

BISPECIFIC FUSION PROTEIN AND APPLICATION THEREOF

FIELD

[0001] The present disclosure relates to a bispecific fusion protein with a novel structure and application thereof. The present disclosure also relates to a polynucleotide, a nucleic acid construct and a host cell encoding or preparing the fusion protein.

BACKGROUND

[0002] With the development of biotechnology, antibodies, as mature therapeutic drugs, play an important role in the treatment of tumors and immune diseases. Currently, most of the antibody drugs on the market target a single antigen and can only act on one target molecule. However, complex diseases are often multifactorial in nature, involving the overexpression or synergy of disease mediators, or the upregulation of different receptors, including molecular interactions in their signaling networks. Therefore, regulating multiple signal pathways can improve the efficacy of therapeutic antibodies. Bispecific antibody (BsAb) therapy using dual targeting strategies has become one of the effective treatments for tumors and immune diseases (Kontermann R. Dual targeting strategies with bispecific antibodies [C]/MAbs. Taylor & Francis, 2012, 4(2): 182-197.).

[0003] Bispecific antibodies can specifically bind to two different antigens or epitopes (Carter P. Bispecific human IgG by design [J]. Journal of immunological methods, 2001, 248(1-2): 7-15.). As early as the 1980s, Morrison et al. first prepared the first truly tetravalent bispecific antibody against dextran and dansyl group by linking single-chain antibodies with different specificities using flexible peptides for fusion expression (Coloma M J, Morrison S L. Design and production of novel tetravalent bispecific antibodies [J]. Nature biotechnology, 1997, 15(2): 159.). However, due to the bottleneck of antibody preparation technology and the lack of basic research on signal pathways, the development of bispecific antibodies has been hindered. With the in-depth understanding of disease pathogenesis and the rapid development of therapeutic monoclonal antibodies (McAbs)-related technologies, the technologies of antibody's construction, expression and purification has greatly improved, enabling the development of bispecific antibodies has the

technology and motivation to overcome the limiting factors. At present, the mechanisms of bispecific or multispecific antibodies are mainly as follows: (1) bridging cells (in trans); (2) receptor inhibition (in-cis) or receptor activation (in-cis); (3) co-factor mimetic; (4) PIgGyback approaches (Aran F. Labrijn, et al. Bispecific antibodies: a mechanistic review of the pipeline, Nature Reviews Drug Discovery volume 18, pages 585–608(2019)).

[0004] In the development of bispecific antibodies, the choice of molecular structure is very important. Different bispecific antibody designs have their own advantages and disadvantages, but the design of bispecific antibodies for clinical treatment purposes must solve the following problems: first, ensuring the correct coupling or pairing of two pairs (or more) of different light chains and heavy chains; second, maintaining the independence of each binding domain of each monoclonal antibody, and when binding different epitopes at the same time, there will be no steric interference between each other; third, the antibody molecules should be easy to express in mammalian cells without complicated protein modification process; fourthly, good druggability is required, such as high thermal stability, high chemical stability, high solubility, not easy to polymerize, low viscosity, high expression, suitable half-life, and the like (Spiess C, Zhai Q, Carter P J. Alternative molecular formats and therapeutic applications for bispecific antibodies [J]. Molecular immunology, 2015, 67(2): 95-106.).

[0005] According to different molecular structure forms (i.e., components and construction methods), bispecific antibodies can be divided into many types. For example, according to the left-right symmetry of the structure, they are divided into symmetrical and asymmetrical structures; according to the presence or absence of Fc region, they can be divided into bispecific antibodies containing Fc region and bispecific antibodies devoid of Fc region; and according to the number of antigen-binding regions, they are divided into bivalent, trivalent, tetravalent or multivalent configurations, etc.. At present, bispecific antibodies have developed dozens of structures (Spiess C, Zhai Q, Carter P J. Alternative molecular formats and therapeutic applications for bispecific antibodies [J]. Molecular immunology, 2015, 67(2): 95-106.), these dozens of bispecific antibodies have diverse structures, roughly divided into 5 categories, including IgG-like bispecific antibodies, IgG-like bispecific antibodies with additional functional regions, bispecific antibodies with different antigen-binding fragments linked in series, fusion protein-type bispecific antibodies, and chemically coupled bispecific antibodies. Among them,

the construction of IgG-like bispecific antibodies mainly employs recombinant DNA technology for structural modification. The constructed IgG-like bispecific antibodies have complete crystallizable fragments (i.e., Fc fragments), and thus they can bind to different antigens while still retaining Fc fragment-mediated ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-mediated cytotoxicity, complement-dependent cytotoxicity) and the like, and at the same time, this type of antibody also retains the feature of extending the *in vivo* half-life of the antibody by binding to the neonatal Fc receptor (FcRn) (Ridgway J B B, Presta L G, Carter P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization [J]. Protein Engineering, Design and Selection, 1996, 9(7): 617-621.); IgG-like bispecific antibodies with additional functional regions are generally based on traditional IgG antibodies and generated by adding other specific antigen-binding fragments (i.e., additional functional regions, such as single domain antibodies, single chain antibodies, etc.) to the heavy chain and/or light chain by means of fusion proteins (LaFleur D, Abramyan D, Kanakaraj P, et al. Monoclonal antibody therapeutics with up to five specificities: functional enhancement through fusion of target-specific peptides [C]//MAbs. Taylor & Francis, 2013, 5(2): 208-218.); bispecific antibodies with different antigen-binding fragments linked in series are obtained by linking different Fabs, single-chain antibodies, single-domain antibodies, or antigen-binding fragments in a certain sequence through a linking peptide (Stork R, Müller D, Kontermann R E. A novel tri-functional antibody fusion protein with improved pharmacokinetic properties generated by fusing a bispecific single-chain diabody with an albumin-binding domain from streptococcal protein G [J]. Protein Engineering, Design & Selection, 2007, 20(11): 569-576.); fusion protein-type bispecific antibodies are protein molecules that can bind two or more antigens at the same time, formed by linking antibodies/antibody fragments (IgG, Fab, scFv, etc.) with different specificities through a linker such as a polypeptide fragment; and chemically coupled bispecific antibodies are similar to fusion protein-type bispecific antibodies, while for the chemically coupled bispecific antibodies, two antibodies/antibody fragments must be prepared separately first, and then the two are coupled through chemical bonds or both are coupled to a carrier protein.

[0006] In addition, with the development of basic research on signal pathways and the expansion of bi-antibody mechanisms, there are more and more targets and combinations available for bispecific antibody selection, such as PD-1, PD-L1, CTLA-4, LAG-3, FGL1, TIM-3,

Galectin-9, TIGIT, CD155 and TGF- β receptor (TGF- β R), CD80, CD86, VEGFR, VEGF-trap, FGL1, CD70, 4-1BBL, OX40L, and SIRP α ; and tumor antigens including Claudin 18.2, HER-2, Mesothelin, BCMA, SSTR2, GPRC5D, PSMA, FCRH5, CD33, CD123, CD20, A33, CEA, CD28, DLL3, EGFR, VEGFR, VEGFR2, VEGF-A, Nectin-4, FGFR, C-met, RANKL, PDGF, PDGFR, PDGFR α , DLL4, Ang-1, and Ang-2, etc. Combinations of immune checkpoint targets or tumor targets with other targets relating to resisting the development of cancer cells or activating T-cells, or the combination of multiple immune checkpoint targets greatly improves the possibility of treating malignant diseases such as tumors. In the early stage, the combination of two separate checkpoint antibodies was used clinically to improve the efficacy. For example, compared with treatment with ipilimumab alone, the treatment with the combination of ipilimumab (anti-CTLA4) and nivolumab (anti-PD1) can improve the survival rate of patients with melanoma. Currently, the safety and early efficacy of the four PD-1 $\times$ CTLA4 bsAbs are being evaluated in early clinical trials. The concept of inhibitors blocking two immune checkpoints is also used clinically for other target combinations, such as PD-1 $\times$ LAG 3, PD-1 $\times$ TIM3, and PD-L1 $\times$ CTLA 4. (Wolchok, J. D. et al. Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma, *N.Engl. J. Med.* 377(14): 1345–1356, (2017); Dovedi, S. J. et al. MEDI5752: A novel bispecific antibody that preferentially targets CTLA-4 on PD-1 expressing T-cells. *Cancer Res.*78(13), Supplement.Abstract 2776: (2018). Hedvat, M. et al. Simultaneous checkpoint —checkpoint or checkpoint — costimulatory receptortargeting with bispecific antibodies promotes enhanced human T cell activation [abstract P664]. Presented at the 2018 Society for Immunotherapy of Cancer (SITC) (2018); Aran F. Labrijn, et al. Bispecific antibodies: a mechanistic review of the pipeline, *Nature Reviews Drug Discovery* volume 18, pages 585–608(2019).

[0007] At present, most of the bispecific antibodies are still in the clinical or preclinical research stage, and only three bispecific antibodies have been approved for marketing. They are Trion Pharma's Catumaxomab (delisted due to commercial factors in 2017), Amgen's Blinatumomab and Roche's Emicizumab. Catumaxomab is an IgG-like bispecific antibody in the form of Triomab. It targets T cells and tumor cells on which epithelial cell adhesion molecules (EpCAMs) overexpress, and inhibits tumor cells through T cell activation, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). It was

approved by EMA in 2009 for the treatment of malignant ascites caused by EpCAM-positive tumors that were ineffective or unfeasible against the standard treatment, and it is also the first bispecific antibody to be approved for marketing. Blinatumomab is a bispecific antibody without Fc region and adopts the structure form of BiTE (bispecific T cell engager). Blinatumomab binds to T cells through the anti-CD3 part, and to malignant and normal B cells through the anti-CD19 part, thereby inducing T cell-mediated tumor cell killing effect. In 2014, it was approved by FDA for the treatment of Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia. Emicizumab is a modified humanized bispecific IgG4 monoclonal antibody that can bind to factor IXa and factor X. This antibody adopts "Knobs-into-Holes (KiH)" for Fc design, "Common Light Chain-IgG (CLC-IgG)" for LC design, and "Multidimensional Optimization" for variable region optimization, and was approved by FDA in 2017 for routine prevention of hemophilia A in the presence of factor VIII inhibitors.

Table 1. Bispecific antibodies that have been approved for marketing (including delisted)

Bispecific antibody	Trade name	Research unit	Time of first approval
Catumaxomab	Removab	Trion Pharma	2009 (EMA) (delisted in 2017)
Blinatumomab	Blincyto	Amgen	2014 (FDA)
Emicizumab	Hemlibra	Roche	2017 (FDA)

[0008] Although there are currently dozens of bispecific antibodies/platforms and research and development experience from 3 marketed drugs, there are more problems and challenges in the development of bispecific antibodies compared to general antibodies due to their complex structures and diverse functional characteristics. For example, IgG-like bispecific antibodies face the problem of mismatches of heavy chain-heavy chain or heavy chain-light chain during the preparation process, the most typical of which is Catumaxomab. Although Catumaxomab is the first bispecific antibody approved for marketing, there are some very obvious limitations, which are mainly reflected in the complex production process of Triomab antibody and the immunogenicity problem that heterologous antibodies are relatively prone to emerge, and in 2017,

the drug was delisted due to commercial factors; for another example, bispecific antibodies with different antigen-binding fragments linked in series have a short half-life due to the lack of constant regions, especially the lack of Fc fragments, and their expression level and intracellular stability after expression are generally lower than traditional IgG (Stork R, Müller D, Kontermann R E. A novel tri-functional antibody fusion protein with improved pharmacokinetic properties generated by fusing a bispecific single-chain diabody with an albumin-binding domain from streptococcal protein G [J]. Protein Engineering, Design & Selection, 2007, 20(11): 569-576.). In addition, most of this type of antibody is difficult to adopt affinity purification means in the purification process, so the purification process and the analysis of impurities in the finished product are also more complicated than traditional IgG (Tan P H, Sandmaier B M, Stayton P S. Contributions of a highly conserved VH/VL hydrogen bonding interaction to scFv folding stability and refolding efficiency [J]. Biophysical journal, 1998, 75(3): 1473-1482.), the most typical of which is Blinatumomab. Blinatumomab has a small molecular weight because of lacking Fc fragments, and its half-life in the blood is only 1.25 ± 0.63 h, which is much shorter than the half-life of an intact antibody, so it requires continuous intravenous injection for 4 weeks to reach the effective dose, and the reagent needs to be additionally equipped with a continuous infusion device when used. Besides, it has poor stability and easily forms aggregates during production, which also affects the quality of the product and increases production costs. The current bispecific antibodies have many problems, such as low expression, poor stability, complex production processes, and significantly higher research and development costs than monoclonal antibodies, all of which limit the development of bispecific antibodies. Therefore, there is an urgent need to develop bispecific antibodies with new structures to provide more clinical options.

SUMMARY

[0009] In response to the above problems, the present disclosure provides a bispecific fusion protein with a novel structure, which belongs to an IgG-like bispecific fusion protein with additional functional regions. Specifically, the fusion protein has a structure in which a second binding domain is inserted in the hinge region of a full-length immunoglobulin G (IgG) through an optional peptide linker, and the Fab region of the IgG is a first binding domain of the fusion

protein; after inserting the second binding domain, the heavy chain of the IgG is the heavy chain of the fusion protein, and the light chain of the IgG is the light chain of the fusion protein; the second binding domain is selected from a human receptor or ligand, an extracellular region fragment of the receptor or ligand, a binding domain fragment, and a fragment combination of the receptor or ligand; wherein the receptor or ligand forms a dimerization or multimerization structure in a natural signaling pathway to activate or inhibit the signaling pathway, and the second binding domain and the first binding domain target different targets. The bispecific fusion protein with the above structure was named as Hibody (Hinge-insertion bispecific antibody) structure fusion protein (hereinafter referred to as Hibody structure fusion protein or Hibody).

[0010] Further, the first binding domain targets and binds to an immune checkpoint molecule or a tumor antigen.

