Anti-PD-L1 and CD40 Bispecific Antibodies and Uses Thereof

Technical field

The present invention relates to the field of antibody drugs, and in particular to a bispecific antibody specifically binding to PD-L1 and CD40, a pharmaceutical composition thereof, and use thereof, especially use thereof in the treatment of tumors.

Background

PD-L1 (programmed death ligand 1, also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1)) is a type I transmembrane protein expressed on immune cells such as T cells and B cells, as well as tumor cells. When PD-L1 on the tumor cell membrane binds to PD-1 on immune cells such as T cells, the tumor cells emit inhibitory signals that prevent T cells from recognizing and killing the tumor cells (Chamoto, K., Al-Habsi, M., & honjo, T. (2017). "Role of PD-1 in Immunity and Diseases". Current topics in microbiology and immunology, 410, 75–97). Therefore, anti-PD-1/PD-L1 monoclonal antibodies (e.g., atezolizumab) have been proposed for the treatment of a variety of solid tumors, including, for example, NSCLC, head and neck squamous cell carcinoma, and melanoma (Radvanyi, et al, P. (2013). "Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer"--letter. Clinical cancer research, 19(19), 5541). However, existing clinical trials have shown that the response rate of PD-1/PD-L1 antibodies is affected by various factors such as the expression level of PD-L1 and the number and quality of T cells in the tumor, and only less than 40% of solid tumors are currently responsive to treatment.

CD40 is a type I transmembrane glycoprotein of a member of the tumor necrosis factor receptor superfamily. It is widely expressed on the surface of immune cells, including B cells, monocytes, macrophages, and dendritic cells, and is also expressed on the surface of non-immune cells, such as epithelial cells, endothelial cells, adipocytes, interstitial cells, and platelets. The ligand of human CD40 is also known as CD40L or CD154. Current evidence indicates that CD40, upon activation, can permit dendritic cells to promote the activation of anti-tumor T cells and exert an influence on the tumor microenvironment, converting a so-called "cold tumor" into a hot tumor (i.e., a tumor with significant T cell infiltration), thereby increasing the sensitivity of the tumor to immune checkpoint inhibition therapy. Agonist CD40 antibodies under clinical development based on this mechanism of action include selicrelumab (RO7009789) and CDX-1140 of the IgG2 isotype, and sotigalimab (APX005M), ChiLob7/4, ADC-1013, dacetuzumab, SEA-CD40, etc. of the IgG1 isotype. The activation potency of these CD40 mAbs varied from strong efficacy (sotigalimab and selicrelumab) to weak efficacy (dacetuzumab). In addition, different CD40 mAbs have different requirements and capabilities for Fc receptor (FcR) crosslinking. At present, there is no consensus on the optimal format for CD40 mAb. See, e.g., Vonderheide, R. H. CD40 Agonist Antibodies in Cancer Immunotherapy. Annu. Rev. Med. 71, 47–58 (2020).

In view of the fact that anti-CD40 agonist antibodies have the function of activating antigen-presenting cells, the combination of anti-CD40 agonist antibodies with anti-PD-1/PD-L1 antibodies and bispecific antibodies targeting PD-1/PD-L1 and CD40 have been proposed as a promising anti-cancer development direction (see, e.g., WO2023232036A1 and WO2023186113A1). However, from the current study (see, e.g., Padrón, L.J. et al. Sotigalimab and/or nivolumab with chemotherapy in first-line metastatic pancreatic cancer: clinical and immunologic analyses from the randomized phase 2 PRINCE trial. Nat Med 28, 1167-1177 (2022). https://doi.org/10.1038/s41591-022-01829-9), the search for suitable drug combinations and bispecific antibody forms to maximize the efficacy of anti-tumor drugs while limiting the toxic and side effects of the molecules remains a challenge that needs to be met in the art.

Summary of the Invention

In view of the above, the inventors constructed a novel anti-PD-L1/CD40 bispecific antibody through diligent research, further explored preferred embodiments with optimized antibody configurations and Fc regions, and confirmed the anti-tumor therapeutic efficacy of the constructed bispecific antibody in a variety of cold tumor models. Based on these studies, the inventors have established the antibody of the present invention and its application in anti-tumor therapy.

Therefore, in a first aspect, the present invention provides an anti-PD-L1/CD40 bispecific antibody and a pharmaceutical composition and use thereof, wherein the antibody comprises at least one antigen-binding site that specifically binds to PD-L1 and at least one antigen-binding site that specifically binds to CD40. In some embodiments, the PD-L1 antigen-binding site comprises a VHH domain comprising CDR1, CDR2 and CDR3 having amino acid sequences set forth in SEQ ID NOs: 2–4, respectively. In some embodiments, the CD40 antigen-binding site comprises a VH domain comprising HCDR1, HCDR2, and HCDR3 having amino acid sequences set forth in SEQ ID NOs: 21-23, respectively, and a VL domain comprising LCDR1, LCDR2, and LCDR3 having amino acid sequences set forth in SEQ ID NOs: 18-20, respectively. In some preferred embodiments, the antibody is a trivalent bispecific antibody comprising one PD-L1 antigen-binding site and two CD40 antigen-binding sites per molecule.

In a second aspect, the present invention provides an anti-PD-L1 antibody, and a pharmaceutical composition and use thereof.

In a third aspect, the present invention provides an immunoconjugate or an immunofusion comprising the antibody of the present invention, and a pharmaceutical composition and use thereof.

The present invention is further illustrated in the following figures and embodiments. The drawings and embodiments, however, should not be considered limiting to the scope of the present invention, and modifications readily conceivable by those skilled in the art will be included within the spirit of the present invention and the protection scope of the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the reporter assay of Dace-hz03C06.g6-2+1 and Selicrelumab molecules in MC38 cells (PD-L1-Negative).

FIG. 2 shows the reporter assay of Dace-hz03C06.g6-2+1 and Selicrelumab molecules on MC38 PD-L1 cells (PD-L1-positive).

FIG. 3 shows the activation of DCs by the molecules Selicrelumab, Dace-hz03C06.g6-2+1, and Dace-hz03C06.g6-2+2 under the condition of high PD-L1 expression.

FIG. 4 shows the anti-tumor effect of the Dace-hz03C06.g6-2+1 antibody drug on the B16F10-hPD-L1 tumor-bearing mouse model.

FIG. 5 shows the effect of the Dace-hz03C06.g6-2+1 antibody drug on body weight changes in mice.

FIG. 6 shows the anti-tumor effect of the Dace-hz03C06.g6-2+1 antibody drug on the B16F10-hPD-L1 tumor-bearing mouse model.

FIG. 7 shows the effect of the Dace-hz03C06.g6-2+1 antibody drug on body weight changes in mice.

FIG. 8 shows the anti-tumor effect of the Dace-hz03C06.g6-2+1 antibody drug on the KPC-hPD-L1 tumor-bearing mouse model.

FIG. 9 shows the effect of the Dace-hz03C06.g6-2+1 antibody drug on body weight changes in mice.

FIG. 10 shows the effect of Dace-hz03C06.g6-2+1 and Selicrelumab antibody drugs on serum ALT levels in mice .

FIG. 11 shows the effect of Dace-hz03C06.g6-2+1 and Selicrelumab antibody drugs on serum AST levels in mice.

FIG. 12 shows the effect of Dace-hz03C06.g6-2+1 and Selicrelumab antibody drugs on serum TNF-α levels in mice.

FIG. 13 shows the effect of Dace-hz03C06.g6-2+1 and Selicrelumab antibody drugs on serum IL-6 levels in mice.

FIG. 14 shows a schematic diagram of an exemplary 2+1 format anti-PD-L1/CD40 bispecific antibody molecule of the present invention.

Detailed description of the invention

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the present invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples described herein are illustrative only and are not intended to be limiting. Other features, objects, and advantages of the present invention will be apparent from the description and drawings, and from the appended claims.

Definitions

The term "about", when used in combination with a numerical value, is intended to encompass numerical values ranging from a lower limit that is 5% less than the specified numerical value to an upper limit that is 5% greater than the specified numerical value.

As used herein, the term "and/or", when used in combination with a plurality of options, refers to any one of the options or any two or more or all of the options.

The term "comprise" or "include", when used herein, unless otherwise specified, also encompasses the situation where the entirety consists of the recited elements, integers or steps. For example, when referring to an antibody variable region "comprising" a particular sequence, it is also intended to encompass an antibody variable region consisting of the particular sequence.

As used herein, the term "antigen-binding molecule" refers to a molecule comprising an antigen-binding domain or an antigen-binding site capable of binding to a target antigen, e.g., a protein or polypeptide or a molecule derived therefrom. In the present invention, when the target antigen is PD-L1 and/or CD40, the antigen-binding molecule binding to PD-L1 and/or CD40 is also referred to as a PD-L1-binding molecule, a CD40-binding molecule, or a PD-L1/CD40-binding molecule. The antigen-binding molecule includes, for example, antibodies and antigen-binding fragments thereof, and various fusions and conjugates constructed based on the antibodies or the antigen-binding fragments, such as VHH-Fc antibodies, immunoconjugates, antibody-drug conjugates (ADCs), multi/bispecific antibodies, and chimeric antigen receptors (CARs). As will be appreciated by those skilled in the art, the antigen-binding site of an antibody generally comprises amino acid residues from a "complementarity determining region" or a "CDR".

As used herein, the term "antibody" refers to a polypeptide comprising at least an immunoglobulin light chain variable region or heavy chain variable region that specifically recognizes and binds to an antigen.The term encompasses a variety of antibody structures, including but not limited to monoclonal antibodies, single-chain or multi-chain antibodies, monospecific or multispecific antibodies (e.g., bispecific antibodies), single-domain antibodies, heavy-chain antibodies, chimeric or humanized antibodies, intact antibodies, and antibody fragments so long as they exhibit the desired antigen-binding activity.

The terms "full-length antibody", "intact antibody", and "whole antibody" are used interchangeably herein to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains

that comprise an Fc-region as defined herein. In some embodiments, a "full-length antibody" is an antibody comprising an antigen-binding variable region and an Fc region. In some other embodiments, a "full-length antibody" is an antibody comprising an antigen-binding variable region, a light chain constant domain (CL), and heavy chain constant domains CH1, CH2, and CH3. Preferably, a "full-length antibody" comprises two antibody light chains (each comprising a light chain variable domain and a light chain constant domain) and two antibody heavy chains (each comprising a heavy chain variable domain, a hinge region, and heavy chain constant domains CH1, CH2, and CH3).The two antibody heavy chains of the full-length antibody may independently of each other have the presence or absence of a C-terminal amino acid residue K or GK.The constant domains included in the antibody may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variants thereof. Herein, correspondingly, the expression "anti-CD40 full-length antibody" refers to an antibody comprising a CD40 antigen-binding variable region and an Fc region. In some embodiments, preferably, the anti-CD40 full-length antibody consists of two Fab or scFv domains specifically binding to CD40 and one dimerized Fc region at its C-terminus.

As used herein, the terms "antibody fragment" and "antigen-binding fragment" are used interchangeably and refer to a molecule different from an intact antibody, which comprises a portion of the intact antibody and is capable of binding to an antigen to which the intact antibody binds. Examples of antibody fragments include, but are not limited to, Fv, Fab, crossFab, Fab', Fab'-SH, $F(ab')_2$; a diabody; a triabody; a linear antibody; a single-chain antibody (e.g., scFv or scFab); a single-domain antibody (sdAb); a camelid antibody (a heavy-chain antibody) or a fragment thereof (e.g., VHH); and a monospecific antibody, a bispecific antibody, or a multispecific antibody formed from an antibody fragment. Unless otherwise stated herein or clearly contradicted by context, reference herein to the term "antibody" is equivalent to "antibody or antibody fragment thereof."

As used herein, the terms "antigen-binding site" and "antigen-binding domain" are used interchangeably to refer to a region in an antibody molecule that actually binds to an antigen, which may include, but is not limited to, a variable domain from a heavy chain antibody (i.e., a "VHH") and a pair of heavy- and light-chain variable domains from a conventional antibody (i.e., a "VH" and a "VL").

As used herein, the term "bispecific" refers to an antigen-binding molecule (e.g., an antibody) having at least two antigen-binding sites, wherein at least one of the at least two antigen-binding sites binds to a first antigen or a first antigenic epitope, while the remaining antigen-binding sites bind to a different second antigen or a different second antigenic epitope, e.g., to a different epitope on a different antigen or a different epitope on the same antigen. In contrast, "monospecific" refers to the ability to bind to only one type of epitope.

The expression "valency", as used herein in connection with an antibody, refers to either the total number of antigen-binding sites in an antibody molecule, or the number of antigen-binding sites having the same antigen-binding specificity. For example, a tetravalent antibody refers to an antibody molecule comprising a total of four antigen-binding sites; the antibody molecule may be a "2+2" format bispecific antibody, i.e., the antibody has two different antigen-binding specificities, wherein for each antigen-binding specificity, there are 2 identical antigen-binding sites. As another example, a trivalent antibody refers to an antibody molecule comprising a total of three antigen-binding sites; the antibody molecule may be a "2+1" format bispecific antibody, i.e., the antibody has two different antigen-binding specificities, wherein for one antigen-binding specificity, there are two identical antigen-binding sites, and for the other antigen-binding specificity, there is only one antigen-binding site.

As used herein, the term "PD-L1" refers to programmed death ligand-1. Unless otherwise stated, the term includes any variants of human PD-L1, including sequence variants, particularly naturally occurring variants, allelic variants, and post-translationally modified variants and conformational variants, and encompasses species homologs thereof. In addition, it should be understood that the term encompasses not only a PD-L1 naturally or recombinantly expressed by a cell or a PD-L1 expressed on a natural or recombinant cell, but also a recombinantly expressed fusion

protein comprising an extracellular domain of a PD-L1. An example of PD-L1 is a human PD-L1 protein comprising the amino acid sequence under UniProtKB accession No. Q9NZQ7, or a recombinant protein comprising an extracellular domain of the protein (particularly, the amino acid sequence of amino acids 19-238). Another example of PD-L1 is a monkey PD-L1 protein, or a recombinant protein comprising an extracellular domain of the protein. As used herein, unless otherwise specified, the term "PD-L1" refers to PD-L1 derived from human. Herein, the "antigen-binding specificity against PD-L1", i.e., the "antigen-binding site that specifically binds to PD-L1", is preferably provided by a VHH domain.

As used herein, the term "PD-L1-positive" cell refers to a cell that is positive for PD-L1 cell-surface expression, such as a dendritic cell, a cancer cell, or an engineered non-tumor cell. The expression level of PD-L1 on the cell surface can be determined by any conventional method known in the art for determining the expression level of cell surface antigens, e.g., FACS assay, immunofluorescence staining, or immunohistochemical staining.

As used herein, the term "CD40" refers to the B-cell surface antigen CD40, also known as tumor necrosis factor receptor superfamily member 5. Unless otherwise stated, the term includes any variants of human CD40, including sequence variants, particularly naturally occurring variants, allelic variants, and post-translationally modified variants and conformational variants, and encompasses species homologs thereof. In addition, it should be understood that the term encompasses not only a CD40 naturally or recombinantly expressed by a cell or a CD40 expressed on a natural or recombinant cell, but also a recombinantly expressed fusion protein comprising an extracellular domain of CD40. An example of CD40 is a human CD40 protein comprising the amino acid sequence under UniProtKB- P25942, or a recombinant protein comprising an extracellular domain of the protein. Another example of CD40 is a monkey CD40 protein, or a recombinant protein comprising an extracellular domain of the protein. Herein, unless otherwise specified, the term "CD40" refers to CD40 derived from human. In the antibody of the present invention, the "antigen-binding specificity against CD40", i.e., the "antigen-binding site that specifically binds to CD40", is preferably provided by a VH and VL domain pair.

As used herein, the term "CD40-positive" cell refers to a cell that is positive for CD40 cell-surface expression, such as a dendritic cell, a B cell, or a modified non-tumor cell. The expression level of CD40 on the cell surface can be determined by any conventional method known in the art for determining the expression level of cell surface antigens, e.g., FACS assay or immunofluorescence staining.

As used herein, the expression "CPS" (combined positive score), which relates to PD-L1 scoring in a tumor, is defined as the percentage of PD-L1-positive viable tumor cells (partial or complete membrane staining of any intensity) and PD-L1-positive lymphocytes and macrophages (cell membrane or cytoplasmic staining of any intensity) relative to all viable tumor cells. The CPS score can be calculated using the following formula: (sum of PD-L1-stained tumor cells + tumor-associated immune cells)/total number of tumor cells × 100, and is expressed as a number from 0 to 100.

The term "binding" or "specifically binding" used herein means that the binding is selective for an antigen and can be distinguished from unwanted or nonspecific interactions.The ability of an antigen-binding site to bind to a specific antigen can be determined by enzyme-linked immunosorbent assay (ELISA) or conventional binding assays known in the art, for example, by ELISA to detect the binding ability of an antibody to an antigen, or by FACS to detect the binding ability of an antibody to cells expressing an antigen on their surfaces, or by SPR technology or BLI technology to detect the affinity constant KD.

As used herein, the term "affinity" or "binding affinity" refers to the strength of the sum of all non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigenic epitope). As used herein, "binding affinity" reflects the intrinsic binding affinity of a 1:1 interaction between members of a binding pair. The binding affinity can be generally expressed in terms of an equilibrium dissociation constant (KD) and can be measured by common methods known in the art, such as surface plasmon resonance (SPR)

or biolayer interferometry (BLI). In contrast, the term "avidity" or "binding avidity " used herein refers to the combined strength of the interactions of multiple binding sites of a molecule (antibody) with the same target.

As used herein, the term "high affinity" refers to a binding affinity KD value of an antigen-binding domain for its target epitope of less than about 100×10^{-9} M, preferably less than about 50×10^{-9} M, about 40×10^{-9} M, about 30×10^{-9} M, or about 20×10^{-9} M, and more preferably less than about 10×10^{-9} M, as determined, for example, by surface plasmon resonance (SPR) technique or bio-layer interferometry (BLI) technique.

As used herein, an antibody having "blocking activity" against PD-L1 refers to the function of the antibody to block the binding of PD-L1 to the receptor PD-1, and/or to reduce the PD-L1/PD-1 signaling . An assay for determining the blocking activity may be, for example, a reporter gene-based signaling pathway blocking assay, such as the reporter gene-based assay described in Example 2. The blocking activity of a test antibody against PD-L1 can be determined by referring to the level of PD-L1/PD-1 binding or PD-L1/PD-1 signaling in the absence of the antibody and/or in the presence of a positive antibody.

