

BIFUNCTIONAL PROTEINS COMBINING CHECKPOINT BLOCKADE FOR TARGETED THERAPY

CROSS - REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to US Provisional Application Serial Number 62/636,825, filed February 28, 2018, which is herein incorporated by reference in its entirety.

BACKGROUND

Field of Invention

[0002] The present invention relates to an antibody. More particularly, the present invention relates to the antibody for cancer therapy.

Description of Related Art

[0003] The two major types of lymphocytes in humans are T (thymus-derived) and B (bone marrow derived). These cells are derived from hematopoietic stem cells in the bone marrow and fetal liver that have committed to the lymphoid development pathway. The progeny of these stem cells follow divergent pathways to mature into either B or T lymphocytes. Human B-lymphocyte development takes place entirely within the bone marrow. T cells, on the other hand, develop from immature precursors that leave the marrow and travel through the bloodstream to the thymus, where they proliferate and differentiate into mature T lymphocytes.

[0004] T cells

[0005] T-cells are the most abundant (about 75% of blood lymphocytes) and potent immune killer cells. The role of effector T-cells in the anti-tumor immune response is strongly supported by in vitro studies and the observation

that a high infiltration of CD8⁺ T cells in several types of tumors correlates with a favorable clinical prognostic (Fridman et al., 2012). The activation of effector naive T-cells requires at least three complementary signals: (i) TCR-CD3/Ag-MHC interaction with the assistance of co-receptors (CD4 or CD8); (ii) binding of co-stimulatory molecules such as CD80 or CD86 to CD28, CD40/CD40L; and (iii) accessory molecules such as cytokines.

[0006] Co-stimulation or the provision of two distinct signals to T-cells is a widely accepted model of lymphocyte activation of resting T lymphocytes by antigen-presenting cells (APCs) (Lafferty and Cunningham, 1975). This model further provides for the discrimination of self from non-self and immune tolerance (Bretscher and Cohn, 1970; Bretscher, 1999; Jenkins and Schwartz, 1987). The primary signal, or antigen specific signal, is transduced through the T-cell receptor (TCR) following recognition of foreign antigen peptide presented in the context of the major histocompatibility-complex (MHC). The second or co-stimulatory signal is delivered to T-cells by co-stimulatory molecules expressed on antigen-presenting cells (APCs), and induce T-cells to promote clonal expansion, cytokine secretion and effector function (Lenschow et al., 1996). In the absence of costimulation, T-cells can become refractory to antigen stimulation, do not mount an effective immune response, and further may result in exhaustion or tolerance to foreign antigens.

[0007] Immune checkpoint protein: PD-L1

[0008] Immune checkpoints refer to a group of inhibitory and stimulatory pathways mostly initiated by ligand-receptor interaction hardwiring the immune system, specifically T-cell mediated immunity, to maintain self-tolerance and

modulate the duration and amplitude of physiological responses in peripheral tissues in order to minimize collateral tissue damages normally (Pardoll, 2012). Tumor cells co-opt certain checkpoint pathways as a major mechanism of immune resistance. For example, programmed cell death protein 1 ligand, PD-L1, is commonly up-regulated on tumor cell surface of human cancers. The interaction of PD-L1 with its receptor, PD-1, expressed on tumor infiltrated lymphocytes (TILs), specifically on T cells, inhibits local T cell-mediated response to escape the immune surveillance (Liang et al., 2006; Sznol and Chen, 2013). Thus, the inhibition of immunosuppressive signals on cancer cells, or direct agonistic stimulation of T cells, results in and/or induces a strong sustained anti-tumor immune response. Recent clinical studies strongly suggested blockage of immune checkpoint proteins via antibody or modulated by soluble ligands or receptors are the most promising approaches to activating therapeutic antitumor immunity (Topalian et al., 2014). Currently, anti-PD-1 and anti-CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) antibodies have been approved by FDA to treat diseases such as melanomas.