[0011] Further, the immune checkpoint molecule includes PD-1 (*Programmed cell death protein 1*), PD-L1 (*Programmed cell death 1 ligand 1*), CTLA-4 (*Cytotoxic T lymphocyte-associated antigen-4*), LAG-3 (*Lymphocyte activation gene-3*), FGL1 (*Fibrinogen-like protein 1*), TIM-3 (*T cell immunoglobulin-3*), Galectin-9, TIGIT (*T-cell immunoreceptor with Ig and ITIM domains*), CD155, CD47; the tumor antigen includes Claudin 18.2, Her-2 (*Human epidermal growth factor receptor-2*), Mesothelin, BCMA (*B Cell Maturation Antigen*), SSTR2 (*Somatostatin receptor 2*), GPRC5D (*G-protein coupled receptor family C group 5 member D*), PSMA (*Prostate specific membrane antigen*), FcRH5 (*Fc receptor-like protein 5*), CD33, CD123, CD20, A33, CEA (*Carcino-embryonic antigen*), CD28, DLL3 (*Delta-like protein 3*), EGFR (*Epidermal growth factor receptor*), VEGFR (*Vascular Endothelial Growth Factor Receptor*), VEGFR2 (*Vascular Endothelial Growth Factor Receptor 2*), VEGF-A (*Vascular endothelial growth factor- A*), Nectin-4, FGFR (*Fibroblast growth factor receptors*), C-met, RANKL (*Receptor activator of nuclear factor kappa-B ligand*), PDGF (*Platelet Derived Growth Factor*), PDGFR (*Platelet Derived Growth Factor Receptor*), PDGFR α (*Platelet Derived Growth Factor Receptor α*), DLL4 (*Delta-like ligand 4*), Ang-1 (*Angiopoietin-1*), and Ang-2 (*Angiopoietin-2*).

[0012] Further, the second binding domain targets and binds to human TGF- β (*Transforming growth factor- β*), CTLA-4, VEGF, LAG3, CD27, 4-1BB (*CD137*), OX40 (*CD134*), CD47, FGL1 (*Fibrinogen Like Protein 1*), TLT-2 (*Trem-like transcript 2*), CD28, HGF (*Hepatocyte growth*

factor), CSF1 (*Colony-stimulating factor 1*), CXCL1 (*CXC chemokine ligand 1*), CXCL2 (*CXC chemokine ligand 2*), CXCL3 (*CXC chemokine ligand 3*), CXCL5 (*CXC chemokine ligand 5*), CXCL6 (*CXC chemokine ligand 6*), CXCL7 (*CXC chemokine ligand 7*), CXCL8 (*CXC chemokine ligand 8*), CXCL9 (*CXC chemokine ligand 9*), CXCL10 (*CXC chemokine ligand 10*), CXCL12 (*CXC chemokine ligand 12*), GPCR (*Glucocorticoid-induced tumor necrosis factor receptor*), EGF (*Epidermal Growth Factor*), and ICOSL (*Inducible co-stimulator ligand*).

[0013] Further, the receptor or ligand is human TGF- β receptor (TGF- β R), CD80, CD86, VEGFR, VEGF-trap, FGL1, CD70, 4-1BBL, OX40L, SIRP α (*Signal regulatory protein α*), B7-H3 (CD276), C-met, CSF1R (*Colony-stimulating factor 1 receptor*), CXCR2 (*CXC chemokine receptor 2*), CXCR3 (*CXC chemokine receptor 3*), CXCR4 (*CXC chemokine receptor 4*), GITRL (*Glucocorticoid-induced TNF receptor ligand*), EGFR, and ICOS (*Inducible co-stimulator*).

[0014] Among them, CD80 and CD86 are transmembrane glycoproteins, which are members of the immunoglobulin superfamily (IgSF). The mature CD80 molecule consists of 254 amino acids, including 208 amino acids in the extracellular domain (ECD), 25 amino acids in the transmembrane domain, and 21 amino acids in the intracellular domain. Similarly, the mature CD86 molecule consists of 303 amino acids, including 222 amino acids in the extracellular domain, 20 amino acids in the transmembrane domain, and 61 amino acids in the intracellular domain. The extracellular domains of CD80 and CD86 include immunoglobulin V (IgV) and immunoglobulin C (IgC) regions. CD80 and CD86 bind to their ligands CD28 and CTLA-4 through the immunoglobulin V (IgV) region. In the case of CD80 and CD86 binding to CD28, CD80 and CD86 have important regulatory effects on antigen-induced T cell activation, proliferation, and production of effector function, acting as positive regulators; while in the case of CD80 and CD86 binding to CTLA-4, CD80 and CD86 down-regulates the immune response, acting as negative regulators. Therefore, CD80 and CD86 are co-stimulatory factors when T lymphocytes are activated, and they play an important role in autoimmune monitoring, humoral immune response and transplantation response. Different from using agonistic anti-CD28 antibodies to activate T cells, using CD28's ligand CD80 as an activating factor to activate T cells does not trigger a severe cytokine storm, thereby greatly reducing the possibility of endangering the life of the patient.

[0015] In some embodiments, CD80 ECD is selected from human CD80 (such as the human CD80 of SEQ ID NO: 134) or human CD80 ECD derived from CD80 isotype 2 or isotype 3 (SEQ ID NO: 135 and 136). The CD80 ECD comprises CD80 immunoglobulin V (IgV) region (CD80-IgV, SEQ ID NO: 133). In one embodiment, the CD80 ECD comprises human CD80 immunoglobulin V region and C region (CD80-IgVIgC, SEQ ID NO: 32). In one embodiment, the CD80 ECD is human CD80 ECD. In one embodiment, the CD80 ECD comprises human CD80 IgV.

[0016] CD80-IgV (SEQ ID NO: 133):

	VIHVTKEVKE VATLSCGHNV SVEELAQTTRI YWQKEKKMVL TMMSGDMNIW PEYKNRTIFD	60
10	ITNNLSIVIL ALRPSDEGTY ECVVLKYEKD AFKREHLAEV TLSVKADFPT PS	112

[0017] CD80-IgVIgC (SEQ ID NO: 32):

	VIHVTKEVKE VATLSCGHNV SVEELAQTTRI YWQKEKKMVL TMMSGDMNIW PEYKNRTIFD	60
	ITNNLSIVIL ALRPSDEGTY ECVVLKYEKD AFKREHLAEV TLSVKADFPT PSISDFEIPT	120
	SNIRRIICST SGGFPEPHLS WLENGEELNA INTTVSQDPE TELYAVSSKL DFNMTTNHSF	180
15	MCLIKYGHRLR VNQTFNWN	198

[0018] CD80 isotype 1 (SEQ ID NO: 134):

	MGHTRRQGTS PSKCPYLNFF QLLVLAGLSH FCSGVIHVTK EVKEVATLSC GHNVSVEELA	60
	QTRIWQKEK KMLVTMMSGD MNIWPEYKNR TIFDITNNLS IVILALRPSD EGTYESCVVLK	120
	YEKDAFKREH LAEVTLSVKA DFPTPSISDF EIPTSNIRRI ICSTSGGFPE PHLSWLENGE	180
20	ELNAINTTVS QDPETELYAV SSKLDFNMTT NHSFMCLIKY GHRLRVNQTFN WNTTKQEHFP	240
	DNLLPSWAIT LISVNGIFVI CCLTYCFAPR CRERRRNERL RRESVRPV	288

[0019] CD80 isotype 2 (SEQ ID NO: 135):

	MGHTRRQGTS PSKCPYLNFF QLLVLAGLSH FCSGVIHVTK EVKEVATLSC GHNVSVEELA	60
	QTRIWQKEK KMLVTMMSGD MNIWPEYKNR TIFDITNNLS IVILALRPSD EGTYESCVVLK	120
25	YEKDAFKREH LAEVTLSVKA DFPTPSISDF EIPTSNIRRI ICSTSGGFPE PHLSWLENGE	180
	ELNAINTTVS QDPETELYAV SSKLDFNMTT NHSFMCLIKY GHRLRVNQTFN WNTSFAPRCR	240
	ERRRNERLRR ESVRPV	256

[0020] CD80 isotype 3 (SEQ ID NO: 136):

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MGHTRRQGTS PSKCPYLNFF QLLVLAGLSH FCSGVIHVTK EVKEVATLSC GHNVSVEELA      60
QTRIIYWQKEK KMLVTMMMSGD MNIWPEYKNR TIFDITNNLS IVILALRPSD EGTYESCVVLK    120
YEKDAFKREH LAEVTLSVKG FAPRCRERRR NERLRRESVR PV                          162
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5 **[0021]** Further, the first binding domain and the second binding domain are selected from the following combinations:

[0022] (1) The first binding domain targets and binds to PD-L1, and the second binding domain targets and binds to human TGF- β , VEGF, FGL1, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL; or

10 **[0023]** (2) The first binding domain targets and binds to PD-1, and the second binding domain targets and binds to human CTLA-4, VEGF, HGF, EGF, CD28, LAG3, CD27, 4-1BB or OX40; or

[0024] (3) The first binding domain targets and binds to CTLA-4, and the second binding domain targets and binds to human CD28, VEGF, HGF, EGF, LAG3, CD27, 4-1BB or OX40; or

15 **[0025]** (4) The first binding domain targets and binds to LAG-3, and the second binding domain targets and binds to human CD28, VEGF, HGF, EGF, CTLA-4, CD27, 4-1BB or OX40; or

[0026] (5) The first binding domain targets and binds to FGL1, and the second binding domain targets and binds to human TGF- β , VEGF, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, 20 CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL; or

[0027] (6) The first binding domain targets and binds to TIM-3, and the second binding domain targets and binds to human VEGF, HGF, EGF, LAG3, CD27, 4-1BB or OX40; or

[0028] (7) The first binding domain targets and binds to Galectin-9, and the second binding domain targets and binds to human TGF- β , VEGF, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, 25 CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL; or

[0029] (8) The first binding domain targets and binds to TIGIT, and the second binding domain targets and binds to human TGF- β , HGF, EGF or VEGF; or

[0030] (9) The first binding domain targets and binds to CD155, and the second binding domain targets and binds to human TGF- β , VEGF, HGF, EGF, CD47 or CD155; or

[0031] (10) The first binding domain targets and binds to Claudin 18.2, HER-2, mesothelin, BCMA, SSTR2, GPRC5D, PSMA, FCRH5, CD33, CD123, CD20, A33, CEA, CD28, DLL3, EGFR, VEGFR, VEGFR2, VEGF-A, Nectin-4, FGFR, C-met, RANKL, PDGF, PDGFR, PDGFR α , DLL-4, Ang-1 or Ang-2, and the second binding domain targets and binds to human CTLA-4, TGF- β , VEGF, FGL1, LAG3, 4-1BB, OX40, CD27, CD28, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL.

[0032] Further, the first binding domain and the receptor or ligand are selected from the following combinations:

[0033] (1) The first binding domain targets and binds to PD-L1, and the receptor or ligand is selected from TGF- β R2, VEGFR, LAG-3, SIRP α , TIGIT, C-MET, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS; or

[0034] (2) The first binding domain targets and binds to PD-1, and the receptor or ligand is selected from human CD80, CD86, VEGFR, c-MET, EGFR, FGL1, CD70, 4-1BBL or OX40L; or

[0035] (3) The first binding domain targets and binds to CTLA-4, and the receptor or ligand is selected from human CD80, CD86, VEGFR, c-MET, EGFR, FGL1, CD70, 4-1BBL or OX40L; or

[0036] (4) The first binding domain targets and binds to LAG-3, and the receptor or ligand is selected from human VEGFR, c-MET, EGFR, CD80, CD86, CD70, 4-1BBL or OX40L; or

[0037] (5) The first binding domain targets and binds to FGL1, and the receptor or ligand is selected from human TGF- β R2, VEGFR, SIRP α , TIGIT, c-MET, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS; or

[0038] (6) The first binding domain targets and binds to TIM-3, and the receptor or ligand is selected from human VEGFR, c-MET, EGFR, FGL1, CD70, 4-1BBL or OX40L; or

[0039] (7) The first binding domain targets and binds to Galectin-9, and the receptor or ligand

is selected from human TGF- β RII, VEGFR, SIRP α , TIGIT, c-MET, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS; or

[0040] (8) The first binding domain targets and binds to TIGIT, and the receptor or ligand is selected from human TGF- β RII, c-MET, EGFR or VEGFR; or

5 **[0041]** (9) The first binding domain targets and binds to CD155, and the receptor or ligand is selected from human TGF- β RII, VEGFR, c-MET, EGFR, SIRP α or TIGIT; or

[0042] (10) The first binding domain targets and binds to Claudin 18.2, HER-2, mesothelin, BCMA, SSTR2, GPRC5D, PSMA, FCRH5, CD33, CD123, CD20, A33, CEA, CD28, DLL3, EGFR, VEGFR, VEGFR2, Nectin-4, FGFR, c-MET, RANKL, PDGF, PDGFR, PDGFR α , DLL4,
10 Ang-1 or Ang-2, and the receptor or ligand is selected from human CTLA-4, CD80, CD86, TGF- β RII, VEGFR, FGL1, LAG3, 4-1BB, OX40, CD27, CD28, CD70, c-MET, SIRP α , TIGIT, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS.

[0043] Further, the fragment of the receptor or ligand comprises a fragment of the extracellular region and a fragment of the binding domain of the receptor or ligand.

15 **[0044]** Further, the first binding domain targets and binds to PD-L1; the second binding domain is a fragment of human TGF- β RII; or the first binding domain targets and binds to PD-1, and the second binding domain comprises human CD80 ECD, CD80 IgV region, human CD80 IgV IgC, VEGFR1 ECD, VEGFR2 ECD or a combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2; or the first binding domain targets and binds to
20 Claudin 18.2, HER-2 or EGFR, and the second binding domain is selected from human CD80 ECD, CD80 IgV region, human CD80 IgV IgC, VEGFR1 ECD, VEGFR2 ECD or a combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2.

[0045] Further, the second binding domain is selected from:

[0046] (1) Human TGF- β RII comprising the sequence as shown in SEQ ID NO: 31, 131 or 132,
25 or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with the sequence as shown in SEQ ID NO: 31, 131 or 132, or an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions or deletions on the sequence as shown in SEQ ID NO: 31, 131 or 132; or

[0047] (2) CD80 ECD comprising the sequence as shown in SEQ ID NO: 32 or 133, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with the sequence as shown in SEQ ID NO: 32 or 133, or an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions or deletions on the sequence as shown in SEQ ID NO: 32 or 133; or

[0048] (3) A combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2, comprising the sequence as shown in SEQ ID NO: 33, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with the sequence as shown in SEQ ID NO: 33, or an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions or deletions on the sequence as shown in SEQ ID NO: 33.

[0049] Further, the C-terminus or/and N-terminus of the second binding domain has a peptide linker, and the peptide linker consists of 2-30 amino acids.