As used herein, the "activation activity" of an antibody for CD40 refers to the function of the antibody to activate the CD40 signaling pathway. An assay for determining the activation activity may be, for example, a reporter gene-based signaling pathway activation assay, such as the reporter gene-based assay described in Example 6. The CD40 activation activity of a test antibody can be determined by referring to the CD40 signaling level in the absence of the antibody and/or in the presence of a positive antibody.

As used herein, the expression "selective" or "PD-L1-dependent", in connection with the CD40 activation activity of an antibody, means that the antibody preferentially activates the CD40 signaling pathway when PD-L1-mediated antibody cross-linking is present (or when PD-L1-mediated antibody cross-linking is present at a high level), as compared with when PD-L1-mediated antibody cross-linking is absent (or when PD-L1-mediated antibody cross-linking is present at only a low level). The selectivity of an antibody can be characterized by a selectivity index (i.e., the ratio of the EC50 values for activation of CD40-expressing cells by the antibody under the two conditions described above).

As used herein, the term "immunoglobulin" refers to a protein having a structure of a naturally occurring antibody. For example, an IgG immunoglobulin is a heterotetrameric glycoprotein of about 150,000 Daltons consisting of two light chains and two heavy chains which are disulfide-bonded. Each immunoglobulin heavy chain has, from N-terminus to C-terminus, a heavy chain variable region (VH), also known as a heavy chain variable domain, followed by three heavy chain constant domains (CH1, CH2, and CH3). Similarly, from N-terminus to C-terminus, each immunoglobulin light chain has one light chain variable region (VL), also known as a light chain variable domain, followed by one light chain constant domain (CL).The heavy chain of an immunoglobulin can be assigned to one of five classes, α (IgA), δ (IgD), ε (IgE), γ (IgG), or μ (IgM), based on the type of its constant region, wherein some classes can be further divided into subclasses such as γ1 (IgG1), γ2 (IgG2), γ3 (IgG3), γ4 (IgG4), α1 (IgA1), and α2 (IgA2).The light chain of an immunoglobulin can also be assigned to one of two classes, called κ or λ, based on the amino acid sequence of its constant domain.

As used herein, the term "isotype" refers to an antibody type determined by the heavy chain constant region of the antibody. For example, the antibody according to the present invention may be an IgA (e.g., IgA1 or IgA2), IgG1, IgG2 (e.g., IgG2a or IgG2b), IgG3, IgG4, IgE, IgM, or IgD antibody, and have a heavy chain constant region of the immunoglobulin type. Herein, it should be understood that when an antibody is described as having a particular isotype, it encompasses not only antibodies having a native sequence constant region of that isotype, but also antibodies comprising a few mutations in the constant region and still belonging to that isotype.

As used herein, the term "variable region" or "variable domain" refers to a domain of a heavy chain or light chain of an antibody involved in the binding of the antibody to an antigen. In the case of a conventional antibody, the pairing of a heavy chain variable domain (VH) with a light chain variable domain (VL) confers antigen-binding

specificity. In the case of a heavy-chain antibody, e.g., a heavy-chain antibody from Camelidae, a single VH domain (also referred to herein as a VHH domain) may be sufficient to provide antigen-binding specificity. VHH domains, like heavy and light chain variable regions of conventional IgG antibodies, comprise four conserved framework regions (FRs) and three complementarity determining regions (CDRs), which are arranged in the order of FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. According to some aspects of the present invention, one or more residues in the variable regions of an antibody can be modified, for example, one or more CDRs and/or one or more framework regions may undergo residue modifications, particularly conservative residue substitutions, to obtain an antibody variant that substantially retains at least one biological property (e.g., antigen-binding ability) of the parent antibody. In still other aspects, the variable regions of an antibody can be modified by CDR grafting. Since CDR sequences are responsible for most of the antibody-antigen interactions, antibody variants that mimic the properties of known antibodies can be constructed. In such antibody variants, CDR sequences from known antibodies are grafted onto the framework regions of different antibodies with different properties, and one to several residue mutations, e.g., back mutations, may be introduced as needed to refine the desired properties of the antibodies. The properties of mutated and/or modified antibodies, such as target antigen-binding properties or other desired functional properties, such as CD40 activation activity and/or *in vivo* tumor killing activity, can be assessed in an *in vitro* or *in vivo* assay. Therefore, variants of any of the variable regions (e.g., VHH and VH/VL regions) described herein are also contemplated in the present invention. In some cases, for a therapeutic use of an antibody or a derivative molecule thereof, it is desirable to reduce the immunogenicity. Thus, in some embodiments, preferably, the antibody comprises a humanized variable domain (e.g., a VHH domain and/or a VH/VL domain).

As used herein, "complementarity determining region" is used interchangeably with "CDR region", "CDR" and "hypervariable region", and refers to a region in an antibody variable domain which is highly variable in sequence and forms a structurally defined loop ("hypervariable loop") and/or comprises antigen-contacting residues ("antigen-contacts"). The CDRs are primarily responsible for binding to antigenic epitopes. In a VHH domain, the CDRs are numbered sequentially from the N-terminus and are generally referred to as CDR1, CDR2, and CDR3. In a VH domain and a VL domain, the CDRs are numbered sequentially from the N-terminus and are generally referred to as HCDR1, HCDR2, and HCDR3, and LCDR1, LCDR2, and LCDR3, respectively. The CDR sequences in a given variable region (e.g., a VHH domain or a VH/VL domain) can be determined using schemes well known in the art, e.g., CDR boundaries defined according to the Kabat, AbM, Chothia, Contact, IMGT scheme, or a combination thereof. Unless otherwise stated, the term "CDR" or "CDR sequence" used herein encompasses a CDR sequence determined in any one of the ways described or a combination thereof. In addition, it is known in the art that, although CDRs vary from antibody to antibody, only a limited number of amino acid positions within a CDR are directly involved in antigen binding. A smallest overlapping region according to at least two of the Kabat, Chothia, AbM, and Contact schemes may be identified, thereby providing a “minimal binding unit” for antigen binding. Such a minimal binding unit may be a sub-portion of a CDR. As will be appreciated by those skilled in the art, residues in the remainder of the CDR sequence can be determined by antibody structure and protein folding. Accordingly, variants of any of the CDRs described herein are also contemplated in the present invention. For example, in a variant of a CDR, the amino acid residues within the minimal binding unit may remain unchanged, while the remaining CDR residues may be substituted.

Unless otherwise stated, in the present invention, residue positions in antibody variable regions and CDRs (including those in heavy-chain variable regions) are numbered according to the Kabat numbering system (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991)).

As used herein, the terms "VHH" and "VHH domain" are used interchangeably to refer to a heavy chain variable domain derived from a heavy-chain antibody lacking a light chain, and are also sometimes referred to as a

single variable domain fragment (sVD) or a single-domain antibody (sdAb). Thus, a VHH differs from the conventional VH domain of a four-chain immunoglobulin in that it does not require pairing with a light-chain variable domain to form an antigen-binding site. Such a VHH may be derived from an antibody produced by Camelidae species, such as camel, alpaca, dromedary, llama, or guanaco. Species outside the family Camelidae may also produce heavy-chain antibodies naturally lacking light chains, and VHHs derived from such heavy-chain antibodies are also within the scope of the present invention.

As used herein, the term "Fab domain" is used to refer to a structure similar to that of a conventional four-chain IgG antibody formed by pairing a heavy chain variable region VH and a heavy chain constant region CH1 (VH-CH1) with a complementary light chain variable region VL and a light chain constant region CL (VL-CL). The term also encompasses structures in which CH1 is exchanged with CL, i.e., structures formed by pairing of VH-CL with VL-CH1. In some embodiments, as shown in FIG. 14, an Fab domain may be fused at the N-terminus of an immunoglobulin Fc region.

As used herein, the term "scFv domain" is used to refer to a single-chain polypeptide comprising a VH domain and a VL domain linked together by a flexible linker, wherein the VH domain and the VL domain on the polypeptide chain pair to form an antigen-binding domain responsible for antigen binding. In some embodiments, an scFv domain may be fused at the N-terminus or C-terminus of an immunoglobulin Fc region.

As used herein, the terms "immunoglobulin Fc region", "Fc region" and "Fc domain" are used interchangeably herein to define a C-terminal region of an immunoglobulin heavy chain, which comprises at least a portion of a heavy chain constant region. Herein, the term "Fc region" or "Fc domain" does not comprise a heavy chain variable region VH and a light chain variable region VL as well as a heavy chain constant region CH1 and a light chain constant region CL of an immunoglobulin; but may comprise a CH2 domain and a CH3 domain, and may or may not comprise an immunoglobulin hinge region. For example, in some instances, the Fc region may comprise or consist of, from N-terminus to C-terminus, a CH2 domain and a CH3 domain. In other examples, the Fc region may comprise or consist of, from N-terminus to C-terminus, an immunoglobulin hinge region or a portion thereof (or a linker peptide in place of the hinge region), a CH2 domain, and a CH3 domain. The sequence of the Fc region may be a native sequence or a variant sequence. Thus, the term "Fc region" as used herein encompasses native sequence Fc regions and variant Fc regions. Fc regions that may be used in the antibody of the present invention include, but are not limited to, Fc regions of IgG1, IgG2, IgG3, or IgG4 and having a native sequence or a variant sequence.

As used herein, unless otherwise specified, amino acid residues in Fc regions and heavy-chain constant regions are numbered according to the EU numbering system (also known as the EU index) as described in Kabat et al., Sequences of Proteins of Immunological Interes, 5th Ed., Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

As used herein, the term "native sequence Fc region" encompasses naturally occurring Fc region sequences of various immunoglobulins, such as Fc region sequences of various Ig subtypes and allotypes thereof (Gestur Vidarsson et al., IgG subclasses and allotypes: from structure to effector functions, 20 October 2014, doi: 10.3389/fimmu.2014.00520.). In some embodiments, a heavy chain Fc region of human IgG has an amino acid sequence extending from Cys226 or from Pro230 to the carboxyl-terminus of the heavy chain. However, lysine (Lys447) or glycine-lysine (Gly446Lys447) at the C-terminal end of the Fc region may or may not be present. In some embodiments, a human IgG heavy chain Fc region carries a hinge sequence or a partial hinge sequence of a native immunoglobulin at the N-terminus, e.g., according to EU numbering, the sequence from E216 to T225, or the sequence from D221 to T225.

As used herein, the term "variant sequence Fc region" refers to an Fc region polypeptide comprising a modification relative to a native sequence Fc region polypeptide. Modifications may be additions, deletions and/or substitutions of amino acid residues. Substitutions may include naturally-occurring amino acid substitutions and

non-naturally-occurring amino acid substitutions. Modifications may be intended to alter the binding of an Fc region to its receptor and the effector function resulting therefrom, or to prevent undesired heavy-chain mispairing, or to introduce an amino acid mutation useful for interchain disulfide bond formation at a specific site.

As used herein, the term "effector function" refers to biological activities attributed to an immunoglobulin Fc region, which vary with immunoglobulin isotypes. Examples of immunoglobulin effector functions include: Fc receptor binding, C1q binding, complement-dependent cytotoxicity (CDC), and antibody-dependent cell-mediated cytotoxicity (ADCC). Depending on the intended use of an antibody molecule, the Fc region of the antibody may be modified to have an altered effector function, such as a reduced or eliminated Fcγ receptor binding, relative to an antibody molecule with a wild-type Fc region.

As used herein, when an antibody comprises an Fc region, an antibody polypeptide chain comprising the Fc region is also referred to as a heavy chain, whereas an antibody polypeptide chain lacking the Fc region is also referred to as a light chain. Accordingly, the term "heavy chain" in relation to a full-length antibody refers to an antibody chain comprising an Fc region in a full-length antibody, whereas "light chain" refers to an antibody chain lacking an Fc region in a full-length antibody. In some cases, the full-length antibody may or may not have an antigen-binding domain, e.g., a VHH domain, covalently linked to the C-terminus of the Fc region of the heavy chain thereof.

As used herein, the terms "flexible linker peptide", "linker", and "linker peptide" are used interchangeably and refer to a short amino acid sequence consisting of amino acids, such as a peptide of glycine (G) and/or serine (S) and/or threonine (T) residues used alone or in combination, or a hinge region from an immunoglobulin.

As used herein, the "percent identity (%)" of an amino acid sequence refers to the percentage of amino acid residues in a candidate sequence that are determined to be identical to the amino acid residues at corresponding positions of a specific amino acid sequence described herein when the candidate sequence is aligned with the specific amino acid sequence, with gaps introduced if necessary to achieve maximum percent sequence identity and without considering any conservative substitution as part of sequence identity. In some embodiments, the present invention contemplates variants of an antibody molecule of the present invention that have a considerable degree of sequence identity to an antibody molecule specifically disclosed herein, e.g., at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% or more identity. The variant may comprise conservative modifications.

For polypeptide sequences, “conservative modification” includes a substitution in a polypeptide sequence that results in a replacement of an amino acid residue with a chemically similar amino acid residue. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following 8 groups comprise amino acids that are conservatively substituted with each other: 1) alanine (A) and glycine (G); 2) aspartic acid (D) and glutamic acid (E); 3) asparagine (N) and glutamine (Q); 4) arginine (R) and lysine (K); 5) isoleucine (I), leucine (L), methionine (M), and valine (V); 6) phenylalanine (F), tyrosine (Y), and tryptophan (W); 7) serine (S) and threonine (T); and 8) cysteine (C) and methionine (M). In some embodiments, the term "conservative modification" is used to refer to, inter alia, an amino acid modification that does not significantly affect or alter the desired properties (e.g., binding characteristics and/or CD activation characteristics) of a parent polypeptide (e.g., a parent antibody) into which the modification is to be introduced.

As used herein, the "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human CDRs and amino acid residues from human FRs. In some embodiments, all or substantially all CDRs in the humanized antibody correspond to CDRs of a non-human antibody, and all or substantially all FRs correspond to FRs of a human antibody. The humanized antibody may optionally comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody (e.g., a non-human antibody) refers to an antibody that has been humanized. In some embodiments herein, a humanized antibody of the present invention has a framework region sequence "derived from" a particular human germline sequence. As used herein,

"derived from" means that the amino acid sequence of the framework region of the antibody has at least 90%, 93%, 95%, 96%, 97%, 98%, or more identity to the amino acid sequence of the corresponding framework region encoded by the human germline immunoglobulin gene, and that the antibody retains antigen-binding activity.

As used herein, an "isolated" antibody refers to an artificial antibody, a recombinantly produced antibody, and an antibody that has been at least partially separated from components in the natural environment where it was produced. In some embodiments, an antibody of the present invention is an "isolated" antibody. In some embodiments, the isolated antibody is purified to a purity greater than 90%, 95%, or 99%, as determined by, for example, electrophoresis (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatography (e.g., ion exchange or reverse-phase HPLC).

As used herein, a variable region amino acid sequence (e.g., a VHH or a VH/VL pair) is said to be "cross-reactive" if it is specific for two different antigens or antigenic determinants (e.g., PD-L1 or CD40 from different mammalian species, such as human PD-L1 and cynomolgus monkey PD-L1, or human CD40 and cynomolgus monkey CD40). It would be advantageous for an antibody to have human-monkey species cross-reactivity, especially similar binding affinities for human and monkey antigens, which may facilitate preclinical drug development for the antibody. In some embodiments, an antibody of the present invention preferably has cross-reactivity with human and monkey PD-L1 and/or human and monkey CD40.

As used herein, the term “host cell” refers to a cell into which an exogenous polynucleotide has been introduced, including the progeny of such cells. Host cells include “transformants” and “transformed cells,” including primary transformed cells and progeny derived therefrom. A host cell may be any type of cell system used to produce the antibody molecule of the present invention, including eukaryotic cells, e.g., mammalian cells, insect cells, and yeast cells; and prokaryotic cells, e.g., *E. coli* cells. Host cells include cultured cells, and also include cells inside a transgenic animal, a transgenic plant, a cultured plant tissue or cultured animal tissue.

As used herein, the term “expression vector” refers to a vector capable of directing the expression of a nucleotide sequence operably linked thereto. An expression vector generally comprises a cis-acting element for expression of the nucleotide sequence, while other elements required for expression may be provided by a host cell or in an *in vitro* expression system. Expression vectors include, for example and without limitation, cosmids, plasmids (e.g., naked plasmids or plasmids contained in liposomes), and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses).

As used herein, the term “immunoconjugate” generally refers to a molecule formed by conjugating or fusing one or more immunoglobulin-related molecules or fragments thereof (e.g., an antibody or an antibody fragment) to one or more other molecules. In some cases, the other molecules may be proteinaceous molecules, such as peptides, polypeptides, or proteins. In other cases, the other molecules may be non-proteinaceous molecules, e.g., chemical toxins. In some cases, the other molecules may be immunoglobulin-related molecules or fragments thereof. In some cases, the other molecules may be molecules other than immunoglobulin-related molecules or fragments thereof. The one or more other molecules may be the same as or different from one another. For example, the other molecules may be target-binding elements and/or effector elements, such as chemotherapeutic agents, toxins, drugs (e.g., immunotherapeutic agents), radioelements, probes, or signaling molecules.

The terms “individual” and “subject” are used interchangeably herein to refer to a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates, such as monkeys), rabbits, and rodents (e.g., mice and rats). In some embodiments, an individual is, in particular, a human individual.

As used herein, the term “treatment” refers to a clinical intervention intended to alter the natural course of a disease in an individual being treated. Desired therapeutic effects include, but are not limited to, preventing the occurrence or recurrence of diseases, alleviating symptoms, reducing any direct or indirect pathological

consequences of diseases, preventing metastasis, delaying disease progression, improving or alleviating conditions, and alleviating or improving prognosis. In the context of tumor or cancer treatment, "treatment" encompasses anti-tumor biological effects that result from human intervention (e.g., administration of a drug), including, but not limited to, a reduction in tumor volume, a reduction in the number of tumor cells, a reduction in tumor cell proliferation, or a reduction in tumor cell viability.

As used herein, the term "prevention" refers to a medical intervention performed before the onset of at least one symptom of a disease to inhibit, delay, or prevent the onset or progression of the disease or a particular symptom of the disease. Thus, in some embodiments, prevention includes administration of a drug to a subject before the onset of a disease or symptom.

The terms "cancer" and "tumor" are used interchangeably herein to refer to or describe a physiological condition in mammals typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinomas, solid tumors, and liquid tumors.

