

NO: C2015105.

The inventors surprisingly found that:

Hybridoma cell line LT002 is capable of secreting a specific monoclonal antibody (named 4G10) that binds specifically to CTLA4, and the monoclonal antibody can effectively block the interaction of CTLA4 to B7.

Hybridoma cell line LT003 is capable of secreting a specific monoclonal antibody (named 14C12) that specifically binds to PD-1, and the monoclonal antibody can effectively block the interaction of PD-1 to PDL1.

Furthermore, the inventors generated humanized antibodies against CTLA4 (named 4G10H1L1, 4G10H3L3, 4G10H4L3 and 8D2H14L2, respectively) and humanized antibodies against PD-1 (named 14C12H1L1) in a creative way.

Furthermore, the inventors created a series of new humanized bispecific antibodies (named BiAb001 BiAb002 BiAb003, BiAb004, BiAb007 and BiAb010, respectively) via recombining the two kinds of humanized antibodies, which can bind both CTLA4 and PD-1, and block interactions of CTLA4 with B7, and PD-1 with PDL1. The bispecific antibodies can effectively bind and activate human T cells, induce lymphocytes to secrete IFN- γ and IL-2, with the potential to be prepared into drugs for prevention and treatment of cancers, such as lung cancer, melanoma, renal cancer, ovarian cancer and leukemia.

The following are provided by the present disclosure:

In one example, there is provided a bispecific antibody thereof, wherein,
the first protein functional area targets PD-1,
and

the second protein functional area targets CTLA4.

In one aspect, the present disclosure refers to a bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a

light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

In another aspect, the present disclosure refers to a bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises comprising a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

In another aspect, the present disclosure refers to a bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain

variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

In one example of the disclosure, the said bispecific antibody, wherein the said first and second protein functional areas are connected directly or via connecting fragments; Preferably, the connecting fragments are (GGGGS) n , and n is a positive integer, such as 1, 2, 3, 4, 5 or 6.

In one example of the disclosure, the said bispecific antibody, wherein,
the said first and second protein functional areas are respectively immunoglobulins

or their antigen-binding fragments, such as half antibody, Fab, F(ab')₂ or single-chain antibody.

Preferably, the said first protein functional area is an immunoglobulin, and the said second protein functional area is a single-chain antibody;

or,

Preferably, the said first protein functional area is a single-chain antibody, and the said second protein functional area is an immunoglobulin.

In one example of the disclosure, the said bispecific antibody, wherein, the quantity of the first protein functional area or the second protein functional area is one, two, or more, independently.

In one example of the disclosure, the said bispecific antibody, wherein, the said immunoglobulin is IgG, IgA, IgD, IgE or IgM; Preferably IgG, such as IgG1, IgG2, IgG3, or IgG4.

In one example of the disclosure, the said bispecific antibody, wherein, the said single-chain antibody is attached at the c-terminal of the heavy chain of the immunoglobulin. Since one immunoglobulin consists of two heavy chains, thus one immunoglobulin molecule is linked to two single-chain antibody molecules. Preferably, the two said single-chain antibody molecules are the same.

In one example of the disclosure, the said bispecific antibody, wherein,
the heavy chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 29-31, and the light chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 32-34;

And/or,

the heavy chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 35 – 37, or SEQ ID NO: 35, SEQ ID NO: 41 and SEQ ID NO: 37, or SEQ ID NO: 42-44; and the light chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 38-40, or SEQ ID NO: 45-47.

In one example of the disclosure, the said bispecific antibody, wherein,

the heavy chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 35-37, or SEQ ID NO: 35, SEQ ID NO: 41 and SEQ ID NO: 37, or SEQ ID NO: 42-44; and the light chain variable region of the said immunoglobulin comprises CDRs with the amino acid sequences of SEQ ID NO: 38-40, or SEQ ID NO: 45-47;

and/or,

the heavy chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 29-31; and the light chain variable region of the said single-chain antibody comprises CDRs with the amino acid sequences of SEQ ID NO: 32-34.

In one example of the disclosure, the said bispecific antibody, wherein,

the amino acid sequence of the heavy chain variable region of the said immunoglobulin is selected from SEQ ID NO. 16 or SEQ ID NO. 20; the amino acid sequence of the light chain variable region of the said immunoglobulin is selected from SEQ ID NO: 18 or SEQ ID NO: 22;

And/or,

the amino acid sequence of the heavy chain variable region of the said single-chain antibody is selected from SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 10, SEQ ID NO: 14 or SEQ ID NO: 25; the amino acid sequence of the light chain variable region of the said single chain antibody is selected from SEQ ID NO: 4, SEQ ID NO: 8, SEQ ID NO: 12 or SEQ ID NO: 27.