[0050] Further, the peptide linker may be:

(1) (GGGGS)_n; or

(2) AKTTPKLEEGEFSEAR (SEQ ID NO: 80); or

(3) AKTTPKLEEGEFSEARV (SEQ ID NO: 81); or

(4) AKTTPKLGG (SEQ ID NO: 82); or

(5) SAKTTPKLGG (SEQ ID NO: 83); or

(6) SAKTTP (SEQ ID NO: 84); or

(7) RADAAP (SEQ ID NO: 85); or

(8) RADAAPTVS (SEQ ID NO: 86); or

(9) RADAAAAGGPGS (SEQ ID NO: 87); or

(10) RADAAAA (SEQ ID NO: 88); or

(11) SAKTTPKLEEGEFSEARV (SEQ ID NO: 89); or

(12) ADAAP (SEQ ID NO: 90); or

(13) DAAPTVSIFPP (SEQ ID NO: 91); or

(14) TVAAP (SEQ ID NO: 92); or

(15) TVAAPSVFIFPP (SEQ ID NO: 93); or

(16) QPKAAP (SEQ ID NO: 94); or

5 (17) QPKAAPSVTLFPP (SEQ ID NO: 95); or

(18) AKTTPP (SEQ ID NO: 96); or

(19) AKTTPPSVTPLAP (SEQ ID NO: 97); or

(20) AKTTAP (SEQ ID NO: 98); or

(21) AKTTAPSVYPLAP (SEQ ID NO: 99); or

10 (22) ASTKGP (SEQ ID NO: 100); or

(23) ASTKGPSVFPLAP (SEQ ID NO: 101); or

(24) GENKVEYAPALMALS (SEQ ID NO: 102); or

(25) GPAKELTPLKEAKVS (SEQ ID NO: 103); or

(26) GHEAAAVMQVQYPAS (SEQ ID NO: 104); or

15 (27) GGGGSGGGGSGGGGSA (SEQ ID NO: 105),

wherein n is equal to 1, 2, 3 or 4;

when n=1, the (GGGS)_n is GGGS (SEQ ID NO: 120);

when n=2, the (GGGS)_n is GGGSGGGGS (SEQ ID NO: 121) or (GGGS)₂;

when n=3, the (GGGS)_n is GGGSGGGSGGGGS (SEQ ID NO: 122) or (GGGS)₃;

20 when n=4, the (GGGS)_n is GGGSGGGSGGGSGGGGS (SEQ ID NO: 123) or (GGGS)₄.

[0051] Further, the size of the inserted second binding domain does not exceed 235, 240, 250, 260, 270, 280, 290 or 300 amino acids.

[0052] Further, the insertion site of the second binding domain is located in a middle front part

of the hinge region, and the insertion site does not affect the formation of disulfide bonds of the immunoglobulin, wherein the middle front part of the hinge region generally refers to the part before 231A. Further, part of the amino acids in the hinge region before and after the insertion site are substituted or deleted. For example, the hinge region contains D221G and/or C220V mutations.

[0053] Further, the IgG is selected from mammalian IgG, humanized IgG, and human IgG, and the mammal includes mouse and rat; preferably, the IgG is IgG1, IgG2, IgG3 or IgG4.

[0054] Further, the Fc region of the fusion protein is aglycosylated or deglycosylated, or has reduced fucosylation or is afucosylated.

[0055] Further, the IgG is Atezolizumab, Avelumab, Durvalumab, Nivolumab, Pembrolizumab, Cemiplimab or Ipilimumab. Preferably, the IgG is Atezolizumab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 106, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 107; or the IgG is Avelumab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 108, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 109; or the IgG is Durvalumab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 110, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 111; or the IgG is Nivolumab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 112, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 113; or the IgG is Pembrolizumab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 114, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 115; or the IgG is Ipilimumab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 116, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 117; or the IgG is Cemiplimab, the amino acid sequence of the heavy chain of the IgG is shown in SEQ ID NO: 118, and the amino acid sequence of the light chain of the IgG is shown in SEQ ID NO: 119.

[0056] Specifically, the present disclosure provides a Hibody structure fusion protein with a first binding domain targeting human PD-L1, the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab of the IgG have the same CDR sequence as

the antibody defined by the following sequence, or has 1-2 amino acid substitutions on the CDRs of the antibody defined by the following sequence, the antibody is defined as follows:

[0057] (1) The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 66; and/or

5 **[0058]** (2) The amino acid sequence of the light chain variable region is shown in SEQ ID NO: 67.

[0059] Further, the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab of the IgG are as follows:

10 **[0060]** (1) For the heavy chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 1-5 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 1-5; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 6-10 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 6-10; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 11-15 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 11-15; and/or

15 **[0061]** (2) For the light chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 16-20 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 16-20; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 21-25 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 21-25; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 26-30 and amino acid sequences having 1
20 or 2 amino acid substitutions on SEQ ID NOs: 26-30.

[0062] In a particular embodiment, according to different measurement methods or system identifications used, the complementarity determining regions CDRs 1-3 of the corresponding heavy chain and light chain variable regions are shown in Table 2.

Table 2. Amino acid sequences of heavy chain and light chain variable region CDRs 1-3 of the anti-PD-L1 antibody

Category	System	CDR1	CDR2	CDR3
Heavy chain	Chothia	SEQ ID NO: 1 GFSLSR Y	SEQ ID NO: 6 WGVGT	SEQ ID NO: 11 NWGTADYFDY

	AbM	SEQ ID NO: 2 GFSLSRYSVH	SEQ ID NO: 7 MIWGVGTTD	SEQ ID NO: 12 NWGTADYFDY
	Kabat	SEQ ID NO: 3 RYSVH	SEQ ID NO: 8 MIWGVGTTDYNALKS	SEQ ID NO: 13 NWGTADYFDY
	Contact	SEQ ID NO: 4 SRYSVH	SEQ ID NO: 9 WLGMIWGVGTTD	SEQ ID NO: 14 ARNWGTADYFD
	IMGT	SEQ ID NO: 5 GFSLSRYS	SEQ ID NO: 10 IWGVGTT	SEQ ID NO: 15 ARNWGTADYFDY
Light chain	Chothia	SEQ ID NO: 16 RASKSVHTSGYSYMH	SEQ ID NO: 21 LASNLES	SEQ ID NO: 26 QHSGELPYT
	AbM	SEQ ID NO: 17 RASKSVHTSGYSYMH	SEQ ID NO: 22 LASNLES	SEQ ID NO: 27 QHSGELPYT
	Kabat	SEQ ID NO: 18 RASKSVHTSGYSYMH	SEQ ID NO: 23 LASNLES	SEQ ID NO: 28 QHSGELPYT
	Contact	SEQ ID NO: 19 HTSGYSYMHY	SEQ ID NO: 24 LLIYLASNLE	SEQ ID NO: 29 QHSGELPY
	IMGT	SEQ ID NO: 20 KSVHTSGYSY	SEQ ID NO: 25 LAS	SEQ ID NO: 30 QHSGELPYT

[0063] Further, the fusion protein comprises the following CDR sequences,

[0064] (1) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 1, 6, 11, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 1, 6, 11; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 16, 21, 26, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 16, 21, 26; or

[0065] (2) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 2, 7, 12, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 2, 7, 12; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 17, 22, 27, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 17, 22, 27; or

[0066] (3) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ

ID NOs: 3, 8, 13, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 3, 8, 13; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 18, 23, 28, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 18, 23, 28; or

5 **[0067]** (4) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 4, 9, 14, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 4, 9, 14; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 19, 24, 29, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 19, 24, 29; or

10 **[0068]** (5) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 5, 10, 15, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 5, 10, 15; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 20, 25, 30, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 20, 25, 30.

15 **[0069]** Further, in the heavy chain of the fusion protein, the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 3, 8, 13; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 18, 23, 28.

[0070] Further, the sequences of the heavy chain variable region and the light chain variable region of the fusion protein are as follows,

20 **[0071]** (1) The heavy chain variable region has a sequence as shown in SEQ ID NO: 66, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 66 and has an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 66; and/or

[0072] (2) The light chain variable region has a sequence as shown in SEQ ID NO: 67, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 67 and has an identity of greater than 80%,
25 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 67.

[0073] More specifically, the amino acid sequences of the heavy chain and light chain of the fusion protein are as follows,

[0074] (1) The heavy chain of the fusion protein has an amino acid sequence as shown in SEQ

ID NO: 72, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 72;

[0075] (2) The light chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 73, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 73.

[0076] Further, the amino acid sequences of the heavy chain and light chain of the fusion protein are as follows,

[0077] (1) The amino acid sequence of the heavy chain of the fusion protein has 1-15 amino acid site mutations or has an alternative peptide linker compared with SEQ ID NO: 72;

[0078] (2) The amino acid sequence of the light chain of the fusion protein has 1-10 amino acid site mutations compared with SEQ ID NO: 73.

[0079] Specifically, the present disclosure provides a Hibody structure fusion protein with a first binding domain targeting human PD-1, wherein the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab have the same CDR sequence as the antibody defined by the following sequence, or has 1-2 amino acid substitutions on the CDRs of the antibody defined by the following sequence, the antibody is defined as follows:

[0080] (1) The amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 64; and/or

[0081] (2) The amino acid sequence of the light chain variable region is shown in SEQ ID NO: 65.

[0082] Further, the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab in the fusion protein are as follows:

[0083] (1) For the heavy chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 34-38 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 34-38; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 39-43 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 39-43; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 44-48 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 44-48; and/or

[0084] (2) For the light chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 49-53 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 49-53; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 54-58 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 54-58; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 59-63 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 59-63

[0085] In a particular embodiment, according to different measurement methods or system identifications used, the complementarity determining regions CDRs 1-3 of the corresponding heavy chain and light chain variable regions are shown in Table 3.

Table 3. Amino acid sequences of heavy chain and light chain variable region CDRs 1-3 of the anti-PD-1 antibody

Category	System	CDR1	CDR2	CDR3
Heavy chain	Chothia	SEQ ID NO: 34 GITFSNS	SEQ ID NO: 39 WYDGSK	SEQ ID NO: 44 NDDY
	AbM	SEQ ID NO: 35 GITFSNSGMH	SEQ ID NO: 40 VIWYDGSKRY	SEQ ID NO: 45 NDDY
	Kabat	SEQ ID NO: 36 NSGMH	SEQ ID NO: 41 VIWYDGSKRYYADSVKG	SEQ ID NO: 46 NDDY
	Contact	SEQ ID NO: 37 SNSGMH	SEQ ID NO: 42 WVAVIWYDGSKRY	SEQ ID NO: 47 ATNDD
	IMGT	SEQ ID NO: 38 GITFSNSG	SEQ ID NO: 43 IWYDGSKR	SEQ ID NO: 48 ATNDDY
Light chain	Chothia	SEQ ID NO: 49 RASQSVSSYLA	SEQ ID NO: 54 DASNRAT	SEQ ID NO: 59 QQSSNWPRT
	AbM	SEQ ID NO: 50 RASQSVSSYLA	SEQ ID NO: 55 DASNRAT	SEQ ID NO: 60 QQSSNWPRT
	Kabat	SEQ ID NO: 51 RASQSVSSYLA	SEQ ID NO: 56 DASNRAT	SEQ ID NO: 61 QQSSNWPRT
	Contact	SEQ ID NO: 52 SSYLAWY	SEQ ID NO: 57 LLIYDASNRA	SEQ ID NO: 62 QQSSNWPR
	IMGT	SEQ ID NO: 53 QSVSSY	SEQ ID NO: 58 DAS	SEQ ID NO: 63 QQSSNWPRT

[0086] Further, the CDRs 1-3 of the variable region of the fusion protein is defined as follows,

[0087] (1) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 34, 39, 44, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 34, 39, 44; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 49, 54, 59, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 49, 54, 59; or

[0088] (2) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 35, 40, 45, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 35, 40, 45; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 50, 55, 60, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 50, 55, 60; or

[0089] (3) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 36, 41, 46, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 36, 41, 46; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 51, 56, 61, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 51, 56, 61; or

[0090] (4) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 37, 42, 47, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 37, 42, 47; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 52, 57, 62, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 52, 57, 62; or

[0091] (5) The amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 38, 43, 48, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 38, 43, 48; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 53, 58, 63, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 53, 58, 63.

[0092] Further, the CDRs 1-3 of the variable region of the fusion protein is defined as follows: the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 36,

41, 46; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 51, 56, 61.

[0093] Further, the amino acid sequences of the heavy chain variable region and the light chain variable region of the fusion protein are as follows,

5 **[0094]** (1) The heavy chain variable region has a sequence as shown in SEQ ID NO: 64, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 64 and has an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 64; and/or

[0095] (2) The light chain variable region has a sequence as shown in SEQ ID NO: 65, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 65 and has an identity of greater than 80%,
10 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 65.

[0096] More specifically, the amino acid sequences of the heavy chain and light chain of the fusion protein are defined as follows:

[0097] (1) The heavy chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 68, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%,
15 98%, 99% compared with SEQ ID NO: 68;

[0098] (2) The light chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 69, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 69.

[0099] Further, the amino acid sequences of the heavy chain and light chain of the fusion
20 protein are defined as follows:

[00100] (1) The amino acid sequence of the heavy chain of the fusion protein has 1-15 amino acid site mutations or has an alternative peptide linker compared with SEQ ID NO: 68;

[00101] (2) The amino acid sequence of the light chain of the fusion protein has 1-10 amino acid site mutations compared with SEQ ID NO: 69.

25 **[00102]** More specifically, the amino acid sequences of the heavy chain and light chain of the fusion protein are defined as follows:

[00103] (1) The heavy chain of the fusion protein has an amino acid sequence as shown in SEQ

ID NO: 70, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 70;

[00104] (2) The light chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 71, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 71.

[00105] Further, the amino acid sequences of the heavy chain and light chain of the fusion protein are defined as follows:

[00106] (1) The amino acid sequence of the heavy chain of the fusion protein has 1-15 amino acid site mutations or has an alternative peptide linker compared with SEQ ID NO: 70;

[00107] (2) The amino acid sequence of the light chain of the fusion protein has 1-10 amino acid site mutations compared with SEQ ID NO: 71.

[00108] The present disclosure also provides an isolated polynucleotide encoding the above fusion protein.

[00109] The present disclosure also provides a nucleic acid construct comprising the above polynucleotide, preferably the nucleic acid construct is a vector.

[00110] The present disclosure also provides a host cell comprising the above polynucleotide or the above nucleic acid construct or the vector, preferably the cell is a prokaryotic cell, eukaryotic cell, yeast cell, mammal cell, E. coli cell or CHO cell, NS0 cell, Sp2/0 cell, or BHK cell.