Embodiments

With the introduction of novel immunotherapeutic drugs, cancer treatment has been revolutionized. However, the design and development of successful anticancer therapies remain challenging. In the course of research on antibody drugs targeting inhibitory and co-stimulatory checkpoints, the inventors developed novel bispecific antibodies targeting PD-L1 and CD40. Accordingly, in some aspects, the present invention provides novel anti-PD-L1/CD40 bispecific antibodies. In some embodiments, the antibody comprises a high-affinity PD-L1 antigen-binding domain, e.g., a PD-L1 antigen-binding domain comprising the CDR sequences set forth in SEQ ID NOs: 2–4; and a CD40 antigen-binding domain having weak agonistic activity in the absence of crosslinking, e.g., a CD40 antigen-binding domain comprising the CDR sequences set forth in SEQ ID NOs: 18–23. In some embodiments, an antibody of the invention exhibits a PD-L1-dependent CD40 activation effect and/or an enhanced ability to selectively activate antigen-presenting cells (e.g., dendritic cells), as compared with a non-PD-L1-dependent agonistic anti-CD40 antibody.

On the basis of the antibodies described above, in some aspects of the present invention, the inventors further investigated and developed preferred embodiments of the antibodies of the present invention having different antibody configurations. According to previous reports, bispecific antibodies against CD40 and PD-L1 predominantly adopte a 2+2 format molecular design. To further optimize the antibodies, the inventors investigated the effects of different antibody configurations on the efficacy of the bispecific antibodies of the present invention. Studies have shown that, as compared with the reported 2+2 format molecular configuration, an antibody of the present invention having a 2+1 configuration (i.e., each molecule comprises two anti-CD40 antigen-binding sites and one anti-PD-L1 antigen-binding site) can more fully utilize PD-L1 anchor molecules in target tissues at the same PD-L1 abundance and antibody dosage, thereby maximizing efficacy. Accordingly, in some preferred embodiments, the present invention provides a 2+1 format anti-PD-L1 and CD40 bispecific antibody of the invention. In some more preferred embodiments, the antibody comprises two anti-CD40 Fabs and one anti-PD-L1 VHH. In some still more preferred embodiments, the bispecific antibody comprises or consists of a full-length anti-CD40 IgG antibody and an anti-PD-L1 VHH domain conjugated thereto, e.g., to the C-terminus of one antibody heavy chain thereof, for example, having a structure shown in FIG. 14.

On the basis of the antibodies described above, in som other aspects, the inventors further investigated and established preferred embodiments of the antibodies of the present invention having different Fc regions. One of the major challenges faced by antibodies with CD40 agonistic activity is greater systemic toxicity and side effects and lower tolerated doses. Selicrelumab, an antibody of IgG2 isotype, has been reported to cause toxicity and side effects, such as cytokine release syndrome and hepatotoxicity, in some patients at doses exceeding 0.2 mg/kg in

clinical trials (WO 2023/232036). It is speculated that the Fc region of the antibody may be a factor contributing to the greater toxicity and side effects. Previous studies (Xiaojie Yu et al., "Isotype Switching Converts Anti-CD40 Antagonism to Agonism to Elicit Potent Antitumor Activity," *Cancer Cell* 2020 Jun 8;37(6):850–866.e7. doi: 10.1016/j.ccell.2020.04.013) have suggested that an Fc region of IgG2 isotype has the potential to promote the agonistic activity of CD40 antibodies, but since it can induce CD40 clustering and activation independently of Fcγ receptors, it also has a greater potential to induce non-tumor-specific immune activation *in vivo*, resulting in increased toxicity and side effects, thereby limiting the clinically tolerated dose and, consequently, the efficacy of the antibody (Xiaojie Yu et al., Cancer Cell. 2020 Jun 8;37(6):850-866.e7. doi: 10.1016/j.ccell.2020.04.013). In view of the foregoing, the inventors investigated the effects of different Fc regions on the efficacy and safety of the bispecific antibody molecules of the present invention, and proposed an optimized Fc-region design that is advantageous for the antibodies of the present invention. In some embodiments, the optimized Fc region has an IgG1 isotype and comprises a modification, such as an amino acid mutation or a glycosylation modification, that reduces or eliminates binding to an Fcγ receptor. Compared with IgG2 Fc regions previously reported in the literature, studies have shown that the optimized Fc region of the present invention can, on the one hand, eliminate interaction between the antibody Fc region and Fcγ receptors, and, on the other hand, avoid the nonspecific CD40 agonistic activity associated with IgG2, thereby substantially reducing the nonspecific toxicity of the bispecific antibody molecules of the present invention outside tumors, improving their safety and tolerated dose and consequently their efficacy. Therefore, in some preferred embodiments, the present invention provides a bispecific antibody having an optimized Fc-region design, wherein the Fc region is an IgG1 Fc region comprising a mutation that reduces or eliminates Fcγ-receptor binding activity, wherein the mutation may be, in particular, a LALA mutation (L234A, L235A).

In pharmacodynamic studies of the antibody molecules of the present invention, various cold tumor models relevant to the potential clinical applications of the antibody molecules were used to evaluate the bispecific antibodies of the present invention, including B16F10-hPD-L1 (Urska Kamensek et al., *Bioelectrochemistry* 140 (2021) 107831; doi:10.1016/j.bioelechem.2021.107831) and KPC-hPD-L1 (doi:10.3390/cancers13030440) cold tumor models. The results showed that the antibodies of the present invention exhibited favorable antitumor efficacy in tumors resistant to PD-1/PD-L1 immune checkpoint inhibitors, and have broad potential for clinical application as anticancer drug candidates.

The components of the bispecific antibodies of the present invention are described in detail below. Those skilled in the art will appreciate that, unless expressly stated otherwise, any combination of any technical features of these components is contemplated by the present invention. Moreover, it will be understood by those skilled in the art that, unless expressly stated otherwise, an antibody of the present invention, including any form thereof, may comprise any such combination of features.

Antigen-binding site

In the course of their studies, the inventors found that, in some instances, combining a PD-L1 antigen-binding site having high binding affinity with a CD40 antigen-binding site exhibiting only weak agonistic activity in the absence of crosslinking can advantageously improve the efficacy and safety of the bispecific antibody of the present invention.

Accordingly, in some embodiments, the present invention provides a bispecific antibody comprising a PD-L1 antigen-binding site that binds PD-L1 with high affinity and a CD40 antigen-binding site that exhibits only weak agonistic activity in the absence of crosslinking. Preferably, the CD40 antigen-binding site a CD40 antigen-binding site that selectively activates the CD40 signaling pathway in a PD-L1-dependent manner.

In some embodiments, the PD-L1 antigen-binding site binds to PD-L1 with a KD value of less than about 30

$\times 10^{-9}$ M, less than about 20×10^{-9} M, less than about 15×10^{-9} M, or less than 10×10^{-9} M. In some embodiments, the PD-L1 antigen-binding site binds to PD-L1 with a KD value of about 10×10^{-9} M to 0.1×10^{-9} M, e.g., about 9×10^{-9} M, about 8×10^{-9} M, about 7×10^{-9} M, about 6×10^{-9} M, about 5×10^{-9} M, about 4×10^{-9} M, about 3×10^{-9} M, about 2×10^{-9} M, or about 1×10^{-9} M or less. In some embodiments, the PD-L1 antigen-binding site binds to PD-L1 preferably with a KD value of about 5×10^{-9} M to 0.5×10^{-9} M, and more preferably with a KD value of about 1 nM to about 5 nM. In some embodiments, the KD value is determined using bio-layer interferometry, e.g., as described in Example 8.

In some embodiments, the CD40 antigen-binding site selectively activates the CD40 signaling pathway in a PD-L1-dependent manner. Preferably, the selectivity index, defined as the ratio of the EC50 value of the antibody in the absence of PD-L1-expressing cells to the EC50 value of the antibody in the presence of PD-L1-expressing cells, is greater than 100, e.g., greater than 150, greater than 200, or greater than 300, or, e.g., about 300-1000, 500-1000, or 700-1000. In some embodiments, the EC50 values are determined using a fluorescent reporter assay in the presence and absence of PD-L1(high)-expressing cells, for example, as described in Example 6, and the selectivity index is calculated therefrom.

In some embodiments, the PD-L1 antigen-binding site has cross-reactivity with human and monkey PD-L1. In some other embodiments, the CD40 antigen-binding site has cross-reactivity with human and monkey CD40.

In some embodiments, the PD-L1 antigen-binding site is provided by an antigen-binding domain that specifically binds to PD-L1; the antigen-binding domain may be selected from, for example, but is not limited to, a VHH domain, a single-domain antibody, a VH/VL domain pair, a Fab domain, and an scFv domain; preferably, the antigen-binding domain comprises or consists of a VHH domain.

In some embodiments, the CD40 antigen-binding site is provided by an antigen-binding domain that specifically binds to CD40; the antigen-binding domain may be selected from, for example, but is not limited to, a VHH domain, a single-domain antibody, a VH/VL domain pair, a Fab domain, and an scFv domain; preferably, the antigen-binding domain comprises or consists of a Fab domain or an scFv domain.

In some embodiments, the PD-L1 antigen-binding site is provided by a VHH domain; and the CD40 antigen-binding site is provided by a Fab domain.

Exemplary PD-L1 antigen-binding site

The PD-L1 antigen-binding site according to the present invention is also referred to herein as the anti-PD-L1 domain according to the present invention. In the bispecific antibody according to the present invention, in some embodiments, preferably, the PD-L1 antigen-binding site according to the present invention is provided by a VHH domain (or abbreviated as "anti-PD-L1 VHH domain").

In some embodiments, the anti-PD-L1 VHH domain according to the present invention comprises CDR1, CDR2, and CDR3 sequences contained within a variable region having the amino acid sequence set forth in SEQ ID NOs: 5 or 6. Preferably, the CDR sequences contained within the variable region are defined according to AbM, Chothia, Kabat, IMGT, or any combination thereof. More preferably, the CDRs are defined according to Kabat. However, it should be understood that the CDRs may also be defined in any other way known in the art.

In some embodiments, the anti-PD-L1 VHH domain according to the present invention comprises CDR1, CDR2, and CDR3 sequences, wherein

(i) the CDR1 comprises or consists of the amino acid sequence of SEQ ID NO: 2;

(ii) the CDR2 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 3;

(iii) the CDR3 comprises or consists of the amino acid sequence set forth in SEQ ID NO: 4.

In some embodiments, the anti-PD-L1 VHH domain according to the present invention comprises a VHH variable region sequence of any one of the exemplary anti-PD-L1 antibodies of the present invention, or a variant thereof. For example, the VHH domain may have the same CDR sequences as one of the exemplary antibodies, and

the same or different framework region sequences, for example, a VHH domain having a humanized sequence. The maintenance or improvement of the PD-L1 antigen-binding properties or other functional properties of the VHH domain can be assessed in an *in vitro* or *in vivo* assay.

In some embodiments, the anti-PD-L1 VHH domain according to the present invention comprises or consists of an amino acid sequence of SEQ ID NO: 5 or 6. In still other embodiments, the VHH domain comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence set forth in SEQ ID NO: 5 or 6 and retaining the ability to specifically bind to PD-L1. In still other embodiments, the VHH domain comprises an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions (e.g., conservative substitutions) compared to the amino acid sequence set forth in SEQ ID NO: 5 or 6, and retaining the ability to specifically bind to PD-L1. preferably, the addition, deletion and/or substitution of the amino acids do not occur in the CDRs.

In some preferred embodiments, the anti-PD-L1 VHH domain according to the present invention comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6.

Exemplary CD40 antigen-binding site

The CD40 antigen-binding site according to the present invention is also referred to herein as the anti-CD40 domain according to the present invention. In some embodiments, preferably, the CD40 antigen-binding site according to the present invention is provided by a VH domain and a VL domain which are paired. In some embodiments, the CD40 antigen-binding site according to the present invention is provided by a Fab domain (or abbreviated as "anti-CD40 Fab domain") or an scFv domain (or abbreviated as "anti-CD40 scFv domain").

In some embodiments, the anti-CD40 domain according to the present invention is derived from an anti-CD40 antibody, and preferably comprises a VH/VL domain pair from the antibody. Examples of anti-CD40 antibodies that can be used in the present invention include, for example, but are not limited to, S2C6 antibody (U.S. Patent No. 6,946,129) or humanized forms thereof (see, e.g., WO 2006/128103 and WO2008/091954).

In some embodiments, the anti-CD40 antibody has one or more or all of the following properties:

- immunological cross-reactivity with human CD40 protein and cynomolgus monkey CD40 protein;
- activate CD40 signaling pathway.

In some embodiments, the anti-CD40 domain according to the present invention comprises a VH domain and a VL domain, wherein the VH domain comprises HCDR1, HCDR2, and HCDR3 sequences contained within a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 17; and the VL domain comprises LCDR1, LCDR2, and LCDR3 sequences contained within a light chain variable region having the amino acid sequence set forth in SEQ ID NO: 16. Preferably, the CDR sequences contained within the variable region are defined according to AbM, Chothia, Kabat, IMGT, or any combination thereof. More preferably, the CDRs are defined according to Kabat. However, it should be understood that the CDRs may also be defined in any other way known in the art.

In some embodiments, the antigen-binding site that specifically binds to CD40 according to the present invention comprises:

(i) HCDR1 comprising or consisting of SEQ ID NO: 21;

(ii) HCDR2 comprising or consisting of SEQ ID NO: 22;

(iii) HCDR3 comprising or consisting of SEQ ID NO: 23;

(iv) LCDR1 comprising or consisting of SEQ ID NO: 18;

(v) LCDR2 comprising or consisting of SEQ ID NO: 19; and

(vi) LCDR3 comprising or consisting of SEQ ID NO: 20.

In some embodiments, the CD40 antigen-binding site according to the present invention comprises the VH/VL variable region sequences of any one of the exemplary anti-CD40 antibodies of the present invention, or variants

thereof. For example, the CD40 antigen-binding site may have the same CDR sequences as one of the exemplary antibodies and the same or different framework region sequences, for example, an anti-CD40 antibody fragment having a humanized sequence. The maintenance or improvement of the antigen-binding properties or other functional properties of the CD40 antigen-binding site can be assessed in an *in vitro* or *in vivo* assay.

In some embodiments, the CD40 antigen-binding site according to the present invention comprises a heavy chain variable region VH and a light chain variable region VL. In some embodiments, the VH comprises or consists of the amino acid sequence set forth in SEQ ID NO: 17. In some other embodiments, the VH comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence set forth in SEQ ID NO: 17 and retaining the ability to specifically bind to CD40. In some other embodiments, the VH comprises an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions (e.g., conservative substitutions) compared to the amino acid sequence set forth in SEQ ID NO: 17 and retaining the ability to specifically bind to CD40, wherein preferably, the amino acid additions, deletions, and/or substitutions do not occur in the CDRs. In some embodiments, the VL comprises or consists of an amino acid sequence of SEQ ID NO: 16. In some other embodiments, the VL comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% identity to an amino acid sequence of SEQ ID NO: 16 and retaining the ability to specifically bind to CD40. In some other embodiments, the VL comprises an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions (e.g., conservative substitutions) compared to the amino acid sequence set forth in SEQ ID NO: 16 and retaining the ability to specifically bind to CD40, wherein preferably, the amino acid additions, deletions, and/or substitutions do not occur in the CDRs.

In some preferred embodiments, the CD40 antigen-binding site according to the present invention comprises a heavy chain variable region VH and a light chain variable region VL, wherein the VH comprises or consists of the amino acid sequence set forth in SEQ ID NO: 17, and the VL comprises or consists of the amino acid sequence set forth in SEQ ID NO: 16.

Immunoglobulin Fc region

In some embodiments, the bispecific antibody of the present invention may comprise an immunoglobulin Fc region. A native Fc region is a C-terminal constant domain of an immunoglobulin, and interacts with cell surface Fc receptors and some proteins of the complement system. An immunoglobulin Fc region generally comprises two or three heavy chain constant domains (referred to as CH2, CH3, and CH4) and optionally a hinge region, and is generally present in a dimerized form.

The immunoglobulin Fc region suitable for use in the bispecific antibody of the present invention may be an Fc region derived from any immunoglobulin. In some embodiments, the immunoglobulin Fc region comprises at least an immunoglobulin CH2 domain and an immunoglobulin CH3 domain. In some embodiments, the immunoglobulin Fc region further comprises a hinge region or a portion thereof. In some embodiments, the immunoglobulin Fc region comprises or consists of, from N-terminus to C-terminus, an immunoglobulin hinge region or a portion thereof, a CH2 domain, and a CH3 domain. In some instances, an immunoglobulin Fc region comprising a hinge region sequence is preferred, which may, for example, promote dimerization of antibody polypeptide chains.

The immunoglobulin Fc region may be fused to the C-terminus or N-terminus of another domain (i.e., PD-L1 or CD40 binding domain). The immunoglobulin Fc region may be fused to another domain via a linker, or fused to another domain directly. In embodiments in which the immunoglobulin Fc region is fused to another domain at its N-terminus, the fusion is preferably effected via an immunoglobulin hinge region sequence. In embodiments in which the immunoglobulin Fc region is fused to another domain at its C-terminus, the fusion is preferably effected

via a flexible linker peptide, such as the linker peptide of SEQ ID NO: 30.

The immunoglobulin Fc region suitable for use in the bispecific antibody of the present invention may be a native Fc region sequence. Alterantively, the Fc region may comprise a mutation relative to a native Fc sequence. Mutations include substitutions, insertions, and/or deletions. Such mutations may be introduced to confer desired therapeutic properties. For example, to promote heterodimerization, Knob-into-Hole (KiH) mutations may be introduced into CH3 domains. In such instances, one Fc chain is designed to contain a larger protruding residue (i.e., a Knob), while the other Fc chain is designed to cotain a complementary pocket (i.e., a Hole). Suitable positions for KiH mutations are known in the art. Exemplary KiH mutations include, but are not limited to, a combination of the Knob mutation T366W and the Hole mutations T366S, L368A, and Y407V; and a combination of the Knob mutation T366Y and the Hole mutation Y407T. Regarding dimerized Fc regions comprising KiH mutations and their use in engineered antibodies, reference may be made, for example, to Carter P.; ridgway J.B.B.; presta L.G.: Immunotechnology, Volume 2, Feb. 01, 1996, pp. 73-73. When the bispecific antibody of the present invention comprises an asymmetric two-chain structure, the Fc regions preferably comprise KiH mutations that promote the correct heterodimerization of the antibody polypeptide chains.

The immunoglobulin Fc region suitable for use in the bispecific antibody of the present invention may comprise a cysteine residue capable of forming an interchain disulfide bond between the heavy chains, for example, a cysteine residue mutation introduced into the Fc region. Exemplary cysteine residue mutations include, but are not limited to, introduction of a Y359C mutation into one Fc chain and an S354C mutation into the other Fc chain of a dimerized Fc region.