[0009] Angiogenesis and VEGF inhibition domain (VID)

[0010] Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is a normal and vital process involved in fetal development and tissue repair. The process is highly regulated by both angiogenic and anti-angiogenic factors, and it involves endothelial cell migration and proliferation, vessel maturation and remodeling, and degradation of the extracellular matrix. Although it is an important process in normal growth and development, angiogenesis also plays a key role in tumor growth. Tumors require a vascular

supply to grow and can achieve this via the expression of pro-angiogenic growth factors, including members of the vascular endothelial growth factor (VEGF) family of ligands (Hicklin and Ellis, 2005). When VEGF and other endothelial growth factors bind to their receptors on endothelial cells, signals within these cells are initiated that promote the growth and survival of new blood vessels. Blocking VEGF activity with VEGF specific antibody (Avastin), soluble VEGF receptors (aflibercept), or inhibitors of VEGF tyrosine kinase activity (sunitinib) are strategies that have been used to treat tumor or angiogenic-type disorders, such as AMD.

[0011] Bi-specific/bi-functional antibody

[0012] The idea of using bispecific antibodies to efficiently retarget effector immune cells toward tumor cells emerged in the 1980s (Karpovsky et al., 1984; Perez et al., 1985; Staerz et al., 1985). Bispecific scaffolds are generally classified in two major groups with different pharmacokinetic properties, based on the absence or presence of an Fc fragment, IgG-like molecules and small recombinant bispecific formats, most of them deriving from single chain variable fragment (scFv). Through their compact size, antibody fragments usually penetrate tumors more efficiently than IgG-like molecules but this benefit is mitigated by a short serum half-life (few hours) limiting their overall tumor uptake and residence time (Goldenberg et al., 2007). By contrast, the presence of an Fc fragment, which binds to the neonatal Fc receptors, provides a long serum half-life (>10 days) to the IgG-like formats, favoring tumor uptake and retention, but limits tumor penetration.

[0013] Recent studies have highlighted the therapeutic efficacy of immunotherapy, a class of cancer treatments that utilize the patient's own immune system to destroy cancerous cells. Within a tumor the presence of a family of negative regulatory molecules, collectively known as "checkpoint inhibitors," can inhibit T cell function to suppress anti-tumor immunity. Checkpoint inhibitors, such as CTLA-4 and PD-1, attenuate T cell proliferation and cytokine production. Targeted blockade of CTLA-4 or PD-1 with antagonist monoclonal antibodies (mAbs) releases the "brakes" on T cells to boost anti-tumor immunity. Also, recent studies have reported the associations between PD-L1 or PD-L2/PD-1 pathways and pro-angiogenic genes including hypoxia inducible factors (HIFs) and vascular endothelial growth factor (VEGF) in several malignancies, such as classical Hodgkin lymphoma (cHL) (Koh et al., 2017) and glioma (Xue et al., 2017). Koh et al. confirmed the positive correlations between PD-L1, VEGF, or MVD. Their findings provided evidence supporting new therapeutic approaches including combinations of anti-PD-L1/PD-1 and anti-VEGF therapy in addition to the current standard regimen for cHL (Koh et al., 2017). VEGF also evidenced its ability to disrupt a key step in the cancer immunity cycle: T-cell infiltration into the tumor (Kim and Chen, 2016; Terme et al., 2012). Targeting VEGF may help restore part of the cancer immunity cycle by increasing T-cell infiltration into the tumor microenvironment (Hughes et al., 2016; Terme et al., 2012; Wallin et al., 2016). VEGF pathway inhibition may lead to increased expression of cell adhesion molecules on endothelial cells, increasing intratumoral T cells to create an immune inflamed tumor microenvironment.

SUMMARY

[0014] The present disclosure designed to investigate the bispecific antibody with immunomodulatory aiming and angiogenesis inhibition for the treatment of patient with cancers, such as prostate cancer, lung cancer, NSCLC, melanoma, lymphoma, breast cancer, head and neck cancer, RCC, or ovarian cancer were examined.

[0015] The present disclosure provides a bispecific antibody or antigen-binding portion thereof comprising at least one of polypeptide chain, wherein the polypeptide chain comprising: a binding domain binding cell surface protein; and a vascular endothelial growth factor (VEGF) inhibiting domain.