[00111] The present disclosure also provides a pharmaceutical composition comprising the above fusion protein and a pharmaceutically acceptable carrier.

[00112] The present disclosure also provides a method for treating tumor or cancer, comprising the step of administering the above fusion protein or the pharmaceutical composition to a subject in need of a treatment or relief.

[00113] The present disclosure also provides use of the above fusion protein, the polynucleotide, the nucleic acid construct or the vector, or the pharmaceutical composition in the manufacture of a medicament for the treatment or prevention of a tumor or cancer. The tumor or cancer includes a solid tumor or a non-solid tumor.

[00114] The present disclosure also provides a diagnostic kit comprising the above fusion protein.

[00115] The present disclosure also provides a method of manufacturing the fusion protein, comprising culturing the host cell under conditions allowing the expression of the above nucleic acid construct, and recovering the produced fusion protein from the culture.

[00116] The Hibody structure fusion protein provided in the present disclosure has the same advantages of expression and production as IgG because the second binding domain is inserted in the hinge region, and can be seamlessly applicable to the existing IgG expression platform and purification platform, which greatly reduces the subsequent development cost; compared with the bispecific antibody constructed by the existing bispecific antibody platform, the bispecific antibody constructed by the Hibody platform has a higher expression level and does not have the problem of light and heavy chain mismatches, which also greatly reduces the cost of antibody production. In addition, it is surprising to find that inserting a second binding domain of a certain size in the two hinge regions not only does not affect the binding activity of the Fab region of the fusion protein and the stability of the protein, but also further improves the stability and obtains a longer half-life. More notably, the binding activity of the second binding domain to the target binding site is significantly improved compared to that of the corresponding soluble natural binding fragment monomer to the corresponding target, which may lie in that since the receptor or ligand of the selected second binding domain will form a dimerization or multimerization structure in the natural signaling pathway to activate or inhibit the signaling pathway, when the second binding domain is inserted into the hinge region, its spatial positional relationship in the hinge region promotes the formation of the dimerization of the corresponding receptor or ligand or its fragment, so that by imitating the natural signal pathway, a more significantly improved binding activity is generated, which makes the fusion protein have a greatly increased activation or inhibition ability compared with a soluble receptor or ligand or its fragment. Besides, through a reasonable combination of the two binding domains, the target of receptor inhibition or receptor activation can be flexibly selected to achieve an effective synergistic effect. The bispecific fusion protein provided in the present disclosure has a more significant disease treatment effect to effectively treat or control the disease process, especially in various tumors and cancers, compared with the separate administration of two target drugs or the bispecific antibody of the

prior art.

[00117]

BRIEF DESCRIPTION OF DRAWINGS

[00118] FIG. 1 Schematic diagram of Hibody structure fusion protein.

5 **[00119]** FIG. 2 shows the inhibition curve of Hib-PDV and its control group on mouse colon cancer model tumor.

[00120] FIG. 3 shows the inhibition curve of Hib-PDC and its control group on mouse colon cancer tumor.

10 **[00121]** FIG. 4 is a comparison chart of Hib-PLT and its control group on promoting cell secretion of IFN- γ factor at the level of 10-100 nM.

DETAILED DESCRIPTION

Definitions

15 **[00122]** 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 related to the present disclosure. Those skilled in the art can refer to the Dictionary of Cell and Molecular Biology, third edition, 1999, Academic Press; the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, second edition, 2002, CRC Press; and the Oxford Dictionary of Biochemistry and Molecular Biology, revised edition, 2000, Oxford University Press.

20 **[00123]** The amino acids involved in the present disclosure can be represented by their commonly known three-letter symbols or by the single-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Likewise, nucleotides can be represented by their generally recognized one-letter codes.

25 **[00124]** In the present disclosure, the determination or numbering method of the complementarity determining region (CDR) of the antibody variable domain includes IMGT, Kabat (such as stated in Kabat et al., Sequences of Proteins of Immunological Interest, 5th edition, Public Health Service, National Institutes of Health, Bethesda MD. (1991)), Chothia, AbM and

Contact method.

[00125] For the purpose of the present invention, "identity", "consistency" or "similarity" between two nucleic acid or amino acid sequences refers to the percentage of the same nucleotides or amino acid residues between the two sequences to be compared obtained after the optimal alignment. The percentage is purely statistical and the differences between the two sequences are randomly distributed and cover their full length. Comparisons between two nucleic acid or amino acid sequences are usually performed by comparing these sequences after aligning them in an optimal manner, and can be performed over a segment or over a "comparison window". In addition to manual implementation, the optimal alignment for comparing sequences can also performed through the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2: 482], through the local homology algorithm of Neddleman and Wunsch (1970) [J. MoI. Biol. 48: 443], through the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85: 2444], and through the computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or through BLAST N or BLAST P comparison software).

[00126] As broadly defined, the immunoglobulin involved in the present disclosure refers to an animal protein with antibody activity, consisting of two identical light chains and two identical heavy chains. It is an important class of immune effector molecules and a protein produced by lymphocytes of higher animal immune system, and can be transformed into antibodies by induction of antigens. Due to different structures, it can be divided into 5 types: IgG, IgA, IgM, IgD and IgE. Preferably, the immunoglobulin involved in the present disclosure is IgG.

[00127] As broadly defined, the receptor involved in the present disclosure is a type of special protein that exists in the cell membrane or in the cell and can bind to a specific signal molecule outside the cell to activate a series of biochemical reactions in the cell to cause the cell to produce a corresponding effect under external stimuli. The biologically active substances that bind to receptors are collectively referred to as ligands. The binding of the receptor and the ligand results in a molecular conformation change, which causes cellular responses, such as mediating intercellular signal transduction, intercellular adhesion, endocytosis, and the like.

[00128] The bispecific fusion protein involved in the present disclosure is based on the structure

of an immunoglobulin with a second binding domain inserted in its hinge region. Therefore, the bispecific fusion protein involved in the present disclosure has two binding domains.

Examples

5 **[00129]** The present disclosure will be further illustrated by way of the following examples. It should be noted that the following examples are for further elaboration and explanation of the present disclosure, and should not be regarded as limiting the present disclosure.

[00130] Example 1 Construction of proteins

10 **[00131]** In this example, the following protein molecules were constructed using conventional methods in the art, and expressed transiently or stably according to conventional methods in the art:

- (1) Three Hibody bispecific fusion proteins: Hib-PLT, Hib-PDC, and Hib-PDV;
- (2) Control proteins 1-3: constructed according to the bifunctional molecular structure in WO2015/118175;
- 15 (3) Other control proteins: TGF- β -R-His, VEGF-R-His, CD80-His, PD-L1 control antibodies.

Table 4. Target combinations of fusion protein

Fusion protein code	First binding domain target	Second binding domain target	Second binding domain fragment inserted	Peptide linker
Hib-PLT	PD-L1	TGF- β	TGF- β RII extracellular region fragment	(GGGGS) ₃
Hib-PDC	PD-1	CD28	CD80	(GGGGS) ₃
Hib-PDV	PD-1	VEGF	The combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2	(GGGGS) ₃
Control protein 1	PD-L1	TGF- β	TGF- β RII extracellular region fragment	(GGGGS) ₃

Control protein 2	PD-1	CD28	CD80	(GGGS) ₃
Control protein 3	PD-1	VEGF	The combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2	(GGGS) ₃

• The nucleic acid sequences and amino acid sequences of the heavy chain and light chain of the fusion protein Hib-PLT are as follows:

[00132] Nucleic acid sequence of fusion protein Hib-PLT heavy chain (SEQ ID NO: 78):

5 CAGGTGCAGCTGCAGGAGTCCGGACCAGGACTGGTGAAGCCATCCGAGACCCTGAGCCTGAC
CTGTACAGTGTCCGGCTTCAGCCTGTCTAGGTACAGCGTGCCTGGATCAGACAGCCACCTGG
CAAGGGACTGGAGTGGCTGGGCATGATCTGGGGCGTGGGCACCACAGACTACAACCTCTGCTC
TGAAGTCCAGACTGACCATCAGCAAGGATACATCTAAGAATCAGTTCAGCCTGAAGCTGTCCA
GCGTGACCGCCGCTGACACAGCCGTGTACTATTGCGCTCGCAACTGGGGCACC GCCGACTACT
10 TCGACTATTGGGGCCAGGGCACCACAGTGACAGTGTCTTCCGCTAGCACCAAGGGCCCATCG
GTCTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCT
GGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCCGGCTGTCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGA
CCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGC
15 AACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAGGCGGGGGAGGCAGCG
GCGGCGGAGGAAGCGGGGGCGGAGGTAGCACCATCCCTCCACACGTGCAGAAGAGCGTG
AACAATGACATGATCGTCACCGACAACAACGGCGCCGTCAAGTTCCCCCAGCTGTGCAA
ATTCTGCGACGTGAGGTTCTCCACGTGCGACAACCAGAAGAGCTGTATGAGCAACTGCA
GCATCACATCCATCTGCGAAAAACCCAGGAAGTGTGCGTCGCCGTCTGGCGGAAGAA
20 CGACGAGAACATCACACTGGAGACCGTGTGCCACGACCCCAAAGTGCCTTACCACGACT
TCATCCTGGAGGACGCCGCCAGCCCAAAGTGCATCATGAAAGAGAAGAAGACCGGG
CGAGACTTTCTTCATGTGCTCCTGCAGCTCCGACGAGTGCAACGATAATATCATCTTCAG
CGAAGAATACAACACATCTAACCAGACGGAGGGGGCGGATCCGGGGGGCGGCGGAAGCGG
CGGGGGGGGCGAGCACTCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGT
25 CAGTCTTCCTCTTCCCCCAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCA
CATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC
GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACC

GTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC
AAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGCA
GCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGG
TCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGC
5 AATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTT
CTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCAT
GCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG
GTAAATGA

[00133] Note: The underlined and bold sequence is the TGF- β RII nucleic acid sequence.

10 **[00134]** Amino acid sequence of fusion protein Hib-PLT heavy chain (SEQ ID NO: 72):

QVQLQESGPG LVKPSETLSL TCTVSGFSLS RYSVHWIRQP PGKGLEWLGM IWGVGTTDYN	60
SALKSRLTIS KDTSKNQFSL KLSSVTAADT AVYYCARNWG TADYFDYWQ GTTVTVSSAS	120
TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL	180
YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKV EPKS CDK GGGGSGG GGSGGGGSTI	240
15 PPHVQKSVNN DMIVTDNNGA VKFPQLCKFC DVRFSTCDNQ KSCMSNCSIT SICEKPQEV	300
VAVWRKNDEN ITLETVCHDP KLPYHDFILE DAASPKCIMK EKKKPGETFF MCSCSSDECN	360
DNIIFSEEYN TSNPD GGGGS GGGGSGGGGS THTCPPCPAP ELLGGP SVFL FPPKPKDTLM	420
ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD	480
WLNQKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLF PSREEMTKNQ VSLTCLVKGF	540
20 YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL	600
HNHYTQKSLS LSPGK	615

[00135] Note: The bold and framed fragment is the hinge region sequence, the peptide linker is in italics, and the underlined and bold sequence is the TGF- β RII amino acid sequence.

[00136] Nucleic acid sequence of fusion protein Hib-PLT light chain (SEQ ID NO: 79):

25 GATATCGTGCTGACCCAGTCTCCAGCTTCCTGGCCGTGTCCCCAGGACAGAGGGGCCACCATC
ACATGTCGGGCTTCCAAGAGCGTGACACAAAGCGGCTACTCTTATATGCATTGGTACCAGCAG
AAGCCCGGCCAGCCCCCTAAGCTGCTGATCTATCTGGCTTCCAACCTGGAGAGCGGAGTGCCA
GCTAGGTTCTCTGGCTCCGGCAGCGGCACCGACTTTACCCTGACAATCAATCCTGTGGAGGCC

AACGATACAGCTAATTACTATTGCCAGCACTCCGGAGAGCTGCCATACACCTTCGGCGGAGGC
 ACAAAGGTGGAGATCAAGCGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGAT
 GAGCAGTTGAAATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAG
 GCCAAAGTACAGTGGAAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTAC
 5 AGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCA
 GACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGT
 CACAAAGAGCTTCAACAGGGGAGAGTGTTAG

[00137] Amino acid sequence of fusion protein Hib-PLT light chain (SEQ ID NO: 73):

DIVLTQSPAS LAVSPGQRAT ITCRASKSVH TSGYSYMHVY QQKPGQPPKL LIYLASNLES 60
 10 GVPARFSGSG SGTDFTLTIN PVEANDTANY YCQHSGLPY TFGGGTKVEI KRTVAAPSVF 120
 IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSLS 180
 STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC 218

• The nucleic acid sequences and amino acid sequences of the heavy chain and light chain of the
 15 fusion protein Hib-PDC are as follows:

[00138] Nucleic acid sequence of fusion protein Hib-PDC heavy chain (SEQ ID NO: 74):

CAGGTGCAGCTCGTGGAGAGCGGGGAGGCGTTCGTCCAGCCTGGCCGTAGCCTGCGGCTGGA
 CTGTAAGGCTAGCGGAATCACGTTACAGCAACAGCGGAATGCACTGGGTCAGACAGGCTCCTG
 GCAAAGGCCTCGAATGGGTCGCCGTGATCTGGTACGACGGGAGCAAGAGATACTACGCAGAT
 20 TCAGTGAAGGGCCGTTTCACAATCAGCCGTGACAACTCGAAGAACACACTGTTCTCCTGCAGAT
 GAACAGCCTGAGGGCAGAAGACACAGCAGTCTACTACTGCGCTACAAATGACGACTACTGGG
 GCCAGGGAACACTGGTCACCGTGAGCTCAGCTTCCACCAAGGGCCCATCCGTCTTCCCCCTG
 GCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTA
 CTTCCCCGAACCGGTGACGGTGTCGTGGAAGTACAGGCGCCCTGACCAGCGGCGTGACACCT
 25 TCCCGGCTGTCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCA
 GCAGCTTGGGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTG
 GACAAGAGAGTTGAGTCCGGTGCGGCGGAAGCGGCGGAGGCAGCGGCGGAGGCGGC
 AGCGTGATCCATGTCACGAAGGAGGTCAAAGAAGTGGCCACCCTCAGCTGCGGTCACA
ACGTCAGCGTGGAAGAGTTGGCCCAGACCAGAATCTACTGGCAGAAGGAGAAGAAGAT
 30 GGTCTTGACCATGATGAGCGGCGACATGAACATCTGGCCAGAGTACAAGAATAGAATA

TCTTCGACATCACCAATAACCTGAGCATCGTGATCCTCGCTTTGCGTCCGAGCGACGAA
GGCACCTACGAGTGCGTAGTGCTAAAGTACGAGAAAGACGCCTTCAAGCGGGAGCACC
TGGCAGAAGTAACCCTGAGCGTGAAGGCAGACTTCCCAACGCCTAGCATCTCCGACTTC
GAGATCCCCACAAGCAACATCCGGCGGATCATCTGTAGCACCTCCGGGGGTTTCCCCGA
5 GCCTCACCTGTCCTGGCTCGAGAACGGCGAGGAGCTGAACGCCATCAACACCACCGTG
AGCCAGGACCCCGAGACAGAACTGTACGCCGTGAGCTCCAAGCTCGACTTCAACATGA
CCACAAATCACAGCTTCATGTGCCTCATCAAGTACGGACACCTGCGGGTGAATCAGACG
TTCAACTGGAACGGCGGGGGGGGTAGCGGCGGCGGGGGCTCAGGTGGGGGGGGCTCAAAAT
ATGGTCCCCCATGCCACCATGCCAGCACCTGAGTTCCTGGGGGGACCATCAGTCTTCCTGT
10 TCCCCCAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTCACGTGCGTGGTGG
TGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGGCGTGAGGTG
CATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGTGGTCAGCGT
CCTCACCGTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACA
AAGGCCTCCCGTCCTCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAGCCA
15 CAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTG
CCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGG
AGAACA ACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCA
GGCTAACCGTGGAACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATGCAT
GAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAATAG

20 **[00139]** Note: The underlined and bold sequence is the CD80 nucleic acid sequence.