Depending on the specific uses of the antibody or antibody-based molecule, the immunoglobulin Fc region suitable for use in the bispecific antibody of the present invention may comprise a mutation that alters an effector function. For example, where an effector function is not required, the Fc region may comprise s mutation that reduced or eliminated such effector function. Such useful mutations can be found, for example, in Ian Wilkinson et al., Fc-engineered antibodies with immune effector functions completely immobilized, PLoS ONE 16(12), https://doi.org/10.1371/journal.pone.0260954. In humans, three types of FcγRs have been characterized:

- FcγRI (CD64), which binds monomeric IgG with high affinity and is expressed on macrophages, monocytes, neutrophils, and eosinophils. An IgG Fc region having mutations at at least one of amino acid residues E233-G236, P238, D265, N297, A327, and P329 (numbering according to the EU index of Kabat) may have reduced binding to FcγRI.

- FcγRII (CD32), which binds IgG complexes with moderate to low avidity and is widely expressed. This receptor can be divided into two subclasses, FcγRIIA and FcγRIIB. FcγRIIA is expressed on many cells involved in killing activity (e.g., macrophages, monocytes, and neutrophils) and is capable of activating the killing process. FcγRIIB plays a role in the inhibitory process and is found on B cells, macrophages, mast cells, and eosinophils. An IgG Fc region having mutations at at least one of the amino acid residues E233-G236, P238, D265, N297, A327, P329, D270, Q295, A327, R292, and K414 (numbering according to the EU index of Kabat) has been found to have reduced binding to FcγRIIA.

- FcγRIII (CD16), which binds IgG with moderate to low avidity and exists in two forms, FcγRIIIA and FcγRIIIB. FcγRIIIA, which mediates ADCC, is found on NK cells, macrophages, eosinophils, and some monocytes and T cells. FcγRIIIB is highly expressed on neutrophils. An IgG Fc region having mutations at at least one of the amino acid residues E233-G236, P238, D265, N297, A327, P329, D270, Q295, A327, S239, E269, E293, Y296, V303, A327, K338, and D376 (numbering according to the EU index of Kabat) has been found to have reduced binding to FcγRIIIA.

In some instances, the immunoglobulin Fc region suitable for use in the bispecific antibody of the present invention preferably comprises a mutation that reduces or eliminates binding of the Fc region to an Fcγ receptor,

for example, LALA mutations in which leucine (L) residues at positions 234 and 235 of the Fc region are replaced with alanine (A). Additionally or alternatively, the Fc region may comprise a mutation that increases binding to FcRn and/or an amino acid modification that removes a protease site. Additionally or alternatively, the Fc region may be mutated for antibody production purposes, for example, to remove or replace amino acids that may undergo post-translational modifications (e.g., glycosylation), thereby conferring improved druggability and developability on a therapeutic antibody.

In some embodiments, the bispecific antibody according to the present invention comprises an Fc region from IgG. In some embodiments, preferably, the immunoglobulin Fc region is of IgG1 isotype or a subtype thereof. In some embodiments, preferably, the immunoglobulin Fc region comprises an Fc region sequence from a human. The Fc region sequence that may be used in the antibody of the present invention includes, but is not limited to, an amino acid sequence selected from SEQ ID NOs: 25-27, or an amino acid sequence having at least 90%, 95%, 96%, 97%, 98%, 99% or higher identity thereto.

In some embodiments, the bispecific antibody according to the present invention comprises a dimerized Fc region, wherein the dimerized Fc region consists of a first Fc region and a second Fc region. In some embodiments, one or both of the first and second Fc regions comprise a mutation that reduces or eliminates the binding to an Fcγ receptor, preferably an L234AL235A mutation (numbering according to Kabat EU index).

In some preferred embodiments, the first and second Fc regions are identical. In some embodiments, the first and second Fc regions have an IgG1 isotype.

In some other preferred embodiments, the first and second Fc regions are different from each other. In some embodiments, the first and second Fc regions comprise KiH mutations, and optionally a cysteine residue mutation used for forming an interchain disulfide bond between the first and second Fc regions. In some embodiments, the KiH mutations are a combination of the Knob mutation T366W and the Hole mutations T366S, L368A, and Y407V (numbering according to Kabat EU index). In some preferred embodiments, the first and second Fc regions, independently from each other, comprise a combination of mutations selected from: (a) T366W; and (b) T366S, L368A, and Y407V. In some other preferred embodiments, the first and second Fc regions, independently from each other, comprise a combination of mutations selected from: (a) S354C and T366W; and (b) Y349C, T366S, L368A, and Y407V. In some other preferred embodiments, the first Fc region and the second Fc region, independently from each other, comprise a combination of mutations selected from: (a) S354C, T366W and L234AL235A; and (b) Y349C, T366S, L368A, Y407V and L234AL235A mutations. In some embodiments, the first and second Fc regions have an IgG1 isotype.

In some embodiments, the bispecific antibody of the present invention comprises a dimerized Fc region formed by two different Fc domains, wherein a first Fc domain comprises the amino acid sequence set forth in SEQ ID NO: 25 or an amino acid sequence having at least 90%, 95%, 96%, 97%, 98%, 99%, or greater identity thereto, and a second Fc domain comprises the amino acid sequence set forth in SEQ ID NO: 26 or an amino acid sequence having at least 90%, 95%, 96%, 97%, 98%, 99%, or greater identity thereto; preferably, the first Fc domain comprises the amino acid sequence set forth in SEQ ID NO: 25, and the second Fc domain comprises the amino acid sequence set forth in SEQ ID NO: 26. Preferably, the bispecific antibody is a trivalent bispecific antibody having an asymmetric structure, particularly a trivalent bispecific antibody having the structure shown in FIG. 14.

In some other embodiments, the bispecific antibody of the present invention comprises a dimerized Fc region formed by two identical Fc domains, wherein each of said Fc domains comprises the amino acid sequence set forth in SEQ ID NO: 27. Preferably, the bispecific antibody has a symmetric structure, for example, a “2+2” format tetravalent bispecific antibody.

Linker peptide

In the bispecific antibody according to the present invention, antibody components (i.e., antigen-binding domains and optionally immunoglobulin Fc regions) may be linked via a linker peptide.

The linker peptide suitable for use in the antibody of the present invention is not particularly limited. The linker peptide sequence generally has flexibility. It may be primarily composed of amino acids, such as glycine, alanine, and serine, that lack bulky side chains that may restrict flexibility. Alternatively, the linker peptide may be composed of a sequence derived from an immunoglobulin hinge region. Depending on the site of connection and the components to be connected, a person skilled in the art can readily determine an appropriate sequence and optimal length for the linker peptide.

A suitable linker peptide may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids in length, or longer. In some embodiments, the linker peptide may be relatively short, for example, less than about 20 or 15 amino acids in length, such as from 2 to 15 amino acids in length or from 5 to 10 amino acids in length.

Suitable linker peptide sequences include, but are not limited to, G4S (SEQ ID NO: 32); (G4S)2 (SEQ ID NO: 33); (G4S)3 (SEQ ID NO: 34); GGGSG (SEQ ID NO: 35); GGSGG (SEQ ID NO: 36); GSGGG (SEQ ID NO: 37); GSGGGP (SEQ ID NO: 38); GGEPS (SEQ ID NO: 39); GGEGGGP (SEQ ID NO: 40) and GGEGGGSEGGGS (SEQ ID NO: 41); and (G4S)n (SEQ ID NO: 42), wherein n is an integer equal to or greater than 1; TS(G4S)n (SEQ ID NO: 43), wherein n is an integer equal to or greater than 1; G(G4S)n (SEQ ID NO: 44), wherein n is an integer equal to or greater than 1; (G4)n (SEQ ID NO: 45), wherein n is an integer equal to or greater than 1; (GRPGS)n (SEQ ID NO: 46), wherein n is an integer equal to or greater than 1. The linker peptides that may be used in the antibody molecule of the present invention may also be, for example, but not limited to, the following amino acid sequences: (G3S)2 (SEQ ID NO: 47), (G4S)2 (SEQ ID NO: 48), (G3S)3 (SEQ ID NO: 49), (G4S)3 (SEQ ID NO: 50), (G3S)4 (SEQ ID NO: 51), (G4S)4 (SEQ ID NO: 52), (G3S)5 (SEQ ID NO: 53), (G4S)5 (SEQ ID NO: 54), (G3S)6 (SEQ ID NO: 55), (G4S) (SEQ ID NO: 56), GGG (SEQ ID NO: 57), DGGGS (SEQ ID NO: 58), TGEKP (SEQ ID NO: 59), GGRR (SEQ ID NO: 60), EGKSSGSGSESKVD (SEQ ID NO: 61), KESGSVSSEQLAQFRSLD (SEQ ID NO: 62), GGRRGGGS (SEQ ID NO: 63), LRQRDGERP (SEQ ID NO: 64), LRQKDGGGSERP (SEQ ID NO: 65), and GSTSGSGKPGSGEGSTKG (SEQ ID NO: 66). Alternatively, suitable flexible linker peptides may be rationally designed by computational modeling of the three-dimensional structures of proteins and peptides, or by phage display.

In some embodiments, the linker peptide used in the antibody of the present invention is a flexible linker peptide of 5-50 amino acids, preferably a linker peptide comprising glycine (G) and/or serine (S) and/or threonine (T) residues. In one embodiment, the linker peptide has a length of 5-50 amino acids, such as 5, 10, 15, 20, 25, or 30 amino acids, or has an amino acid length falling between any two of the integers. In some embodiments, the linker peptide comprises an amino acid sequence (G)n, wherein n is an integer equal to or greater than 5, for example, n is an integer of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15. In still some other embodiments, the linker peptide is a hinge region derived from an immunoglobulin.

In some preferred embodiments, the bispecific antibody of the present invention comprises a linker peptide having the amino acid sequence set forth in SEQ ID NO: 30. In some embodiments, the linker peptide is used to link an anti-PD-L1 VHH domain of the present invention to the C-terminus of an Fc region, preferably to the C-terminus of one or both (preferably one) heavy chains of an anti-CD40 full-length antibody of the present invention comprising the Fc region.

Format of bispecific antibody

The bispecific antibody according to the present invention may be in any suitable format, for example, single-chain, two-chain, triple-chain, or tetrachained, symmetric or asymmetric format. Preferably, the bispecific antibody

according to the present invention is a 2+2 format antibody comprising two PD-L1-binding sites and two CD40-binding sites per molecule. More preferably, the bispecific antibody according to the present invention is a 2+1 format antibody comprising one PD-L1-binding site and two CD40-binding sites per molecule. In some embodiments, the bispecific antibody according to the present invention further comprises an Fc region.

In some embodiments, the present invention provides a bispecific antibody comprising:

(a) an anti-CD40 full-length antibody comprising a dimerized Fc region linked to two CD40 antigen-binding sites of the present invention; and

(b) one or two, preferably one, PD-L1 antigen-binding sites of the present invention,

wherein the PD-L1 antigen-binding site is linked to the full-length antibody, optionally via a linker peptide,

preferably, the CD40 antigen-binding site is linked to the N-terminus of the dimerized Fc region, and the PD-L1 antigen-binding site is linked to the C-terminus of the dimerized Fc region,

more preferably, the CD40 antigen-binding site is selected from a Fab, a VH/VL pair, an scFab, an scFv, and a CrossFab, preferably a Fab or scFv domain, and more preferably a Fab domain; and the PD-L1 antigen-binding site is a VHH domain.

In some preferred embodiments, the full-length antibody comprises two heavy chains, and the anti-PD-L1 VHH domain is fused to the C-terminus of one heavy chain of the full-length antibody. In one embodiment,

(a) the heavy chain fused to the anti-PD-L1 VHH domain in the full-length antibody has the tripeptide LSP as C-terminal amino acid residues, wherein the proline residue is fused to the VHH domain via a linker peptide; and

(b) the heavy chain not fused to the VHH domain in the full-length antibody has the tripeptide LSP, or SPG, or PGK as C-terminal amino acid residues.

Exemplary bispecific antibodies

In some aspects, the present invention provides a bispecific antibody comprising first, second, and third polypeptide chains, wherein:

- the first polypeptide chain comprises, from N-terminus to C-terminus, a VH domain, a CH1 domain, an Fc region, a linker peptide, and a VHH domain;

- the second polypeptide chain comprises, from N-terminus to C-terminus, a VH domain, a CH1 domain, and an Fc region; and

- the third polypeptide chain comprises, from N-terminus to C-terminus, a VL domain and a CL domain,

wherein the VHH domain specifically binds to PD-L1, and wherein the VH domain pairs with the VL domain to specifically bind to CD40. Preferably, the VHH domain is an anti-PD-L1 domain of the present invention, and the VH and VL domains pair to form an anti-CD40 domain of the present invention.

In some embodiments, the VH domain comprises HCDR1, HCDR2 and HCDR3 having amino acid sequences set forth in SEQ ID NOs: 21, 22 and 23, respectively, and the VL domain comprises LCDR1, LCDR2 and LCDR3 having amino acid sequences set forth in SEQ ID NOs: 18, 19 and 20, respectively,

preferably, the VH domain comprises the amino acid sequence set forth in SEQ ID NO: 17, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 17, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions compared to SEQ ID NO: 17; and

the VL domain comprises the amino acid sequence set forth in SEQ ID NO: 16, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 16, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions compared to SEQ ID NO: 16;

most preferably, the VH domain comprises the amino acid sequence set forth in SEQ ID NO: 17, and the VL

domain comprises the amino acid sequence set forth in SEQ ID NO: 16.

In some embodiments, the VHH domain comprises a CDR1, a CDR2, and a CDR3 having the amino acid sequences set forth in SEQ ID NOs: 2, 3, and 4, respectively. Preferably, the VHH domain comprises the amino acid sequence set forth in SEQ ID NO: 5 or 6, or an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% identity to SEQ ID NO: 5 or 6, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions as compared to SEQ ID NO: 5 or 6. Most preferably, the VHH domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6.

In some embodiments, the CH1 domain is a human IgG CH1 domain. In some embodiments, the CH1 domain comprises the amino acid sequence set forth in SEQ ID NO: 29, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity thereto, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions as compared to SEQ ID NO: 29. Most preferably, the CH1 domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 29.

In some embodiments, the CL domain is a human immunoglobulin kappa or lamda light chain constant domain. In some embodiments, the CL domain comprises the amino acid sequence set forth in SEQ ID NO: 24, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 24, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions as compared to SEQ ID NO: 24. Most preferably, the CH1 domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 24.

In some embodiments, the Fc regions of the first polypeptide chain and the second polypeptide chain comprise a Knob mutation and a Hole mutation promoting heterodimerization of the first polypeptide chain and the second polypeptide chain, respectively, and optionally a cysteine residue mutation for forming an interchain disulfide bond between the first polypeptide chain and the second polypeptide chain. In some embodiments, the knob mutation is T366W; and the hole mutation is T366S, L368A, and Y407V. In some embodiments, the cysteine residue mutation is a Y349C introduced into the Fc region of the first polypeptide chain and a S354C introduced into the Fc region of the second polypeptide chain, or is a S354C introduced into the Fc region of the first polypeptide chain and a Y349C introduced into the Fc region of the second polypeptide chain. Still more preferably, the Fc regions of the first and second polypeptide chains, independently from each other, comprise an amino acid sequence selected from: SEQ ID NO: 25 or an amino acid sequence having at least 95% identity thereto, and SEQ ID NO: 26 or an amino acid sequence having at least 95% identity thereto. More preferably, the Fc region of the first polypeptide chain comprises the amino acid sequence of SEQ ID NO: 25, and the Fc region of the second polypeptide chain comprises the amino acid sequence of SEQ ID NO: 26; or vice versa.

More preferably, the bispecific antibody comprises a first polypeptide chain, a second polypeptide chain, and a third polypeptide chain, wherein:

the first polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 9 or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity thereto;

the second polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 8, or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto; and

the third polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 7, or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto.

Still more preferably, the bispecific antibody comprises first, second, and third polypeptide chains having the amino acid sequences set forth in SEQ ID NOs: 9, 8, and 7, respectively. Most preferably, the bispecific antibody is a trivalent bispecific antibody consisting of one first polypeptide chain, one second polypeptide chain, and two light chains.

In some other aspects, the present invention provides a bispecific antibody or an antigen-binding fragment thereof, wherein the bispecific antibody comprises a first polypeptide chain and a second polypeptide chain, wherein:

- the first polypeptide chain comprises, from N-terminus to C-terminus, a VH domain, a CH1 domain, an Fc region, a linker peptide, and a VHH domain;

- the second polypeptide chain comprises, from N-terminus to C-terminus, a VL domain and a CL domain;

wherein the VHH domain specifically binds to PD-L1, and the VH domain is paired with the VL domain and specifically binds to CD40. Preferably, the VHH domain is an anti-PD-L1 domain of the present invention, and the VH and the VL domain are paired to form an anti-CD40 domain of the present invention.

In some embodiments, the VH domain comprises HCDR1, HCDR2 and HCDR3 having amino acid sequences set forth in SEQ ID NOs: 21, 22 and 23, respectively, and the VL domain comprises LCDR1, LCDR2 and LCDR3 having amino acid sequences set forth in SEQ ID NOs: 18, 19 and 20, respectively,

preferably, the VH domain comprises the amino acid sequence set forth in SEQ ID NO: 17, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 17, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions compared to SEQ ID NO: 17; and

the VL domain comprises the amino acid sequence set forth in SEQ ID NO: 16, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 16, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions compared to SEQ ID NO: 16;

most preferably, the VH domain comprises the amino acid sequence set forth in SEQ ID NO: 17, and the VL domain comprises the amino acid sequence set forth in SEQ ID NO: 16.

In some embodiments, the VHH domain comprises a CDR1, a CDR2, and a CDR3 having the amino acid sequences set forth in SEQ ID NOs: 2, 3, and 4, respectively. Preferably, the VHH domain comprises the amino acid sequence set forth in SEQ ID NO: 5 or 6, or an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% identity to SEQ ID NO: 5 or 6, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions as compared to SEQ ID NO: 5 or 6. Most preferably, the VHH domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6.

In some embodiments, the CH1 domain is a human IgG CH1 domain. In some embodiments, the CH1 domain comprises the amino acid sequence set forth in SEQ ID NO: 29, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 29, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions, and/or substitutions as compared to SEQ ID NO: 29. Most preferably, the CH1 domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 29.

In some embodiments, the CL domain is a human immunoglobulin kappa or lamda light chain constant domain. In some embodiments, the CL domain comprises the amino acid sequence set forth in SEQ ID NO: 24, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 24, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions as compared to SEQ ID NO: 24. Most preferably, the CH1 domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 24.