In one example of the disclosure, the said bispecific antibody, wherein,

the amino acid sequence of the heavy chain variable region of the said immunoglobulin is selected from SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 10, SEQ ID NO: 14 or SEQ ID NO: 25; the amino acid sequence of the light chain variable region of the single-chain antibody is selected from SEQ ID NO: 4, SEQ ID NO:8, SEQ ID NO:12 or SEQ ID NO:27;

and/or,

The amino acid sequence of the heavy chain variable region of the said single-chain antibody is selected from SEQ ID NO: 16 or SEQ ID NO: 20; the amino acid sequence of

the light chain variable region of the said immunoglobulin is selected from SEQ ID NO: 18 or SEQ ID NO: 22.

In one example of the disclosure, the said bispecific antibody, wherein,
the said immunoglobulin contains non-CDR regions from species other than mouse, for example, from human.

In one example of the disclosure, the constant region of the said immunoglobulin is humanized. For example, the constant region of the heavy chain is Ig gamma-1 chain C region, ACCESSION: P01857; the constant region of light chain is Ig kappa chain C region, ACCESSION: P01834.

In one example of the disclosure, the said bispecific antibody, wherein, the said bispecific antibody binds to CTLA4 protein and/or PD-1 protein with a K_D less than approximately 10^{-5} M, such as less than approximately 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less.

The present disclosure also relates to a bispecific antibody, whose heavy chain variable region comprises CDRs with amino acid sequences of SEQ ID NO: 29-31, SEQ ID NO: 35-37, or SEQ ID NO: 35, SEQ ID NO: 41, and SEQ ID NO: 37, or SEQ ID NO: 42-44;

and,

of SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47;

And light chain variable region comprises CDRs with amino acid sequences of SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47;

Preferably, the CDRs in the light chain variable region and the heavy chain variable region are not the same.

Disclosed is an isolated nucleic acid molecule comprising a nucleotide sequence capable of encoding the heavy chain variable region of the antibody, wherein,

the heavy chain variable region of the said antibody comprises CDRs with amino acid sequences selected from SEQ ID NO: 29-31, SEQ ID NO: 35-37, or SEQ ID NO: 35, SEQ ID NO: 41, and SEQ ID NO: 37, or SEQ ID NO: 42-44;

and,

selected from SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47.

In another aspect, the present disclosure refers to an isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31, and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

In another aspect, the present disclosure refers to an isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

In another aspect, the present disclosure refers to an isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein

the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,

and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

Disclosed is an isolated nucleic acid molecule comprising a nucleotide sequence capable of encoding the light chain variable region of the antibody, wherein,

the light chain variable region of the said antibody comprises CDRs with amino acid sequences selected from SEQ ID NO: 32-34, or SEQ ID NO: 38-40, or SEQ ID NO: 45-47.

In another aspect, the present disclosure refers to an isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein,

the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

Disclosed is a vector comprising the isolated nucleic acid molecule described in the present disclosure.

In another aspect, the present disclosure refers to a vector, comprising the isolated nucleic acid molecule as described herein or any combination thereof.

Disclosed is a host cell comprising the isolated nucleic acid molecule described in the present disclosure, or the vector described in the present disclosure.

In another aspect, the present disclosure refers to a host cell line, comprising the isolated nucleic acid molecule as described herein or any combination thereof, or the vector as described herein.

Disclosed is a method for preparing the bispecific antibodies described in the present disclosure, by culturing the host cells in the present disclosure under appropriate conditions, and recovering the said bispecific antibodies from the cell culture.

In another aspect, the present disclosure refers to a method for preparing the bispecific antibody as described herein, the method comprising culturing the host cell line as described herein under appropriate conditions, and recovering the bispecific antibody from the cell culture.

Disclosed are conjugates, including the bispecific antibodies described in the present disclosure and a conjugating partner as a detectable marker. Specifically, the said conjugating partners are radioactive isotopes, fluorescein, luminescent materials, colorful substances, or enzymes.

In another aspect, the present disclosure refers to a conjugate comprising the bispecific antibody as described herein and a conjugating partner as a detectable marker.

Disclosed are reagent kits, consisting of the bispecific antibodies or the conjugates described in the disclosure.

In another aspect, the present disclosure refers to a kit comprising the bispecific antibody as described herein, or the conjugate as described herein.

Specifically, the reagent kits may contain a secondary antibody, which specifically recognizes the said bispecific antibody; optionally, such secondary antibody may contain detectable markers such as radioactive isotopes, fluorescein, luminescent materials, colorful substances, or enzymes.

Disclosed is usage of the said bispecific antibodies described in the present disclosure to prepare reagent kits for detection of the existence or the levels of CTLA4 and/or PD-1 in samples.

In another example, there is provided use of the bispecific antibody as described herein in the preparation of a kit for the detection of the existence or the levels of CTLA4 and/or PD-1 in a sample.

Disclosed is a pharmaceutical composition comprising the said

bispecific antibodies or the conjugates described in the disclosure. Optionally, it may also comprise a pharmaceutically acceptable carrier or excipient.