[0016] In one embodiment, the cell surface protein comprising programmed cell death protein 1 ligand (PD-L1), programmed cell death protein 1 (PD-1), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG3), B- and T-lymphocyte attenuator (BTLA), OX40 (cluster of differentiation 134, CD134), CD27, CD28, tumor necrosis factor receptor superfamily member 9 (TNFRSF9 or CD137), inducible T cell costimulator (ICOS or CD278), CD40, or a combination thereof.

[0017] In one embodiment, the binding domain binds the PD-L1, and the binding domain comprises: a heavy chain variable domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of SEQ ID NOs. 4 and 6; and a light chain variable domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the

group consisting of amino acid 1-111 of SEQ ID NO. 3 and 1-110 of SEQ ID NO. 5.

[0018] In one embodiment, the VEGF inhibiting domain is from human VEGF receptor 1 (VEGFR-1) or human VEGF receptor 2 (VEGFR-2).

[0019] In one embodiment, the VEGF inhibiting domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of SEQ ID NOs. 1, 2, 9, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, and a combination thereof.

[0020] In one embodiment, the bispecific antibody or antigen-binding portion thereof further comprises: a Fc domain; and a Fab fragment connected to the N-terminus of the Fc domain, and the Fab fragment comprising the binding domain, wherein the VEGF inhibiting domain is connected to the C-terminus of the Fc domain.

[0021] In one embodiment, the bispecific antibody or antigen-binding portion thereof further comprises a linker between the Fc domain and the VEGF inhibiting domain.

[0022] In one embodiment, the bispecific antibody comprises an amino acid sequence set forth in SEQ ID NO. 12 or 13.

[0023] In one embodiment, the bispecific antibody or antigen-binding portion thereof comprises one pairs of polypeptide chains.

[0024] In one embodiment, the bispecific antibody is an IgG, IgE, IgM, IgD, IgA, or IgY antibody.

[0025] In one embodiment, the bispecific antibody is an IgG antibody.

[0026] In one embodiment, the IgG antibody is an IgG1, IgG2, IgG3, or IgG4 antibody.

[0027] In one embodiment, the IgG1 antibody is a reduction of antibody-dependent cell-mediated cytotoxicity of IgG1 antibody.

[0028] In one embodiment, the bispecific antibody is a human antibody.

[0029] The present disclosure also provides a pharmaceutical composition, comprising: a bispecific antibody or an antigen-binding fragment thereof as above mentioned, and at least one pharmaceutically acceptable carrier.

[0030] The present disclosure also provides an antibody-drug conjugate comprising: a therapeutic agent; and a bispecific antibody or an antigen-binding fragment binding PD-L1 and/or a VEGF inhibiting domain, wherein the therapeutic agent is covalently conjugated to the antibody or the antigen-binding fragment by a linker.

[0031] In one embodiment, the bispecific antibody or an antigen-binding fragment is selected from the bispecific antibody or an antigen-binding fragment as above mentioned.

[0032] The present disclosure also provides a method of treating cancer, the method comprising administering to the subject in need thereof an effective amount of the bi-specific antibody or antigen-binding portion as above mentioned.

[0033] In one embodiment, the cancer is selected from the group consisting of prostate cancer, lung cancer, Non-Small Cell Lung Cancer (NSCLC),

melanoma, lymphoma, breast cancer, head and neck cancer, renal cell carcinoma (RCC), and ovarian cancer.

[0034] In one embodiment, the effective amount is from 0.001 $\mu\text{g/kg}$ to 250 mg/kg.

[0035] The present disclosure also provides a nucleic acid molecule encoding the antibody or the antigen-binding portion as above mentioned.

[0036] The present disclosure also provides a method for cancer diagnosis in a subject, comprising: (a) obtaining a body fluid sample or a cell sample from a subject; (b) contacting the sample with one or more antibodies that can detect expression of a panel of cancer markers selected from the group consisting PD-L1 and VEGF; (c) assaying the binding of the one or more antibodies to the cell or the sample; and (d) assessing the cancer status of the subject in an assay by measuring and comparing the level of antibody binding with a normal control to determine the presence of the cancer in the subject.