In some embodiments, the Fc region of the first polypeptide chain comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% identity to SEQ ID NO: 27, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions as compared to SEQ ID NO: 27. Most preferably, the Fc region comprises or consists of the amino acid sequence set forth in SEQ ID NO: 27.

In some embodiments, preferably, the first polypeptide chain comprises the amino acid sequence set forth in

SEQ ID NO: 11 or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto; and

the second polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 10 or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto.

Still more preferably, the bispecific antibody comprises a first polypeptide chain and a second polypeptide chain having the amino acid sequences set forth in SEQ ID NOs: 11 and 10, respectively. Most preferably, the bispecific antibody is a tetravalent bispecific antibody assembled from two first polypeptide chains and two second polypeptide chains.

Properties of the antibody of the present invention

The bispecific antibody of the present invention may have one or more of the following properties:

(i) binding with high affinity to cells expressing PD-L1 (preferably tumor cells or antigen-presenting cells, such as DC cells);

(ii) having species cross-reactivity with cynomolgus monkey PD-L1;

(iii) having species cross-reactivity with cynomolgus monkey CD40; and

(iv) capable of selectively activating CD40 signaling pathway and activating DCs in a PD-L1-dependent manner.

In some aspects, through having the PD-L1-binding site serving as a means for antibody crosslinking, the bispecific antibody of the present invention is capable of selectively activating CD40-expressing cells (e.g., DC cells) in the presence of PD-L1-expressing cells. This selective property of the antibody of the present invention may reduce the likelihood that the antibody induces CD40 activation in normal tissues and subsequently causes toxicity and side effects. The reduction in toxicity and side effects may increase the tolerable dose of the antibody of the present invention in humans. An increased administration dose may increase the blood concentration of the antibody and its accumulation in the tumor microenvironment, thereby enhancing its efficacy.

In some other aspects, by abrogating binding of the Fc region to Fcγ receptors, the bispecific antibody of the present invention has reduced Fc region-mediated effector functions, such as ADCC and CDC, thereby avoiding effector function-mediated killing of antigen-presenting cells (such as DCs) to which the antibody specifically binds and enhancing the efficacy of the antibody.

In some other aspects, by adopting a unique 2+1 format and having a monovalent PD-L1-binding site serving as an anchoring site, the antibody of the present invention may, as compared with a bivalent PD-L1-binding-site design, enable maximal utilization of PD-L1 in target tumor tissues and reduce PD-L1-binding avidity, thereby reducing binding of the antibody to normal tissue cells expressing low levels of PD-L1, increasing selective binding of the antibody to cells expressing high levels of PD-L1 in the tumor microenvironment, facilitating enrichment of the antibody in the tumor microenvironment, and improving the safety and efficacy of the antibody.

II. The anti-PD-L1 antibody of the present invention

In a second aspect, the present invention provides an anti-PD-L1 antibody or an antigen-binding fragment thereof, comprising a VHH domain that specifically binds to PD-L1, wherein the VHH domain comprises CDR1, CDR2, and CDR3 sequences contained in SEQ ID NO: 5 or 6;

preferably, the CDR1, CDR2, and CDR3 sequences comprise or consist of the amino acid sequences set forth in SEQ ID NOs: 2, 3, and 4, respectively;

more preferably, the VHH domain comprises the amino acid sequence set forth in SEQ ID NO: 5 or 6, an amino acid sequence having at least 80%, 85%, 90%, 95%, or 99% sequence identity to SEQ ID NO: 5 or 6, or an amino acid sequence having one or more (preferably 1–10 and more preferably 1–5) amino acid additions, deletions,

and/or substitutions, as compared to SEQ ID NO: 5 or 6;

most preferably, the VHH domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6.

The anti-PD-L1 antibody of the present invention may have any suitable antibody structure, including, but not limited to, a single-chain or multichain antibody, a monospecific or multispecific antibody (e.g., a bispecific antibody), a linear antibody, a single-domain antibody, a heavy-chain antibody, a chimeric antibody, or a humanized antibody. In some embodiments, the anti-PD-L1 antibody of the present invention comprises an immunoglobulin Fc region linked to the VHH domain, i.e., has a VHH-Fc format. In some embodiments, the anti-PD-L1 antibody of the present invention is a heavy-chain antibody. As used herein, the term "heavy-chain antibody" (hcAb) refers to an antibody lacking a light chain, and may comprise, from N-terminus to C-terminus, VHH-CH2-CH3 or VHH-CH1-CH2-CH3; and may form a homodimer, such as a heavy-chain dimer antibody lacking a light chain.

The VHH domain of the anti-PD-L1 antibody of the present invention has good tumor-targeting properties, and exhibits immunologic human-monkey species cross-reactivity. Therefore, the anti-PD-L1 antibody of the present invention, or the VHH domain thereof, may be used as a targeting module of a conjugate, and may be conjugated or coupled to, for example, a chemotherapeutic agent, a toxin, a drug (e.g., an immunotherapeutic agent), a radioelement, a probe, or a signalling molecule, thereby providing improved applications such as tumor killing, immune regulation, or disease detection.

III. Production and purification of antibody of the present invention

In a third aspect, the present invention provides a method for producing an antibody of the present invention. To produce the antibody of the present invention, polypeptide chains of the antibody may be obtained, for example, by solid-phase peptide synthesis (e.g., Merrifield solid-phase synthesis) or recombinant production, and assembled under appropriate conditions.

For recombinant production, polynucleotides encoding any one polypeptide chain and/or multiple polypeptide chains of the antibody may be isolated and inserted into one or more vectors for further cloning and/or expression in host cells. The polynucleotides may be readily isolated and sequenced using conventional methods. In one embodiment, the present invention provides a polynucleotide encoding one or more polypeptide chains of an antibody of the present invention. In another embodiment, the present invention provides a vector, preferably an expression vector, comprising one or more polynucleotides of the present invention. Accordingly, in one embodiment, the present invention provides a method for producing an antibody of the present invention, comprising: culturing a host cell comprising a polynucleotide encoding the polypeptide chains of the antibody under conditions suitable for expression of the polypeptide chains; and optionally assembling the polypeptide chains under conditions suitable for their assembly into the antibody.

The expression vector may be constructed using methods well known to those skilled in the art. The expression vector includes, but is not limited to, a virus, a plasmid, a cosmid, a λ phage, or a yeast artificial chromosome (YAC).

In one embodiment, the present invention further provides a host cell comprising one or more polynucleotides of the present invention. In some embodiments, a host cell comprising an expression vector of the present invention is provided. Suitable host cells include prokaryotic microorganisms, such as *Escherichia coli*; eukaryotic microorganisms, such as filamentous fungi or yeast; and eukaryotic cells, such as Chinese hamster ovary (CHO) cells and insect cells. Mammalian cell lines suitable for suspension culture may be used. Examples of useful mammalian host cell lines include SV40-transformed monkey kidney CV1 cells (COS-7), human embryonic kidney cells (HEK293 or 293F cells), baby hamster kidney cells (BHK), monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical cancer cells (HeLa), canine kidney cells (MDCK), buffalo rat liver cells (BRL 3A), human lung cells (WI-38), human liver cells (HepG2), CHO cells, NS0 cells, and myeloma cell lines,

such as Y0, NS0, P3X63, and Sp2/0. In a preferred embodiment, the host cell is a CHO cell or an HEK293 cell.

The antibody produced by the methods described herein may be purified using methods known in the art, such as high-performance liquid chromatography, ion-exchange chromatography, gel electrophoresis, affinity chromatography, and size-exclusion chromatography. After purification, the purity of the antibody may be determined using any of a variety of well-known analytical methods, including size-exclusion chromatography, gel electrophoresis, high-performance liquid chromatography, and the like. The physicochemical properties and/or biological activity of the antibody provided herein may be identified, screened, or characterized using a variety of assays known in the art.

In a preferred embodiment, when recombinantly produced in a mammalian host cell, such as a CHO cell, the antibody of the present invention exhibits good production properties, particularly good expression yield and a favorable by-product profile.

IV. Immunofusion and immunoconjugate

In a fourth aspect, the present invention provides an immunofusion or an immunoconjugate comprising an antibody of the present invention fused or conjugated to a heterologous molecule.

In one embodiment, in the immunofusion, the antibody, or an antigen-binding fragment thereof, of the present invention is linked, directly or via an amino acid linker, to a heterologous peptide or polypeptide molecule. Such heterologous peptides and polypeptides that may be mentioned include, but not limited to, a protein or polypeptide that imparts an additional functional activity to the immunofusion, or a tag peptide that facilitates purification or detection of the immunofusion.

In one embodiment, in the immunoconjugate, the antibody, or an antigen-binding fragment thereof, of the present invention is conjugated to a therapeutic agent, a diagnostic agent, or a detectable agent. In the immunoconjugate, a linker may be used to covalently link different entities of the conjuate. Suitable linkers include chemical linkers and peptide linkers. Advantageously, the linker may be a “cleavable linker” that facilitates the release of the polypetide upon delivery to a target site. For example, an acid-labile linker, a peptidase-sensitive linker, a photolabile linker, a dimethyl linker, or a disulfide-containing linker may be used.

In embodiments involving conjugation to a therapeutic agent, the therapeutic agent suitable for the immunoconjugate includes, but is not limited to, a cytotoxin (e.g., a cytostatic agent or a cytotoxic agent), a drug, or a radioisotope.

In embodiments involving conjugation to a diagnostic agent or a detectable agent, the immunoconjugate may be used as part of a clinical testing method, for example, to determine the efficacy of a particular therapy, or to monitor or predict the onset, development, progression, and/or severity of a disease or disorder. Diagnosis and detection may be achieved by coupling the antibody to a detectable agent, including, but not limited to, enzymes such as horseradish peroxidase; prosthetic groups such as streptavidin/biotin and avidin/biotin; fluorescent substances; luminescent substances; radioactive substances; positron-emitting metals; and non-radioactive paramagnetic metal ions used in positron emission tomography techniques.

V. Pharmaceutical composition, pharmaceutical combination, and kit

In a fifth aspect, the present invention provides a composition, e.g., a pharmaceutical composition, comprising an antibody, an immunoconjugate, or an immunofusion of the present invention formulated together with a pharmaceutically acceptable carrier. As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, isotonic agents, absorption-delaying agents, and the like that are physiologically compatible. The pharmaceutical composition of the present invention is suitable for intravenous, intramuscular, subcutaneous, parenteral, rectal, spinal, or epidermal administration (e.g., by injection or infusion). In some

embodiments, the antibody, immunoconjugate, or immunofusion of the present invention is the only active ingredient in the pharmaceutical composition. In some other embodiments, the pharmaceutical composition may comprise the antibody, immunoconjugate, or immunofusion of the present invention and one or more additional therapeutic agents.

In another aspect, the present invention further provides a pharmaceutical combination comprising an antibody, an immunoconjugate, or an immunofusion disclosed herein and one or more additional therapeutic agents.

The additional therapeutic agent suitable for use in the pharmaceutical composition or pharmaceutical combination of the present invention may be selected from any one of the following categories (i)-(iv): (i) drugs that enhance antigen presentation (e.g., tumor antigen presentation); (ii) drugs that enhance effector cell responses (e.g., B-cell and/or T-cell activation and/or mobilization); (iii) drugs that reduce immunosuppression; and (iv) drugs having anti-tumor effects.

The pharmaceutical composition of the present invention may comprise a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody, an immunoconjugate, or an immunofusion of the present invention. "Therapeutically effective amount" refers to an amount effective to achieve a desired therapeutic result when administered at a required dose for a required period of time. A therapeutically effective amount may vary depending on a variety of factors, such as the disease state and the age, sex, and weight of the subject. A therapeutically effective amount is an amount for which any toxic or detrimental effects are outweighed by the therapeutically beneficial effects. Preferably, a therapeutically effective amount inhibits a measurable parameter (e.g., tumor growth rate) by at least about 20%, more preferably at least about 40%, even more preferably at least about 60%, and still more preferably at least about 80%, relative to an untreated subject. The ability of an antibody of the present invention to inhibit a measurable parameter (e.g., tumor volume) may be evaluated in an animal model system predictive of efficacy in human tumors. "Prophylactically effective amount" refers to an amount effective to achieve a desired prophylactic result when administered at a required dose for a required period of time. Generally, because a prophylactic dose is administered to a subject prior to or at an early stage of a disease, the prophylactically effective amount is less than the therapeutically effective amount.

A kit comprising an antibody described herein is also within the scope of the present invention. The kit may include one or more additional elements, including, for example, instructions for use; other reagents, such as a labelling reagent or a coupling reagent; a pharmaceutically acceptable carrier; and a device or other materials for administration to a subject.

VI. Uses and Methods

In a sixth aspect, based on the favorable targeting properties of the antibody, immunoconjugate, or immunofusion of the present invention for tumor cells and cells in the tumor microenvironment, as well as the other advantageous properties described above, the present invention further provides uses of, and methods of using, the antibody, immunoconjugate, or immunofusion of the present invention for preventing or treating a tumor. In such uses, the antibody, or an antigen-binding fragment thereof, of the present invention; the immunoconjugate or immunofusion of the present invention; or a pharmaceutically acceptable salt or solvate thereof may be administered to a subject as the sole active agent or in combination with other therapies or therapeutic agents. Such other therapies and therapeutic agents include, for example, drugs that target antigens on the surface of tumor cells and destroy tumors by binding to and/or blocking such antigens; and drugs that activate the immune system of a subject to promote immune-mediated tumor elimination.

In yet another aspect, the present invention also provides a method for preventing or treating a tumor in a subject, comprising administering to a subject in need thereof an antibody, or an antigen-binding fragment thereof, of the present invention; an immunoconjugate or immunofusion of the present invention; or a pharmaceutically

acceptable salt or solvate thereof.

Tumors suitable for the methods and uses of the present invention include various solid tumors and hematological tumors. A tumor suitable for the methods and uses of the present invention may be a cancer at an early, intermediate, or advanced stage, or may be metastatic cancer. In addition, a tumor suitable for the methods and uses of the present invention may be a tumor that has previously undergone treatment but has developed immune escape. In some embodiments, the tumor is resistant to an anti-PD-1 or anti-PD-L1 inhibitor.

In some embodiments, the tumor is a cold tumor characterized by low T-cell infiltration. Studies of tumors resistant to PD-1 or PD-L1 inhibitors (so-called "cold tumors") have found that tumor resistance is associated, to a large extent, with the quantity and quality of T cells infiltrating the tumor. PD-L1 expression analysis of some such tumors shows that, although immune cell infiltration in the tumor microenvironment is low, some tumor cells may exhibit moderate levels of PD-L1 expression, while DCs and macrophages in the tumor microenvironment may exhibit very high levels of PD-L1 expression. Therefore, further promoting T-cell infiltration into tumors and enhancing the anti-tumor function of T cells by activating DCs and/or macrophages in the tumor microenvironment is expected to be beneficial for overcoming drug resistance in such tumors. See, e.g., Rafael Winograd et al., Induction of T-cell immunity overcomes complete resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma, Cancer Immunol. Res. 2015 Apr.; 3(4):399–411, doi: 10.1158/2326-6066.CIR-14-0215; Haidong Tang et al., Facilitating T-cell infiltration in the tumor microenvironment overcomes resistance to PD-L1 blockade, Cancer Cell. 2016 Mar. 14; 29(3):285–296, doi: 10.1016/j.ccell.2016.02.004.

In some embodiments, the tumor treated according to the method of the present invention has at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, 99%, or 100% PD-L1-positive cells, or has a CPS score of 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99, or 100. PD-L1 expression in tumor and tumor-microenvironment biopsy samples may be assessed by immunohistochemistry. The CPS score of the tumor may be determined by a method as described in Karina Kulangara et al. (Clinical Utility of the Combined Positive Score for Programmed Death Ligand-1 Expression and the Approval of Pembrolizumab for Treatment of Gastric Cancer, Arch. Pathol. Lab. Med. (2019) 143(3):330–337, https://doi.org/10.5858/arpa.2018-0043-OA). In some embodiments, the method of the present invention induces tumor regression in said cancer. In some embodiments, the method of the present invention inhibits tumor growth in said cancer.

In any one of the above embodiments of the method of the present invention, administration of the antibody or antigen-binding fragment thereof of the present invention, or the immunoconjugate or immunofusion of the present invention, or a pharmaceutically acceptable salt or solvate thereof, may comprise: (1) a therapeutic measure that cures, slows, or alleviates symptoms of a diagnosed pathological condition or disease and/or halts progression of the diagnosed pathological condition or disease; or (2) a prophylactic or preventive measure that prevents and/or slows progression of a pathological condition or disease. Accordingly, in the methods of the present invention, the subject may be an individual who already has the disease, an individual susceptible to the disease, or an individual seeking to prevent the disease. Such individual will benefit from the therapeutic or prophylactic measure, and exhibit alleviation or amelioration of the occurrence, recurrence, or progression of the disease, disorder, condition, and/or symptom, as compared with an individual not receiving the treatment. In some embodiments, the present invention relates to treatment of a disease or disorder; in other embodiments, the present invention relates to prevention of a disease or disorder.

The antibody or antigen-binding fragment thereof of the present invention, the immunoconjugate or immunofusion of the present invention, and a pharmaceutically acceptable salt or solvate thereof, as well as optional additional therapeutic agent used in combination therewith, may be administered by any suitable route, including parenteral, intratumoral, and intranasal administration. Parenteral administration includes intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Various dosing regimens are

encompassed herein, including, but not limited to, single administration, multiple administrations at multiple time points, bolus injection, and pulse infusion.

For the prevention or treatment of a disease, the appropriate dosage of the antibody or antigen-binding fragment thereof of the present invention, or the immunoconjugate or immunofusion of the present invention, or a pharmaceutically acceptable salt or solvate thereof, when used alone or in combination with one or more additional therapeutic agents, will depend on the type of disease to be treated, the particular therapeutic agent used, the severity and progression of the disease, the prophylactic or therapeutic purpose of administration, previous treatments, the patient's clinical history and response to the antibody, and the judgment of the attending physician.

In some embodiments, the present invention further provides use of the antibody or antigen-binding fragment thereof of the present invention, or the immunoconjugate or immunofusion of the present invention, or a pharmaceutically acceptable salt or solvate thereof, as a medicament or in the preparation of a medicament. In some embodiments, the medicament is for use in the aforementioned therapeutic or prophylactic methods.

Any or all of the features described above and throughout the present application may be combined in various embodiments of the invention. The following Examples further illustrate the present invention. However, it should be understood that the Examples are provided for illustrative purposes only and should not be construed as limiting.