[00140] Amino acid sequence of fusion protein Hib-PDC heavy chain (SEQ ID NO: 68):

QVQLVESGGG VVQPGRSRLR DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY	60
ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTILVT VSSASTKGPS	120
VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS	180
25 VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESGGGGSGG GSGGGGS VI HVTKEVKEVA	240
TLSCGHNVSV EELAQTRIYW QKEKKMVLTM MSGDMNIWPE YKNRTIFDIT NNLSIVILAL	300
RPSDEGTYEC VVLKYEKDAF KREHLAEVTL SVKADFPTPS ISDFEIPSTN IRRICSTSG	360
GFPEPHLSWL ENGEELNAIN TTVSQDPETE LYAVSSKLDF NMTTNHSFMC LIKYGHLRVN	420
QTFNWN GGGG SGGGGSGGGG SKYGPPCPPC PAPEFLGGPS VFLFPPKPKD TLMISRTPEV	480
30 TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY	540

KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV 600
 EWESNGQPEN NYKTTTPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK 660
 SLSLSLGK 668

[00141] Note: The peptide linker is in italics, and the underlined and bold sequence is the CD80
 5 amino acid sequence.

[00142] Nucleic acid sequence of fusion protein Hib-PDC light chain (SEQ ID NO: 75):

GAGATCGTGCTGACACAGAGCCCAGCCACTCTGTCACTGTCCCCAGGAGAAAGGGCTACTCT
 GTCTTGCCGGGCAAGCCAGTCTGTCTCCAGCTACCTGGCCTGGTATCAGCAGAAGCCCGGACA
 GGCTCCTAGACTGCTGATCTACGACGCAAGTAACAGAGCCACCGGCATCCCCGCACGCTTCAG
 10 TGGCTCAGGCTCCGGAACAGACTTTACTCTGACCATCTCTAGTCTGGAGCCTGAAGATTTCGC
 CGTGTACTATTGTCAGCAGAGCTCTAATTGGCCTAGAACCTTCGGCCAGGGCACCAAAGTCGA
 GATCAAGCGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAA
 TCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAG
 TGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAG
 15 CAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAA
 CACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT
 CAACAGGGGAGAGTGTTAG

[00143] Amino acid sequence of fusion protein Hib-PDC light chain (SEQ ID NO: 69):

EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA 60
 20 RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP 120
 SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT 180
 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC 214

• The nucleic acid sequences and amino acid sequences of the heavy chain and light chain of the
 25 fusion protein Hib-PDV are as follows:

[00144] Nucleic acid sequence of fusion protein Hib-PDV heavy chain (SEQ ID NO: 76):

CAGGTGCAGCTCGTGGAGAGCGGGGAGGCGTCGTCCAGCCTGGCCGTAGCCTGCGGCTGGA
 CTGTAAGGCTAGCGGAATCACGTTTCAGCAACAGCGGAATGCACTGGGTCAGACAGGCTCCTG

GCAAAGGCCTCGAATGGGTCGCCGTGATCTGGTACGACGGGAGCAAGAGATACTACGCAGAT
TCAGTGAAGGGCCGTTTCACAATCAGCCGTGACAACTCGAAGAACACACTGTTCCCTGCAGAT
GAACAGCCTGAGGGCAGAAGACACAGCAGTCTACTACTGCGCTACAAATGACGACTACTGGG
GCCAGGGAACACTGGTCACCGTGAGCTCAGCTTCCACCAAGGGCCCATCCGTCTTCCCCCTG
5 GCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGCTGCCTGGTCAAGGACTA
CTTCCCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGACACCT
TCCCGGCTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCA
GCAGCTTGGGCACGAAGACCTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTG
GACAAGAGAGTTGAGTCCGGTGCGGCGGAAGCGGCGGCGGAGGCAGCGGCGGAGGCGGC
10 AGC**GGTAGACCATTTCGTAGAGATGTACAGCGAAATCCCCGAAATCATCCACATGACTGAA**
GGAAGGGAGCTCGTCATCCCCTGCCGGGTACGTACCTAACATCACTGTTACTCTGAA
GAAGTTCCCACTCGACACTTTGATCCCTGATGGAAAACGCATCATCTGGGACAGCAGAA
AGGGCTTCATCATCTCAAATGCAACGTACAAAGAAATCGGACTCCTGACCTGTGAAGCA
ACAGTCAATGGACATTTGTATAAGACAAACTATCTCACACATCGACAGACCAATACAATC
15 **ATCGATGTGGTTCTGAGCCCGTCTCATGGAATCGAACTATCTGTTGGAGAAAAGCTCGT**
CCTGAATTGTACAGCAAGAAGTGAAGTGAATGTGGGAATCGACTTCAACTGGGAATACC
CTTCTTCGAAGCATCAGCATAAGAAACTCGTAAACCGAGACCTAAAGACCCAGTCTGGG
AGCGAGATGAAGAAATTCTTGAGCACCTGACTATCGATGGTGTAACCCGGAGCGACCA
GGGATTGTACACCTGTGCAGCATCCAGCGGGCTGATGACCAAGAAGAACAGCACATTCCG
20 **TCAGGGTCCATGAACCC**GGCGGGGGGGGTAGCGGCGGCGGGGGCTCAGGTGGGGGGGGCT
CAAAATATGGTCCCCCATGCCACCATGCCAGCACCTGAGTTCCTGGGGGGACCATCAGTCT
TCCTGTTCCCCCAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTCACGTGCG
TGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGGCGTG
GAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGTGGT
25 CAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCT
CCAACAAAGGCCTCCCGTCCTCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGA
GAGCCACAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAGCCT
GACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGC
AGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCT
30 ACAGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTG
ATGCATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAATAG

[00145] Note: The underlined and bold sequence is the VEGFR nucleic acid sequence.

[00146] Amino acid sequence of fusion protein Hib-PDV heavy chain (SEQ ID NO: 70):

QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY 60
 ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTTLVT VSSASTKGPS 120
 VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS 180
 VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESGGGGSGG GGSGGGSGR PFVEMYSEIP 240
 5 EIIHMTEGRE LVIPCRVTSP NITVTLKKFP LDTLIPDGKR IIWDSRKGFI ISNATYKEIG 300
LLTCEATVNG HLYKTNYLTH RQTNTIIDVV LSPSHGIELS VGEKLVLNCT ARTELNVGID 360
FNWEYPSSKH QHKKLVRDL KTQSGSEMKK FLSTLTIDGV TRSDQGLYTC AASSGLMTKK 420
NSTFVRVHEP GGGGSGGGGS GGGGSKYGPP CPPCPAPEFL GGPSVFLFPP KPKDTLMISR 480
 TPEVTCVVVD VSQEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTYRVVSV LTVLHQDWLN 540
 10 GKEYKCKVSN KGLPSSIEKT ISKAKGQPRE PQVYTLPPSQ EEMTKNQVSL TCLVKGFYPS 600
 DIAVEWESNG QPENNYKTP PVLDSGDSFF LYSRLTVDKS RWQEGNVFSC SVMHEALHNNH 660
 YTQKSLSLSL GK 672

[00147] Note: The peptide linker is in italics, and the underlined and bold sequence is the VEGF amino acid sequence.

15 **[00148]** Nucleic acid sequence of fusion protein Hib-PDV light chain (SEQ ID NO: 77):

GAGATCGTGCTGACACAGAGCCCAGCCACTCTGTCACTGTCCCCAGGAGAAAGGGCTACTCT
 GTCTTGCCGGGCAAGCCAGTCTGTCTCCAGCTACCTGGCCTGGTATCAGCAGAAGCCCGGACA
 GGCTCCTAGACTGCTGATCTACGACGCAAGTAACAGAGCCACCGGCATCCCCGCACGCTTCAG
 TGGCTCAGGCTCCGGAACAGACTTTACTCTGACCATCTCTAGTCTGGAGCCTGAAGATTTTCGC
 20 CGTGTACTATTGTCAGCAGAGCTCTAATTGGCCTAGAACCTTCGGCCAGGGCACCAAAGTCGA
 GATCAAGCGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAA
 TCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAG
 TGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAG
 CAAGGACAGCACCTACAGCCTCAGCAGACCCTGACGCTGAGCAAAGCAGACTACGAGAAA
 25 CACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT
 CAACAGGGGAGAGTGTTAG

[00149] Amino acid sequence of fusion protein Hib-PDV light chain (SEQ ID NO: 71):

EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA 60

RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP	120
SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT	180
LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEK	214

5 • The amino acid sequences of the heavy chain and light chain of the Control protein 1

[00150] Amino acid sequence of Control protein 1 heavy chain (SEQ ID NO: 124):

QVQLQESGPG LVKPSSETLSL TCTVSGFSL RYSVHWIRQP PGKGLEWLG IWGVGTTDYN	60
SALKSRLTIS KDTSKNQFSL KLSSVTAADT AVYYCARNWG TADYFDYWQ GTTVTVSSAS	120
TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL	180
10 YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTCPPC PAPELLGGPS	240
VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST	300
YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT	360
KNQVSLTCLV KGFYPSTIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ	420
GNVFSCSVMH EALHNHYTQK SLSLSPGKGG <i>GGSGGGGSGG</i> <u>GGSTIPPHVQ KSVNNDMIVT</u>	480
15 <u>DNNGAVKFPQ LCKFCDVRES TCDNQKSCMS NCSITSICEK PQEVCVAVWR KNDENITLET</u>	540
<u>VCHDPKLPYH DFILEDAASP KCIMKEKKKP GETFFMCSCS SDECNDNIIF SEEYNTSNPD</u>	600

[00151] Note: The peptide linker is in italics, and the underlined and bold sequence is the TGF- β RII amino acid sequence.

[00152] Amino acid sequence of Control protein 1 light chain (SEQ ID NO: 125):

20 DIVLTQSPAS LAVSPGQRAT ITCRASKSVH TSGYSYMHY QKPGQPPKL LIYLASNLES	60
GVPARFSGSG SGTDFTLTIN PVEANDTANY YCQHSYGELPY TFGGGTKVEI KRTVAAPSVF	120
IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYLS	180
STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC	218

25 • The amino acid sequences of the heavy chain and light chain of the Control protein 2

[00153] Amino acid sequence of Control protein 2 heavy chain (SEQ ID NO: 126):

	QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY	60
	ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTTLVT VSSASTKGPS	120
	VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS	180
5	VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPPCP PCPAPEFLGG PSVFLFPPKP	240
	KDTLMISRTP EVTCVVVDVS QEDPEVQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT	300
	VLHQDWLNGK EYCKVSNKG LPSSIEKTIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLTC	360
	LVKGFYPSDI AVEWESNGQP ENNYKTTTPV LSDGSFFLY SRLTVDKSRW QEGNVFSCSV	420
	MHEALHNHYT QKSLSLSLGK <i>GGGSGGGGS GGGGS</i> <u>VIHVT KEVKEVATLS CGHNSVEEL</u>	480
10	<u>AQTRIYWQKE KKMVLTMMSG DMNIWPEYKN RTIFDITNNL SIVILALRPS DEGTYESVVL</u>	540
	<u>KYEKDAFKRE HLAEVTLVK ADFPTPSISD FEIPTSNIIR IICSTSGGFP EPHLSWLENG</u>	600
	<u>EELNAINTTV SQDPETELYA VSSKLDENMT TNHSFMCLIK YGHLRVNQTF NWN</u>	653

[00154] Note: The peptide linker is in italics, and the underlined and bold sequence is the CD80 amino acid sequence.

15 **[00155]** Amino acid sequence of Control protein 2 light chain (SEQ ID NO: 127):

	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA	60
	RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP	120
	SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT	180
	LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	214

20

- The amino acid sequences of the heavy chain and light chain of the Control protein 3

[00156] Amino acid sequence of Control protein 3 heavy chain (SEQ ID NO: 128):

	QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY	60
	ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTTLVT VSSASTKGPS	120
25	VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS	180

	VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPPCP PCPAPEFLGG PSVFLFPPKP	240
	KDTLMISRTP EVTCVVVDVS QEDPEVQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT	300
	VLHQDWLNGK EYKCKVSNKG LPSSIEKTIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLTC	360
	LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV	420
5	MHEALHNHYT QKSLSLSLGK <i>GGGSGGGGS GGGSGRPFV EMYSEIPEII HMTEGRELVI</i>	480
	<u>PCRVTSPNIT VTLKKFPLDT LIPDGKRIIW DSRKGFIISN ATYKEIGLLT CEATVNGHLY</u>	540
	<u>KTNYLTHRQT NTIIDVVLSP SHGIELSVGE KLVLNCTART ELNVGIDFNW EYPSSKHQHK</u>	600
	<u>KLVNRLKTQ SGSEMKKFLS TLTIDGVTRS DQGLYTCAAS SGLMTKKNST FVRVHEP</u>	657

10 **[00157]** Note: The peptide linker is in italics, and the underlined and bold sequence is the VEGFR amino acid sequence.

