-1-binding protein, or a unit dose of the pharmaceutical composition comprises 0.1-2000 mg of the PD-1-binding protein; in some specific embodiments, a unit dose of the pharmaceutical composition comprises 1-1000 mg of the PD-1-binding protein.

In some embodiments, provided is a product or kit comprising at least one container, wherein each container independently comprises the aforementioned PD-1-binding protein. Optionally, the kit comprises a container and a label. The container is, for example, a bottle, a syringe, and a test tube. The container accommodates a composition effective in treating a disorder. The label on or associated with the container indicates that the composition is used for treating the disorder of choice. The composition comprises the aforementioned PD-1-binding protein.

Method for Treating Disease and Pharmaceutical Use

The present disclosure provides a method for treating, alleviating, preventing, or diagnosing a disease or disorder by using the aforementioned PD-1-binding protein, polynucleotide, or composition (including the pharmaceutical composition).

In some embodiments, provided is a method for ameliorating, alleviating, treating, or preventing a disease, comprising administering to a subject an amelioratively, alleviatively, therapeutically, or prophylactically effective amount of the aforementioned PD-1-binding protein, polynucleotide, or composition (including the pharmaceutical composition).

In some embodiments, provided is use of the PD-1-binding protein of the present

disclosure in the manufacture of a medicament for ameliorating, alleviating, treating, or preventing a disease.

In some embodiments, the aforementioned disease is inflammation or an immune disease. In some embodiments, the inflammation or the immune disease is selected from the group consisting of the following or a combination thereof: graft versus host disease (GvHD), transplant rejection, rheumatoid arthritis (RA), and arthritis (CIA).

In some embodiments, provided is a method for inhibiting T cell activity, comprising administering to a subject in need thereof an effective amount of the aforementioned PD-1-binding protein, polynucleotide, or composition (including the pharmaceutical composition).

In some specific embodiments, the inhibiting T cell activity is a characteristic selected from the group consisting of at least one of the following:

(a) inhibiting cytokine-releasing activity of T cells, e.g., inhibiting IFNγ-secreting activity of T cells;

(b) inhibiting T cell proliferation, e.g., inhibiting memory CD8+ T cell proliferation; and

(c) reducing PD-1-positive T cells, e.g., reducing T cells highly expressing PD-1.

Detection and Kit

The present disclosure provides use of the PD-1-binding protein, the polynucleotide, and the composition for detection. The present disclosure further provides a method, system, or device for detecting PD-1 *in vivo* or *in vitro*, comprising treating a sample with the aforementioned binding protein, polynucleotide, or composition of the present disclosure.

In some embodiments, the method, system, or device for *in vitro* detection may comprise, for example,

(1) contacting a sample with the PD-1-binding protein, the polynucleotide, or the composition;

(2) detecting a complex formed between the aforementioned binding protein or polynucleotide and the sample; and/or

(3) contacting a reference sample (e.g., a control sample) with the binding protein or nucleic acid; and

(4) determining the extent of formation of the complex by comparison with the reference sample. A change (e.g., a statistically significant change) in the formation of the complex in the sample or subject as compared to a control sample or subject indicates the presence of PD-1 in the sample.

In some other embodiments, the method, system, or device for *in vivo* detection may comprise:

(1) administering to a subject the aforementioned binding protein or polynucleotide; and

(2) detecting the formation of a complex between the aforementioned binding protein or polynucleotide and the subject.

The detection may comprise determining the location or time at which the complex is formed. The aforementioned binding protein or polynucleotide may be labeled with a

detectable substance, and detection of a substance capable of binding to the protein or polynucleotide (e.g., PD-1) is achieved by detection of the label. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent substances, luminescent substances, and radioactive substances. The formation of the complex from the binding protein or polynucleotide and PD-1 can be detected by measuring or visualizing substances that bind or do not bind to PD-1. Conventional detection methods may be used, such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or tissue immunohistochemistry. For detection purposes, the binding protein or polynucleotide of the present disclosure may be labeled with a fluorophore chromophore. In some embodiments, further provided are a diagnostic reagent comprising the polynucleotide or binding protein described above, and related diagnostic use.

In some embodiments, further provided is a kit comprising the aforementioned binding protein or polynucleotide, wherein the kit may further comprise instructions for diagnostic use. The kit may further comprise at least one additional reagent, such as a label or an additional diagnostic agent. For *in vivo* use, the binding protein may be formulated as a pharmaceutical composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A and 1B show assays for assessing the antagonism of antibodies 2E1-A06 and 113.3 against PD-L1, respectively, with camrelizumab as a positive control and peresolimab and IgG1 as negative controls, wherein FIG. 1A shows the results for antibody 2E1-A06, and FIG. 1B shows the results for antibody 113.3.

FIGs. 2A and 2B show Jurkat reporter gene assays for assessing the activation of the PD-1 signaling pathway by antibodies 2E1-A06 and 113.3, respectively, with IgG1 as a negative control, wherein FIG. 2A shows the results for antibody 2E1-A06, and FIG. 2B shows the results for antibody 113.3.

FIG. 3 shows an assay for assessing the antagonistic activity of antibodies 2E1-A06-H4 and 113.3 Hbk2L1+4 against PD-L1, with camrelizumab as a positive control and peresolimab and IgG1 as negative controls.

FIG. 4 shows the inhibitory activity of antibodies 2E1-A06-H4 and 113.3 Hbk2L1+4 in a PBMC activation assay system, with peresolimab as a positive control.

FIG. 5 shows the inhibitory activity of antibodies 2E1-A06-H4 and 113.3 Hbk2L1+4 in a CMV recall assay system, with peresolimab as a positive control and IgG1 as a negative control.

FIG. 6 shows an assay for assessing the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of antibody 2E1-A06-H4, with peresolimab as a positive control and IgG1 as a negative control.

FIG. 7 shows the process of an experiment for evaluating the activity of anti-PD-1 antibodies in a graft versus host disease (GvHD) mouse model.

FIGs. 8A and 8B show the results of administration of antibody 2E1-A06-H4 to a graft

versus host disease (GvHD) mouse model, with peresolimab and a model group (Isotype) as controls, wherein FIG. 8A shows the average body weight change percentages of mice, and FIG. 8B shows the average disease rejection scores of mice.

FIG. 9 shows the process of an experiment for evaluating the activity of anti-PD-1 antibodies in a collagen-induced arthritis (CIA) mouse model.

DETAILED DESCRIPTION

Definitions of Terms

In order to facilitate the understanding of the present disclosure, certain technical and scientific terms are specifically defined below. Unless otherwise specifically defined in the present disclosure, all other technical and scientific terms used in the present disclosure have the meanings generally understood by those of ordinary skill in the art to which the present disclosure pertains.

"Programmed death-1", "programmed cell death-1", "protein PD-1", "PD-1", "PDCD1", and "hPD-1" are used interchangeably and include variants, isotypes, and species homologs of human PD-1, as well as analogs sharing at least one common epitope with PD-1. The complete PD-1 sequence is GenBank Accession # NP_005009.2.

"Bind to PD-1" refers to being able to interact with PD-1 or an epitope thereof, wherein the PD-1 or the epitope thereof may be human-derived. "Antigen-binding site" refers to a discontinuous three-dimensional spatial site on an antigen that is recognized by the antibody or the antigen-binding fragment of the present disclosure.

"PD-1-binding protein" encompasses any protein capable of specifically binding to PD-1 or any molecule capable of specifically binding to its epitopes. The PD-1-binding protein may include an antibody against PD-1 as defined in the present disclosure or a conjugate thereof. The PD-1-binding protein also encompasses immunoglobulin superfamily antibodies (IgSF) or CDR-grafted molecules. The "PD-1-binding protein" of the present disclosure may comprise at least one immunoglobulin single variable domain (such as a VHH) that binds to PD-1. In some embodiments, the "PD-1-binding protein" may comprise 2, 3, 4, or more immunoglobulin single variable domains (such as VHHs) that bind to PD-1. The PD-1-binding protein of the present disclosure may also comprise, in addition to the immunoglobulin single variable domain of PD-1, a linker and/or a moiety with an effector function, such as a half-life extending moiety (e.g., an immunoglobulin single variable domain that binds to serum albumin) and/or a fusion partner (such as serum albumin) and/or a conjugated polymer (such as PEG) and/or an Fc region. The "PD-1-binding protein" of the present disclosure may comprise a heavy chain variable region (VH) and a light chain variable region (VL), such as a Fab, a Fab', a F(ab')2, a single-chain antibody (scFv), or an IgG antibody.

The "PD-1-binding protein" or "anti-PD-1 antibody" of the present disclosure may comprise one or more effector molecules, for example, in a conjugated manner. The "effector molecules" include, for example, small-molecule compounds, polypeptides or

polypeptide fragments, biologically active proteins (such as enzymes), other antibodies or antibody fragments, synthetic or naturally occurring polymers, nucleic acids and fragments thereof (such as DNA, RNA, and fragments thereof), and radionuclides.

"Cytokine" is a general term for proteins that are released by a cell population to act on other cells as intercellular mediators. Examples of such cytokines include lymphokines, monokines, chemokines, and traditional polypeptide hormones. An exemplary cytokine is IFNγ.

"Antibody" encompasses a variety of antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, monospecific antibodies, multispecific antibodies (e.g., bispecific antibodies), full-length antibodies, and antibody fragments (or antigen-binding fragments, or antigen-binding moieties), so long as they exhibit the desired antigen-binding activity. An antibody may refer to an immunoglobulin, which is a four-peptide-chain structure formed by two heavy chains and two light chains linked by interchain disulfide bonds. The heavy chain constant regions of immunoglobulins differ in amino acid composition and arrangement; therefore, they also differ in antigenicity. Accordingly, immunoglobulins can be classified into five classes, also referred to as isotypes of immunoglobulins, namely IgM, IgD, IgG, IgA, and IgE, and their corresponding heavy chains are a μ chain, a δ chain, a γ chain, an α chain, and an ε chain, respectively. Igs of the same class can be classified into different subclasses based on the amino acid composition of their hinge regions and the number and positions of heavy chain disulfide bonds; for example, IgG can be divided into IgG1, IgG2, IgG3, and IgG4. Light chains are classified into κ or λ chains based on their constant regions. Each of the five Ig classes may have a κ chain or λ chain.