Examples

Example 1. Preparation and panning of PD-L1 phage display library

1.1 Two healthy adult alpacas (Chengdu NBbiolab Co., Ltd.) were selected. A recombinant protein (CST, catalog No. 71391S) of the extracellular fragment of human PD-L1 (UniProtKB accession No. Q9NZQ7; Phe19–Arg238) was homogeneously mixed with Freund's adjuvant at a ratio of 1:1. The alpacas were immunized five times by multipoint subcutaneous injection with immunization intervals of 2–3 weeks.

1.2 Peripheral blood was collected from alpacas after the third, fourth, and fifth immunizations. Lymphocytes were isolated, and TRIzol reagent (Thermo) was added to extact total RNA. The extracted RNA was reverse-transcribed using PrimeScript reverse transcription kit (Takara). PCR was performed using the cDNA as a template to amplify antibody fragments. The phage-display vector and the PCR product were subjected to double digestion. The resulting digestion products were mixed in proportion, and T4 DNA ligase (Thermo) was added for ligation. TG1 competent cells were electroporated with the ligation product to construct a VHH antibody immune library.

The library baterial culture described above was inoculated and cultured to the logarithmic growth phase, and M13KO7 helper phage was added for infection to prepare a phage-display library.

1.3 The recombinant phages were subjected to two rounds of panning using biotin-labeled PD-L1-His antigen (ACRO, catalog No. PD1-H82E5-200UG). In each round of panning, the eluted recombinant phages were used to infect TG1 cells at the logarithmic growth phase, and a phage library was prepared for the subsequent round of panning. Meanwhile, the bacterial culture after each round of infection was serially diluted and plated to obtain individual colonies for Binding ELISA analysis.

1.4 Binding ELISA assay

Individual colonies obtained in Section 1.3 were picked and induced with IPTG for expression. The culture supernatants obtained after induction were used for a binding ELISA assay. A 96-well ELISA plate was coated with human PD-L1 antigen (ACRO, catalog No. PD1-H5258-1MG). Then, 80 μL of blocking reagent and 20 μL of bacterial expression supernatant were added to each well, and the plate was incubated at room temperature for 1 hour. The plate was washed three times with PBST, followed by addition of an anti-FLAG/HRP secondary antibody diluted in blocking reagent, and incubated at room temperature for 1 hour. After washing with PBST, TMB chromogenic solution was added for color development in the dark. The reaction was stopped by addition of a stop solution, and absorbance at 450 nm (OD450nm) was measured using a microplate reader. Bacterial clones with an

OD450 value greater than 0.1 were identified as positive clones and subjected to blocking-function screening.

Example 2. Screening for PD-L1 VHH blocking antibodies

2.1 Screening for PD-L1-Positive Clones by Blocking ELISA

A 96-well ELISA plate was coated with human PD-L1-Fc antigen (ACRO, catalog No. PD1-H5258-1MG) diluted in PBS buffer. After blocking the plate, 80 μL of biotin-labeled PD-1 diluted in blocking reagent and 20 μL of diluted bacterial expression supernatant were added to the 96-well plate, and incubated at room temperature for 1 hour. The plate was washed three times with PBST, followed by addition of HRP avidin secondary antibody (Thermo, catalog No. 21126) diluted in blocking reagent, and incubated at room temperature for 1 hour. After washing with PBST, TMB chromogenic solution was added for color development in the dark. The reaction was stopped by addition of a stop solution, and absorbance OD450nm was measured using a microplate reader. Bacterial clones with an OD450 value of less than 0.15 were considered clones having a blocking function.

2.2 Screening for PD-L1-Positive Clones by Reporter Assay

A reporter assay was performed using a method similar to that described in the previous literature (Liu, C. et al., Small molecule inhibitors against PD-1/PD-L1 immune checkpoints and current methodologies for their development: a review. Cancer Cell Int. 21, 239 (2021). https://doi.org/10.1186/s12935-021-01946-4). Briefly, PD-L1 aAPC cells (artificial antigen-presenting cells recombinantly expressing PD-L1) were plated in a 96-well plate using a PD-1/PD-L1 Blockade Bioassay kit (Promega, catalog No. J1252) according to the manufacturer's instructions and cultured overnight. Diluted bacterial expression supernatant and PD-1 effector cells (Jurkat cells stably expressing human PD-1 and NFAT-RE-luciferase reporter) were added to the plate, and the plate was cultured for 6 hours. A chromogenic substrate was added, followed by incubation at room temperature. Luminescence was measured using a multimode microplate reader.

Bacterial clones exhibiting a blocking function in both the blocking ELISA assay and the reporter assay were sent to a supplier for sequencing.

Example 3. Eukaryotic expression and purification of VHH candidate antibodies

The sequences of the anti-PD-L1 VHH antibodies having a blocking function obtained in Example 2 were amplified by PCR and inserted into eukaryotic expression vectors using molecular biology techniques. HEK-293 cells were transfected. After expression and purification, VHH-His recombinant antibodies were obtained.

The purified antibodies were screened using the blocking ELISA and reporter assay described in Example 2, and one lead molecule capable of blocking the binding between PD-1 and PD-L1 was finally obtained.

The full-length nucleotide sequence and amino acid sequence of the anti-PD-L1 VHH antibody LA03C06 obtained in the present invention, together with the corresponding sequence identifier numbers, are shown in the appended Sequence Listing.

Example 4. Determination of Antigen-Binding Kinetics of VHH Antibodies of the Invention by Bio-Layer Interferometry

The equilibrium dissociation constants (KD) of the anti-PD-L1 VHH antibodies of the present invention for human PD-L1 and monkey PD-L1 were determined by bio-layer interferometry (ForteBio). The ForteBio affinity assay was performed according to established method (Estep, P. et al., High throughput solution-based measurement of antibody-antigen affinity and epitope binning. mAbs 5(2), 270–278 (2013)). Half an hour before the assay, an appropriate number of AHC biosensors (18-5060, Sartorius) were taken according to the number of samples and soaked in SD buffer (1× PBS, 0.1% BSA, and 0.05% Tween 20). 100 μL of SD buffer, antibody, and antigen were added to a 96-well black polystyrene half-area microplate (Greiner, 675076). The sensors were arranged according

to the sample layout. The instrument parameters were set as follows: running steps: Baseline 120 s, Loading ~1 nm, Baseline 120 s, Association 100 s, and Dissociation 120 s, rotation speed of 1,000 rpm and temperature of 30°C. The KD values were analyzed using ForteBio analysis software.

The binding affinities of the antibodies are shown in Table 1.

Table 1. Affinity constant (equilibrium dissociation constant) for monovalent binding of antibodies to PD-L1 antigen by ForteBio assay

	Affinity					
Clone ID	Human PD-L1 FC			Cyno PD-L1 FC		
	KD (M)	kon(1/Ms)	kdis(1/s)	KD (M)	kon(1/Ms)	kdis(1/s)
LA03C06	1.19E-10	1.68E+06	2.00E-04	1.11E-10	1.81E+06	2.00E-04
NB-His*	4.63E-09	1.91E+06	8.86E-03	4.73E-09	2.10E+06	9.94E-03

* The control molecule NB is from patent application CN107686520A and has a VHH sequence of SEQ ID NO: 31.

Example 5. Humanization of PD-L1 VHH antibody and Expression and purification of bispecific antibody protein

1. Humanization of PD-L1 VHH antibody

Based on Discovery Studio and PyMOL software, the nanobody LA03C06 obtained by alpaca immunization and screening was humanized according to the following steps:

(1) determining the CDR regions of the nanobody;

(2) screening a human germline sequence database to identify a homologous sequence having the highest similarity to the V/J regions;

(3) grafting the CDRs of the nanobody onto human framework regions;

(4) identifying, based on sequence and structural features, amino acid positions in the framework regions that have a functon of maintaining CDRs, and introducing back-mutations at positions identified as important; and

(5) synthesizing the sequences, preparing single-domain VHH-His antibodies, and determining the affinity of humanized molecules for human PD-L1 by bio-layer interferometry (BLI).

As shown by the affinity data in Table 2, the humanized molecule hz03C06.g6 retained an affinity comparable to that of the camelid-derived single-domain antibody. The amino acid sequences of the chimeric antibody and the humanized molecule hz03C06.g6 are shown in Table 2.

Table 2. Affinity constant (M) for Monovalent Antigen-Antibody Binding by ForteBio assay

antibody	Human PD-L1		
	KD(M)	Kon(1/Ms)	Kdis(1/s)
LA03C06	4.31E-10	1.33E+06	5.73E-04
hz03C06.g6	3.45E-10	1.87E+06	6.46E-04

2. Preparation of bispecific antibody protein

DNAs encoding the sequences of the bispecific antibodies were synthesized and inserted into an expression plasmid pcDNA3.1 to construct light chain plasmids and heavy chain plasmids for expressing the 2+1 format bispecific antibody Dace-hz03C06.g6-2+1 (SEQ ID NOs: 7-9) and 2+2 format bispecific antibody Dace-hz03C06.g6-2+2 (SEQ ID NOs: 10 and 11), respectively. Light-chain plasmids and heavy chain plasmids for expressing the controls, anti-PD-L1 monoclonal antibody atezolizumab (SEQ ID NOs: 12-13) and anti-CD40 monoclonal antibody selicrelumab (SEQ ID NOs: 14-15), were also constructed.

Expi293F cells (purchased from Gibco) were cultured in an Expi293F medium (Gibco, REF# A14351-01).

The cell density was measured the day before transfection (cell viability should be greater than 95%), and the cells were adjusted to 3×10^6 cells/mL with a fresh Expi293F medium and cultured. On the day of transfection, the cell density was adjusted to 3×10^6 cells/mL.

1/10 of the final transfection volume of Opti-MEM medium (Gibco, REF# 31985-070) was used as a transfection buffer, and the DNA to be transfected was added at 1 mg/L, wherein the ratio of light and heavy chains for 2 + 1 format antibody molecules was 2:1:1, and the ratio of light and heavy chains for the other antibodies were 1:1. The mixture was mixed uniformly, and PEIMax (Polysciences Inc. Cat# 24765-1) was added at a DNA:PEI mass ratio of 1:3. The mixture was mixed uniformly and incubated at room temperature for 20 min. Then the mixture was gently added into Expi293F cell suspension with shaking, and the cells were cultured in a shaker at 8% CO2, 36.5 °C, and 120 rpm.

After culturing for 16-18 hours, the cell suspension was supplemented with 200 g/L Feed (100 g/L Phytone Peptone + 100 g/L Difco Select Phytone) at 2% (v/v), and with a glucose solution at a final concentration of 5 g/L, and Valproic acid sodium salt (Merk, Cat# P4543-100G) at a final concentration of 2.2 mM, and gently mixed uniformly, and cultured for another 7 days at 36.5 °C and 120 rpm with 8% CO2. The harvested cell culture liquid was uniformly mixed with celite (Sartorius, Cat. No.: 1000037025) (40 g Celite per liter of cell culture broth), and the mixture was filtered using a 0.22 μm disposable vacuum filtration device.

Purification of target proteins by affinity chromatography: A HiTrap MabSelect PrismA affinity chromatography column (GE Healthcare, Cat. No. 17549853) was used for affinity capture. Before purification, the flow path and affinity chromatography column were treated with 10–20 column volumes of 0.1 M NaOH, followed by washing with 10–20 column volumes of distilled water. The packed column was equilibrated with 5 column volumes of 1× PBS (Gibco). The filtered cell culture supernatant was loaded onto the column, which was then washed with 10 column volumes of 1× PBS to remove nonspecifically bound proteins. The packed column was eluted with 5 column volumes of elution buffer (100 mM sodium citrate, pH 3.5). The eluate was collected, adjusted to pH 6.0 with 2 M Tris, and sterilized by filtration.

Purification of bispecific antibodies by ion-exchange chromatography: A Mono S 5/50 GL ion-exchange chromatography column (GE Healthcare) was installed on an AKTA pure system (GE Healthcare). The AKTApure system equipped with the Mono S 5/50 GL ion-exchange chromatography column were treated with 0.5 M NaOH for endotoxin removal for 2 hours, and then washed with distilled water. The column was equilibrated with 5–10 column volumes of loading buffer (20 mM phosphate, pH 6.0) until the conductivity and pH were stable. The protein obtained by affinity chromatography was diluted 10-fold with loading buffer and loaded onto the column. The column was then re-equilibrated with 5 column volumes of loading buffer. Linear gradient elution was performed using 0–40% elution buffer (20 mM phosphate, 1 M NaCl, pH 6.0) over a total of 30 column volumes. Fractions were collected based on UV absorbance.

Samples of each collected fraction were analyzed by SDS-PAGE electrophoresis. Reducing loading buffer (Yesen Biotech, Cat. No. 20315ES05) or non-reducing loading buffer (Beijing Biolab, Cat. No. WE0289) was added to the ion-exchange-purified protein samples, and were heated at 70 °C for 10 min. An appropriate amount of each sample was loaded onto a 4–20% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Cat. No. 4561095), with a protein marker (Bio-Rad, Cat. No. 1610375) included as a control. Electrophoresis was performed at 150 V for 50 min. The gel was stained with a Protein Staining device (GenScript, Cat. No. L00760C) and imaged using a gel imaging system (Shanghai Tianneng, Tanon-1600).

The purity of each collected fraction sample was determined by size-exclusion chromatography (SEC). Based on the SDS-PAGE and SEC results, fractions having a purity greater than 95% were pooled.

A protein sample meeting the purity requirement was centrifuged in a 15 mL ultrafiltration centrifugal tube at 4,000 rpm for 10 min. The protein was diluted with PBS and centrifuged again at 4,000 rpm for 10 min. This

procedure was repeated several times to exchange the proteins into a storage buffer. After buffer exchange, the protein solution was sterilized by filtration, and the antibody concentration was determined. The composition and content of the bispecific antibody were further identified and quantified by capillary electrophoresis (CE-SDS) and liquid chromatography–mass spectrometry (LC-MS).

Example 6. CD40 Reporter Assay for Target Molecules

1. Detection method

HEK-Blue CD40L cells (HEK293 cells recombinantly expressing CD40 and a SEAP reporter gene under the control of an NF-κB response element; InvivoGen, Cat. No. hkb-cd40), MC38 cells, and MC38 PD-L1 cells (recombinant MC38 cells highly expressing PD-L1) were passaged 2–3 times after recovery. The culture medium was removed, and the cells were washed with 10 mL of PBS and digested with 2 mL of trypsin. After digestion was terminated by addition of culture medium, the cells were centrifuged at 400 × g for 3 min. The supernatant was discarded, and the cells were resuspended in culture medium. A 20 μL aliquot of the cell suspension was transferred to a 1.5 mL EP tube for cell counting.

HEK-Blue CD40L cells were prepared as a cell suspension in a 15 mL centrifuge tube. At 3×10^4 cells/well, the cell suspension was dispensed into two flat-bottom 96-well plates at 50 μL/well using a multichannel pipette. Plate 1 was supplemented with 50 μL of MC38 cells at 0.5×10^4 cells/well and with 50 μL of the serially-diluted test bispecific antibody, and was mixed well. Plate 2 was supplemented with 50 μL of MC38 PD-L1 cells at 0.5×10^4 cells/well and with 50 μL of the serially-diluted test bispecific antibody drug, and was mixed well. To minimize edge effects, 200 μL of PBS was added to the periphery surrounding the cells. The cell plate was incubated in the incubator for another 20 hours.

QUANTI-Blue™ solution (InvivoGen) was prepared according to the manufacturer's instructions. Briefly, 10 mL of ddH_2O was placed in a 50 mL centrifuge tube, followed by sequential addition of 100 μL of buffer and 100 μL of QUANTI-Blue (100×). After thorough mixing, the solution was covered with aluminum foil and stored at 4 °C. The solution was prewarmed for 30 min before use, and QUANTI-Blue was dispensed into a flat-bottom 96-well plate at 180 μL/well.

A new round-bottom 96-well plate was prepared, and 80 μL of culture medium was added. Then 20 μL of the supernatant of HEK-Blue CD40L cells that had been co-cultured with the test drug and MC38 or MC38 PD-L1 for 20 h was added, and the mixture was mixed well (i.e., diluted 5-fold). 20 μL of the mixture was added to the cell plate with QUANTI-Blue, and the plate was incubated at 37 °C for 1 hour. The lid was removed, and the plate was placed in a microplate reader and read at OD635 nm.

2 Results of CD40 reporter assay:

HEK-Blue CD40L cells were stably transfected with CD40 and SEAP reporter gene vectors. The binding of an CD40 ligand to CD40 on the surface of the cells triggers a signaling cascade, resulting in the activation of NF-KB in the cells and the subsequent secretion of SEAP into the cell supernatant. QUANTI-Blue is an enzymatic colorimetric detection reagent. The level of SEAP in the cell supernatant can be detected by QUANTI-Blue to characterize the activity of the CD40 ligand.

The results showed that the CD40 activation induced by Dace-hz03C06.g6-2+1 was about 91-fold weaker than that induced by Selicrelumab in the absence of PD-L1 (as shown in FIG. 1), and was significantly improved in the presence of PD-L1 and about 12-fold stronger than that induced by Selicrelumab (as shown in FIG. 2). The results show that the agonistic activity of the Dace-hz03C06.g6-2+1 molecule on CD40 is significantly PD-L1-dependent; by the design of monovalent PD-L1 binding site, the ability of the antibody molecule to selectively activate the CD40 signaling pathway is significantly improved (compared with the PD-L1-absent condition, where EC_{50} is about

8.6 nM, the EC_{50} in the presence of PD-L1 is about 0.009 nM).