In another aspect, the present disclosure refers to a pharmaceutical composition, comprising the bispecific antibody as described herein, or the conjugate as described herein.

Disclosed is use of the bispecific antibodies or conjugates described in the disclosure for producing drugs that are used for prevention and/or treatment of tumors or anemia, or for diagnosis of tumors or anemia; specifically, the said tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer and leukemia.

In another aspect, the present disclosure refers to use of the bispecific antibody as described herein, or the conjugate as described herein, or the pharmaceutical composition as described herein for the manufacture of a medicament for the treatment of a tumor.

In another aspect, the present disclosure refers to use of the bispecific antibody as disclosed herein, the conjugate as disclosed herein or the pharmaceutical composition as disclosed herein for the manufacture of a medicament for the treatment of anemia.

In another aspect, the present disclosure refers to use of the bispecific antibody as disclosed herein, the conjugate as disclosed herein or the pharmaceutical composition as disclosed herein for the manufacture of a medicament for the treatment of a viral or parasitic infection.

The present inventors have found through animal experiments that, bispecific antibody BiAb004 described in the disclosure can effectively inhibit the growth of MC38 tumor cells inoculated subcutaneously in right side of PD-1 HuGEMM mice, which the growth of tumor volume in PD-1 HuGEMM tumor-bearing mice is significantly inhibited.

Disclosed is the use of the bispecific antibodies or the conjugates described in the present disclosure, to prepare drugs with the following purposes:

- Testing CTLA4 level in samples,
- Blocking CTLA4 binding to B7,
- Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,
- Removing immunosuppression of CTLA4,
- Activating T lymphocytes, or

Increasing the secretion of IL-2 in T lymphocytes;

And/or,

Blocking PD-1 binding to PDL1,

Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

Removing immunosuppression of PD-1, or

Increasing the secretion of IFN- γ in T lymphocytes.

In another example, there is provided use of the bispecific antibody as described herein, or the conjugate as described herein, for the manufacturing of a medicament selected from the following:

a medicament for testing CTLA4 level in samples,

a medicament for blocking CTLA4 binding to B7,

a medicament for regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

a medicament for removing immunosuppression of CTLA4,

a medicament for activating T lymphocytes, or

a medicament for increasing the secretion of IL-2 in T lymphocytes;

And/or,

a medicament for blocking PD-1 binding to PDL1,

a medicament for regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,

a medicament for removing immunosuppression of PD-1, or

a medicament for increasing the secretion of IFN- γ in T lymphocytes.

The present disclosure relates to an in vivo or in vitro method to apply to cells or subjects in need with an effective dose of the bispecific antibodies or the conjugates described in the present disclosure, and the said method is selected from the following:

Testing CTLA4 level in samples,

Blocking CTLA4 binding to B7,
Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,
Removing immunosuppression of CTLA4,
Activating T lymphocytes, or
Increasing the secretion of IL-2 in T lymphocytes;
And/or,
Blocking PD-1 binding to PDL1,
Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,
Removing immunosuppression of PD-1, or
Increasing the secretion of IFN- γ in T lymphocytes.

In another example, there is provided an in vivo or in vitro method, comprising administering to a cell or a subject in need with an effective dose of the bispecific antibody as described herein, or the conjugate as described herein, and for the purpose selected from the following:

Testing CTLA4 level in samples,
Blocking CTLA4 binding to B7,
Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,
Removing immunosuppression of CTLA4,
Activating T lymphocytes, or
Increasing the secretion of IL-2 in T lymphocytes;
And/or,
Blocking PD-1 binding to PDL1,
Regulating (e.g. down-regulating) PD-1 activity or PD-1 levels,
Removing immunosuppression of PD-1, or
Increasing the secretion of IFN- γ in T lymphocytes.

In in vitro experiments in the present disclosure, the anti-CTLA4 antibodies, the anti-PD-1 antibodies, and the anti-CTLA4-anti-PD-1 bispecific antibodies described in the present disclosure all can induce the secretion of IFN- γ , and activate the immune response.

Disclosed is a method for the prevention and/or treatment of tumors or anemia, or for diagnosis of tumors or anemia, including

procedures to apply to subjects in need with an effective dose of the bispecific antibodies or the conjugates described in the present disclosure; specifically, the said tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer or leukemia.

In another example, there is provided use of the bispecific antibody as described herein, or the conjugate as described herein, for the manufacturing of a medicament for the prevention, treatment, adjuvant treatment, and/or diagnosis of tumors or anemia in a subject in need thereof; preferably, the tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer or leukemia.

The use of bispecific antibodies or conjugates thereof described in the present disclosure for the prevention and/or treatment of tumors or anemia, or for diagnosis of tumors or anemia; specifically, the said tumors may be melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer or leukemia.