In the antibody heavy and light chains, the sequences of about 110 amino acids near the N-terminus vary considerably and thus are referred to as variable regions (V regions); the remaining amino acid sequences near the C-terminus are relatively stable and thus are referred to as constant regions (C regions). The variable regions comprise 3 hypervariable regions (HVRs) and 4 framework regions (FRs) with relatively conservative sequences. The 3 hypervariable regions determine the specificity of the antibody and thus are also known as complementarity-determining regions (CDRs). Each of the light chain variable regions (VLs) and the heavy chain variable regions (VHs) consists of 3 CDRs and 4 FRs arranged from the amino-terminus to the carboxyl-terminus as follows: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The 3 CDRs of the light chain refer to LCDR1, LCDR2, and LCDR3, and the 3 CDRs of the heavy chain refer to HCDR1, HCDR2, and HCDR3.

"Antigen-binding fragment" encompasses a single-chain antibody (i.e., full-length heavy and light chains); a Fab, a modified Fab, a Fab', a modified Fab', a F(ab')2, an Fv, a Fab-Fv, a Fab-dsFv, a single-domain antibody (e.g., VH or VL or VHH), an scFv, a bivalent or trivalent or tetravalent antibody, a Bis-scFv, a diabody, a tribody, a triabody, a tetrabody, and an epitope-binding fragment of any one of the above (see, e.g., Holliger and Hudson, 2005, Nature Biotech. 23(9):1126-1136; Adair and Lawson, 2005, Drug Design Reviews-

Online 2(3), 209-217).

Methods for producing and preparing such antigen-binding fragments are well known in the art (see, e.g., Verma et al., 1998, Journal of Immunological Methods, 216, 165-181). Fab-Fv was first disclosed in WO2009/040562, and its disulfide-stabilized form Fab-dsFv was first disclosed in WO2010/035012. The antigen-binding fragment of the present disclosure also includes Fab and Fab' fragments described in WO2005/003169, WO2005/003170, and WO2005/003171. Multivalent antibodies may comprise multiple specificities (e.g., bispecificities) or may be monospecific (see, e.g., WO92/22583 and WO05/113605), and an example of the latter is Tri-Fab (or TFM) described in WO 92/22583.

For the determination or definition of "CDRs", the deterministic depiction of CDRs and identification of residues of antigen-binding sites can be accomplished by resolving the structure of the antibody and/or resolving the structure of the antibody-ligand complex. This can be accomplished by any one of a variety of techniques known to those skilled in the art, such as X-ray crystallography. A variety of analysis methods can be used to identify CDRs, including but not limited to the Kabat numbering scheme, the Chothia numbering scheme, the AbM numbering scheme, the IMGT numbering scheme, the contact definition, and the conformational definition.

The Kabat numbering scheme is a standard for numbering residues in antibodies and is generally used to identify CDRs (see, e.g., Johnson & Wu, 2000, Nucleic Acids Res., 28:214-8). The Chothia numbering scheme is similar to the Kabat numbering scheme, except that it takes into account the positions of certain structural loop regions (see, e.g., Chothia et al., 1986, J. Mol. Biol., 196:901-17; Chothia et al., 1989, Nature, 342:877-83). The AbM numbering scheme adopts a computer program integration suite for modeling antibody structures manufactured by Oxford Molecular Group (see, e.g., Martin et al., 1989, Proc Natl Acad Sci (USA), 86:9268-9272; "AbMTM, A Computer Program for Modeling Variable Regions of Antibodies", Oxford, UK; Oxford Molecular, Ltd.). The AbM numbering scheme adopts a combination of a knowledge database and the de-novo method to model the tertiary structure of antibodies from basic sequences (see those described in Samudrala et al., 1999, "Ab Initio Protein Structure Prediction Using a Combined Hierarchical Approach", PROTEINS, Structure, Function and Genetics Suppl., 3:194-198). The contact definition is based on the analysis of the available complex crystal structures (see, e.g., MacCallum et al., 1996, J. Mol. Biol., 5:732-45). In the conformational definition, the positions of the CDRs can be identified as residues that contribute enthalpy to the antigen binding (see, e.g., Makabe et al., 2008, Journal of Biological Chemistry, 283:1156-1166). Other CDR boundary definitions may not strictly follow one of the above methods, but still overlap with at least a portion of the Kabat CDRs. The boundaries of CDRs may be shortened or lengthened based on predictions or experimental results that a particular residue or group of residues does not significantly affect the antigen binding. As used in the present disclosure, a CDR may refer to a CDR

defined by any method known in the art, including combinations of methods. The correspondence between the various numbering schemes is well known to those skilled in the art.

The CDR amino acid residues of the VL and VH regions of the antibody of the present disclosure accord with the known Kabat numbering scheme in terms of number and positions.

The antibodies of the present disclosure may be polyclonal, monoclonal, xenogeneic, allogeneic, syngeneic, or modified forms thereof, with the monoclonal antibodies being especially applicable in various examples. Generally, the antibodies of the present disclosure are recombinant antibodies.

The term "recombinant" as used herein generally refers to such a product as a cell, a nucleic acid, a protein, or a vector, and indicates that the cell, the nucleic acid, the protein, or the vector has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a natural nucleic acid or protein. For example, recombinant cells express genes that are not found within the natural (non-recombinant) cellular form or express natural genes that are abnormally expressed, lowly expressed, or not expressed at all.

A "domain" of a polypeptide or protein refers to a folded protein structure that is capable of maintaining its tertiary structure independently of the rest of the protein. In general, a domain is responsible for a single functional property of a protein and, in many cases, may be added, deleted, or transferred to other proteins without loss of functions of the rest of the protein and/or the domain of the protein.

"Immunoglobulin domain" refers to a globular region of an antibody chain. Immunoglobulin domains are characterized by their maintenance of the folding feature of the antibody molecule.

"Immunoglobulin variable domain" refers to an immunoglobulin domain substantially consisting of four "framework regions", i.e., "framework region 1" or "FR1", "framework region 2" or "FR2", "framework region 3" or "FR3", and "framework region 4" or "FR4", and three "complementarity determining regions" or "CDRs", i.e., "complementarity determining region 1" or "CDR1", "complementarity determining region 2" or "CDR2", and "complementarity determining region 3" or "CDR3". Thus, the general structure or sequence of an immunoglobulin variable domain can be expressed as follows: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. An immunoglobulin variable domain possesses specificity for an antigen by virtue of having an antigen-binding site.

"Antibody framework (FR)" refers to a portion of a variable domain, which serves as a framework for the antigen-binding loops (CDRs) of the variable domain.

"Immunoglobulin single variable domain" is generally used to refer to an immunoglobulin variable domain (which may be a heavy or light chain domain, including a VH, VHH, or VL domain) that can form a functional antigen-binding site without interacting with other variable domains (e.g., without VH/VL interactions as required

between the VH and VL domains of conventional four-chain monoclonal antibodies). Examples of "immunoglobulin single variable domains" include nanobodies (including VHHs, humanized VHHs, and/or camelized VHs, e.g., camelized human VHs), IgNARs, domains, (single-domain) antibodies as VH domains or derived from VH domains (such as dAbs™) and (single-domain) antibodies as VL domains or derived from VL domains (such as dAbs™). Immunoglobulin single variable domains based on and/or derived from heavy chain variable domains (such as VH or VHH domains) are generally preferred. A specific example of an immunoglobulin single variable domain is a "VHH domain" (or "VHH" for short) as defined below.

"VHH domain", also known as heavy-chain single-domain antibody, VHH, V_HH domain, VHH antibody fragment, VHH antibody, or nanobody, is a variable domain of an antigen-binding immunoglobulin known as a "heavy-chain antibody" (i.e., "an antibody devoid of light chains") (Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R., "Naturally occurring antibodies devoid of light chains"; Nature 363, 446-448 (1993)). The term "VHH domain" is used to distinguish said VHH from the VHs and VLs present in antibodies of the conventional four-peptide-chain structure. VHH domains specifically bind to epitopes without the need for additional antigen-binding domains (however, in antibodies of the conventional four-peptide-chain structure, epitopes are recognized by the VL domains together with the VH domains). A VHH domain is a small, stable, and highly efficient antigen recognition unit formed by a single immunoglobulin domain. The terms "heavy-chain single-domain antibody", "VHH domain", "VHH", "V_{HH} domain", "VHH antibody fragment", "VHH antibody", "Nanobody®", and "Nanobody® domain" ("Nanobody" is a trademark of Ablynx N.V., Ghent, Belgium) are used interchangeably. "VHH domains" include, but are not limited to, natural antibodies produced by camelids, antibodies produced by camelids and then humanized, or antibodies obtained by screening with phage display techniques. The total number of amino acid residues in a VHH domain will usually be in the range of 110 to 120, often between 112 and 115. However, it should be noted that shorter and longer sequences may also be suitable for the purposes described in the present disclosure. Methods for obtaining VHHs that bind to a particular antigen or epitope have been previously disclosed in the following documents: R. van der Linden et al., Journal of Immunological Methods, 240 (2000) 185-195; Li et al., J Biol Chem., 287 (2012) 13713-13721; Deffar et al., African Journal of Biotechnology Vol. 8 (12), pp.2645-2652, 17 June, 2009, and WO94/04678.

As is well known in the art for VH domains and for VHH domains, the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering). This means that, in general, the numbering according to

Kabat may or may not correspond to the actual numbering of the amino acid residues in the actual sequence. Other numbering systems or numbering schemes for VHHs include Chothia, IMGT, and AbM.