Example 7. DC activation assay of bispecific antibody CD40/PD-L1 molecules

1. Detection method

Dendritic cells (DCs) were isolated and induced from human PBMCs. Briefly, hPBMCs (50 million) were removed from a liquid nitrogen tank, thawed in a 37 °C water bath, added with RPMI 1640 complete medium, centrifuged at 300× g for 5 min, and then resuspended with 1 mL of medium, adjusted to a concentration of about 5×10^7 cells/mL, and transferred to a 5 mL flow cytometry tube. Using a human monocyte enrichment kit without CD16 depletion (Stemcell, Cat. No. 19058), 100 μL of enrichment cocktail was added according to the manufacturer's instructions. The mixture was uniformly mixed and then left to stand at 4 °C for 10 min. Magnetic beads were vortexed for 30 s, and 100 μL of magnetic beads were added to the sample. The mixture was uniformly mixed and then left to stand at 4 °C for 5 min, and brought to 2.5 mL by adding medium (RPMI 1640 + 10% FBS). The mixture was gently mixed 2-3 times. The mixture was left to stand on a magnetic rack at room temperature for 2.5 min. The supernatant was transferred to a new 5 mL flow cytometry tube, and the cells were counted and adjusted to the concentration of about 5E5 cells/mL. GM-CSF and IL-4 were added to final concentrations of 40 ng/mL each, and the cells was placed in an incubator at 37 °C for culture. On day 3, the cell suspension was collected and centrifuged at 300× g for 5 min, and the supernatant was discarded. The cells were resuspended in a medium and then counted, and the cell density was adjusted to 5E5 cells/mL. GM-CSF and IL-4 were again added to final concentrations of 40 ng/mL each, and then the mixture was added back to a culture flask. The culture flask was placed in an incubator at 37 °C for continued incubation. On day 6, suspended cells were collected and centrifuged at 300× g for 5 min, and the supernatant was discarded. Then monocyte-derived DC dendritic cells (DCs) were obtained. The cells were resuspended in a culture medium, counted, and plated in a 96-well round-bottom plate at 50 μL/well to provide 1.2E5 cells/well.

A culture of CHOK1 cells not expressing human PD-L1 (CHOK1 WT) and a culture of CHOK1 cells recombinantly expressing human PD-L1 (CHOKI PD-L1) were collected. The culture supernatant was discarded. The cells were rinsed once with 10 mL of PBS and digested with 4 mL of trypsin. After the digestion reaction was stopped with the medium, the cells were centrifuged at 400× g for 3 min. The supernatant was discard, and the cells were resuspended in the medium and counted. The obtained CHOK1 WT cells or CHOK1 PD-L1 cells were added to the wells of the 96-well round-bottom plate described above, at 50 μL each well, to provide 0.8E5 cells/well.

The test antibody was serially diluted at a 1:4 ratio to generate 8 concentrations ranging from 0.009766 nM to 40 nM, and the concentrations were separately added to the wells of the 96-well round-bottom plate at 50 μL/well. The plate was incubated in an incubator for 48 h. The 96-well plate was removed from the incubator, and centrifuged at 300× g for 5 min. 20 μL of cell supernatant was collected and added to 180 μL of culture medium, and the 10-fold diluted cell supernatant was analyzed for IL-12p40 cytokine levels using the Liankebio EK1183-96 ELISA kit according to the manufacturer's instructions.

2. DC activation assay result

CD40 on the surface of DC cells binds to and interacts with its ligand, and transduces signals to activate different signaling pathways, therey promoting DC cell maturation and cytokine IL-12 secretion. The activation of DCs by an antibody molecule can be indicated by measuring the concentration of IL-12p40 in the cell supernatant using an ELISA kit.

As shown in FIG. 3, consistent with previous reports that CD40 activation by antibody Selicrelumab is independent of crosslinking, Selicrelumab exhibited strong DC activation activity under both high- and low-PD-L1 expression conditions. Dace-hz03C06.g6-2+1 and Dace-hz03C06.g6-2+2 both showed dependence on PD-L1-

mediated crosslinking, with stronger DC activation observed under high PD-L1 expression than under low PD-L1 expression. In addition, as can be seen from FIG.3, under high PD-L1-expression conditions, Dace-hz03C06.g6-2+1 showed significantly greater activation activity than Dace-hz03C06.g6-2+2 and demonstrated a more pronounced selective DC activation effect. These results indicate that the 2+1 format molecular configuration is more conducive to enabling the antibody of the present invention to fully leverage PD-L1 and selectively activate DCs.

Example 8. Determination of Antigen-Binding Kinetics of Dace-hz03C06.g6-2+1 by Bio-Layer Interferometry

The equilibrium dissociation constants (KD) of the antibody of the present invention for human, cynomolgus monkey, rat, and mouse CD40 and PD-L1 were determined by bio-layer interferometry (ForteBio). The ForteBio affinity assay was performed according to established method (Estep, P et al., High throughput solution Based measurement of antibody-antigen affinity and epitope binning. MAbs, 2013. 5(2): 270-8). Half an hour before the assay, an appropriate number of AHC biosensors (18-5060, Sartorius) were taken according to the number of samples and soaked in SD buffer (1× PBS containing 0.1% BSA and 0.05% Tween 20). 100 μL of SD buffer, antibody, and antigen were added to a 96-well black polystyrene half-area microplate (Greiner, 675076). The sensors were arranged according to the sample layout. The instrument parameters were set as follows: running steps: Baseline 120 s, Loading ~1 nm, Baseline 120 s, Association 100 s, and Dissociation 120 s, rotation speed of 1,000 rpm and temperature of 30°C. The KD values were analyzed using ForteBio analysis software.

The binding affinities of the antibody are shown in Table 3.

Table 3. Affinity constant for monovalent antigen-antibody binding of Dace-hz03C06.g6-2+1 by ForteBio assay

Species	CD40(K_D, nM)	PD-L1(K_D, nM)
Human	11.9	1.71
Cyno	9.16	3.77
Rat	N.B.	N.B.
Mouse	N.B.	N.B.

N.B. denotes no binding

Example 9. Therapeutic effect of Dace-hz03C06.g6-2+1 in B16F10-hPD-L1 model

In this experiment, C57 KI hPD-1/hPD-L1/hCD40 transgenic mice were inoculated with B16F10-hPD-L1 cells to determine the anti-tumor effect of the Dace-hz03C06.g6-2+1 antibody of the present invention.

C57 KI hPD-1/hPD-L1/hCD40 transgenic mice:

The femal mice with C57 KI hPD-1/hPD-L1/hCD40 transgenic background were purchased from Biocytogen Jiangsu Co., Ltd. (SPF grade, qualified by Suzhou Xishan Biotechnology Co., Ltd., with certificate No. 320726230100116536). The mice were acclimated for 3 days following arrival, after which the study was initiated.

Cells:

B16F10-hPD-L1 melanoma cells were obtained from Innovent Biologics (Suzhou) Co., Ltd. (CAT#: N/A), and were routinely subcultured strictly according to the instructions for subsequent in vivo experiments. The cells were collected by centrifugation, resuspended in sterile PBS and adjusted to a cell density of 5×10^6 cells/mL. On day 0, 0.2 mL of the cell suspension was subcutaneously inoculated into the right abdominal region of the C57 KI hPD-1/hPD-L1/hCD40 transgenic mice to establish B16F10-hPD-L1 tumor-bearing mouse models.

Administration:

The tumor volume in each mouse was measured 7 days after the tumor cell inoculation. Mice with a tumor volume of about 18-89 mm^3 were selected and evenly divided into groups by tumor volume (7 mice per group). The

dose and mode of administration are shown in Table 4, and h-IgG (purchased from EQUITECH-BIO) was used as a negative control. The drug was administered on day 7 after inoculation, and the tumor volume and body weight of the mice were monitored twice a week. The body weight and tumor volume were measured before each administration. On day 18 after inoculation, the relative tumor growth inhibition (TGI%) was calculated using the following formula: TGI% = 100% × (tumor volume in the control group –tumor volume in the treatment group)/(tumor volume in the control group –tumor volume in the control group before administration). Tumor volume measurement: The maximum length of the major axis (L) and the maximum length of the minor axis (W) of tumors were measured with a vernier caliper, and tumor volume was calculated using the following formula: $V = L \times W^2/2$. The mice were weighed using an electronic balance.

Table 4. Experimental design

Group	Dose	Dosing Frequency	Mode of Administration
h-IgG	3 mg/kg	Q3-4D*3	Intraperitoneal injection
Dace-hz03C06.g6-2+1	0.3 mg/kg	Q3-4D*3	Intraperitoneal injection
Dace-hz03C06.g6-2+1	1 mg/kg	Q3-4D*3	Intraperitoneal injection
Dace-hz03C06.g6-2+1	3 mg/kg	Q3-4D*3	Intraperitoneal injection

The results of tumor growth inhibition rate are shown in FIG. 4 and Table 5. On Day 18 after inoculation, the tumor growth inhibition rates in the 0.3 mg/kg Dace-hz03C06.g6-2+1 group, the 1 mg/kg Dace-hz03C06.g6-2+1 group, and the 3 mg/kg Dace-hz03C06.g6-2+1 group were 27%, 63%, and 88%, respectively. Meanwhile, the body weights of the mice were monitored. As shown in FIG. 5, there was no significant difference in body weight of the mice. Therefore, the Dace-hz03C06.g6-2+1 drug in the present disclosure has a significant inhibitory effect on tumors and exhibits a dose-response relationship.

Table 5. Tumor growth inhibition on day 18

Group	Tumor volume (mm^3)	TGI (%)
h-IgG, 3 mg/kg	3094	N/A
Dace-hz03C06.g6-2+1, 0.3 mg/kg	1733	27
Dace-hz03C06.g6-2+1, 1 mg/kg	339	63
Dace-hz03C06.g6-2+1, 3 mg/kg	206	88

Example 10. Therapeutic effect of Dace-hz03C06.g6-2+1 in B16F10-hPD-L1 model

In this experiment, C57 KI hPD-1/hPD-L1/hCD40 transgenic mice were inoculated with B16F10-hPD-L1 cells, and the anti-tumor effect of the Dace-hz03C06.g6-2+1 antibody of the present invention was determined.

C57 KI hPD-1/hPD-L1/hCD40 transgenic mice:

The femal mice with C57 KI hPD-1/hPD-L1/hCD40 transgenic background were purchased from Biocytogen Jiangsu Co., Ltd. (SPF grade, qualified by Suzhou Xishan Biotechnology Co., Ltd., with certificate No. 320726230100038316). The mice were acclimated for 3 days following arrival, after which the study was initiated.

Cells:

B16F10-hPD-L1 melanoma cells were purchased from Innovent Biologics (Suzhou) Co., Ltd. (CAT#: N/A), and were routinely subcultured strictly according to the instructions for subsequent in vivo experiments. The cells were collected by centrifugation, resuspended in sterile PBS and adjusted to a cell density of 5×10^6 cells/mL. On day 0, 0.2 mL of the cell suspension was subcutaneously inoculated into the right abdominal region of the C57 KI hPD-1/hPD-L1/hCD40 transgenic mice to establish B16F10-hPD-L1 tumor-bearing mouse models.

Administration:

The tumor volume in each mouse was measured 6 days after the tumor cell inoculation. Mice with a tumor

volume of about 22-116 mm^3 were selected and evenly divided into groups by tumor volume (7 mice per group). The dose and mode of administration are shown in Table 6, and h-IgG (purchased from EQUITECH-BIO, Cat. No. SLH56-0001) was used as a negative control. The drug was administered on day 6 after inoculation, and the tumor volume and body weight of the mice were monitored twice a week. The body weight and tumor volume were measured before each administration. On day 20 after inoculation, the relative tumor growth inhibition (TGI%) was calculated using the following formula: TGI% = 100% × (tumor volume in the control group –tumor volume in the treatment group)/(tumor volume in the control group –tumor volume in the control group before administration). Tumor volume measurement: The maximum length of the major axis (L) and the maximum length of the minor axis (W) of tumors were measured with a vernier caliper, and tumor volume was calculated using the following formula: $V = L \times W^2/2$. The mice were weighed using an electronic balance.

Table 6. Experimental design

Group	Dose	Dosing Frequency	Mode of administration
h-IgG	10 mg/kg	Q3-4D*3	Intraperitoneal injection
Atezolizumab	10 mg/kg	Q3-4D*3	Intraperitoneal injection
Dace-hz03C06.g6-2+1	3 mg/kg	Q3-4D*3	Intraperitoneal injection
Dace-hz03C06.g6-2+2	3 mg/kg	Q3-4D*3	Intraperitoneal injection

The results of tumor growth inhibition rate are shown in FIG. 6 and Table 7. On day 20 after inoculation, the tumor growth inhibition rates of the anti-PD-L1 monoclonal antibody atezolizumab (10 mg/kg) group, Dace-hz03C06.g6-2 + 1 (3 mg/kg) group, and Dace-hz03C06.g6-2 + 2 (3 mg/kg) group were 4.2%, 97%, and 67%, respectively. The efficacy of the Dace-hz03C06.g6-2 + 1, 3 mg/kg group and the Dace-hz03C06.g6-2 + 2, 3 mg/kg group was significantly stronger than that of the PD-L1 monoclonal antibody atezolizumab, 10 mg/kg group. Meanwhile, the body weight of the mice was measured. As shown in FIG. 7, there was no significant difference in body weight of the mice. Therefore, Dace-hz03C06.g6-2+1 and Dace-hz03C06.g6-2+2 had significant inhibitory effects on B16F10-hPD-L1 tumors, and the efficacy of Dace-hz03C06.g6-2+1 was significantly better than that of Dace-hz03C06.g6-2+2.

Table 7. Tumor growth inhibition on day 20

Group	Tumor volume (mm^3)	TGI (%)
h-IgG	1836	N/A
Atezolizumab, 10mg/kg	1760	4.2
Dace-hz03C06.g6-2+1, 3mg/kg	111	97
Dace-hz03C06.g6-2+2, 3mg/kg	647	67

Example 11. Therapeutic effect of Dace-hz03C06.g6-2+1 in KPC-HPD-L1 model

In this experiment, C57 KI hPD-1/hPD-L1/hCD40 transgenic mice were inoculated with KPC-hPD-L1 cells to determine the anti-tumor effect of Dace-hz03C06.g6-2+1.

C57 KI hPD-1/hPD-L1/hCD40 transgenic mice:

The femal mice with C57 KI hPD-1/hPD-L1/hCD40 transgenic background were purchased from Biocytogen Jiangsu Co., Ltd. (SPF grade, qualified by Suzhou Xishan Biotechnology Co., Ltd., with certificate No. A202311090029). The mice were acclimated for 3 days following arrival, after which the study was initiated.

Cells:

KPC-hPD-L1 pancreatic cancer cells were from Innovent Biologics (Suzhou) Co., Ltd. (CAT#: N/A), and were routinely subcultured strictly according to the instructions for subsequent in vivo experiments. The cells were collected by centrifugation, resuspended in sterile PBS and adjusted to a cell density of 30×10^6 cells/mL. On day 0,

0.2 mL of the cell suspension was subcutaneously inoculated into the right abdominal region of the C57 KI hPD-1/hPD-L1/hCD40 transgenic mice to establish KPC-hPD-L1 tumor-bearing mouse models.

Administration:

The tumor volume in each mouse was measured 4 days after the tumor cell inoculation. Mice with a tumor volume of about 42-121 mm^3 were selected and evenly divided into groups by tumor volume (6 mice per group). The dose and mode of administration are shown in Table 8, and h-IgG (purchased from EQUITECH-BIO) was used as a negative control. The drug was administered on day 4 after inoculation, and the tumor volume and body weight of the mice were monitored twice a week. The body weight and tumor volume were measured before each administration. On day 29 after inoculation, the relative tumor growth inhibition (TGI%) was calculated using the following formula: TGI% = 100% × (tumor volume in the control group –tumor volume in the treatment group)/(tumor volume in the control group –tumor volume in the control group before administration). Tumor volume measurement: The maximum length of the major axis (L) and the maximum length of the minor axis (W) of tumors were measured with a vernier caliper, and tumor volume was calculated using the following formula: $V = L \times W^2/2$. The mice were weighed using an electronic balance.

Table 8. Experimental design

Group	Dose	Dosing Frequency	Mode of administration
h-IgG	20 mg/kg	Q3-4D*5	Intraperitoneal injection
Dace-hz03C06.g6-2+1	10 mg/kg	Q3-4D*5	Intraperitoneal injection

The results of tumor inhibition rate are shown in FIG. 8 and Table 9. On day 29 after inoculation, the tumor growth inhibition rate of Dace-hz03C06.g6-2+1 was 54%. Meanwhile, the body weight of the mice was measured. As shown in FIG. 9, the body weight of the mice had no significant change. Therefore, the Dace-hz03C06.g6-2+1 group had a significant inhibitory effect on the KPC-hPD-L1 tumor.

Table 9. Tumor growth inhibition on day 29

Group	Tumor volume (mm^3)	TGI(%)
h-IgG	965	N/A
Dace-hz03C06.g6-2+1, 10mg/kg	485	54

Example 12. Toxic effects of Dace-hz03C06.g6-2+1 in C57 KI hPD-1/hPD-L1/hCD40 mice

In this experiment, C57 KI hPD-1/hPD-L1/hCD40 transgenic mice were used, and Dace-hz03C06.g6-2+1 drug was injected intraperitoneally once. The ALT, AST, and cytokine release in the serum of mice was observed to determine the toxicity of the drug.

C57 KI hPD-1/hPD-L1/hCD40 transgenic mice:

The femal mice with C57 KI hPD-1/hPD-L1/hCD40 transgenic background were purchased from Biocytogen Jiangsu Co., Ltd. (SPF grade, qualified by Suzhou Xishan Biotechnology Co., Ltd., with certificate No. 320726230100133615). The mice were acclimated for 3 days following arrival, after which the study was initiated.

Administration:

The mice were grouped and weighed according to the experimental design in Table 10. On day 0, h-IgG, Selicrelumab, and Dace-hz03C06.g6-2+1 were injected intraperitoneally, and then blood samples were collected on day 1.

Table 10. Experimental design

Group	Dose	Number of Doese	Mode of administration
h-IgG	50 mg/kg	Single dose	Intraperitoneal injection

Selicrelumab	50 mg/kg	Single dose	Intraperitoneal injection
Dace-hz03C06.g6-2+1	50 mg/kg	Single dose	Intraperitoneal injection

ALT and AST assays:

Following blood collection, the blood samples were centrifuged at 4 °C and 3000 rpm for 10 min, and the supernatants were collected and sent to Suzhou Xishan Zhongke Pharmaceutical Research and Development Co., Ltd. for detection of alanine transaminase (ALT) and aspartate transaminase (AST). As shown in FIGs. 10 and 11, the AST and ALT levels of the Dace-hz03C06.g6-2+1 group were significantly lower than those of the Selicrelumab group and comparable to those of the IgG1 group, indicating that Dace-hz03C06.g6-2+1 caused less liver injury in mice than Selicrelumab, the reference substance.

Cytokine detection:

Serum cytokine levels were determined using a cytometric bead array (CBA) cytokine detection system and a Human Th1/Th2/Th17 CBA Kit (BD, Cat. No. 560484). The Th1/Th2/Th17 standards were reconstituted in 2 mL of assay diluent and serially diluted at 1:2 ratio to obtain standard solutions ranging from 5,000 to 20 pg/mL. The volume of capture beads was determined based on the number of samples (10 μL beads per sample). All beads were combined and vortexed for subsequent use (The beads were prone to settling, and they should be thoroughly mixed before each aspiration). A 96-well plate was prepared, and 50 μL each of the following solutions were added: mixed capture beads, standard and serum sample, and PE detection reagent. The plate was incubated at room temperature for 2 h in the dark. Subsequently, 200 μL of wash buffer was added, followed by centrifugation at 200 × g for 5 min. The supernatant was removed, and the beads were resuspended in 150 μL of wash buffer for analysis on flow cytometer. The flow cytometric results were analyzed using FCAP Array software.