[0037] The present disclosure also provides a method for assessing the risk of a subject suffering from cancer or a method for cancer screening in a subject, comprising: (a) obtaining a body fluid sample or a cell sample from a subject; (b) contacting the sample with one or more antibodies that can detect expression of a panel of cancer markers selected from the group consisting PD-L1 and VEGF; (c) assaying the binding of the one or more antibodies to the cell or the sample; and (d) assessing the cancer status of the subject in an assay by measuring and comparing the level of antibody binding with a normal control to determine whether the subject having the risk of suffering from cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038] The invention can be more fully understood by reading the following detailed description of the embodiment, with reference made to the accompanying drawings as follows:

[0039] Fig. 1 shows immune checkpoints modulating T-cell mediated immunity. Antibody either agonistic or antagonistic against the checkpoints, such as anti-ICOS, anti-CD28, anti-OX40, and anti-CD27, or anti-PD-1, anti-CTLA4, anti-LAG3, anti-BTLA, could be used to construct the bi-functional fusion protein depending on applications.

[0040] Figs. 2A and 2B show the screening of phage clones by direct ELISA for recombinant PD-L1.

[0041] Fig. 3 shows purified antibody leads specific for PD-L1 by SDS-PAGE with non-reducing or reducing reagent to reveal the integrity and purity.

[0042] Fig. 4 shows examples of the direct ligand binding activity of purified anti-immune check point proteins and anti-PD-L1 antibody leads against PD-L1. Ligand pre-coated wells were first incubated with various concentrations of antibody leads as indicated. The bound proteins were then detected with HRP conjugated goat anti-human IgG Fab specific antibody and OD₄₅₀ readings were plotted.

[0043] Fig. 5 shows the flow analysis using PD-L1 expression 293 cells. PD-L1 expression HEK293 cells were first incubated with purified antibody leads, and the bound antibodies were detected with Alexa-488 conjugated goat anti-human IgG (H+L) followed by fluorescence-activated cell sorter (FACS) analysis.

[0044] Fig. 6 shows the blockage of PD-1/PD-L1 interaction with purified anti-PD-L1 antibodies. Purified antibodies as indicated were applied with biotinylated-PD-L1-Fc and pre-coated PD-1/His in 96-well plate to evaluate the inhibition activity of PD-1/PD-L1 interaction. The binding recombinant PD-L1-Fc on recombinant PD-1 was detected by streptavidin-Alexa-488 and analysis by ELISA.

[0045] Figs. 7A and 7B show anti-PD-L1 antibody leads with 1 or 10 µg/mL stimulates IL-2 and/or IFN-γ production in a mixed lymphocyte reaction (MLR) assay after 3 days (Fig. 7A) or 5 days (Fig. 7B) antibody treatment.

[0046] Fig. 8 shows the structure of an antibody heavy chain Fc fused with VEGF inhibition domain (VID) from VEGF receptor.

[0047] Fig. 9 shows examples of PAGE-gel analysis of anti-immune check point antibodies-VID bispecific antibodies. Purified fusion proteins, anti-PD-L1-VID bispecific antibodies were shown to have a molecular weight about 220 kDa (non-reducing), and heavy chain fusion has about 85 kDa and light chain is about 25 kDa (reduced) in both antibody fusions. M is marker, Lane 1 is non-reduced anti-PD-L1-VID/eIgG1, Lane 2 is reduced anti-PD-L1-VID/eIgG1, Lane 3 is non-reduced anti-PD-L1-VID/IgG4, and Lane 2 is reduced anti-PD-L1-VID/IgG4. Each of lanes loads 3 µg.

[0048] Figs. 10A and 10B show the purity of purified anti-PD-L1-VID/IgG4 (Fig. 10A) and anti-PD-L1-VID/eIgG1 (Fig. 10B) bispecific antibodies from HEK293 cells by SEC-HPLC analysis.