"Humanized antibody", also known as a CDR-grafted antibody, refers to an antibody produced by grafting non-human CDR sequences into the framework of variable regions of a human antibody. Therefore, the strong immune responses induced by the large amount of non-human protein components contained in chimeric antibodies can be overcome. To avoid a decrease in activity caused by a decrease in immunogenicity, the variable regions of a fully human antibody can be subjected to minimum reverse mutation to maintain the activity. Examples of "humanization" include "humanization" of VHH domains derived from camelidae by replacing one or more amino acid residues in the amino acid sequence of the original VHH sequence with one or more amino acid residues present at the corresponding positions in a VH domain of a human conventional antibody with a four-peptide-chain structure (also referred to in the present disclosure as "sequence optimization"; in addition to humanization, "sequence optimization" may also encompass other modifications to the sequence by one or more mutations providing improved properties of the VHH, such as removal of potential post-translational modification sites). The humanized VHH domain may contain one or more fully human framework region sequences. Another example of "humanization" includes an antibody produced by grafting mouse CDR sequences into a human antibody variable region framework, i.e., a different type of human germline antibody framework sequence. Therefore, the strong antibody variable antibody responses induced by the large amount of heterologous protein components contained in chimeric antibodies can be overcome. Methods for humanization include, for example, protein surface amino acid humanization (resurfacing) and the universal framework grafting method for antibody humanization (CDR grafting to a universal framework), i.e., "grafting" CDRs onto other "scaffolds" (including but not limited to human scaffolds or non-immunoglobulin scaffolds). Scaffolds and techniques suitable for such CDR grafting are known in the art. For example, germline DNA sequences of genes of the human heavy and light chain variable regions can be found in the "VBase" human germline sequence database, as well as in Kabat, E. A. et al., 1991 Sequences of Proteins of Immunological Interest, 5th Edition. The humanized antibody of the present disclosure also includes humanized antibodies formed by further affinity maturation of the CDRs by phage display. In addition, in order to avoid a decrease in activity caused by a decrease in immunogenicity, the framework sequence in the human antibody variable region can be subjected to minimum reverse mutation or back mutation to maintain activity.

An "affinity-matured" antibody is an antibody that comprises one or more changes in one or more hypervariable regions (HVRs) compared to the parental antibody, and such changes result in an improvement in the affinity of the antibody for the antigen. For example, an "affinity-matured" PD-1-binding protein or anti-PD-1 antibody comprises

one or more changes in one or more CDRs, and the changes result in an increase in the affinity for the antigen compared to its parental antibody. Affinity-matured antibodies can be prepared, for example, by methods known in the art as described below: Marks et al., 1992, Biotechnology 10:779-783 or Barbas et al., 1994, Proc. Nat. Acad. Sci, USA 91:3809-3813; Shier et al., 1995, Gene 169:147-155; Yelton et al., 1995, Immunol. 155:1994-2004; Jackson et al., 1995, J. Immunol. 154(7):3310-9; Hawkins et al., 1992, J. MoI. Biol. 226(3):889896; KS Johnson and RE Hawkins, "Affinity maturation of antibodies using phage display", Oxford University Press 1996.

"Fully human antibody" includes antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The fully human antibody of the present disclosure may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutations *in vivo*). "Fully human antibody" does not include "humanized antibodies".

Generally, "specific binding" or "selective binding" refers to the binding of a binding protein to an epitope on an antigen. The PD-1-binding protein of the present disclosure binds to an antigen to be bound (i.e., PD-1) or an epitope thereof with a dissociation constant (K^D) of 10^{-7} to 10^{-10} mol/L (M), or 10^{-8} to 10^{-10} M, or 10^{-9} to 10^{-10} M, or less, as measured in a Biacore or KinExA or Fortibio assay. Any K_D value greater than 10^{-4} M is generally considered to indicate non-specific binding. The specific binding of a binding protein to an antigen or epitope can be determined in any known suitable manner, including, for example, a surface plasmon resonance (SPR) assay as described in the present disclosure.

"Epitope" refers to a site on an antigen to which an immunoglobulin or an antibody binds. An epitope may be formed from contiguous amino acids, or non-contiguous amino acids juxtaposed by tertiary folding of the protein. An epitope formed from contiguous amino acids is generally retained after exposure to a denaturing solvent, while an epitope formed by tertiary folding is generally lost after treatment with a denaturing solvent. An epitope generally comprises, for example, at least 3-15 amino acids in a distinctive spatial conformation. Methods for determining the binding of an epitope to a given antibody are well known in the art and include an immunoblotting assay, an immunoprecipitation assay, and the like. Methods for determining the spatial conformation of an epitope include techniques in the art and techniques described in the present disclosure, such as X-ray crystallography and two-dimensional nuclear magnetic resonance.

"Binding affinity" or "affinity" is used in the present disclosure as a measure of the strength of a non-covalent interaction between two molecules (e.g., an antibody or a portion thereof and an antigen). The binding affinity between two molecules can be quantified by determining the dissociation constant (K_D). K_D can be determined by measuring the kinetics of complex formation and dissociation by using, e.g., the surface plasmon resonance (SPR) method (Biacore). The rate constants corresponding to the

association and dissociation of a monovalent complex are referred to as the association rate constant ka (or kon) and the dissociation rate constant kd (or koff), respectively. K_D is related to ka and kd by the equation K_D = kd/ka. The value of the dissociation constant can be determined directly by well-known methods, and calculations can even be performed for complex mixtures by, for example, those methods described in Caceci et al (1984, Byte 9:340-362). For example, K_D can be determined by using a dual filtration nitrocellulose filter binding assay such as that disclosed by Wong & Lohman (1993, Proc. Natl. Acad. Sci. USA 90:5428-5432). Other standard assays for assessing the ability of an antibody to bind to a target antigen are known in the art and include, for example, ELISA, western blot, RIA, and flow cytometry, as well as other assays exemplified in the present disclosure. The binding kinetics and binding affinity of the antibody can also be evaluated by standard assays known in the art, such as surface plasmon resonance (SPR), e.g., by using the Biacore™ system or KinExA. The binding affinities associated with different molecular interactions, e.g., the binding affinities of different antibodies for a given antigen, can be compared by comparing the K_D values of antibody/antigen complexes. Similarly, the specificity of an interaction can be evaluated by determining and comparing the K_D value for the interaction of interest (e.g., a specific interaction between an antibody and an antigen) with the K_D value for an interaction not of interest (e.g., a control antibody known not to bind to PD-1).

"Conservative replacement" refers to replacement by another amino acid residue having similar properties to the original amino acid residue. For example, lysine, arginine, and histidine have similar properties in that they have basic side chains, and aspartic acid and glutamic acid have similar properties in that they have acidic side chains. In addition, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, and tryptophan have similar properties in that they have uncharged polar side chains, and alanine, valine, leucine, threonine, isoleucine, proline, phenylalanine, and methionine have similar properties in that they have nonpolar side chains. In addition, tyrosine, phenylalanine, tryptophan, and histidine have similar properties in that they have aromatic side chains. Thus, it will be apparent to those skilled in the art that even when an amino acid residue in a group exhibiting similar properties as described above is replaced, it will not exhibit a particular change in properties.

"Homology", "identity", or "sequence identity" refers to sequence similarity between two polynucleotide sequences or between two polypeptides. When positions in two compared sequences are occupied by identical nucleotides or amino acid monomers, e.g., if the position of each of two DNA molecules is occupied by an identical nucleotide, the molecules are homologous at that position. The percent homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of compared positions × 100%. For example, if 6 out of 10 positions are matched or homologous when two sequences are optimally aligned, the two sequences are 60% homologous. In general, a comparison is performed when two

sequences are aligned to obtain the maximum percent homology.

"Nucleic acid" or "polynucleotide" are used interchangeably herein to refer to any DNA or RNA molecule, either single- or double-stranded, and, if single-stranded, the molecule of its complementary sequence, e.g., a double-stranded DNA. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence.

"Host cell" includes individual cells or cell cultures, which may be or have been the recipient of a vector for incorporation of a polynucleotide insert. The host cell includes progeny of a single host cell, and the progeny are not necessarily completely identical (in morphology or genomic DNA complement) to the parental cell due to natural, accidental, or deliberate mutations. The host cell includes cells transfected and/or transformed *in vivo* with the polynucleotide of the present disclosure. "Cell", "cell line", and "cell culture" are used interchangeably, and all such designations include their progeny. It should also be understood that their progeny may not be precisely identical in DNA content due to deliberate or unintentional mutations. The term includes mutant progeny that have the same function or biological activity as the cell selected in the original transformation.

"Inhibition" and "blocking" are used interchangeably and encompass both partial and complete inhibition/blocking. "Inhibition of growth" (e.g., involving cells) is intended to include any measurable reduction in cell growth.

"Inflammatory or autoimmune disease" refers to a disease or disorder resulting from the inappropriate activation of the immune system, which may include inflammatory diseases or disorders and autoimmune diseases or disorders. In some embodiments, "inflammatory disease" or "inflammatory disorder" refers to a disease or disorder in which a component of the immune system of a mammal causes, mediates, or otherwise contributes to an inflammatory response leading to the development of disease in the mammal. In some embodiments, "autoimmune disease" or "autoimmune disorder" refers to a disease or disorder resulting from the inappropriate activation of immune cells that are reactive with the body's own tissues.

"Immune response" includes T cell-mediated and/or B cell-mediated immune responses. Exemplary immune responses include T cell responses, such as cytokine production and cytotoxicity. In addition, the term immune response includes immune responses indirectly affected by T cell activation, such as antibody production (humoral responses) and activation of cytokine-reactive cells (e.g., macrophages).

"Down-regulate" or "down-regulating" refers to a detectable decrease in the level of an immune response in a subject compared to the level of a response in the subject in the absence of a treatment or compound, and/or compared to the level of a response in an otherwise identical but untreated subject.

"Giving", "administering", and "treating", when applied to animals, humans, experimental subjects, cells, tissues, organs, or biological fluids, refer to contacting an

exogenous drug, a therapeutic agent, a diagnostic agent, or a composition with the animals, humans, subjects, cells, tissues, organs, or biological fluids, e.g., therapeutic, pharmacokinetic, diagnostic, research, and experimental methods. Treatment of cells comprises contact between a reagent and cells, and contact between the reagent and a fluid, wherein the fluid is in contact with the cells. "Giving", "administering", and "treating" also mean treating, for example, a cell with a reagent, diagnostic, or binding composition or with another cell *in vitro* and *ex vivo*. When applied to humans, veterinary medicine, or research subjects, the terms refer to therapeutic treatment, prophylactic or preventive measures, and research and diagnostic applications.