The CBA cytokine detection system detects soluble proteins by using microspheres of known volumn size and fluorescence intensity to capture single or multiple proteins. Each type of capture bead in the detection system is coated with one specific antibody. Following co-incubation of the capture beads, the detection antibody, and the test sample, the three components formed a two-antibody sandwich complex, which can be detected by flow cytometry. The results are shown in FIGs. 12 and 13. Compared with the CD40 agonist Selicrelumab, the levels of both TNF-α and IL-6 in the Dace-hz03C06.g6-2+1 group were substantially reduced and comparable to those in the IgG1 group, indicating that Dace-hz03C06.g6-2+1 presents a lower risk of inducing a cytokine storm.

The combined liver enzyme and cytokine assay results demonstrate that Dace-hz03C06.g6-2+1 exhibited better safety in mice than the CD40 agonist Selicrelumab.

Sequence listing

SEQ ID NO:	Sequence description	sequence
1	LA03C06 base sequence	CAGGTGCAGCTCGTGGAGTCTGGGGGAGGATTGGTGC AGGCTGGGGGCTCTCTGAGACTCTCCTGTGCAGCCTCT GGACACACCTCTAGCAACTATGCCATGGGCTGGTTCCG CCAGGCTCCAGGGAAGGAGCGTGAGTTTGTAGCAACT CGTGGCTGGAAAGGTGATACATACTATGCAGAGTCCG TGAAGGGCCGATTCGTCATCTCCATGGACAACGCCAA GAACACGGTGTTTCTGCAAATGAACAGTCTGAAACCT GAGGACACGGCCGTTTATTACTGTGCAGTTTCCCTGCT GGGCCCTACGGTAGTGCCTGGCGGATATAACTACTGG GGCCAGGGGACCCAGGTCACCGTCTCCTCG
2	CDR1 amino acid sequence VH	GHTSSNYAMG

	CDR1 (AbM) of LA03C06/ hz03C06.g6 single-domain antibody	
3	CDR2 amino acid sequence VH CDR2 (Kabat) of LA03C06/ hz03C06.g6 single-domain antibody	TRGWKGDTYYAESVKG
4	CDR3 amino acid sequence VH CDR3 (Kabat) of LA03C06/ hz03C06.g6 single-domain antibody	SLLGPTVVPGGYNY
5	Amino acid sequence of single-domain antibody LA03C06	QVQLVESGGGLVQAGGSLRLSCAASGHTSSNYAMGWFR QAPGKEREFVATRGWKGDTYYAESVKGRFVISMDNAKN TVFLQMNSLKPEDTAVYYCAVSLLGPTVVPGGYNYWGQ GTQVTVSS
6	Amino acid sequence of single-domain antibody hz03C06.g6	EVQLLESGGGLVQPGGSLRLSCAASGHTSSNYAMGWFR QAPGKGLEFVSTRGWKGDTYYAESVKGRFTISRDNSKNT LYLQMNSLRAEDTAVYYCAVSLLGPTVVPGGYNYWGQ G
7	Light chain amino acid sequence of bispecific antibodyDace-hz03C06.g6-2+1 (CDRs underlined)	DIQMTQSPSSLSASVGDRVTITCRSSQSLVHSNGNTFLHW YQQKPGKAPKLLIYTVSNRFSGVPSRFSGSGSGTDFTLTIS SLQPEDFATYFCSQTTHVPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC
8	Heavy chain 1 amino acid sequence of bispecific antibodyDace-hz03C06.g6-2+1 (CDRs underlined)	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYYIHWVRQ APGKGLEWVARVIPNAGGTSYNQKFKGRFTLSVDNSKNT AYLQMNSLRAEDTAVYYCAREGIYWWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRD ELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK
9	Heavy chain 2 amino acid sequence of bispecific antibodyDace-hz03C06.g6-2+1 (CDRs underlined)	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYYIHWVRQ APGKGLEWVARVIPNAGGTSYNQKFKGRFTLSVDNSKN TAYLQMNSLRAEDTAVYYCAREGIYWWGQGTLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT YICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP CRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGGGGGGGGGGGGEVQLLESGGGLV QPGGSLRLSCAASGHTSSNYAMGWFRQAPGKGLEFVST RGWKGDTYYAESVKGRFTISRDNSKNTLYLQMNSLRAE DTAVYYCAVSLLGPTVVPGGYNYWGQGTLVTVSS
10	Light chain amino acid sequence of bispecific antibodyDace-hz03C06.g6-2+2 (CDRs underlined)	DIQMTQSPSSLSASVGDRVTITCRSSQSLVHSNGNTFLHW YQQKPGKAPKLLIYTVSNRFSGVPSRFSGSGSGTDFTLTIS SLQPEDFATYFCSQTTHVPWTFGQGTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE

		VTHQGLSSPVTKSFNRGEC
11	Heavy chain amino acid sequence of bispecific antibodyDace-hz03C06.g6-2+2 (CDRs underlined)	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYYIHWVRQ APGKGLEWVARVIPNAGGTSYNQKFKGRFTLSVDNSKNT AYLQMNSLRAEDTAVYYCAREGIYWWGQGTLVTVSSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGGGGGGGGGGGEVQLLESGGGLVQPGG SLRLSCAASGHTSSNYAMGWFRQAPGKGLEFVSTRGWK GDTYYAESVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY YCAVSLLGPTVVPGGYNYWGQGTLVTVSS
12	Heavy chain amino acid sequence of atezolizumab antibody	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQ APGKGLEWVAWISPYGGSTYYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEL LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK
13	Light chain amino acid sequence of atezolizumab antibody	DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKP GKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPED FATYYCQQYLYHPATFGQGTKVEIKRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
14	Heavy chain amino acid sequence of Selicrelumab antibody	MDWTWRILFLVAAATGAHSQVQLVQSGAEVKKPGASVK VSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPDSG GTNYAQKFQGRVTMTRDTSISTAYMELNRLRSDDTAVYY CARDQPLGYCTNGVCSYFDYWGQGTLVTVSSASTKGPS VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDH KPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHN AKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVS NKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVS LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGK
15	Light chain amino acid sequence of Selicrelumab antibody	MRLPAQLLGLLLLWFPGSRCDIQMTQSPSSVSASVGDRVT ITCRASQGIYSWLAWYQQKPGKAPNLLIYTASTLQSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQANIFPLTFGGG TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
16	Light chain variable region of anti-CD40 Fab (Dace)	DIQMTQSPSSLSASVGDRVTITCRSSQSLVHSNGNTFLHW YQQKPGKAPKLLIYTVSNRFSGVPSRFSGSGSGTDFTLTIS SLQPEDFATYFCSQTTHVPWTFGQGTKVEIK
17	Heavy chain variable region of	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYYIHWVRQ

	anti-CD40 Fab (Dace)	APGKGLEWVARVIPNAGGTSYNQKFKGRFTLSVDNSKNT AYLQMNSLRAEDTAVYYCAREGIYWWGQGTLVTVSS
18	Light chain variable region LCDR1 of anti-CD40 Fab (Dace)	RSSQSLVHSNGNTFLH
19	Light chain variable region LCDR2 of anti-CD40 Fab (Dace)	TVSNRFS
20	Light chain variable region LCDR3 of anti-CD40 Fab (Dace)	SQTTHVPWT
21	Heavy chain variable region HCDR1 of anti-CD40 Fab (Dace)	GYSFTGYYIH
22	Heavy chain variable region HCDR2 of anti-CD40 Fab (Dace)	RVIPNAGGTSYNQKFKG
23	Heavy chain variable region HCDR3 of anti-CD40 Fab (Dace)	EGIYW
24	human light chain constant region kappa (CL-κ)	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ WKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSFNRGEC
25	Human IgG1 Fc LALA + disulfide mutation (Y349C) + Hole mutation (T366S, L368A, Y407V)	EPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
26	Human IgG1 Fc LALA + disulfide mutation (S354C) + Knob mutation (T366W)	EPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
27	Human IgG1 Fc LALA	EPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
28	Human IgG2 Fc	ERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTIS KTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
29	Human IgG1 CH1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT YICNVNHKPSNTKVDKKV
30	Linker	GGGGGGGGGGG
31	control NB molecule	QVQLQESGGGLVQPGGSLRLSCAASAYTISRNSMGWFRQ APGKGLEGVAAIESDGSTSYSDSVKGRFTISLDNSKNTLY LEMNSLRAEDTAVYYCAAPKVGLGPRTALGHLAFMTLPA LNYWGQGTLVTVSS
32	Linker peptide	G4S
33	Linker peptide	(G4S)2
34	Linker peptide	(G4S)3
35	Linker peptide	GGGSG
36	Linker peptide	GGSGG
37	Linker peptide	GSGGG
38	Linker peptide	GSGGGP

39	Linker peptide	GGEPS
40	Linker peptide	GGEGGGP
41	Linker peptide	GGEGGGSEGGGS
42	Linker peptide	(G4S)n, where n is an integer equal to or greater than 1
43	Linker peptide	TS(G4S)n, where n is an integer equal to or greater than 1
44	Linker peptide	G(G4S)n, where n is an integer equal to or greater than 1
45	Linker peptide	(G4)n, where n is an integer equal to or greater than 1
46	Linker peptide	(GRPGS)n, where n is an integer equal to or greater than 1
47	Linker peptide	(G3S)2
48	Linker peptide	(G4S)2
49	Linker peptide	(G3S)3
50	Linker peptide	(G4S)3
51	Linker peptide	(G3S)4
52	Linker peptide	(G4S)4
53	Linker peptide	(G3S)5
54	Linker peptide	(G4S)5
55	Linker peptide	(G3S)6
56	Linker peptide	G4S
57	Linker peptide	GGG
58	Linker peptide	DGGGS
59	Linker peptide	TGEKP
60	Linker peptide	GGRR
61	Linker peptide	EGKSSGSGSESKVD
62	Linker peptide	KESGSVSSEQLAQFRSLD
63	Linker peptide	GGRRGGGS
64	Linker peptide	LRQRDGERP
65	Linker peptide	LRQKDGGGSERP
66	Linker peptide	GSTSGSGKPGSGEGSTKG

Claims

1. A bispecific antibody or antigen-binding fragment thereof that specifically binds to PD-L1 and CD40, wherein the antibody comprises at least one antigen-binding site that specifically binds to PD-L1 and at least one antigen-binding site that specifically binds to CD40.

2. The bispecific antibody or antigen-binding fragment thereof of claim 1, wherein

- the PD-L1 antigen-binding site binds to human PD-L1 with high affinity, and preferably, with a KD value of about 5×10^{-9} M to 0.5×10^{-9} M;

- the CD40 antigen-binding site selectively activates CD40 signaling pathway in a PD-L1-dependent manner, and preferably, the selectivity index, as defined by the ratio of the EC50 value of the antibody in the absence of PD-L1-expressing cells to the EC50 value of the antibody in the presence of PD-L1-expressing cells, is greater than 100, e.g., about 300-1000; and/or

- the antibody is a trivalent bispecific antibody comprising one PD-L1 antigen-binding site and two CD40 antigen-binding sites.

3. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-2, wherein the PD-L1 antigen-binding site comprises or consists of a VHH domain, and the CD40 antigen-binding site comprises or consists of a Fab or scFv domain.

4. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-3, wherein the PD-L1 antigen-binding site comprises CDR1-3 sequences contained within a VHH domain set forth in SEQ ID NO: 5 or 6, and the CD40 antigen-binding site comprises LCDR1-3 sequences contained within a light chain variable domain set forth in SEQ ID NO: 16 and HCDR1-3 sequences contained within a heavy chain variable domain set forth in SEQ ID NO: 17.

5. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-4, wherein the antibody further comprises an immunoglobulin Fc region; preferably, the Fc region is an IgG1 Fc region; more preferably, the Fc region has a mutation that reduces or eliminates Fcγ receptor binding activity.

6. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-5, wherein the antibody comprises or consists of an anti-CD40 full-length antibody and one or two (preferably one) anti-PD-L1 VHH domains conjugated thereto.

7. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-6, wherein the PD-L1 antigen-binding site comprises a VHH domain, wherein the VHH domain comprises CDR1, CDR2 and CDR3 comprising or consisting of amino acid sequences set forth in SEQ ID NOs: 2, 3 and 4, respectively,

preferably, the VHH domain comprises the amino acid sequence set forth in SEQ ID NO: 5 or 6, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 5 or 6, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions compared to SEQ ID NO: 5 or 6,

most preferably, the VHH domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6.

8. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-7, wherein the CD40 antigen-binding site comprises a heavy chain variable domain (VH) and a light chain variable domain (VL), wherein the VH domain comprises HCDR1, HCDR2, and HCDR3 comprising or consisting of amino acid sequences set forth in SEQ ID NOs: 21, 22, and 23, respectively, and the VL domain comprises LCDR1, LCDR2, and LCDR3 comprising or consisting of amino acid sequences set forth in SEQ ID NOs: 18, 19, and 20, respectively,

preferably, the VH domain comprises the amino acid sequence set forth in SEQ ID NO: 17, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 17, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions compared to SEQ ID NO: 17; and

the VL domain comprises the amino acid sequence set forth in SEQ ID NO: 16, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 16, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions compared to SEQ ID NO: 16;

most preferably, the VH domain comprises the amino acid sequence of SEQ ID NO: 17, and the VH domain comprises the amino acid sequence of SEQ ID NO: 16.

9. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-8, wherein the bispecific antibody comprises a linker peptide having a length of 10-20 amino acids, and preferably comprising the amino acid sequence set forth in SEQ ID NO: 30.

10. The bispecific antibody or antigen-binding fragment thereof according to any one of claims 1-9, wherein the bispecific antibody comprises a first, a second, and a third polypeptide chain, wherein

- the first polypeptide chain comprises, from N-terminus to C-terminus, a VH domain, a CH1 domain, an Fc region, a linker peptide, and a VHH domain;

- the second polypeptide chain comprises, from N-terminus to C-terminus, a VH domain, a CH1 domain and an Fc region;

- the third polypeptide chain comprises, from N-terminus to C-terminus, a VL domain and a CL domain;

wherein the VHH domain specifically binds to PD-L1, and the VH domain is paired with the VL domain and specifically binds to CD40,

preferably, the Fc regions of the first and second polypeptide chains comprise a Knob mutation and a Hole mutation that promote heterodimerization of the first and second polypeptide chains, respectively, and optionally a cysteine residue mutation for forming an interchain disulfide bond between the first and second polypeptide chains;

more preferably, the Fc regions of the first and second polypeptide chains each independently comprise an amino acid sequence selected from: SEQ ID NO: 25 or an amino acid sequence having at least 95% identity thereto, and SEQ ID NO: 26 or an amino acid sequence having at least 95% identity thereto,

more preferably, the bispecific antibody comprises a first polypeptide chain, a second polypeptide chain, and a third polypeptide chain, wherein:

the first polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 9 or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity thereto;

the second polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 8, or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto; and

the third polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 7, or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto.

11. The bispecific antibody or the antigen-binding fragment thereof according to any one of claims 1-9, wherein the bispecific antibody comprises a first polypeptide chain and a second polypeptide chain, wherein

- the first polypeptide chain comprises, from N-terminus to C-terminus, a VH domain, a CH1 domain, an Fc region, a linker peptide, and a VHH domain;

- the second polypeptide chain comprises, from N-terminus to C-terminus, a VL domain and a CL domain;

wherein the VHH domain specifically binds to PD-L1, and the VH domain is paired with the VL domain and specifically binds to CD40,

preferably, the first polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 11 or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto;

the second polypeptide chain comprises the amino acid sequence set forth in SEQ ID NO: 10 or an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity thereto.

12. An anti-PD-L1 antibody or an antigen-binding fragment thereof, comprising a VHH domain that specifically binds to PD-L1, wherein the VHH domain comprises CDR1, CDR2, and CDR3 sequences contained within SEQ ID NO: 5 or 6;

preferably, the CDR1, CDR2, and CDR3 sequences comprise or consist of amino acid sequences set forth in SEQ ID NOs: 2, 3, and 4, respectively;

more preferably, the VHH domain comprises the amino acid sequence set forth in SEQ ID NO: 5 or 6, or an amino acid sequence having at least 80%, 85%, 90%, 95% or 99% identity to SEQ ID NO: 5 or 6, or an amino acid sequence having one or more (preferably 1-10, and more preferably 1-5) amino acid additions, deletions and/or substitutions compared to SEQ ID NO: 5 or 6,

most preferably, the VHH domain comprises or consists of the amino acid sequence set forth in SEQ ID NO: 6.

13. A polynucleotide, encoding the antibody or the antigen-binding fragment thereof according to any one of claims 1 to 12.

14. A vector, preferably an expression vector, comprising the polynucleotide according to claim 13.

15. A host cell, comprising the polynucleotide according to claim 13 or the vector according to claim 14, wherein optionally, the host cell is a mammalian cell.

16. A method for producing the antibody or the antigen-binding fragment thereof according to any one of claims 1-12, comprising: culturing a host cell comprising a polynucleotide encoding a polypeptide chain of the antibody or the antigen-binding fragment thereof under conditions suitable for expressing the polypeptide chain.

17.An immunoconjugate or an immunofusion comprising the antibody or the antigen-binding fragment thereof according to any one of claims 1 to 12.

18. A pharmaceutical composition, comprising the antibody or the antigen-binding fragment thereof according to any one of claims 1-12 or the immunoconjugate or immunofusion according to claim 17, and a pharmaceutically acceptable carrier.

19. Use of the antibody or the antigen-binding fragment thereof according to any one of claims 1-12 or the

immunoconjugate or the immunofusion according to claim 17 as a medicament or for the preparation of a medicament.

20. The use of claim 19, wherein the medicament is for the treatment and/or prevention of a cancer in an individual, or as a diagnostic tool for a disease, preferably, the individual is a mammal, more preferably a human.

21. A method for treating or preventing cancer, comprising: administering to a subject in need thereof an effective amount of the antibody or the antigen-binding fragment thereof according to any one of claims 1 to 12, or the immunoconjugate or the immunofusion according to claim 17.

22. The use or method according to any one of claims 19-21, wherein the cancer is a solid tumor or a hematological tumor, and preferably, the tumor is a PD-L1 positive tumor.