[0049] Figs. 11A and 11B show examples of the PD-L1 (Fig. 11A) and VEGF₁₆₅ (Fig. 11B) binding activity of purified anti-PD-L1-VID bispecific antibodies by

direct ELISA. Ligand pre-coated wells were first incubated with various concentrations of test samples as indicated. The bound Abs were then detected with HRP conjugated goat anti-human IgG Fc or F(ab')₂ specific antibody and OD₄₅₀ readings were plotted.

[0050] Fig. 12 shows the inhibition of VEGF₁₆₅-stimulated HUVEC proliferation with by purified anti-PD-L1-VID bispecific antibodies. VEGF₁₆₅ were pre-incubated with test samples as indicated for one day and then applied for HUVEC cells to monitor VEGF₁₆₅-stimulated HUVEC cell proliferation. After 3 days culture, the cell proliferation was determined by MTS reagent (Promega). The absorbance was plotted against the Abs concentration of the test sample, and the concentration at which the cell proliferation was inhibited by 50% (IC₅₀) was determined.

[0051] Figs. 13A and 13B show bispecific antibody synergic stimulates T-cell activation for IL-2 (Fig. 13A) and IFN- γ (Fig. 13B) production in a mixed lymphocyte reaction (MLR) assay after 3 or 5 days with isotype IgG, reference antibody (MPDL3280A) or anti-PD-L1-VID/elgG1 bispecific antibody treatment.

[0052] Figs. 14A, 14B and 14C show the *in vitro* serum stability of anti-PD-L1-VID/elgG1 bispecific antibody from different species (Fig 14A: Human serum; Fig 14B: Mouse serum; Fig.14C: Cyno serum). Purified antibody was incubated in serum (15 μ g/mL) from different species as indicated at 37°C for 1, 3, 5, 7, and 14 days. After incubation, the collected samples were applied for sandwich ELISA assay to determine the relative binding activity for PD-L1 and VEGF₁₆₅. The half-life were plotted based on the concentration of bispecific antibody in serum.

[0053] Fig. 15A is a graph showing the effect of anti-PD-L1-VID/elgG1 bispecific antibody treatment and monoclonal antibody treatment on the growth of PC-3 tumor in Fox Chase SCID®Beige mice. Fig. 15B shows that the tumor size bispecific antibody treatment is significant smaller than isotype or reference antibody treatment on day 35 post-inoculation.

[0054] Figs. 16A, 16B and 16C show anti-PD-L1-VID Abs sustains its antigen binding specificity as compared with anti-PD-L1 alone in IFN- γ stimulated A549 (Fig. 16A), NCI-H292 (Fig. 16B), or stable PD-L1 expression 293 cell (Fig. 16C); MFI: mean fluorescence intensity.

DETAILED DESCRIPTION

[0055] Reference will now be made in detail to the present embodiments of the invention, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers are used in the drawings and the description to refer to the same or like parts.

[0056] The present invention describes the expression, purification and characterization of bi-functional proteins with isolated functional VEGF inhibition domain to the C-terminal of Fc domain of anti-immune checkpoint protein antibodies. These proteins interact with its corresponding check point target shall transmit the inhibitory signal to modulate T-cell involved immunity and neutralized the VEGF-induced angiogenesis at the same time. The components of Fc fusion proteins in present invention are of all human origins, and thus are expected to be non-immunogenic and can be used as therapeutics in human.

[0057] Bispecific molecules such as bispecific antibodies (BsAbs) provide a means of simultaneously targeting multiple epitopes on the same molecular