[00158] Amino acid sequence of Control protein 3 light chain (SEQ ID NO: 129):

	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA	60
	RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP	120
	SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT	180
15	LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	214

• TGF-β-R-His

20 **[00159]** TGF-β-R-His is the His-tagged TGF-βRII extracellular region fragment, wherein the amino acid sequence of the TGF-βRII extracellular region fragment (SEQ ID NO: 137) is as follows:

	TIPPHVQKSV NNDMIVTDNN GAVKFPQLCK FCDVRFSTCD NQKSCMSNCS ITSICEKPQE	60
	VCAVAVWRKND ENITLETVCH DPKLPYHDFI LEDAASPKCI MKEKKKPGET FFMCS CSSDE	120
	CNDNIIFSEE YNTSNPD	137

• VEGF-R-His amino acid sequence

25 **[00160]** VEGF-R-His is the His-tagged combination fragment of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2, wherein the amino acid sequence of

combination fragment of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2 (SEQ ID NO: 130) is as follows:

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GRPFVEMYSE IPEIIHMTEG RELVIPCRVT SPNITVTLKK FPLDTLIPDG KRIIWDSRKG      60
FIISNATYKE IGLLTCEATV NGHLYKTNLY THRQTNTIID VVLSPSHGIE LSVGEKLVLN      120
5 CTARTELVNG IDFNWEYPSS KHQHKKLVNR DLKTQSGSEM KKFLSTLTID GVTRSDQGLY      180
TCAASSGLMT KKNSTFVRVH EP                                                  202

```

• CD80-His amino acid sequence

[00161] CD80-His is the His-tagged CD80 fragment, wherein the amino acid sequence of the CD80 fragment (SEQ ID NO: 32) is as follows:

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10 VIHVTKEVKE VATLSCGHN VVEELAQTRI YWQKEKKMVL TMSGDMNIW PEYKNRTIFD      60
ITNNLSIVIL ALRPSDEGTY ECVVLKYEKD AFKREHLAEV TLSVKADFPT PSISDFEIPT      120
SNIRRIICST SGGFPEPHLS WLENGEELNA INTTVSQDPE TELYAVSSKL DFNMTTNHSF      180
MCLIKYGHRL VNQTFNWN                                                       198

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[00162] Example 2 CHO expression

15 **[00163]** A. Cell Thawing and Expansion:

[00164] CHO host cells were thawed with a culture volume of 10 mL and culture conditions of 37.0°C, 200 rpm, 8% CO₂, and 80% humidity. After thawing, the cell density was 2.82×10⁵ cells/mL, and the viability was 95.41%.

20 **[00165]** After 72 hours of cell expansion and culture, the density of viable cells was 2.22×10⁶ cells/mL, and the viability was 98.32%.

[00166] The cells were re-seeded at 5×10⁵ cells/mL for cell re-seeding operation. After 48 hours of continuous culture, the density of viable cells was 2×10⁶ cells/mL, and the viability was 98.68%.

25 **[00167]** The cells were seeded into a 6-well plate at 5×10⁵ cells/well, with a culture volume of 2 mL/well, and placed in a carbon dioxide incubator (culture conditions: 37°C, 8% CO₂) overnight for adherence and transfection the next day.

[00168] B. Transfection

[00169] The transfection reagent used Lipofectamine[®]LTX Reagent (Invitrogen, catalog No. 15338-100), containing LTX and Plus reagents.

[00170] Transfection procedure: Changing the medium in the 6-well plate and preparing the transfection complex: LTX: 9 µl/well + plasmid: 2.5 µg/well + plus: 2.5 µl/well. The transfection complex was added at 250 µl/well to the 6-well plate. The medium in each well of the 6-well plate was changed 5 hours after cell transfection. After 48 hours of transfection, the supernatant was taken to detect the antibody content of the supernatant by the double antigen ELISA method.

[00171] C. Batch culture and fed-batch culture

[00172] After 48 hours of transfection, the cells in the six-well plate were digested, collected into a cell culture tube, and adapted to grow in suspension at a cell density of 3×10^5 cells/mL. When the cell viability returned to about 90%, Hib-PLT expressing cell pool and the Control protein 1 expressing cell pool were cultured in batch culture, and Hib-PDC, Control protein 2, Hib-PDV, and Control protein 3 were cultured in fed-batch culture.

[00173] Batch culture: Cells were cultured in a TPP cell culture tube at a cell density of 3×10^5 cells/mL, with a volume of 30 mL and culture conditions at 37.0°C, 200 rpm, 8% CO₂, and 80% humidity, and the cell viability and metabolism were monitored every day. The culture was terminated until the viability was <60%, and the cell supernatant after the completion of the culture was taken for concentration detection.

[00174] Fed-batch culture: Cells were cultured in a TPP cell culture tube at a cell density of 3×10^5 cells/mL, with a volume of 30 mL and culture conditions at 37.0°C, 200 rpm, 8% CO₂, and 80% humidity. The cell concentrated medium was given according to a certain fed-batch strategy (see Table 5), during which Glc was controlled at 2-6 g/L. The culture was terminated until the viability was <60%, and the cell supernatant after the completion of the culture was taken for concentration detection.

[00175]

Table 5. Fed-batch strategy

Day 0	3×10 ⁵ /ml, culture volume 30 ml
Day 4、6、8、10、12	900 μL CellBoost7A、90 μL CellBoost7B
Day 16	Detecting VCD and viability, stocking the sample and terminating the experiment

[00176] Table 6 shows the expression level results of Hibody bispecific fusion proteins Hib-PLT, Hib-PDC and Hib-PDV and their corresponding control proteins. The results show that the relative value of the increase in Hib-PLT expression level increased by 52.85% compared with its corresponding control protein; the relative value of the increase in Hib-PDC expression level increased by 33.43% compared with its corresponding control protein; and the relative value of the increase in Hib-PDV expression level increased by 25.6% compared with its corresponding control protein, indicating the expression level of the Hibody bispecific fusion protein provided in the present disclosure is significantly superior to that of the corresponding control protein, and the bispecific antibody provided in the present disclosure has unexpected technical effects.

Table 6 Expression level of bispecific antibodies

Category	Method	Expression level	Relative value of increase
Hib-PLT	Batch culture	188 mg/L	52.85%
Control protein 1	Batch culture	123 mg/L	
Hib-PDC	Fed-batch culture	349.59 mg/L	33.43%
Control protein 2	Fed-batch culture	262 mg/L	
Hib-PDV	Fed-batch culture	243.95 mg/L	25.6%
Control protein 3	Fed-batch culture	194.22 mg/L	

Note: Relative value of increase = (the expression level of the bispecific fusion protein provided in the present disclosure - the expression level of the corresponding control protein) / the expression level of the corresponding control protein.

[00177] Example 3 Determination of binding activity

[00178] 1. Determination of Hib-PLT binding activity:

(1) Coating antigen: TGF- β 1 was diluted to 2 μ g/ml with coating buffer, added to a 96-well plate at 100 μ l/well, and allowed to stand overnight at 2-8°C;

5 (2) Washing the plate: The plate was automatically washed 3 times at 350 μ l/well with PBST used as the eluent, and was patted to dryness after washed;

(3) Blocking the plate: The plate was blocked at 300 μ l/well with 3% BSA-PBST used as the blocking solution, and incubated at 37°C for 2 hours;

(4) Washing the plate: The plate was automatically washed 3 times at 350 μ l/well with PBST used as the eluent, and was patted to dryness after washed;

10 (5) Adding samples: Each concentration point of samples is as follows. The sample with each concentration point was sequentially added to a 96-well plate at 100 μ l/well, and each concentration point was in parallel with 2 replicate wells, and the plate was incubated at 37°C for 2 hours;

Test group	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11
Hib-PLT	555.556	55.556	5.556	1.852	0.617	0.206	0.069	0.023	0.008	0.001	0.000
TGF- β -R-His	64516.000	6451.600	645.160	215.053	71.684	23.895	7.965	2.655	0.885	0.088	0.009

(Unit: nM)

15 (6) Washing the plate: The plate was automatically washed 3 times at 350 μ l/well with PBST used as the eluent, and was patted to dryness after washed;

(7) Adding the secondary antibody:

- Hib-PLT sample well: Goat-anti-Human-Fc-HRP was diluted at 1:5000, and added at 100 μ l/well. Then the plate was incubated at 37°C for 2 hours;
- 20 • TGF- β -R-His well: Anti-His-HRP was diluted at 1:5000, and added at 100 μ l/well. Then the plate was incubated at 37°C for 2 hours;

(8) Washing the plate: The plate was automatically washed 5 times at 350 μ l/well with

PBST used as the eluent, and was patted to dryness after washed;

(9) Color development: The plate was subjected to color development for 2 minutes at 100 µl/well with TMB used as the color development solution;

5 (10) Terminating the color development: The plate was terminated for color development at 50 µl/well with 2M H₂SO₄ used as the stop solution;

(11) Reading: The absorbance values were read at 450/655 nm respectively using the MD microplate reader. The concentration of the sample to be tested was used as the abscissa and the absorbance value as the ordinate to plot making a 4-PL curve, and the software automatically gave the EC50 value.

10 **[00179]** 2. Determination of Hib-PDV binding activity:

(1) Coating antigen: VEGF was diluted to 1 µg/ml with coating buffer, added to a 96-well plate at 100 µl/well, and allowed to stand overnight at 2-8°C;

(2) Washing the plate: The plate was automatically washed 3 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

15 (3) Blocking the plate: The plate was blocked at 300 µl/well with 3% BSA-PBST used as the blocking solution, and incubated at 37°C for 2 hours;

(4) Washing the plate: The plate was automatically washed 3 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

20 (5) Adding samples: Each concentration point of samples is as follows. The sample with each concentration point was sequentially added to a 96-well plate at 100 µl/well, and each concentration point was in parallel with 2 replicate wells, and the plate was incubated at 37°C for 2 hours;

Test group	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11
Hib-PDV	555.556	55.556	5.556	1.852	0.617	0.206	0.069	0.023	0.005	0.001	0.000
VEGFR-His	43478.300	4347.830	434.783	144.928	48.309	16.103	5.368	1.789	0.358	0.036	0.004

(Unit: nM)

(6) Washing the plate: The plate was automatically washed 3 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

(7) Adding the secondary antibody:

- 5 • Hib-PDV sample well: Goat-anti-Human-Fc-HRP was diluted at 1:5000, and added at 100 µl/well. Then the plate was incubated at 37°C for 2 hours;
- VEGFR-His well: Anti-His-HRP was diluted at 1:5000, and added at 100 µl/well. Then the plate was incubated at 37°C for 2 hours;

10 (8) Washing the plate: The plate was automatically washed 5 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

(9) Color development: The plate was subjected to color development for 2 minutes at 100 µl/well with TMB used as the color development solution;

(10) Terminating the color development: The plate was terminated for color development at 50 µl/well with 2M H₂SO₄ used as the stop solution;

15 (11) Reading: The absorbance values were read at 450/655 nm respectively using the MD microplate reader. The concentration of the sample to be tested was used as the abscissa and the absorbance value as the ordinate to plot making a 4-PL curve, and the software automatically gave the EC₅₀ value.

[00180] 3. Determination of Hib-PDC binding activity:

20 (1) Coating antigen: CTLA-4 Protein was redissolved with 1 mL of sterilized water at a concentration of 200 µg/mL, diluted to 20 µg/ml with coating buffer, added to a 96-well plate at 100 µl/well, and allowed to stand overnight at 2-8°C;

(2) Washing the plate: The plate was automatically washed 3 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

25 (3) Blocking the plate: The plate was blocked at 300 µl/well with 3% BSA-PBST used as the blocking solution, and incubated at 37°C for 2h±20min;

(4) Washing the plate: The plate was automatically washed 3 times at 350 µl/well with

PBST used as the eluent, and was patted to dryness after washed;

(5) Adding samples: The biotinylated sample was diluted with PBST. Each concentration point of samples was as follows. The sample with each concentration point was sequentially added to a 96-well plate at 100 µl/well, and each concentration point was in parallel with 2 replicate wells, with the blank well being PBST, and the plate was incubated at 37°C for 2h±20min;

Test group	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11
Hib-PDC	1000000	100000	10000	3333.333	1111.111	370.370	123.457	41.152	13.717	1.372	0.137
CD80-His	1000000	100000	10000	3333.333	1111.111	370.370	123.457	41.152	13.717	1.372	0.137

(Unit: nM)

(6) Washing the plate: The plate was automatically washed 3 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

10 (7) Adding the secondary antibody:

- Hib-PDC sample well and CD80-His well: Streptavidin-HRP was diluted at 1:2000, and added at 100 µl/well. Then the plate was incubated at 37°C for 1h±10min;

(8) Washing the plate: The plate was automatically washed 5 times at 350 µl/well with PBST used as the eluent, and was patted to dryness after washed;

15 (9) Color development: TMB Double Slow substrate was added at 100 µl/well and the plate was subjected to color development for 6 minutes at room temperature shielded from light;

(10) Terminating the color development: The plate was terminated for color development at 50 µl/well with 2M H₂SO₄ used as the stop solution;

20 (11) Reading: The absorbance values were read at 450/655 nm respectively using the MD microplate reader. The concentration of the sample to be tested was used as the abscissa and the absorbance value as the ordinate to plot making a 4-PL curve, and the software automatically gave the EC₅₀ value. (see table 7).

Table 7. EC50 value of each test group

Test group	EC50 value
Hib-PLT	0.164 nM
TGF- β -R-His	11.60 nM
Hib-PDV	0.061 nM
VEGFR-His	2154 nM
Hib-PDC	24.36 nM
CD80-His	50.69 nM

[00181] The results of determination of binding activity showed that, comparison between Hib-PLT and TGF- β receptor monomer: Hib-PLT has an EC50 value of 0.164 nM, TGF- β receptor monomer has an EC50 value of 11.60 nM, and the binding activity of Hib-PLT is about 70 times better than that of TGF- β receptor monomer; comparison between Hib-PDV and VEGF receptor monomer: Hib-PDV has an EC50 value of 0.061 nM, VEGF receptor monomer has an EC50 value of 2154 nM, and the binding activity of Hib-PDV binding activity is about 35,311 times better than that of VEGF receptor monomer; comparison between Hib-PDC and CD80 monomer: Hib-PDC has an EC50 value of 24.36 nM, CD80 monomer has an EC50 value of 50.69 nM, and the binding activity of Hib-PDC is about 2 times better than that of CD80 monomer. That is, the binding activities of Hib-PLT, Hib-PDV, and Hib-PDC constructed with the bi-antibody model provided by the present disclosure are much better than that of their corresponding monomers.

[00182] Example 4 The efficacy of Hib-PDV on subcutaneously transplanted tumor of colon cancer MC38 cells in C57BL/6J-hPD-1 mice

[00183] Thirty-two 7-week-old, C57BL/6J humanized PD-1 transgenic mice (from Shanghai Model Organisms Center, Inc.) were collected. 0.1 mL of colon cancer MC38 cell suspension (1×10^6 cells/mouse) was inoculated subcutaneously into the right flank of mice. When the tumor grew to about 100 mm³, mice were randomly divided into groups according to the tumor size,