"Treat", "treated", "treating", or "treatment" refers to giving a therapeutic agent, such as a therapeutic agent comprising any one of the binding proteins of the present disclosure or a pharmaceutical composition thereof, either internally or externally, to a subject who has had, is suspected of having, or is predisposed to having one or more immune disorders; the therapeutic agent is known to have a therapeutic effect on these symptoms. Typically, the therapeutic agent is given in an amount that is effective in alleviating one or more disease symptoms in the subject or population being treated, whether by inducing regression of such symptoms or inhibiting the progression of such symptoms to any clinically measurable degree. The amount of therapeutic agent effective in alleviating any specific disease symptom (also referred to as the "therapeutically effective amount") may vary depending on a variety of factors such as the disease state, age, and body weight of the subject, and the ability of the drug to produce the desired therapeutic effect in the subject. Whether a disease symptom has been palliated can be evaluated by any clinical test method commonly used by doctors or other health care professionals to evaluate the severity or progression of the symptom. Although embodiments of the present disclosure (e.g., treatment methods or products) may be ineffective in alleviating the disease symptom of interest in a certain subject, they shall palliate the disease symptom of interest in a statistically significant number of subjects, as determined by any statistical testing method known in the art, such as a Student's t-test, a Chi-square test, a U-test by Mann and Whitney, a Kruskal-Wallis test (H-test), a Jonckheere-Terpstra test, and a Wilcoxon test.

"Effective amount" comprises an amount sufficient to ameliorate or prevent a symptom or sign of a medical disorder. An effective amount also means an amount sufficient to allow or facilitate diagnosis. The effective amount for a subject may vary depending on factors such as the disorder to be treated, the general health of the subject, the method, route, and dose of administration, and the severity of side effects. An effective amount may be the maximum dose or administration regimen to avoid significant side effects or toxic effects. A subject of the present disclosure may be an animal or a human subject.

"Optional" or "optionally" means that the event or circumstance subsequently described may, but does not necessarily, occur; this description includes the instance where the event or circumstance occurs or does not occur. "And/or" should be taken as a specific

disclosure of each of the two specified features or components, with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" in the present disclosure includes "A and B", "A or B", "A" (alone), and "B" (alone). Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "have", "include", etc., are to be understood in an inclusive sense as opposed to an exclusive or exhaustive sense, that is to say, in the sense of "including but not limited to". The "subject" or "patient" in the present disclosure means a mammal, particularly a primate, and especially a human.

Examples

The present disclosure is further described with reference to the following examples; however, these examples are not intended to limit the scope of the present disclosure. Experimental procedures without specific conditions indicated in the examples or test examples of the present disclosure are generally conducted under conventional conditions, or under conditions recommended by the manufacturers of the starting materials or commercial products. See Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press; Current Protocols in Molecular Biology, Ausubel et al., Greene Publishing Association, Wiley Interscience, NY. Reagents without specific sources indicated were commercially available conventional reagents.

Example 1: Anti-PD-1 Nanobody (VHH) Screening and Preparation

1.1. Sequences and preparation of screening and detection antigens

Programmed death receptor 1 (PD-1) is an important immunosuppressive molecule. A C-terminally His-tagged human PD-1 protein extracellular region fragment (Leu25-Gln167) was selected as an immunogen for immunizing animals (camelids). The sequence, source, and uses of the recombinant human PD-1 protein used in the present disclosure are shown in Table 1. The protein reagent can be used in the experiments in the following examples.

Table 1. The sources and uses of the amino acid sequences of recombinant proteins

Name	Start and end of amino acid sequence	ACRO Catalog No.	Use
human-PD-1-His	Leu 25 - Gln 167 (Accession#NP_005009.2)	PD1-H5221	Camelid immunization Phage display solid-phase magnetic bead screening
cyno-PD-1-His	Leu 25 - Gln 167 Accession # B0LAJ3	PD1-C52H5	ELISA screening for positive clones

The complete PD-1 protein sequence is described below. In the sequence, 1-23 aa form the signal peptide, 24-170 aa form the extracellular region of PD-1 (underlined), 171-191 aa form the transmembrane region, and 192-288 aa form the intracellular region.

> The amino acid sequence of human PD-1

MQIPQAPWPVVWAVLQLGWRPGWFLDSPDRPWNPPTFSPALLVVTEGDNATFT
CSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVTQLPNGRDFH
MSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRP
AGQFQTLVVGVVGGLLGSLVLLVWVLA*VI*CSRAARGTIGARRTGQPLKEDPSAV
PVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGP
RSAQPLRPEDGHCSWPL (SEQ ID NO: 1)

1.2. Immunization, phage library construction, and panning

After an alpaca was immunized four times, the serum titer was determined. After the titer was determined to be acceptable, PBMCs were isolated, total RNA was extracted, the purity was measured, and the RNA was reverse-transcribed into DNA for subsequent antibody gene amplification. A purified vector and the target VHH fragment were digested with an enzyme and ligated. Electroporation was performed, and clones were selected to obtain a phage library.

A solid phase was coated with the PD-1 antigen, and screening was performed by trypsin elution; specific phages were eluted. After three rounds of panning, 384 clones were randomly picked from the titer determination plates of the second round and the third round and screened by phage ELISA for positive clones, and the optical density at 450 nm was measured. According to the sequencing results, alignment and phylogenetic tree analysis of amino acid sequences were performed. Finally, 25 unique sequences were obtained.

The activity of the above antibodies was assessed, including: determining the activation activity of the antibodies on the PD-1 signaling pathway, determining the inhibitory activity of the antibodies on memory T cells using a CMV recall assay system, determining the inhibitory activity of the antibodies against the release of cytokines from T cells using a PBMC activation assay system, and assessing the binding of the antibodies to human and monkey PD-1 using FACS. In addition, the drug developability (aggregates, cleavage, accelerated stability, post-translational modifications (PTMs), etc.) of the antibodies was assessed. An anti-PD-1 antibody having significant advantages in activity and drug developability was selected from the antibodies, and the obtained antibody 2E1-A06-VHH was further tested.

The sequence of 2E1-A06-VHH is shown below:

> 2E1-A06-VHH

QVQLVESGGGSVQAGGSLRLSCATSGYVIS*RNCMA*WFRQAPGKEREWVA*LTPG*
*GGTTYYTNSVKG*RFTDSQDKNTVYLQMTSLKPEDTAMYYCAA*RIGFGYCTGTDR*
*DYTH*WGQGTQVTVSS

(SEQ ID NO: 2)

In the sequence, the underlined parts are CDR sequences determined according to the Kabat numbering scheme. See Table 2 for details.

Table 2. The CDR sequences of 2E1-A06-VHH (according to the Kabat numbering scheme)

Antibody	Name	Sequence	No.
2E1-A06-VHH	CDR1	RNCMA	SEQ ID NO:3
	CDR2	LTPGGGTTYYTNSVKG	SEQ ID NO:4
	CDR3	RIGFGYCTGTDRDYTH	SEQ ID NO:5

1.3. Nanobody preparation

The obtained antibody 2E1-A06-VHH was fused to wild-type IgG1-Fc or an IgG1-Fc region with a mutation to obtain a nanobody. For example, 2E1-A06-VHH was fused to a human wild-type IgG1-Fc region (Glu216-Lys447) with mutation C220A (numbered according to the EU numbering system) to obtain a VHH-Fc chimeric antibody. The sequence of the human wild-type IgG1-Fc region with mutation C220A is shown below:

> Human wild-type IgG1-Fc (Glu216-Lys447), with mutation C220A

EPKSADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

(SEQ ID NO: 6)

> Human wild-type IgG1-Fc

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK

(SEQ ID NO: 45)

The sequence of antibody 2E1-A06 is shown below:

> 2E1-A06

QVQLVESGGGSVQAGGSLRLSCATSGYVISRNCMAWFRQAPGKEREWVALTPG
GGTTYYTNSVKGRFTDSQDKNTVYLQMTSLKPEDTAMYYCAARIGFGYCTGT
DRDYTHWGQGTQVTVSSEPKSADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLM
ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

(SEQ ID NO: 7)

In the sequence, the underlined part is the human IgG1 Fc region with mutation C220A. A gene sequence encoding the above antibody was synthesized and subcloned into a pcDNA3.1 expression vector. The expression vector and the transfection reagent PEI were transfected into CHO cells in a 1:2 ratio, and the cells were incubated in a CO_2 incubator for 4-5 days. For the expressed antibody, centrifugation was performed, and the supernatant was collected and then conventionally purified. Analysis showed that the target antibody had been obtained.

Example 2: Anti-PD-1 Monoclonal Antibody (IgG) Screening and Preparation

2.1. Sequences and preparation of screening and detection antigens

His-tagged human PD-1 (h-PD-1-His) recombinant protein and His-tagged monkey PD-1 (cyno-PD-1-His) recombinant protein were both commercially available purified protein reagents purchased from Acrobiosystems. Their respective sequence sources are shown in Table 3. The protein reagents can be used in the experiments in the following examples.

Table 3. The sources and uses of the amino acid sequences of recombinant proteins

Name	Start and end of amino acid sequence	ACRO Catalog No.	Use
human-PD-1-His	Leu 25 - Gln 167 (Accession#NP_005009.2)	PD1-H5221	Mouse immunization Hybridoma fusion and screening
cyno-PD-1-His	Leu 25 - Gln 167 Accession # B0LAJ3	PD1-C52H5	Hybridoma fusion and screening

2.2. Antibody hybridoma preparation and screening

Anti-human PD-1 monoclonal antibodies were produced by immunization of mice. Experimental Balb/C mice, 6 to 8 weeks old. Housing environment: SPF.

The immunizing antigen was His-tagged human recombinant PD-1 protein (h-PD-1-His, 1 μg/μL in phosphate-buffered saline). Emulsification with Freund's adjuvants: complete Freund's adjuvant (CFA) was used for the first immunization, and incomplete Freund's adjuvant for the remaining ones. The injections for immunization were performed on days 0, 14, 28, 42, and 70. Blood was collected and tested on days 21, 35, 49, 63, and 77. Mouse serum was assayed by ELISA to determine the antibody titer in the mouse serum. After the third immunization, mice in which the serum antibody titer was high and was reaching a plateau were selected for splenocyte fusion. Boost immunization was performed 3 days before the fusion. Lymphocytes and Sp2/0 myeloma cells were fused by electrofusion to obtain hybridoma cells, and the hybridoma supernatant was subjected to *in vitro* activity screening. Clones with good *in vitro* activity were sequenced. According to the sequencing results, alignment and phylogenetic tree analysis of

sequences were performed. Finally, murine-derived monoclonal antibody sequences were obtained. The heavy chain variable region (HCVR) and light chain variable region (LCVR) sequences of monoclonal antibody m113.3 are shown below:

> m113.3-HCVR

QIQLQQSGPELVKPGASVKISCKASGYTFT*DYYIN*WVKQKPGQGLEWIG*WIYPGS GNTKYNEKFKG*KATLTVDTSSSTAYMQLSSLTSEDTAVYFCAR*YSNFFMDY*WGQ GASVTVSS

(SEQ ID NO: 8)

> m113.3-LCVR

DIQMTQSPSSLSASLGERVSLTC*RASQEISGYLS*WLQQKPDGTIKRLIY*VASTLHS*G VPKRFSGSRSGSDYSLTISSLESEDFADYYC*LQYASYPYT*FGGGTKLEIK

(SEQ ID NO: 9)

In the sequences, the underlined parts are CDR sequences determined according to the Kabat numbering scheme. See Table 4.