target or different targets with a single therapeutic agent. As cancer therapeutics, they have the potential to confer novel or more potent activities, lower the cost of goods and facilitate the development of new therapeutic regimens in contrast to a mixture of two mAbs (Chames and Baty, 2009; Hollander, 2009; Thakur and Lum, 2010). Recently, catumaxomab, a trifunctional bispecific antibody targeting human epithelial cell adhesion molecule (EpCAM) and CD3 has shown a clear clinical benefit in patients with peritoneal carcinomatosis of epithelial cancers (Heiss et al., 2010), and a bispecific T-cell engaging (BiTE) antibody with dual specificity for CD19 and CD3 has also demonstrated encouraging clinical activity in patients with CD19 expressing hematological malignancies (Bargou et al., 2008). Despite strong interest in the development of bispecific molecules as cancer therapeutics, technical challenges in the production of stable and active bispecific molecules have in the past hindered the clinical evaluation of most bispecific formats. Many engineered antibody formats, including an IgG-like bispecific antibody have compromised stability or solubility (Bargou et al., 2008; Demarest and Glaser, 2008; Lu et al., 2005). Furthermore, several strategies have been taken to increase the product quality and in vivo stability of bispecific molecules, including PEGylation, conjugation with human serum albumin and Fc engineering (Muller et al., 2007; Ridgway et al., 1996). Bispecific antibodies of the general form described above have the advantage that the nucleotide sequence encoding the two V-domains, single linker or one spacer can be incorporated into a suitable host expression organism under the control of a single promoter. This increases the flexibility with which these constructs can be designed as well as the degree of experimenter control during their production.

In addition, the Fc of IgG is a very another attractive scaffold for designing novel therapeutics because it contains all antibody functions except the binding ability. Fc engineering is important for improving the effectiveness of the bispecific antibodies. Therefore, the IgG-based conformation is using in present invention for two independent target on immune cells or pro-angiogenic proteins in cancer therapy.

[0058] Targeting immune-check point proteins are promising approaches to activate antitumor immunity. Anti-check point proteins, such as PD-1, PD-L1, CTLA-4, LAG3, etc., are currently evaluated clinically (Fig. 1). Preliminary data with blockers of immune checkpoint proteins have been shown to be able to enhance antitumor immunity with the potential to produce durable clinical responses. However, despite the remarkable clinical efficacy of these agents in a number of malignancies, it has become clear that they are not sufficiently active for many patients. Numerous additional immunomodulatory pathways as well as inhibitory factors expressed or secreted by myeloid and stromal cells in the tumor microenvironment are potential targets for synergizing with immune checkpoint blockade. Therefore, combining anticancer or bispecific antibody therapies has been essential to achieve complete remission and cures for patients with cancer. Meanwhile, targeting VEGF already know to be reduced the angiogenesis by tumor (Hicklin and Ellis, 2005) and may help restore part of the cancer immunity cycle by increasing T-cell infiltration into the tumor microenvironment (Hughes et al., 2016; Terme et al., 2012; Wallin et al., 2016).

[0059] The extracellular ligand binding domain (SEQ ID NOs. 1 and 2) of a human VEGF receptor is capable of binding to a VEGF ligand, and comprises

one or more of Ig-like domains D1-D7 (Table 1) of one or more VEGF receptors. Preferably, the extracellular ligand binding domain of the VEGF receptor comprises an Ig-like domain D2 of a first VEGF receptor and an Ig-like domain D3 of a second VEGF receptor, wherein the first and second VEGF receptors are the same or different VEGF receptors. In present invention, VEGF inhibition domain (VID), the extracellular ligand binding domain of the VEGF receptor comprises an Ig-like domain D2 of a VEGFR1 and an Ig-like domain D3 of a VEGFR2 to block the VEGF and reduce the angiogenesis.