Use of bispecific antibodies or the conjugates thereof described in the present disclosure, with the following purposes:

Blocking CTLA4 binding to B7,

Regulating (e.g. down-regulating) CTLA4 activity or CTLA4 levels,

HCDR1: GFAFSSYD (SEQ ID NO: 29)

HCDR2: ISGGGRYT (SEQ ID NO: 30)

HCDR3: ANRYGEAWFAY (SEQ ID NO: 31)

HCDR4: GFTFSDNW (SEQ ID NO: 42)

HCDR5: IRNKPYN YET (SEQ ID NO: 43)

HCDR6: TAQFAY (SEQ ID NO: 44)

HCDR7: ENIYGG (SEQ ID NO: 45)

HCDR8: GAT (SEQ ID NO: 46)

HCDR9: QNVLRSPFTF (SEQ ID NO: 47)

The amino acid sequences of three CDRs of the light chain variable region are the same as those of BiAb001.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Furthermore, laboratory techniques of cell and tissue culture, molecular genetics, oligo- or polynucleotide chemistry, and immunology described herein are those well-known and commonly used in the art. Meanwhile, to better understand the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

As used herein, the term "amino acid sequence of CTLA4 (Cytotoxic T-Lymphocyte Antigen 4)" refers to not only the full-length CTLA4 protein, but also, the extracellular fragment of CTLA4 (CTLA4ECD), or fragments containing CTLA4ECD, or fusion proteins of CTLA4ECD, such as fragments of fusions with mouse or human IgG Fc fragments (mFc or hFc). However, understood by those of ordinary skill in the art, the amino acid sequence of CTLA4 protein can have natural or artificial mutations or variation (including but not limited to substitutions, deletions, and/or additions), not affecting its biological functions. Thus, in the present disclosure, the term "CTLA4 protein" also includes these amino acid sequences containing natural or artificial variants. Additionally, when referring to sequence fragments of CTLA4 protein, the sequence fragments containing natural or artificial variants are also included.

As used herein, the term “amino acid sequence of PD-1 (Programmed cell death protein 1, NCBI GenBank: 005018 NM)” refers to not only the full-length PD-1 protein, but also, the extracellular fragment of PD-1, PD-1ECD), or fragments containing PD-1ECD, or fusion proteins of PD-1ECD, such as fragments of fusions with mouse or human IgG Fc fragments (mFc or hFc). However, understood by those of ordinary skill in the art, the amino acid sequence of PD-1 protein can have natural or artificial mutations or variation (including but not limited to substitutions, deletions, and/or additions), not affecting its biological functions. Thus, in the present disclosure, the term “PD-1 protein” also includes these amino acid sequences containing natural or artificial variants. Additionally, when referring to sequence fragments of PD-1 protein, the sequences fragments containing natural or artificial variants are also included.

As used in this disclosure, if not specifically stated, B7 protein described herein is B7-1 and/or B7-2 protein whose amino acid sequences are well known in the prior art, which can be referenced from the existing literature or sequences disclosed in GenBank. For example, B7-1 (CD80, NCBI Gene ID: 941) and B7-2 (CD86, NCBI Gene ID: 942).

As used herein, the term “EC₅₀” refers to the concentration of 50% of maximal effect.

As used herein, the term "antibody" refers to an immunoglobulin molecule normally composed of two pairs of peptides (each pair with a "light" (L) chain and a "heavy" (H) chain). In general, the heavy chain can be comprehended as the polypeptide chain with a higher molecular weight, while the light chain refers to the polypeptide chain with a lower molecular weight. The light chains of an antibody are classified as either κ or λ light chains, while the heavy chains of an antibody are classified as μ , δ , γ , α or ϵ heavy chains, which define the antibody isotypes as IgM, IgD, IgG, IgA, and IgE, respectively.

Within a light and a heavy chain, the variable region and the constant region are connected through a "J" region consisting of about 12 or more amino acids, and a heavy chain also contains a "D" region consisting of about three or more amino acids. A heavy chain consists of a heavy chain variable region (V_H) and a heavy chain constant region (C_H). A heavy chain constant region consists of three structural domains (C_{H1}, C_{H2}, and

C_{H3}). A light chain consists of a light chain variable region (V_L) and a light chain constant region (C_L). The constant region of a light chain consists of a structural domain C_L. The constant region of an antibody mediates the binding of an immunoglobulin to host tissues or factors, including various immune cells (e.g. effector cells) and the complement component 1q (C1q) of the classical complement system. V_H and V_L regions can further be subdivided into regions with high variability (known as complementarity determining region (CDR)), separated by relatively conservative regions called framework region (FR). Each V_H and V_L comprises three CDRs and four FRs in the order of FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4 from the amino terminus to the carboxyl terminus. The variable regions (V_H and V_L) of each heavy/light chain form the antibody binding sites respectively. Distribution of amino acids to the regions or domains follows the definitions by Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md.) (1987 and 1991), or Chothia & Lesk (1987) *J. Mol. Biol.* 196:901-917; Chothia et al. (1989) *Nature* 342:878-883. In particular, heavy chains can also contain more than three CDRs, such as 6, 9, or 12. For example, in the case of the bispecific antibodies in the present disclosure, the heavy chain can be a heavy chain of an IgG antibody with a scFv of another antibody connected to its C terminus, thus this heavy chain contains 9 CDRs. The term "antibody" is not restricted by any particular method of making antibodies. For example, it includes, in particular, recombinant antibodies, monoclonal antibodies or polyclonal antibodies. Antibodies can be of different isoforms, such as IgG (for example, IgG1, IgG2, IgG3 or IgG4 subtypes), IgA1, IgA2, IgD, IgE or IgM antibodies.