Table 4. The CDR sequences of m113.3 (according to the Kabat numbering scheme)

Antibody	Name	Sequence	No.
m113.3	HCDR1	DYYIN	SEQ ID NO:10
	HCDR2	WIYPGSGNTKYNEKFKG	SEQ ID NO:11
	HCDR3	YSNFFMDY	SEQ ID NO:12
	LCDR1	RASQEISGYLS	SEQ ID NO:13
	LCDR2	VASTLHS	SEQ ID NO:14
	LCDR3	LQYASYPYT	SEQ ID NO:15

2.3. Anti-PD-1 monoclonal antibody preparation

The obtained heavy chain variable region and light chain variable region were linked to the human IgG1 heavy chain constant region and the human κ light chain constant region, respectively, to construct a human-mouse chimeric full-length antibody, 113.3. The sequences are shown below:

> Human IgG1 heavy chain constant region

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV*EPKSCDKTHTCP PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*

(SEQ ID NO: 16)

> Human κ light chain constant region

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

(SEQ ID NO: 17)

> 113.3-HC

QIQLQQSGPELVKPGASVKISCKASGYTFTDYYINWVKQKPGQGLEWIGWIYPG
SGNTKYNEKFKGKATLTVDTSSSTAYMQLSSLTSEDTAVYFCARYSNFFMDYWG
QGASVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA
LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPGK

(SEQ ID NO: 18)

> 113.3-LC

DIQMTQSPSSLSASLGERVSLTCRASQEISGYLSWLQQKPDGTIKRLIYVASTLHS
GVPKRFSGSRSGSDYSLTISSLESEDFADYYCLQYASYPYTFGGGTKLEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
DSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

(SEQ ID NO: 19)

Peresolimab is a PD-1 agonist developed by Eli Lilly and Company and was used as a control antibody in subsequent examples. Its heavy and light chain sequences are shown below:

> Peresolimab HC

QVQLVQSGAEVKKPGASVKVSCKVSGYSLSKYDMSWVRQAPGKGLEWMGIIY
TSGYTDYAQKFQGRVTMTEDTSTDTAYMELSSLRSEDTAVYYCATGNPYYTNG
FNSWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE
YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
MHEALHNHYTQKSLSLSPGK

(SEQ ID NO: 20)

> Peresolimab LC

DIQMTQSPSSLSASVGDRVTITCQASQSPNNLLAWYQQKPGKAPKLLIYGASDL
PSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNNYYVGPVSYAFGGGTKVEI
KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

(SEQ ID NO: 21)

Camrelizumab is a PD-1 antagonist developed by Hengrui Pharmaceuticals. It can block the binding of PD-L1 to PD-1 and be used for anti-tumor treatment. It was used as a control antibody in subsequent examples. Its specific sequences are shown below:

> Camrelizumab HC

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYMMSWVRQAPGKGLEWVATISG
GGANTYYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARQLYYFDY
WGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKR
VESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPE
VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEAL
HNHYTQKSLSLSLGK

(SEQ ID NO: 22)

> Camrelizumab LC

DIQMTQSPSSLSASVGDRVTITCLASQTIGTWLTWYQQKPGKAPKLLIYTATSLA
DGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQVYSIPWTFGGGTKVEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

(SEQ ID NO: 23)

In the above sequences, the underlined part in the heavy chain is the human IgG1 Fc region, and the underlined part in the light chain is the human Cκ region.

Gene sequences encoding the above antibodies were synthesized and subcloned into pcDNA3.1 expression vectors. Each expression vector and the transfection reagent PEI were transfected into CHO cells in a 1:2 ratio, and the cells were incubated in a CO_2 incubator for 4-5 days. For the expressed antibodies, centrifugation was performed, and the supernatants were collected and then conventionally purified. Analysis showed that the target antibodies had been obtained.

Example 3: Affinities of Anti-PD-1 Antibodies

3.1. Determination of affinities of anti-PD-1 antibodies for PD-1 protein by SPR

Experimental method: An assay was performed using a Biacore 8K instrument (GE Healthcare). A Protein A sensor chip was used, and an HBS-EP+ buffer solution (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20) was used as the mobile phase. The antibodies to be tested were dissolved in the HBS-EP+ buffer solution as ligands and captured using the Protein A protein on the channels of the chip. Human PD-1 antigen protein (ACRO, PD1-H5221) was used as the analyte and dissolved in the HBS-EP+ buffer solution. The analyte was serially diluted 2-fold from 100 nM, and a total of 7 concentration points were obtained. The analyte was left to flow through the

experimental channel and the reference channel at a flow rate of 30 μL/min, with an association time of 90 s and a dissociation time of 500 s. A regeneration buffer (10 mM glycine pH 1.5, GE Healthcare, 29238268-AA) was run at a flow rate of 10 μL/min for 30 s. Data were processed using Biacore 8K Evaluation analysis software. The corresponding reference channel (Fc 1) signal values were subtracted from the detection channel (Fc 2) signal values to obtain corrected signal curves. Affinity kinetic curves were fitted according to the 1:1 Langmuir binding model, and the association rate Ka, the dissociation rate Kd, and the dissociation constant (i.e., the affinity KD value) were calculated.

The results show that antibodies 2E1-A06 and 113.3 bound to human PD-1 protein; their affinities are shown in Table 5.

Table 5. The affinities of anti-PD-1 antibodies for recombinant PD-1 protein

Antibody	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)
2E1-A06	1.11E+05	3.85E-03	3.48E-08
113.3	1.37E+05	1.80E-04	1.31E-09

3.2. Assay for assessing antagonistic activity of anti-PD-1 antibodies against PD-L1

The antagonistic activity of chimeric antibodies against PD-L1 was assessed using an assay system in which PD-L1-overexpressing cells were co-incubated with a Jurkat reporter gene cell line.

Experimental method: 40,000 PD-L1 aAPC/CHO-K1 cells (Promega) were plated in a 96-well plate and cultured overnight. The next day, the cells were washed with PBS buffer and 1640 culture medium, and 50,000 human PD-1-overexpressing Jurkat PD-1 NFAT reporter gene cells and the antibodies to be tested (different concentrations) were added. After the cells were cultured in an incubator at 37 °C for 6 h, the expression of the reporter gene was measured using Bright-Glo™ (Promega).

As shown in FIGs. 1A and 1B, the PD-1 antagonistic control antibody camrelizumab exhibited the function of antagonizing PD-L1 and induced significant luciferase expression, while 2E1-A06, 113.3, and peresolimab did not antagonize the binding of PD-L1 to PD-1.

3.3. Reporter gene assay for assessing activation of PD-1 signaling pathway by anti-PD-1 antibodies

The activation activity of chimeric antibodies for PD-1 was investigated using an assay system in which THP-1 cells were co-incubated with Jurkat reporter gene cells.

Experimental method: 100,000 human PD-1-overexpressing Jurkat PD-1 NFAT reporter gene cells were plated in a 96-well plate, and the antibodies to be tested (different concentrations) were added. In addition, 50,000 mitomycin C-treated, CD32a-overexpressing THP-1 cells were added. The plate was incubated at 37 °C for 30 min.

Subsequently, 2 μg/mL anti-CD3 (Biolegend, OKT3) and 1 μg/mL anti-CD28 (Biolegend) were added to activate Jurkat cells to express the luciferase reporter gene. After the co-incubated cells were incubated at 37 °C for another 5 h, the expression of the reporter gene was measured using Bright-Glo™ (Promega).

As shown in FIGs. 2A and 2B, both 2E1-A06 and 113.3 significantly inhibited the expression of the luciferase reporter gene, indicating that both antibodies can significantly activate the PD-1 signaling pathway.

Example 4: Humanization Engineering of Anti-PD-1 Antibody

4.1. Humanization of anti-PD-1 nanobody

The humanization of the nanobody used CDR grafting. The sequence of the parental PD-1 nanobody was aligned with the fully human germline genes in the IMGT database using the IMGT or NCBI website. Human germline genes highly homologous with the PD-1 nanobody were selected as the framework region (FR) templates for humanization, and the CDR sequences of the parental camelid antibody were grafted into the framework regions (FRs) of the corresponding germline genes.

In addition, during humanization, according to the structural analysis results of the sequence and antibody homology modeling, the key core residues near the CDRs were back-mutated to finally obtain humanized antibody mutants. The humanized sequences of 2E1-A06-VHH are shown below:

> 2E1-A06-VHH-H1

EVQLVESGGGVVQPGGSLRLSCAASGYVISRNCMAWFRQAPGKGREWVALTP
GGGTTYYTNSVKGRFTISRDKNTLYLQMNSLRAEDTAVYYCAKRIGFGYCTGT
DRDYTHWGQGTLVTVSS

(SEQ ID NO: 24)

> 2E1-A06-VHH-H2

EVQLVESGGGVVQPGGSLRLSCAASGYVISRNCMAWFRQAPGKGREWVALTP
GGGTTYYTNSVKGRFTISQDKNTVYLQMNSLRAEDTAVYYCAARIGFGYCTGT
DRDYTHWGQGTLVTVSS

(SEQ ID NO: 25)

> 2E1-A06-VHH-H3

EVQLVESGGGVVQPGGSLRLSCATSGYVISRNCMAWFRQAPGKEREWVALTPG
GGTTYYTNSVKGRFTDSQDKNTVYLQMNSLRAEDTAVYYCAARIGFGYCTGT
DRDYTHWGQGTLVTVSS

(SEQ ID NO: 26)

> 2E1-A06-VHH-H4

EVQLVESGGGVVQPGGSLRLSCATSGYVISRNCMAWFRQAPGKEREWVALTPG
GGTTYYTNSVKGRFTDSQDKNTVYLQMNSLKPEDTAVYYCAARIGFGYCTGTD
RDYTHWGQGTLVTVSS

(SEQ ID NO: 27)

> 2E1-A06-VHH-H5

EVQLVESGGGVVQPGGSLRLSCAASGYVISRNCMAWFRQAPGKGREWVALTP
GGGTTYYTNSVKGRFTISQDNSKNTVYLQMNSLRAEDTAVYYCAARIGFGYCT
GTDRDYTHWGQGTLVTVSS

(SEQ ID NO: 28)

> 2E1-A06-VHH-H6

EVQLVESGGGVVQPGGSLRLSCATSGYVISRNCMAWFRQAPGKEREWVALTPG
GGTTYYTNSVKGRFTDSQDNSKNTVYLQMNSLRAEDTAVYYCAARIGFGYCT
GTDRDYTHWGQGTLVTVSS

(SEQ ID NO: 29)

In the above sequences, the underlined parts are CDRs (numbered according to the Kabat scheme).