namely Vehicle (PBS) group, Hib-PDV (1.25 mg/kg) group, Hib-PDV (2.5 mg/kg) group and Hib-PDV (5 mg/kg) group, respectively, a total of 4 groups, 8 tumor-bearing mice in each group. They were administered intraperitoneally once a week, a total of 3 administrations, during the administration period, the long diameter and short diameter of the tumor and body weight were measured twice a week. On the 21st day after administration, the tumor growth inhibition rate (TGI_{RTV}) was calculated for drug efficacy evaluation, as shown in Table 8.

Table 8. Dosage regimen of Hib-PDV on tumor inhibitory activity of mouse colon cancer

Group	Administration group	Dosage (mg/kg)	Administration route	Administration frequency	Administration volume
1	Vehicle	--	i.p.	QW×3	10 mL/kg
2	Hib-PDV	5	i.p.	QW×3	10 mL/kg
3	Hib-PDV	2.5	i.p.	QW×3	10 mL/kg
4	Hib-PDV	1.25	i.p.	QW×3	10 mL/kg

[00184] Calculation formula:

[00185] Tumor volume: $TV = D_1 \times D_2^2 / 2$, where D1 and D2 represent the long diameter and short diameter of the tumor, respectively;

[00186] Relative tumor volume: $RTV = T_{VT} / T_{V1}$, where T_{V1} is the tumor volume before administration, and T_{VT} is the tumor volume at each measurement;

[00187] Relative tumor inhibition rate: $TGI_{RTV}(\%) = (1 - T_{RTV} / C_{RTV}) \times 100\%$, where T_{RTV} represents the RTV of the administration group or the positive control group, and C_{RTV} represents the RTV of the negative control group.

[00188] Results and conclusions: The tumor inhibitory effects of Hib-PDV on mouse colon cancer model are shown in Table 9 and FIG. 2, where Table 9 shows the tumor volume and tumor growth inhibition rate of Hib-PDV on mouse colon cancer model, and FIG. 2 is a graph of tumor growth after administration. Experimental results show that Hib-PDV has a significant inhibitory effect on mouse colon cancer model tumor.

Table 9. effect of Hib-PDV on the tumor volume and tumor inhibition rate of mouse colon cancer model (mean+SEM)

Administration group	Tumor volume (TV, mm ³)		TGIRTV (%)
	D0	D21	
Vehicle	86+10	1299+174	--
Hib-PDV (5 mg/kg)	86+10	675+218	46
Hib-PDV (2.5 mg/kg)	86+8	755+305	47
Hib-PDV (1.25 mg/kg)	85+8	1088+259	14

[00189] Example 5 The efficacy of Hib-PDC on subcutaneously transplanted tumor of transgenic hPD-L1 MC38 cells in C57BL/6J-hPD-1 mice

[00190] Thirty-two 7-9 week-old, humanized C57BL/6J humanized PD-1 transgenic female mice (from Shanghai Model Organisms Center, Inc.) were collected. 0.1 mL of transgenic hPD-L1 MC38 cell suspension (1×10^6 cells/mouse) was subcutaneously implanted in the back of the right forelimb of the mouse. When the tumor grew to about 100 mm³, mice were randomly divided into groups according to the tumor size, namely Vehicle (PBS) group, Hib-PDC (1.25 mg/kg) group, Hib-PDC (2.5 mg/kg) group and Hib-PDC (5 mg/kg) group, respectively, a total of 4 groups, 8 tumor-bearing mice in each group. They were administered intraperitoneally once a week, a total of 3 administrations, during the administration period, the long diameter and short diameter of the tumor and body weight were measured twice a week. On the 21st day after administration, the tumor growth inhibition rate (TGI_{RTV}) was calculated for drug efficacy evaluation, as shown in Table 10.

Table 10. Dosage regimen of Hib-PDC on tumor inhibitory activity of mouse colon cancer

Group	Administration group	Dosage (mg/kg)	Administration route	Administration volume	Administration frequency
1	Vehicle(PBS)	--	i.p.	10 ml/kg	QW×3

2	Hib-PDC	1.25	i.p.	10 ml/kg	QW×3
3	Hib-PDC	2.5	i.p.	10 ml/kg	QW×3
4	Hib-PDC	5	i.p.	10 ml/kg	QW×3

[00191] Calculation formula:

[00192] Tumor volume: $TV = D_1 \times D_2^2 / 2$, where D_1 and D_2 represent the long diameter and short diameter of the tumor, respectively;

5 **[00193]** Relative tumor volume: $RTV = T_{VT} / T_{V1}$, where T_{V1} is the tumor volume before administration, and T_{VT} is the tumor volume at each measurement;

[00194] Relative tumor inhibition rate: $TGI_{RTV}(\%) = (1 - T_{RTV} / C_{RTV}) \times 100\%$, where T_{RTV} represents the RTV of the administration group or the positive control group, and C_{RTV} represents the RTV of the negative control group.

10 **[00195]** Results and conclusions: The tumor inhibitory effects of Hib-PDC on mouse colon cancer are shown in Table 11 and FIG. 3, where Table 11 shows the effect of Hib-PDC on the tumor volume and tumor inhibition rate on mouse colon cancer model, and FIG. 3 is a graph of tumor growth after administration. Experimental results show that Hib-PDC has a significant inhibitory effect on subcutaneously transplanted tumors of colorectal cancer in mice.

15 **Table 11. effect of Hib-PDC on the tumor volume and tumor inhibition rate of mouse colon cancer model (mean+SEM)**

Group	Dosage (mg/kg)	Tumor volume (TV, mm ³)		TGIRTV (%)
		D0	D21	
PBS	--	101±10	2319±517	--
Hib-PDC	1.25	101±8	1577±211	31
Hib-PDC	2.5	101±9	813±74	63

Hib-PDC	5	101±9	541±179	77
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[00196] Example 6 Effect of Hib-PLT on the cytokine secretion in the mixed reaction of allogeneic lymphocytes

[00197] ELISA method was used to detect the concentration of cytokine IFN- γ in the cell supernatant to evaluate the role of Hib-PLT in activating CD4⁺ T cells. The mononuclear lymphocytes from donor 1 were induced into mature dendritic cells *in vitro* by DC cell rapid maturation in vitro kit (cat: CT-004, StemEry), the cells were collected after 48 h and diluted to 2×10⁵ cells/mL. The CD4⁺ T cells from donor 2 were enriched with Magnetic beads (EasySep™ Human CD4⁺ T Cell Enrichment Kit, StemCell, cat: 19052) and diluted to 1×10⁶ cells/mL. The mature dendritic cells and CD4⁺ T cells were mixed at a volume ratio of 1:1, and the mixed cell suspension was added to a 96-well plate at a volume of 100 μ L/well. Hib-PLT, PD-L1 control antibody + TGF- β Trap (combination medication), PD-L1 control antibody, TGF- β Trap, and human IgG1 (Biolegend) were added to a 96-well plate containing mixed cell suspension at a final concentration of 0.01-100 nM. The plate was then placed in a 37°C, 5% CO₂ incubator for 120 h, centrifuged at 2000 rpm for 5 min, and 150-200 μ L of the cell supernatant was collected. ELISA kits (Human IL-2 Quantikine ELISA Kit, R&D, cat: S2050; Human IFN-gamma Quantikine ELISA Kit, R&D, cat: SIF50C) were used to detect IFN- γ factor in the supernatant of the mixed reaction of allogeneic lymphocytes. One-way ANOVA was used to analysis the differences between groups.

[00198] Results and conclusions: As shown in FIG. 4, the level of Hib-PLT at a concentration of 10-100 nM promoting cell secretion of IFN- γ factor was significantly higher than that of the combination medication group of PD-L1 control antibody and TGF- β trap ($p < 0.05$). The results show that at the concentration level of 10-100 nM, Hib-PLT is better than the combination medication group in promoting the activation of CD4⁺ T cells in the mixed lymphocytes reaction.

[00199] The present disclosure has been exemplified by various specific examples. However, a person of ordinary skill in the art can understand that the present disclosure is not limited to each specific embodiments, and a person of ordinary skill can make various changes or modifications within the scope of the present disclosure, and each technical feature mentioned in various places

in this specification can be combined with each other without departing from the spirit and scope of the present disclosure. Such changes and modifications are within the scope of the present disclosure.

CLAIMS

1. A bispecific fusion protein, wherein the fusion protein has a structure in which a second binding domain is inserted in the hinge region of a full-length immunoglobulin G (IgG) through an optional peptide linker, and the Fab region of the IgG is a first binding domain; after inserting the second binding domain, the heavy chain of the IgG is the heavy chain of the fusion protein, and the light chain of the IgG is the light chain of the fusion protein; the second binding domain is selected from a human receptor or ligand, a fragment of the receptor or ligand, and a fragment combination of the receptor or ligand; wherein the receptor or ligand forms a dimerization or multimerization structure in a natural signaling pathway to activate or inhibit the signaling pathway, and the second binding domain and the first binding domain target different targets.

2. The fusion protein according to claim 1, wherein the first binding domain targets and binds to an immune checkpoint molecule or a tumor antigen.

3. The fusion protein according to claim 2, wherein the immune checkpoint molecule includes PD-1, PD-L1, CTLA-4, LAG-3, FGL1, TIM-3, Galectin-9, TIGIT, CD155 and CD47; the tumor antigen includes Claudin 18.2, HER-2, Mesothelin, BCMA, SSTR2, GPRC5D, PSMA, FCRH5, CD33, CD123, CD20, A33, CEA, CD28, DLL3, EGFR, VEGFR, VEGFR2, VEGF-A, Nectin-4, FGFR, C-met, RANKL, PDGF, PDGFR, PDGFR α , DLL4, Ang-1 and Ang-2.

4. The fusion protein according to claim 3, wherein the second binding domain targets and binds to human TGF- β , CTLA-4, VEGF, LAG3, CD27, 4-1BB, OX40, CD47, FGL1, TLT-2, CD28, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, GITR, EGF and ICOSL.

5. The fusion protein according to claim 4, wherein the receptor or ligand is human TGF- β receptor (TGF- β R), CD80, CD86, VEGFR, VEGF-trap, FGL1, CD70, 4-1BBL, OX40L, SIRP α ,

B7-H3, C-met, CSF1R, CXCR2, CXCR3, CXCR4, GITRL, EGFR and ICOS.

6. The fusion protein according to claim 4, wherein the first binding domain and the second binding domain are selected from the following combinations:

5 (1) the first binding domain targets and binds to PD-L1, and the second binding domain targets and binds to human TGF- β , VEGF, FGL1, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL; or

(2) the first binding domain targets and binds to PD-1, and the second binding domain targets and binds to human CTLA-4, VEGF, HGF, EGF, CD28, LAG3, CD27, 4-1BB or OX40;

10 or

(3) the first binding domain targets and binds to CTLA-4, and the second binding domain targets and binds to human CD28, VEGF, HGF, EGF, LAG3, CD27, 4-1BB or OX40; or

(4) the first binding domain targets and binds to LAG-3, and the second binding domain targets and binds to human CD28, VEGF, HGF, EGF, CTLA-4, CD27, 4-1BB or OX40; or

15 (5) the first binding domain targets and binds to FGL1, and the second binding domain targets and binds to human TGF- β , VEGF, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL; or

(6) the first binding domain targets and binds to TIM-3, and the second binding domain targets and binds to human VEGF, HGF, EGF, LAG3, CD27, 4-1BB or OX40; or

20 (7) the first binding domain targets and binds to Galectin-9, and the second binding domain targets and binds to human TGF- β , VEGF, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL; or

(8) the first binding domain targets and binds to TIGIT, and the second binding domain targets and binds to human TGF- β , HGF, EGF or VEGF; or

25 (9) the first binding domain targets and binds to CD155, and the second binding domain targets and binds to human TGF- β , VEGF, HGF, EGF, CD47 or CD155; or

(10) the first binding domain targets and binds to Claudin 18.2, HER-2, mesothelin, BCMA,

SSTR2, GPRC5D, PSMA, FCRH5, CD33, CD123, CD20, A33, CEA, CD28, DLL3, EGFR, VEGFR, VEGFR2, VEGF-A, Nectin-4, FGFR, C-met, RANKL, PDGF, PDGFR, PDGFR α , DLL-4, Ang-1 or Ang-2, and the second binding domain targets and binds to human CTLA-4, TGF- β , VEGF, FGL1, LAG3, 4-1BB, OX40, CD27, CD28, CD47, CD155, HGF, CSF1, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL12, EGF or ICOSL.

7. The fusion protein according to claim 6, wherein the first binding domain and the receptor or ligand are selected from the following combinations:

(1) the first binding domain targets and binds to PD-L1, and the receptor or ligand is selected from TGF- β R2, VEGFR, LAG-3, SIRP α , TIGIT, C-MET, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS; or

(2) the first binding domain targets and binds to PD-1, and the receptor or ligand is selected from human CD80, CD86, VEGFR, c-MET, EGFR, FGL1, CD70, 4-1BBL or OX40L; or

(3) the first binding domain targets and binds to CTLA-4, and the receptor or ligand is selected from human CD80, CD86, VEGFR, c-MET, EGFR, FGL1, CD70, 4-1BBL or OX40L; or

(4) the first binding domain targets and binds to LAG-3, and the receptor or ligand is selected from human VEGFR, c-MET, EGFR, CD80, CD86, CD70, 4-1BBL or OX40L; or

(5) the first binding domain targets and binds to FGL1, and the receptor or ligand is selected from human TGF- β R2, VEGFR, SIRP α , TIGIT, c-MET, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS; or

(6) the first binding domain targets and binds to TIM-3, and the receptor or ligand is selected from human VEGFR, c-MET, EGFR, FGL1, CD70, 4-1BBL or OX40L; or

(7) the first binding domain targets and binds to Galectin-9, and the receptor or ligand is selected from human TGF- β R2, VEGFR, SIRP α , TIGIT, c-MET, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS; or

(8) the first binding domain targets and binds to TIGIT, and the receptor or ligand is selected

from human TGF- β RII, c-MET, EGFR or VEGFR; or

(9) the first binding domain targets and binds to CD155, and the receptor or ligand is selected from human TGF- β RII, VEGFR, c-MET, EGFR, SIRP α or TIGIT; or

(10) the first binding domain targets and binds to Claudin 18.2, HER-2, mesothelin, BCMA, SSTR2, GPRC5D, PSMA, FCRH5, CD33, CD123, CD20, A33, CEA, CD28, DLL3, EGFR, VEGFR, VEGFR2, Nectin-4, FGFR, c-MET, RANKL, PDGF, PDGFR, PDGFR α , DLL4, Ang-1 or Ang-2, and the receptor or ligand is selected from human CTLA-4, CD80, CD86, TGF- β RII, VEGFR, FGL1, LAG3, 4-1BB, OX40, CD27, CD28, CD70, c-MET, SIRP α , TIGIT, CSF1R, CXCR2, CXCR3, CXCR4, EGFR or ICOS.

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8. The fusion protein according to any one of claims 1-7, wherein the fragment of the receptor or ligand comprises a fragment of the extracellular region and a fragment of the binding domain of the receptor or ligand.

15 9. The fusion protein according to claim 7, wherein the first binding domain targets and binds to PD-L1; the second binding domain is a fragment of human TGF- β RII; or the first binding domain targets and binds to PD-1, and the second binding domain is selected from human CD80 ECD, CD80 IgV region, human CD80 IgVIgC, VEGFR1 ECD, VEGFR2 ECD or a combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2; or the first binding domain targets and binds to Claudin 18.2, HER-2 or EGFR, and the second binding domain is selected from human CD80 ECD, CD80 IgV region, human CD80 IgVIgC, VEGFR1 ECD, VEGFR2 ECD or a combination of the second extracellular region of VEGFR1 and the third extracellular region of VEGFR2.

25 10. The fusion protein according to claim 9, wherein the second binding domain is selected from:

(1) human TGF- β RII comprising the sequence as shown in SEQ ID NO: 31, 131 or 132, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%