As used herein, the term "antigen-binding fragments" refers to polypeptides containing fragments of a full-length antibody, maintaining the ability to bind specifically to the same antigen, and/or to compete with the full length antibody against the antigen, which is also called "the antigen binding portion". See *Fundamental Immunology*, Ch. 7 (Paul, W., 2nd edition, Raven Press, N.Y. (1989)), including the entire article and references in this disclosure for all purposes. Antigen-binding fragments can be produced by recombinant DNA techniques or by cleaving intact antibodies with proteolytic enzymes or chemicals. In some cases, the antigen-binding fragments include Fab, Fab',

antibody, and screened for specificity in the same manner as for the full antibody.

In the present disclosure, unless specified otherwise, the term "antibody" refers to not only the intact antibody, but also the antigen binding fragments of the antibody.

As used in this disclosure, the terms "mAb" and "monoclonal antibodies" refers to an antibody or a fragment of an antibody that is derived from a group of highly homologous antibodies, i.e. from a group of identical antibody molecules, except for mutations that may arise spontaneously. Monoclonal antibody has high specificity against a single epitope on the antigen. Polyclonal antibodies are different from monoclonal antibodies, containing at least 2 or more different antibodies, which usually recognize different epitopes on the antigen. Monoclonal antibodies can be obtained with hybridoma technology reported originally by Kohler et al., (Nature, 256: 495, (1975)), as well as recombinant DNA Technology (see U.S. Patent 4,816,567).

As used in this disclosure, the term "chimeric antibody" refers to an antibody in which parts of the light chain and/or heavy chain are from one antibody (can be from a particular species or belong to a specific antibody class or subclass), and the other parts of the light chain and/or heavy chain are from another antibody (can be from the same or different species or belong to the same or different antibodies classes or subclass). Nevertheless, it retains antigen binding activity (U.S.P to Cabilly et al., 4816567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851 6855 (1984)).

As used in this disclosure, the term "humanized antibody" refers to an antibody or its fragments, derived from a human immunoglobulin (receptor antibody), whose CDRs or parts of CDRs are replaced by CDRs from a non-human antibody (donor antibody), where the donor antibody may be a non-human antibody (for example, from mice, rats, or rabbits) with predictable specificity, binding affinity, and reactivity. In addition, to further improve or optimize the performance of the antibody, some amino acid residues in framework regions (FR) of the receptor antibody can also be replaced by the corresponding amino acid residues of non-human species, or replaced by the corresponding amino acid residues of other antibodies. For more details on humanized antibodies, see for example Jones, et al., Nature, 321: 522-525 (1986); Reichmann et al., Nature, 332: 323-329 (1988); Presta, Curr. Op. Struct. Biol., 2: 593-596 (1992); and

Clark, Immunol. Today, 21: 397-402 (2000).

As used in this disclosure, the term "Epitope" refers to a site on the antigen that the immunoglobulin or antibody can specifically bind to. "Epitope" is also known as the "antigenic determinant" in this field. Epitope or antigenic determinants usually consist of chemically active surface groups of molecules, such as amino acids, carbohydrates or glycoside chains, and usually have specific three dimensional structures, as well as specific charge characteristics. For example, epitopes typically consist of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 consecutive or non-consecutive amino acids in a unique spatial conformation, which can be "linear" or "conformational". See, for example, Epitope Mapping Protocols in Methods in Molecular Biology, volume 66, G. E. Morris, Ed. (1996). In a linear epitope, the interacting points between the protein and interacting molecule (e.g., antibodies) exist linearly along the primary amino acid sequence; while in a conformational epitope, the interacting points are separated along the primary amino acid sequence.

As used in this disclosure, the term "isolate" or "isolated" refers to obtained by artificial means in the natural state. If there is an "isolated" substance or component in nature, it may be due to the change in its natural environment, or isolated from the natural environment, or both. For example, polynucleotide or polypeptide in a natural existence in a living animal will be called "isolated" if it was separated with high purity in the same natural state. The term "isolate" or "isolated" does not exclude existence of artificial or synthetic material, or other impurities that does not affect the activity.

As used in this disclosure, the term "*E. coli* expression system" refers to the expression system composed of *Escherichia coli* (strain) and vector, where *E. coli* (strain) is commercially available, including but not limited to: GI698, ER2566, BL21 (DE3), B834 (DE3), and BLR (DE3).