Each of the above humanized VHHs was fused to a human wild-type IgG1-Fc region (Glu216-Lys447) with mutation C220A (numbered according to the EU numbering system) to obtain a full-length sequence. For example, 2E1-A06-VHH-H4 (SEQ ID NO: 27) was fused to the human wild-type IgG1-Fc region (Glu216-Lys447) to obtain 2E1-A06-H4 (SEQ ID NO: 30).

> 2E1-A06-H4

EVQLVESGGGVVQPGGSLRLSCATSGYVISRNCMAWFRQAPGKEREWVALTPG
GGTTYYTNSVKGRFTDSQDKNTVYLQMNSLKPEDTAVYYCAARIGFGYCTGTD
RDYTHWGQGTLVTVSSEPKSADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

(SEQ ID NO: 30)

In the sequence, the underlined part is the human IgG1 Fc region with mutation C220A.

Gene sequences encoding the above antibodies were synthesized and subcloned into pcDNA3.1 expression vectors. Each expression vector and the transfection reagent PEI were transfected into CHO cells in a 1:2 ratio, and the cells were incubated in a CO_2 incubator for 4-5 days. For the expressed antibodies, centrifugation was performed, and the supernatants were collected and then conventionally purified. Analysis showed that the target antibodies had been obtained.

4.2. Humanization of anti-PD-1 murine monoclonal antibody

On the basis of the typical structure of the obtained murine monoclonal antibody 113.3, the heavy and light chain variable region sequences were compared with an antibody

germline database to obtain human germline templates with high homology. For antibody 113.3, IGHV1-3 was selected as the human germline heavy chain template, and IGkV1-16 as the human germline light chain template. The CDRs of the murine monoclonal antibody were grafted onto the selected humanization templates to replace the original CDRs of the humanization templates, thus constructing variable regions. Then, based on the three-dimensional structure of the murine monoclonal antibody, embedded residues, residues directly interacting with the CDRs, and residues having important influence on the conformations of the VH and VL were back-mutated to produce a range of humanized antibodies.

The heavy chain variable regions of the humanized antibodies of 113.3 are shown below:

> 113.3 H1CVR

EIQLVQSGAEVKKPGASVKVSCKASGYTFTDYYINWVRQAPGQRLEWMGWIYPGSGNTKYNEKFKGRVTITVDTSASTAYMELSSLRSEDTAVYYCARYSNFFMDYWGQGTLVTVSS

(SEQ ID NO: 31)

> 113.3 H2CVR

EIQLVQSGAEVKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYPGSGNTKYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYFCARYSNFFMDYWGQGTLVTVSS

(SEQ ID NO: 32)

> 113.3 H3CVR

EIQLVQSGAEVKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYPGSGNTKYNEKFKGRVTLTVDTSSSTAYMELSSLRSEDTAVYFCARYSNFFMDYWGQGTLVTVSS

(SEQ ID NO: 33)

> 113.3 HbkCVR

EIQLVQSGSELKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYPGSGNTKYNEKFKGRVTITVDTSASTAYMELSSLRSEDTAVYYCARYSNFFMDYWGQGTTVTVSS

(SEQ ID NO: 34)

> 113.3 Hbk2CVR

EIQLVQSGSELKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYPGSGNTKYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYFCARYSNFFMDYWGQGTTVTVSS

(SEQ ID NO: 35)

> 113.3 Hbk3CVR

EIQLVQSGSELKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYPGSGNTKYNEKFKGRVTLTVDTSSSTAYMELSSLRSEDTAVYFCARYSNFFMDYWGQGTTVTVSS

(SEQ ID NO: 36)

In the above sequences, the underlined parts are heavy chain CDRs.

The light chain variable regions of the humanized antibodies of 113.3 are shown below:

> 113.3 L1CVR

DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWFQQKPGKAPKRLIYVASTLH
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIK

(SEQ ID NO: 37)

> 113.3 L2CVR

DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKPGKAPKRLIYVASTLH
SGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIK

(SEQ ID NO: 38)

> 113.3 L3CVR

DIQMTQSPSSLSASVGDRVTLTCRASQEISGYLSWLQQKPGKTPKRLIYVASTLH
SGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIK

(SEQ ID NO: 39)

> 113.3 L4CVR

DIQMTQSPSSLSASVGDRVTLTCRASQEISGYLSWLQQKPGKTIKRLIYVASTLHS
GVPSRFSGSRSGTDYTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIK

(SEQ ID NO: 40)

> 113.3 L1+4CVR

DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWFQQKPGKAIKRLIYVASTLHS
GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIK

(SEQ ID NO: 41)

> 113.3 L2+4CVR

DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWLQQKPGKAIKRLIYVASTLHS
GVPSRFSGSRSGTDYTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIK

(SEQ ID NO: 42)

In the above sequences, the underlined parts are light chain CDRs.

Each of the heavy chain variable regions described above and each of the light chain variable regions described above were linked to the human IgG1 heavy chain constant region and the human Cκ light chain constant region, respectively, to obtain full-length sequences.

The heavy chain full-length sequence and light chain full-length sequence of 113.3 Hbk2L1+4 are shown below as an example:

> 113.3 Hbk2C

EIQLVQSGSELKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYP
GSGNTKYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYFCARYSNFFMDY
WGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC

KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

(SEQ ID NO: 43)

> 113.3 L1+4C

DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWFQQKPGKAIKRLIYVASTLHSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

(SEQ ID NO: 44)

In the above sequences, the underlined part in the heavy chain is the human IgG1 Fc region, and the underlined part in the light chain is the human Cκ region.

The humanized heavy chain full-length sequences and light chain full-length sequences described above were combined to obtain the following humanized molecules. See Table 6:

Table 6. Anti-PD-1 humanized antibodies

Antibody	Heavy chain variable region	Light chain variable region
113.3	m113.3-HCVR (SEQ ID NO:8)	m113.3-LCVR (SEQ ID NO:9)
113.3 H1L1	113.3 H1CVR (SEQ ID NO:31)	113.3 L1CVR (SEQ ID NO:37)
113.3 H1L2		113.3 L2CVR (SEQ ID NO:38)
113.3 H1L3		113.3 L3CVR (SEQ ID NO:39)
113.3 H1L4		113.3 L4CVR (SEQ ID NO:40)
113.3 H2L1	113.3 H2CVR (SEQ ID NO:32)	113.3 L1CVR (SEQ ID NO:37)
113.3 H2L2		113.3 L2CVR (SEQ ID NO:38)
113.3 H2L3		113.3 L3CVR (SEQ ID NO:39)
113.3 H2L4		113.3 L4CVR (SEQ ID NO:40)
113.3 H3L1	113.3 H3CVR (SEQ ID NO:33)	113.3 L1CVR (SEQ ID NO:37)
113.3 H3L2		113.3 L2CVR (SEQ ID NO:38)
113.3 H3L3		113.3 L3CVR (SEQ ID NO:39)
113.3 H3L4		113.3 L4CVR (SEQ ID NO:40)
113.3 Hbk2L4	113.3 Hbk2CVR (SEQ ID NO:35)	113.3 L4CVR (SEQ ID NO:40)
113.3 Hbk2L1+4		113.3 L1+4CVR (SEQ ID NO:41)

113.3 Hbk2L2+4		113.3 L2+4CVR (SEQ ID NO:42)
113.3 Hbk3L3	113.3 Hbk3CVR (SEQ ID NO:36)	113.3 L3CVR (SEQ ID NO:39)
113.3 Hbk3L4	113.3 Hbk3CVR (SEQ ID NO:36)	113.3 L4CVR (SEQ ID NO:40)

Gene sequences encoding the above antibodies were synthesized and subcloned into pcDNA3.1 expression vectors. Each expression vector and the transfection reagent PEI were transfected into CHO cells in a 1:2 ratio, and the cells were incubated in a CO_2 incubator for 4-5 days. For the expressed antibodies, centrifugation was performed, and the supernatants were collected and then conventionally purified. Analysis showed that the target antibodies had been obtained.

Example 5: Determination of Affinities of Anti-PD-1 Antibodies

The affinities of anti-PD-1 antibodies for recombinant human PD-1 protein were determined by SPR.

Experimental method: An assay was performed using a Biacore 8K instrument (GE Healthcare). A Protein A sensor chip was used, and an HBS-EP+ buffer solution (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20) was used as the mobile phase. The antibodies to be tested were dissolved in the HBS-EP+ buffer solution as ligands and captured using the Protein A protein on the channels of the chip. Human PD-1 antigen protein (ACRO, PD1-H5221) was used as the analyte and dissolved in the HBS-EP+ buffer solution. The analyte was serially diluted 2-fold from 100 nM, and a total of 7 concentration points were obtained. The analyte was left to flow through the experimental channel and the reference channel at a flow rate of 30 μL/min, with an association time of 90 s and a dissociation time of 500 s. A regeneration buffer (10 mM glycine pH 1.5, GE Healthcare, 29238268-AA) was run at a flow rate of 10 μL/min for 30 s. Data were processed using Biacore 8K Evaluation analysis software. The corresponding reference channel (Fc 1) signal values were subtracted from the detection channel (Fc 2) signal values to obtain corrected signal curves. Affinity kinetic curves were fitted according to the 1:1 Langmuir binding model, and the association rate Ka, the dissociation rate Kd, and the dissociation constant (i.e., the affinity KD value) were calculated.