compared with the sequence as shown in SEQ ID NO: 31, 131 or 132, or an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions or deletions on the sequence as shown in SEQ ID NO: 31, 131 or 132; or

(2) CD80 ECD comprising the sequence as shown in SEQ ID NO: 32 or 133, or a sequence
5 having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with the sequence as shown in SEQ ID NO: 32 or 133, or an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions or deletions on the sequence as shown in SEQ ID NO: 32 or 133; or

(3) a combination of the second extracellular region of VEGFR1 and the third extracellular
10 region of VEGFR2, comprising the sequence as shown in SEQ ID NO: 33, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with the sequence as shown in SEQ ID NO: 33, or an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid substitutions or deletions on the sequence as shown in SEQ ID NO: 33.

15 11. The fusion protein according to claim 9 or 10, wherein the first binding domain targets and binds to PD-L1, and the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab of the IgG have the same CDR sequence as the antibody defined by the following sequence, or have 1-2 amino acid substitutions on the CDRs of the antibody defined by the following sequence, wherein the antibody is defined as follows:

20 (1) the amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 66; and/or

(2) the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 67.

25 12. The fusion protein according to claim 11, wherein the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab of the IgG are as follows:

(1) for the heavy chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 1-5 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 1-5; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 6-10 and amino acid

sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 6-10; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 11-15 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 11-15; and/or

(2) for the light chain variable region, the amino acid sequence of CDR1 is selected from
5 SEQ ID NOs: 16-20 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 16-20; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 21-25 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 21-25; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 26-30 and amino acid sequences having 1 or 2 amino acid substitutions on SEQ ID NOs: 26-30.

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13. The fusion protein according to claim 12, wherein:

(1) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 1, 6, 11, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 1, 6, 11; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ
15 ID NOs: 16, 21, 26, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 16, 21, 26; or

(2) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 2, 7, 12, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 2, 7, 12; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ
20 ID NOs: 17, 22, 27, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 17, 22, 27; or

(3) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 3, 8, 13, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 3, 8, 13; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ
25 ID NOs: 18, 23, 28, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 18, 23, 28; or

(4) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 4, 9, 14, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 4, 9, 14; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ

ID NOs: 19, 24, 29, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 19, 24, 29; or

(5) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 5, 10, 15, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 5, 10, 15; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 20, 25, 30, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 20, 25, 30.

14. The fusion protein according to claim 13, wherein the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 3, 8, 13; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 18, 23, 28.

15. The fusion protein according to claim 14, wherein:

(1) the heavy chain variable region has a sequence as shown in SEQ ID NO: 66, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 66 and has an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 66; and/or

(2) the heavy chain variable region has a sequence as shown in SEQ ID NO: 67, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 67 and has an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 67.

16. The fusion protein according to claim 15, wherein:

(1) the heavy chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 72, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 72;

(2) the light chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 73, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 73.

17. The fusion protein according to claim 16, wherein:

(1) the amino acid sequence of the heavy chain of the fusion protein has 1-15 amino acid site mutations or has an alternative peptide linker compared with SEQ ID NO: 72;

5 (2) the amino acid sequence of the light chain of the fusion protein has 1-10 amino acid site mutations compared with SEQ ID NO: 73.

18. The fusion protein according to claim 9, wherein the first binding domain targets and binds to PD-1, and the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab of the IgG have the same CDR sequence as the antibody defined by the following sequence, or have 1-2 amino acid substitutions on the CDRs of the antibody defined by the following sequence, wherein the antibody is defined as follows:

(1) the amino acid sequence of the heavy chain variable region is shown in SEQ ID NO: 64; and/or

15 (2) the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 65.

19. The fusion protein according to claim 18, wherein the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region in the Fab of the IgG are as follows:

20 (1) for the heavy chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 34-38 and an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 34-38; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 39-43 and an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 39-43; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 44-48 and an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 44-48; and/or

25 (2) for the light chain variable region, the amino acid sequence of CDR1 is selected from SEQ ID NOs: 49-53 and an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 49-53; the amino acid sequence of CDR2 is selected from SEQ ID NOs: 54-58 and an

amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 54-58; the amino acid sequence of CDR3 is selected from SEQ ID NOs: 59-63 and an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 59-63.

5 20. The fusion protein according to claim 19, wherein:

(1) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 34, 39, 44, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 34, 39, 44; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 49, 54, 59, or an amino acid sequence having 1 or 2 amino acid substitutions on
10 SEQ ID NOs: 49, 54, 59; or

(2) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 35, 40, 45, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 35, 40, 45; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 50, 55, 60, or an amino acid sequence having 1 or 2 amino acid substitutions on
15 SEQ ID NOs: 50, 55, 60; or

(3) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 36, 41, 46, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 36, 41, 46; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 51, 56, 61, or an amino acid sequence having 1 or 2 amino acid substitutions on
20 SEQ ID NOs: 51, 56, 61; or

(4) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 37, 42, 47, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 37, 42, 47; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 52, 57, 62, or an amino acid sequence having 1 or 2 amino acid substitutions on
25 SEQ ID NOs: 52, 57, 62; or

(5) the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 38, 43, 48, or an amino acid sequence having 1 or 2 amino acid substitutions on SEQ ID NOs: 38, 43, 48; and/or an amino acid sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 53, 58, 63, or an amino acid sequence having 1 or 2 amino acid substitutions on

SEQ ID NOs: 53, 58, 63.

21. The fusion protein according to claim 20, wherein the amino acid sequences of CDRs 1-3 of the heavy chain variable region are SEQ ID NOs: 36, 41, 46; and/or an amino acid
5 sequence of CDRs 1-3 of the light chain variable region are SEQ ID NOs: 51, 56, 61.

22. The fusion protein according to claim 21, wherein:

(1) the heavy chain variable region has a sequence as shown in SEQ ID NO: 64, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 64 and has an identity of greater than 80%,
10 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 64; and/or

(2) the light chain variable region has a sequence as shown in SEQ ID NO: 65, or a sequence that has the same CDRs 1-3 as SEQ ID NO: 65 and has an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 65.

15 23. The fusion protein according to claim 22, wherein:

(1) the heavy chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 68, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 68;

(2) the light chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO:
20 69, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 69.

24. The fusion protein according to claim 23, wherein:

(1) the amino acid sequence of the heavy chain of the fusion protein has 1-15 amino acid site
25 mutations or has an alternative peptide linker compared with SEQ ID NO: 68;

(2) the amino acid sequence of the light chain of the fusion protein has 1-10 amino acid site mutations compared with SEQ ID NO: 69.

25. The fusion protein according to claim 22, wherein:

(1) the heavy chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 70, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 70;

(2) the light chain of the fusion protein has an amino acid sequence as shown in SEQ ID NO: 71, or a sequence having an identity of greater than 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% compared with SEQ ID NO: 71.

26. The fusion protein according to claim 25, wherein:

(1) the amino acid sequence of the heavy chain of the fusion protein has 1-15 amino acid site mutations or has an alternative peptide linker compared with SEQ ID NO: 70;

(2) the amino acid sequence of the light chain of the fusion protein has 1-10 amino acid site mutations compared with SEQ ID NO: 71.

27. The fusion protein according to any one of claims 1-10, wherein the IgG is Atezolizumab, Avelumab, Durvalumab, Nivolumab, Pembrolizumab, Cemiplimab or Ipilimumab.

28. The fusion protein according to any one of claims 1-27, wherein the C-terminus or/and N-terminus of the second binding domain has a peptide linker, and the peptide linker consists of 2-30 amino acids.

29. The fusion protein of claim 28, wherein the peptide linker is:

(1) (GGGGS)_n; or

(2) AKTTPKLEEGEFSEAR (SEQ ID NO: 80); or

(3) AKTTPKLEEGEFSEARV (SEQ ID NO: 81); or

- (4) AKTTPKLGG (SEQ ID NO: 82); or
- (5) SAKTTPKLGG (SEQ ID NO: 83); or
- (6) SAKTTP (SEQ ID NO: 84); or
- (7) RADAAP (SEQ ID NO: 85); or
- 5 (8) RADAAPTVS (SEQ ID NO: 86); or
- (9) RADAAAAGGPGS (SEQ ID NO: 87); or
- (10) RADAAAA (SEQ ID NO: 88); or
- (11) SAKTTPKLEEGEFSEARV (SEQ ID NO: 89); or
- (12) ADAAP (SEQ ID NO: 90); or
- 10 (13) DAAPTVSIFPP (SEQ ID NO: 91); or
- (14) TVAAP (SEQ ID NO: 92); or
- (15) TVAAPSVFIFPP (SEQ ID NO: 93); or
- (16) QPKAAP (SEQ ID NO: 94); or
- (17) QPKAAPSVTLFPP (SEQ ID NO: 95); or
- 15 (18) AKTTPP (SEQ ID NO: 96); or
- (19) AKTTPPSVTPLAP (SEQ ID NO: 97); or
- (20) AKTTAP (SEQ ID NO: 98); or
- (21) AKTTAPSVYPLAP (SEQ ID NO: 99); or
- (22) ASTKGP (SEQ ID NO: 100); or
- 20 (23) ASTKGPSVFPLAP (SEQ ID NO: 101); or
- (24) GENKVEYAPALMALS (SEQ ID NO: 102); or
- (25) GPAKELTPLKEAKVS (SEQ ID NO: 103); or
- (26) GHEAAVMQVQYPAS (SEQ ID NO: 104); or
- (27) GGGGSGGGGSGGGGSA (SEQ ID NO: 105),

wherein n is equal to 1, 2, 3 or 4.

30. The fusion protein according to any one of claims 1-29, wherein the size of the inserted second binding domain does not exceed 300 amino acids.

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31. The fusion protein according to any one of claims 1-30, wherein the insertion site of the second binding domain is located in a middle front part of the hinge region, and the insertion site does not affect the formation of disulfide bonds of the immunoglobulin.

10 32. The fusion protein according to claim 31, wherein the middle front part of the hinge region refers to the part before 231A.

33. The fusion protein according to any one of claims 1-32, wherein part of the amino acids in the hinge region before and after the insertion site are substituted or deleted.

15

34. The fusion protein according to claim 33, wherein the hinge region contains D221G and/or C220V mutations.

20 35. The fusion protein according to any one of claims 1-34, wherein the IgG is selected from mammalian IgG, humanized IgG, and human IgG, and the mammal includes mouse, rat and rabbit; preferably, the IgG is IgG1, IgG2, IgG3 or IgG4.

36. The fusion protein according to any one of claims 1-35, wherein the Fc region of the fusion protein is aglycosylated or deglycosylated, or has reduced fucosylation or is afucosylated.

25

37. An isolated polynucleotide encoding the fusion protein according to any one of claims

1-36.

38. A nucleic acid construct comprising the polynucleotide according to claim 37, preferably the nucleic acid construct is a vector.

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39. A host cell comprising the polynucleotide according to claim 37 or the nucleic acid construct or the vector according to claim 38, preferably the cell is a prokaryotic cell, eukaryotic cell, yeast cell, mammal cell, E. coli cell or CHO cell, NS0 cell, Sp2/0 cell, or BHK cell.

10 40. A pharmaceutical composition comprising the fusion protein according to any one of claims 1-36 and a pharmaceutically acceptable carrier.

41. A method for treating tumor or cancer, comprising the step of administering the fusion protein according to any one of claims 1-36 or the pharmaceutical composition according to claim 40 to a subject in need of a treatment or relief.

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42. Use of the fusion protein according to any one of claims 1-36, the polynucleotide according to claim 37, the nucleic acid construct or the vector according to claim 38, or the pharmaceutical composition according to claim 40 in the manufacture of a medicament for the treatment or prevention of a tumor or cancer.

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43. The use according to claim 42, wherein the tumor or cancer includes a solid tumor or a non-solid tumor.

25 44. A diagnostic kit comprising the fusion protein according to any one of claims 1-36.

45. A method of manufacturing the fusion protein according to any one of claims 1-36, comprising culturing the host cell according to claim 39 under a condition allowing the expression of the nucleic acid construct according to claim 38, and recovering the produced fusion protein from the culture.