As used in this disclosure, the term "vector" refers to a nucleic acid delivery vehicle that can be inserted with a polynucleotide. The vector that can have the protein that is encoded by the inserted polynucleotide expressed is called an expression vector. Vectors can be inserted into the host cell by transformation, transduction, or transfection, so that the genetic substances carried by the vector can be expressed in the host cell. Vectors are

well known to the technical personnel in the field, including but not limited to: plasmid; phasmid; cosmid; artificial chromosome such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1 derived artificial chromosome (PAC); phage such as λ phage or M13 phage and animal viruses etc. Animal viruses may include but not limited to, reverse transcriptase virus (including lentivirus), adenovirus, adeno-associated virus, herpes virus (e. g. herpes simplex virus), chicken pox virus, baculovirus, papilloma virus, and papova virus (such as SV40). A vector can contain multiple components that control expression, including but not limited to, promoter, transcription initiation factor, enhancer, selection element, and reporter gene. In addition, the vector may also contain replication initiation site.

As used in this disclosure, the term "host cell" refers to cells that can import vectors, including but not limited to, prokaryotic cells such as *E. coli* and *Bacillus subtilis*, fungal cells such as yeast and *Aspergillus*, insect cells such as S2 drosophila cells and Sf9, or animal cells such as fibroblast cells, CHO cells, COS cells, NSO cells, HeLa cells, BHK cells, HEK293 cells or human cells.

As used in this disclosure, "Homologous" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in the two compared sequences is occupied by the same base or amino acid, e.g., if a position in two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The "percent homology" of two sequences is calculated by the function of the numbers of matched positions of the two sequences divided by the total numbers of positions that are compared multiplied by 100. For example, if 6 out of 10 positions of two sequences are matched, thus the homology of the two sequences is 60%. For example, homology of DNA sequences CTGACT and CAGGTT is 50% (3 out of 6 positions are matched). Generally, a comparison is made when two sequences are aligned to give maximum identity. Such alignment can be simply generated using computer programs, for instance, the ALIGN program (DNASTar, Inc.), implemented by the method of Needleman et al. (1970) J. Mol. Biol. 48: 443-453. Or, using the algorithm proposed by E. Meyers and W. Miller (Comput. Appl Biosci. , 4:11-17 (1988)) that has been integrated into ALIGN program (version 2.0), in which the percent homology of

two sequences is calculated by using PAM120 residue weight table, a gap length penalty of 12 and gap penalty of 4. In addition, GAP program that has been integrated into the GCG software package (available on www.gcg.com) implemented with algorithm of Needleman and Wunsch (J Mol Biol. 4-453 (1970)), Blossum 62 matrix, PAM250 matrix as well as 16, 14, 12, 10, 8, 6 or 4 GAP weight and 1, 2, 3, 4, 5 or 6 length weight can be used to measure the percent homology of two amino acid sequences.

As used in this disclosure, the term "specific binding" refers to non-randomly binding between two molecules, i.e., interaction between antibodies and antigen. In some examples, the antibody specifically binding to the antigen (or antibody with specificity to an antigen) refers that the antibody binds the antigen with an affinity (K_D) smaller approximately than 10^{-5} M, such as smaller than 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M or even less. In some examples of the disclosure, the term "target(ed)" refers to specific binding.

As used in this disclosure, the term " K_D " refers to the dissociation equilibrium constant for specific antibody-antigen interactions, to describe the binding affinity between antibodies and antigens. The smaller the equilibrium dissociation constant is, the tighter the antibody binds antigen, the higher the affinity between the antibody and the antigen is. Generally, antibodies bind antigens with a dissociation equilibrium constant (K_D) less than approximately 10^{-5} M, in particular, less than approximately 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, or 10^{-10} M, or less, for example, as measured with a BIACORE instrument by surface plasmon resonance (SPR).

As used in this disclosure, the terms "monoclonal antibodies" and "mAb" have the same meaning and are used interchangeably; the terms "polyclonal antibodies" and "PcAb" have the same meaning and are used interchangeably; the terms "polypeptide" and "protein" have the same meaning and are used interchangeably. Also in the present disclosure, amino acids are usually represented by single letter or three letter abbreviations known in the field. For example, alanine can be represented by A or Ala.

As used in this disclosure, the terms "hybridoma" and "hybridoma cell line" are used interchangeably, and when the terms "hybridoma" and "hybridoma cell line" are used, they also include subclones and progenies of the hybridoma cell line. For example, when

referring to the hybridoma cell lines LT002 or LT003, it also refers to the subclones and progenies of the hybridoma cell lines LT002 or LT003.