The results show that both the humanized antibodies 2E1-A06-H4 and 113.3 Hbk2L1+4 could bind to human PD-1 protein; their affinities are shown in Table 7.

Table 7. The affinities of anti-PD-1 antibodies for recombinant PD-1 protein

Antibody	K_{on} (1/Ms)	K_{off} (1/s)	K_D (M)

2E1-A06-H4	1.49E+05	4.20E-03	2.82E-08
113.3 Hbk2L1+4	9.82E+04	4.34E-04	4.42E-09

Example 6: Determination of Activity of Anti-PD-1 Antibodies

6.1. Assay for assessing antagonistic activity of anti-PD-1 antibodies against PD-L1

The antagonistic activity of humanized antibodies against PD-L1 was assessed using an assay system in which PD-L1-overexpressing cells were co-incubated with a Jurkat reporter gene cell line.

Experimental method: 40,000 PD-L1 aAPC/CHO-K1 cells (Promega) were plated in a 96-well plate and cultured overnight. The next day, the cells were washed with PBS buffer and 1640 culture medium, and 50,000 human PD-1-overexpressing Jurkat PD-1 NFAT reporter gene cells and the antibodies to be tested (different concentrations) were added. After the cells were cultured in an incubator at 37 °C for 6 h, the expression of the reporter gene was measured using Bright-Glo™ (Promega).

The results are shown in FIG. 3. Only the PD-1 antagonist camrelizumab induced significant expression of the luciferase reporter gene, and none of 2E1-A06-H4, 113.3 Hbk2L1+4, and peresolimab blocked the binding of PD-L1 to PD-1.

6.2. Inhibitory activity of anti-PD-1 antibodies in PBMC activation assay system

The inhibitory activity of humanized antibodies against the release of a cytokine from T cells was evaluated using a PBMC activation assay system.

Experimental method: PBMCs were incubated with 0.05 μg/mL PHA for 72 h and plated at a density of 200,000 cells/well in a 96-well plate pre-coated with 0.01 μg/mL anti-CD3 and the humanized antibodies (different concentrations). In addition, 0.2 μg/mL anti-CD28 was added, and incubation was performed. After the cells were cultured overnight at 37 °C, the secretion levels of IFNγ in the supernatants were measured using an HTRF human IFN gamma kit (Cisbio).

The results are shown in FIG. 4. Both 2E1-A06-H4 and 113.3 Hbk2L1+4 inhibited IFNγ secretion in a dose-dependent manner, and their activity was significantly superior to that of the agonist molecule peresolimab. The conclusions of this assay have been validated for reproducibility in 4 different PBMC donors. See Table 8.

Table 8. The inhibitory activity of anti-PD-1 antibodies against the secretion of IFNγ by T cells from different PBMC donors in a PBMC activation assay system

	2E1-A06-H4	113.3 Hbk2L1+4	Peresolimab
	Inhibition rate at 300 nM		
Donor 1	100%	96%	83%
Donor 2	100%	99%	62%

Donor 3	89%	75%	46%
Donor 4	78%	82%	54%

6.3. Inhibitory activity of humanized antibodies in CMV recall assay system

The inhibitory activity of humanized antibodies against memory T cells was evaluated using a CMV recall assay system.

Experimental method: 200,000 thawed PBMCs were co-incubated with a 3-50 ng/mL CMV peptide pool (Mabtech) and the humanized antibodies (different concentrations). After the cells were cultured for 5 days, the expression of the proliferation marker Ki67 (BD) in memory CD8+ T cells was measured using different fluorescently labeled antibodies and the fluorescent secondary antibody anti-Human IgG Fc (Invitrogen).

The results are shown in FIG. 5. Both 2E1-A06-H4 and 113.3 Hbk2L1+4 could inhibit memory CD8+ T cell proliferation, and their inhibitory activity was superior to that of the agonist molecule peresolimab. The conclusions of this assay have been validated for reproducibility in different PBMC donors. See Table 9.

Table 9. The inhibitory activity of humanized antibodies against memory T cells from different PBMC donors in a CMV recall assay system

Maximum inhibition rate	2E1-A06-H4	113.3Hbk2L1+4	Peresolimab
Donor 1-assay 1	50%	/	18%
Donor 1-assay 2	/	35%	17%
Donor 2	66%	51%	31%
Donor 3	72%	/	36%

Example 7: Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Activity of Anti-PD-1 Antibodies

The ability of humanized antibodies to mediate the killing of PD-1-positive cells by NK cells was evaluated using an ADCC assay.

Experimental method: CD4+ T cells were sorted and enriched from fresh primary human peripheral blood PBMCs using a sorting kit (Stem cell), activated for 48 h by pre-coating with 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28, and then labeled with CellTrace™ Violet (Invitrogen) in a dark place. After 20 min of incubation for labeling, the CD4+ T cells were further incubated with an antibody to be tested (100 nM), and fresh primary NK cells (NK:CD4+ T = 5:1) were added. After thorough mixing, the mixture was incubated at 37 °C for 4 h. The proportion of apoptotic cells among CD4+ T cells was determined using PI (Beyotime) staining.

The results are shown in FIG. 6 or Table 10. 2E1-A06-H4 could induce ADCC killing in two different donors, and its killing activity was superior to that of peresolimab.

Table 10. The antibody-dependent cell-mediated cytotoxicity (ADCC) activity of anti-PD-1 antibodies

Maximum killing rate	2E1-A06-H4	Peresolimab	IgG
Donor 1	23%	20%	11%
Donor 2	18%	12%	9%

Example 8: Verification of *In Vivo* Function and Effect of Anti-PD-1 Antibodies

8.1. Experiment for evaluating activity of anti-PD-1 antibodies in graft versus host disease (GvHD) model

Experimental method: Female NOG mice aged 6-8 weeks (Vital River) were acclimatized in an SPF environment. The evenly grouped mice were intravenously injected with primary human peripheral blood PBMCs (5,000,000 cells) to establish a model. From the day of modeling onward, each group of mice was treated with an equimolar dose of a drug, and the body weight and rejection score (evaluated based on skin, coat color, posture, and activity level) of each mouse were measured twice weekly. The mice were euthanized 35 days after induction. The specific experimental process is shown in FIG. 7, and the administration regimen is shown in Table 11. The mice were dosed according to the following regimen, and the agonistic activity of the humanized antibodies was assessed based on mouse body weight change and rejection score.

Table 11. The administration regimen for the graft versus host disease (GvHD) model

Group No.	Group	Number	Dose (mg/kg)	Route of administration	Administration time
1	Control group	3	/	/	/
2	Model group (Isotype)	5	1	Intraperitoneally	Twice weekly
3	Peresolimab	5	1	Intraperitoneally	Twice weekly
4	2E1-A06-H4	5	0.53	Intraperitoneally	Twice weekly

The results are shown in FIGs. 8A and 8B and Table 12. Both 2E1-A06-H4 and the control PD-1 agonist peresolimab significantly inhibited mouse body weight loss and inhibited rejection scores (the statistical differences between the data of each group and the data of the model group were calculated; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). 2E1-A06-H4 significantly outperformed the control peresolimab in maintaining mouse body weight and inhibiting rejection scores (the statistical differences between the data of the 2E1-A06-H4 group and the data of the peresolimab group were calculated; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; ####, $p < 0.0001$), indicating that in this model, 2E1-A06-H4 more significantly inhibited human PBMC activation and exhibited better

in vivo agonistic activity than peresolimab.

Table 12. The average body weight change percentages and average clinical scores of the graft versus host disease (GvHD) model

	Days after modeling	Control group	Model group	Peresolimab	2E1-A06-H4
Body weight change percentage	28	+15.0%	-8.9%	+1.5%	+11.8%
	32	+14.3%	-17.6%	-3.1%	+8.0%
	35	+17.2%	-15.5%	-5.3%	+7.8%
Clinical score	28	0.0	5.4	2.6	0.6
	32	0.0	7.6	2.6	1.0
	35	0.0	7.6	3.4	1.6

8.2. Experiment for evaluating activity of anti-PD-1 antibodies in collagen-induced arthritis (CIA) mouse model

In order to better simulate in mice the mechanism of action of antibodies in humans, we changed the Fc region of 2E1-A06-H4 to mouse IgG2a and designated the resulting antibody as 2E1-A06-H4-mIgG2a. For the antibodies peresolimab and 113.3 Hbk2L1+4, only their VH and VL sequences were kept unchanged, their heavy chain CH1 and Fc regions were changed to mIgG2a, and their light chain constant regions were changed to mouse Kappa; the resulting antibodies were designated as peresolimab-mIgG2a and 113.3 Hbk2L1+4-mIgG2a. The specific sequences are shown below:

> Peresolimab-mIgG2a HC

QVQLVQSGAEVKKPGASVKVSCKVSGYSLSKYDMSWVRQAPGKGLEWMGIIY
TSGYTDYAQKFQGRVTMTEDTSTDTAYMELSSLRSEDTAVYYCATGNPYYTNG
FNSWGQGTLVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLT
WNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDK
KIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSED
DPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKC
KVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPE
DIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSV
VHEGLHNHHTTKSFSRTPGK

(SEQ ID NO: 46)

> Peresolimab-mIgG2a LC

DIQMTQSPSSLSASVGDRVTITCQASQSPNNLLAWYQQKPGKAPKLLIYGASDL
PSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNNYYVGPVSYAFGGGTKVEI
KRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVL

NSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

(SEQ ID NO: 47)

> 2E1-A06-H4-mIgG2a

EVQLVESGGGVVQPGGSLRLSCATSGYVISRNCMAWFRQAPGKEREWVALTPG
GGTTYYTNSVKGRFTDSQDKNTVYLQMNSLKPEDTAVYYCAARIGFGYCTGTD
RDYTHWGQGTLVTVSSEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMIS
LSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPI
QHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKK
QVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVE
KKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

(SEQ ID NO: 48)

> 113.3 Hbk2L1+4-mIgG2a HC

EIQLVQSGSELKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWMGWIYP
GSGNTKYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYFCARYSNFFMDY
WGQGTTVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNS
GSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEP
RGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDV
QISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNN
KDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVE
WTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGL
HNHHTTKSFSRTPGK

(SEQ ID NO: 49)

> 113.3 Hbk2L1+4-mIgG2a LC

DIQMTQSPSSLSASVGDRVTITCRASQEISGYLSWFQQKPGKAIKRLIYVASTLHS
GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCLQYASYPYTFGGGTKVEIKRADA
APTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTD
QDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

(SEQ ID NO: 50)

Experimental method: Male C57BL/6 human PD-1 transgenic mice aged 8-9 weeks (Biocytogen) were acclimatized in an SPF environment. On days 0 and 21, an emulsion prepared from 0.2 mg of type II collagen and an adjuvant was intradermally injected into the base of the tail of each mouse. From the second induction onward, arthritis in the four limbs of the mice was scored three times a week. On day 30 after the first induction, the mice were evenly grouped according to mouse arthritis score and peripheral C-reactive protein level, and drug treatment was started at the same time. Thereafter, the scoring frequency was maintained at three times weekly. The mice were euthanized at the end of the experiment on day 57, and the right posterior joint was examined for pathological changes. The specific experimental process is shown in FIG. 9. The mice were dosed according to the following regimen, and the agonistic activity of the humanized antibodies was assessed based on mouse arthritis score and endpoint joint pathology score.