As used in this disclosure, the term "pharmaceutically acceptable carrier and/or excipient" refers to a carrier and/or excipient that is compatible with the subject and active ingredients in pharmacology and/or physiology and is known to this field (e.g. Remington's Pharmaceutical Sciences. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company, 1995), including but not limited to: pH regulator, surfactant, adjuvant, and ionic strength enhancer. For example, pH regulators include but not limited to phosphate buffers; Surfactants include but not limited to cations, anions or non-ionic surfactants such as Tween 80; Ionic strength enhancers include but not limited to sodium chloride.

As used in this disclosure, the term "adjuvant" refers to a nonspecific immune booster that, when delivered into the body together or beforehand with an antigen enhances or changes the body's immune response to the antigen. There are many kinds of adjuvants, including but not limited to aluminum adjuvants (such as aluminum hydroxide), Freund's adjuvants (such as complete and incomplete Freund's adjuvants), corynebacterium parvum, lipopolysaccharides, cytokines, etc. Freund's adjuvant is currently the most commonly used adjuvant in animal experiments. Aluminum hydroxide adjuvant is used mostly in clinical trials.

As used in this disclosure, the term "effective dose" refers to the quantity that is sufficient to partially or completely achieve the desired effect. For example, effective prevention dose (e.g., diseases associated with CTLA4 binding to B7 or hyperactivity of CTLA4, such as tumors) is defined as the amount of a therapeutic sufficient to prevent, stop, or delay the diseases (e.g., diseases associated with hyperactivity of CTLA4 binding to B7 or diseases associated with hyperactivity of CTLA4, such as tumors); effective treatment dose is the amount of a therapeutic to cure, or at least partially stop, the disease and its complications in sick patients. Determination of such an effective dose is entirely within the scope of the capabilities of the technical personnel in the field. For example, the effective treatment dose will depend on the severity of the disease, the overall state of the patient's own immune system, the general background of patients such as age, weight

and sex, administration of drugs, and other treatments at the same time.

Effects of the Disclosure

The monoclonal antibodies in the present disclosure, 4G10H1L1 and 4G10H3L3, are capable of binding to CTLA4 specifically, effectively blocking the interaction of CTLA4 and B7, and removing the immunosuppression of CTLA4 specifically to activate T lymphocytes.

The monoclonal antibody 14C12H1L1 is capable of binding to CTLA4 specifically, effectively blocking the interaction of CTLA4 and B7, and removing the immunosuppression of CTLA4 specifically to activate T lymphocytes.

The bispecific antibodies of the present disclosure have the potential to be prepared for drugs for the prevention and/or treatment of tumors, such as melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer and leukemia.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 SDS-PAGE Results of Monoclonal Antibody 4G10. From left to right: 1 μ g antibody in non-reduced loading buffer; 1 μ g antibody in reduced loading buffer; 5 μ L Marker; 1 μ g BSA.

Figure 2 SDS-PAGE Results of Monoclonal Antibody 4G10H1L1. From left to right: 1 μ g antibody in non-reduced loading buffer; 1 μ g antibody in reduced loading buffer; 5 μ L Marker.

Figure 3 SDS-PAGE Results of Monoclonal Antibody 4G10H3L3. From left to right: 1 μ g antibody in reduced loading buffer; 5 μ L Marker.

Figure 4 SDS-PAGE Results of Monoclonal Antibody 14C12H1L1. From left to right: 1 μ g antibody in non-reduced loading buffer; 1 μ g antibody in reduced loading buffer; 5 μ L Marker; 1 μ g BSA.

Figure 5 SDS-PAGE Results of Bispecific Antibody BiAb001. From left to right: 5 μ L Marker; 1 μ g antibody in non-reduced loading buffer; 1 μ g antibody in reduced loading buffer; 1 μ g BSA.

Claims

1. A bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

2. A bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises comprising a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

3. A bispecific antibody comprising at least one first protein functional area that binds to PD-1 and at least one second protein functional area that binds to CTLA4, wherein the

at least one first and at least one second protein functional areas each comprise an immunoglobulin or antigen-binding fragment thereof,

wherein the at least one first protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 29-31 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 32-34,

wherein the at least one second protein functional area comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

4. The bispecific antibody of any one of claims 1-3, wherein the heavy chain variable region of the at least one first protein functional area comprises the amino acid sequence of SEQ ID NO: 16 or SEQ ID NO: 20 and the light chain variable region of the at least one first protein functional area comprises the amino acid sequence of SEQ ID NO: 18 or SEQ ID NO: 22.

5. The bispecific antibody of claim 1, wherein the heavy chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 6 or SEQ ID NO: 10 and light chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 8 or SEQ ID NO: 12.

6. The bispecific antibody of claim 2, wherein the heavy chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 14 and light chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 12.

7. The bispecific antibody of claim 3, wherein the heavy chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID

NO: 25 and light chain variable region of the at least one second protein functional area comprises the amino acid sequence of SEQ ID NO: 27.

8. The bispecific antibody of any one of claims 1-7, wherein,
the at least one first protein functional area and the at least one second protein functional area are directly connected or connected by a connecting fragment.
9. The bispecific antibody of claim 8, wherein the connecting fragment is (GGGGS)_n, wherein n is a positive integer.
10. The bispecific antibody of claim 9, wherein n is 1, 2, 3, 4, 5, or 6.
11. The bispecific antibody of any one of claims 1-10, wherein,
the antigen-binding fragment comprises a half antibody, a Fab, a F(ab')₂ or a single-chain antibody.
12. The bispecific antibody of any one of claims 1-11, wherein the at least one first protein functional area comprises an immunoglobulin and the at least one second protein functional area comprises a single-chain antibody.
13. The bispecific antibody of any one of claims 1-11, wherein the at least one first protein functional area comprises a single-chain antibody and the at least one second protein functional area comprises an immunoglobulin.
14. The bispecific antibody of any one of claims 1-13, wherein the bispecific antibody comprises at least two or more first protein functional areas, at least two or more second protein functional areas or any combination thereof.
15. The bispecific antibody of any one of claims 1-14, wherein the immunoglobulin is IgG, IgA, IgD, IgE or IgM.

16. The bispecific antibody of claim 15, wherein the immunoglobulin is IgG.
17. The bispecific antibody of claim 16, wherein the immunoglobulin is IgG1, IgG2, IgG3 or IgG4.
18. The bispecific antibody of any one of claims 12-17, wherein the single-chain antibody is attached at the c-terminus of the heavy chain of the immunoglobulin.
19. The bispecific antibody of any one of claims 1-18, wherein
the immunoglobulin or antigen-binding fragment thereof contains non-CDR regions from species other than mouse, and optionally, from human.
20. The bispecific antibody of any one of claims 1-19, wherein the bispecific antibody binds to CTLA4 protein and/or PD-1 protein with a K_D less than approximately 10^{-5} M, and optionally, less than approximately 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M or less.
21. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein
the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,
and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35-37 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.
22. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein
the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,
and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 35, 41 and 37 and a

light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 38-40.

23. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain variable region of an antibody and a single-chain antibody, wherein
the heavy chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 29-31,
and wherein the single-chain antibody comprises a heavy chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 42-44 and a light chain variable region comprising CDRs with the amino acid sequences of SEQ ID NOs: 45-47.

24. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of an antibody, wherein,
the light chain variable region of the antibody comprises CDRs with amino acid sequences selected from SEQ ID NOs: 32-34.

25. A vector, comprising the isolated nucleic acid molecule of claim 21, claim 22, claim 23, claim 24 or any combination thereof.

26. A host cell line, comprising the isolated nucleic acid molecule of claim 21, claim 22, claim 23 claim 24 or any combination thereof, or the vector of claim 25.

27. A method for preparing the bispecific antibody of any one of claims 1-20, the method comprising culturing the host cell line of claim 26 under appropriate conditions, and recovering the bispecific antibody from the cell culture.

28. A conjugate comprising the bispecific antibody of any one of claims 1-20 and a conjugating partner as a detectable marker.

29. The conjugate of claim 28, wherein the conjugating partner is a radioactive isotope, fluorescein, a luminescent material, a colorful substance, or an enzyme.
30. A kit comprising the bispecific antibody of any one of claims 1-20 or the conjugate of any one of claims 28-29.
31. The kit of claim 30, wherein the kit further comprises a secondary antibody, wherein the secondary antibody specifically recognizes the bispecific antibody.
32. The kit of claim 31, wherein the secondary antibody comprises a detectable marker.
33. The kit of claim 32, wherein the detectable marker is a radioactive isotope, fluorescein, a luminescent material, a colorful substance, or an enzyme.
34. A pharmaceutical composition, comprising the bispecific antibody of any one of claims 1 to 20 or the conjugate of any one of claims 28-29.
35. The pharmaceutical composition of claim 34, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier or excipient.
36. Use of the bispecific antibody of any one of claims 1 -20, the conjugate of any one of claims 28-29 or the pharmaceutical composition of any one of claims 34-35 for the manufacture of a medicament for the treatment of a tumor.
37. The use of claim 36, wherein the tumor is selected from the group consisting of melanoma, renal cancer, prostate cancer, bladder cancer, colorectal cancer, gastrointestinal cancer, liver cancer, non-small cell lung cancer, ovarian cancer and leukemia.

38. Use of the bispecific antibody of any one of claims 1-20, the conjugate of any one of claims 28-29 or the pharmaceutical composition of any one of claims 34-35 for the manufacture of a medicament for the treatment of anemia.

39. Use of the bispecific antibody of any one of claims 1-20, the conjugate of any one of claims 28-29 or the pharmaceutical composition of any one of claims 34-35 for the manufacture of a medicament for the treatment of a viral or parasitic infection.

40. The use of any one of claims 36-39, wherein the bispecific antibody or the conjugate is to be administered to the subject in at least one therapeutically effective amount.