Table 13. The administration regimen for the collagen-induced arthritis (CIA) mouse model

Group No.	Group	Number	Dose (mg/kg)	Route of administration	Administration time
1	Control group	3	/	/	/
2	Model group (Isotype)	6	20	Intraperitoneally	Twice weekly
3	Peresolimab-mIgG2a	6	20	Intraperitoneally	Twice weekly
4	2E1-A06-H4-mIgG2a	6	3.5	Intraperitoneally	Twice weekly

The results show that 2E1-A06-H4-mIgG2a significantly inhibited mouse arthritis scores, while the control peresolimab-mIgG2a improved the total score of the disease to a relatively small extent. Individual analysis of the differences between the scores of the mice after administration and the scores at the time of grouping on day 30 showed that 2E1-A06-H4-mIgG2a could induce arthritis score changes of less than 0 in most animals, indicating that their arthritis was alleviated; in contrast, the control peresolimab-mIgG2a group exhibited individual disease alleviation in the later stage of the disease (Table 14). The above data indicate that in the mouse arthritis model, 2E1-A06-H4-mIgG2a had the pharmacodynamic advantage of significantly inhibiting joint damage over peresolimab-mIgG2a.

Table 14. The arthritis scores and arthritis alleviation rate of each group of mice in the arthritis (CIA) mouse model

Days after first induction		Model group	Peresolimab-mIgG2a 20 mg/kg	2E1-A06-H4-mIgG2a 3.53 mg/kg
Day 30	Score	7.00±1.03	6.83±1.01	6.67±0.95
Day 53	Score	8.67±1.98	7.33±1.31	4.33±0.84
	Alleviation rate	17%	33%	100%

The score data are presented as Mean ± SEM. The arthritis alleviation rate is defined as the proportion of individuals exhibiting an arthritis score change of <0 compared to the arthritis score at the time of grouping and administration.

CLAIMS

1. A PD-1-binding protein, comprising at least one immunoglobulin single variable domain, wherein the immunoglobulin single variable domain comprises a CDR1, a CDR2, and a CDR3 from the amino acid sequence set forth in any one of SEQ ID NOs: 2 and 24-29, and the CDR1, the CDR2, and the CDR3 are defined according to the Kabat, IMGT, Chothia, AbM, or Contact numbering scheme.

2. The PD-1-binding protein according to claim 1, wherein the immunoglobulin single variable domain comprises a CDR1, a CDR2, and a CDR3, and the CDR1, the CDR2, and the CDR3 comprise the amino acid sequences set forth in SEQ ID NOs: 3-5, respectively.

3. The PD-1-binding protein according to claim 1 or 2, wherein the immunoglobulin single variable domain is camelid-derived, engineered by humanization, and/or engineered by affinity maturation.

4. The PD-1-binding protein according to any one of claims 1 to 3, wherein the immunoglobulin single variable domain comprises the amino acid sequence set forth in any one of SEQ ID NOs: 2 and 24-29 or an amino acid sequence having at least 90% sequence identity thereto.

5. The PD-1-binding protein according to any one of claims 1-4, comprising the amino acid sequence set forth in SEQ ID NO: 30, 7, or 48 or an amino acid sequence having at least 90% sequence identity thereto.

6. The PD-1-binding protein according to any one of claims 1-5, wherein the PD-1-binding protein is an anti-PD-1 antibody; preferably, the anti-PD-1 antibody is a camelid antibody, a chimeric antibody, a humanized antibody, or an antigen-binding fragment thereof.

7. A PD-1-binding protein, comprising a heavy chain variable region (VH) and a light chain variable region (VL), wherein:
the VH comprises a HCDR1, a HCDR2, and a HCDR3 from the amino acid sequence set forth in any one of SEQ ID NOs: 8 and 31-36, and the VL comprises a LCDR1, a LCDR2, and a LCDR3 from the amino acid sequence set forth in any one of SEQ ID NOs: 9 and 37-42;
the CDRs are defined according to the Kabat, IMGT, Chothia, AbM, or Contact numbering scheme.

8. The PD-1-binding protein according to claim 7, wherein the heavy chain variable region comprises a HCDR1, a HCDR2, and a HCDR3, and the HCDR1, the HCDR2, and the HCDR3 comprise the amino acid sequences set forth in SEQ ID NOs: 10-12, respectively; and the light chain variable region comprises a LCDR1, a LCDR2, and a LCDR3, and the LCDR1, the LCDR2, and the LCDR3 comprise the amino acid sequences set forth in SEQ ID NOs: 13-15, respectively.

9. The PD-1-binding protein according to claim 7 or 8, wherein the PD-1-binding protein is an anti-PD-1 antibody, and the anti-PD-1 antibody is a murine-derived antibody, a chimeric antibody, or a humanized antibody or an antigen-binding fragment thereof;
preferably, the humanized antibody uses IGHV1-3 as a human germline heavy chain template and/or IGkV1-16 as a human germline light chain template.

10. The PD-1-binding protein according to any one of claims 7 to 9, wherein:
the VH comprises the amino acid sequence set forth in any one of SEQ ID NOs: 8 and 31-36 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in any one of SEQ ID NOs: 9 and 37-42 or an amino acid sequence having at least 90% sequence identity thereto;
preferably,
the VH comprises the amino acid sequence set forth in SEQ ID NO: 35 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 41 or an amino acid sequence having at least 90% sequence identity thereto;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37 or an amino acid sequence having at least 90% sequence identity thereto;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity thereto;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 31 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid

sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 32 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 37 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 38 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 33 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 35 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 35 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 42 or an amino acid sequence having at least 90% sequence identity thereto;

the VH comprises the amino acid sequence set forth in SEQ ID NO: 36 or an amino acid

sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 39 or an amino acid sequence having at least 90% sequence identity thereto;
the VH comprises the amino acid sequence set forth in SEQ ID NO: 36 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 40 or an amino acid sequence having at least 90% sequence identity thereto; or
the VH comprises the amino acid sequence set forth in SEQ ID NO: 8 or an amino acid sequence having at least 90% sequence identity thereto, and the VL comprises the amino acid sequence set forth in SEQ ID NO: 9 or an amino acid sequence having at least 90% sequence identity thereto.

11. The PD-1-binding protein according to any one of claims 1 to 10, further comprising an immunoglobulin Fc region, wherein preferably, the Fc region is an Fc region of human IgG1, IgG2, or IgG4.

12. The PD-1-binding protein according to claim 11, comprising a heavy chain and a light chain, wherein:
the heavy chain comprises the amino acid sequence set forth in SEQ ID NO: 43 or an amino acid sequence having at least 90% sequence identity thereto, and the light chain comprises the amino acid sequence set forth in SEQ ID NO: 44 or an amino acid sequence having at least 90% sequence identity thereto;
the heavy chain comprises the amino acid sequence set forth in SEQ ID NO: 49 or an amino acid sequence having at least 90% sequence identity thereto, and the light chain comprises the amino acid sequence set forth in SEQ ID NO: 50 or an amino acid sequence having at least 90% sequence identity thereto; or
the heavy chain comprises the amino acid sequence set forth in SEQ ID NO: 18 or an amino acid sequence having at least 90% sequence identity thereto, and the light chain comprises the amino acid sequence set forth in SEQ ID NO: 19 or an amino acid sequence having at least 90% sequence identity thereto.

13. A polynucleotide, encoding the PD-1-binding protein according to any one of claims 1 to 12.

14. A host cell, comprising the polynucleotide according to claim 13.

15. A pharmaceutical composition, comprising the PD-1-binding protein according to any one of claims 1 to 12 and at least one pharmaceutically acceptable excipient, diluent, or carrier.

16. A method for preparing a PD-1-binding protein, comprising:
1) culturing the host cell according to claim 14, and
2) isolating the expressed PD-1-binding protein from the host cell.

17. Use of the PD-1-binding protein according to any one of claims 1 to 12 or the pharmaceutical composition according to claim 15 in the manufacture of a medicament for treating an inflammatory or autoimmune disease, wherein:
preferably, the inflammatory or autoimmune disease is selected from the group consisting of graft versus host disease (GvHD), transplant rejection, arthritis (CIA), and rheumatoid arthritis (RA).

18. A method for treating an inflammatory or autoimmune disease, comprising administering to a subject a therapeutically effective amount of the PD-1-binding protein according to any one of claims 1 to 12, the polynucleotide according to claim 13, or the pharmaceutical composition according to claim 15, wherein:
preferably, the inflammatory or autoimmune disease is selected from the group consisting of graft versus host disease (GvHD), transplant rejection, arthritis (CIA), and rheumatoid arthritis (RA).

19. A method for down-regulating an immune response, comprising administering to a subject a therapeutically effective amount of the PD-1-binding protein according to any one of claims 1 to 12, the polynucleotide according to claim 13, or the pharmaceutical composition according to claim 15.

20. A method for inhibiting T cell activity, comprising administering to a subject in need thereof an effective amount of the PD-1-binding protein according to any one of claims 1 to 12, the polynucleotide according to claim 13, or the pharmaceutical composition according to claim 15, wherein:
preferably, the inhibiting T cell activity is selected from the group consisting of at least one of the following characteristics:
(a) inhibiting cytokine-releasing activity of T cells, e.g., inhibiting IFNγ-secreting activity of T cells;
(b) inhibiting T cell proliferation, e.g., inhibiting memory CD8+ T cell proliferation; and
(c) reducing PD-1-positive T cells.