Table 1. The amino acid sequence of Ig-like domains of human VEGF receptors

Name	SEQ ID NO.	Sequence
D1 of human VEGF receptor 1	14	PELSLKGTQHIMQAGQTLHLQCRGEAAHK WSLPPEMVSKESERLSITKSA
D2 of human VEGF receptor 1	15	GRELVIPCRVTSPNITVTLKKFPLDTLIPDG KRIIWDSRKGFIISNATYKEIGLLTCEATVN GH
D3 of human VEGF receptor 1	16	IDVQISTPRPVKLLRGHTLVLNCTATTPLNT RVQMTWSYPDEKNKRASVRRRIDQSNH ANIFYSVLTIDKMQNKDKGLYTCRVRSGPS FKSVNTSVH
D4 of human VEGF receptor 1	17	TVKHRKQQVLETVAGKRSYRLSMKVKAFF SPEVWVLKDGLPATEKSARYLTRGYSIIK DVTEEDAGNYTILLSIKQSNVFNLTAT
D5 of human VEGF receptor 1	18	PQIYEKAVSSFPDPALYPLGSRQILTCTAY GIPQPTIKWFWHPCNNHSEARCDFCN NEESFILDADSNMGNRIESITQRMIIIEGKN KMASTLVVADSRISGIYICIASNKVGTVGRN ISFYIT
D6 of human VEGF receptor 1	19	PNGFHVNLEKMPTEGEDLKLSTVNKFLY RDVTWILLRTVNNRTMHYSISKQKMAITKE HSITLNLTIMNVSLQDSGTYACRARNVYTG EELQ
D7 of human VEGF receptor 1	20	PYLLRNLSDHTVAISSSTTLDCANGVPEP QITWFKNNHKIQQEPGIILGPGSSTLFIERV TEEDEGVYHCKATNQKGSVESSAYLT
D1 of human VEGF receptor 2	21	NTTLQITCRGQRDLWLWPNNQSGSEQR VEVTECSDFLCKTLTIPKVIGNDTGAYKC FYRETDL
D2 of human VEGF receptor 2	22	NKNKTVVIPCLGSISNLNVSLCARYPEKRF VPDGNRISWDSKKGFTIPSYMISYAGMVF

		CEAKINDE
D3 of human VEGF receptor 2	23	YDVVLSPSHGIELSVGEKLVNCTARTELN VGIDFNWEYPSSKHQHKLVNRDLKTQSG SEMKKFLSTLTIDGVTRSDQGLYTCAASS GLMTKKNST
D4 of human VEGF receptor 2	24	FVAFGSGMESLVEATVGERVRIPAKYLG PPPEIKWYKNGIPLESNHTIKAGHVLT SERDTGNYTVILTNPISKEKQSHVVS
D5 of human VEGF receptor 2	25	PQIGEKSLISPVDSYQYGTTQTLTCTVYAIP PPHHIHWYWQLEEECANEPSQAVSVTNP YPCEEWRSVEDFQGGNKIEVNKNQFALIE GKNKTVSTLVIQAANVSALYKCEAVNKG RGERVISFHVT
D6 of human VEGF receptor 2	26	PEITLQPDMPQTEQESVSLWCTADRSTFE NLTWYKLGPPQPLPIHVGELPTPVCKNLDTL WKLNATMFSNSTNDILIMELKNASLQDQG DYVCLAQDRKTKKRHCVRQLT
D7 of human VEGF receptor 2	27	PTITGNLENQTTSIGESIEVSCTASGNPPP QIMWFKDNETLVEDSGIVLKDGNRNLTIRR VRKEDEGLYTCCQACSVLGCCKVEAFFI

[0060] In some embodiments, the VEGF inhibiting domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of SEQ ID NOs. 1, 2, 9, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, and a combination thereof. In some examples, the VEGF inhibiting domain comprises an amino acid sequence of at least about 85%, 90%, or 95% sequence homology to the amino acid sequence as above mentioned.

[0061] The present invention describes the construction, expression and characterization of anti-immune checkpoint protein antibody Fc fused with VEGF inhibition domain (VID) from human VEGF receptor. The N-terminally positioned anti-PD-L1 antibody in fusion constructs shall allow expanding the power of fusion proteins beyond immune potentiating agent if the fusion counterpart is replaced by other immune checkpoints, such as anti-CTLA-4,

CD3, OX40 antibodies or cell surface targeting molecule such as anti-EGFR, anti-HER2, anti-CD40 antibodies for example.

[0062] The present disclosure provides bispecific antibody or antigen-binding portion thereof, comprising a binding domain binding cell surface protein; and a vascular endothelial growth factor (VEGF) inhibiting domain. In some embodiments, the binding domain binding the PD-L1 comprises: a heavy chain variable domain and a light chain variable domain. The heavy chain variable domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of SEQ ID NOs. 4 and 6. In some examples, the heavy chain variable region comprises an amino acid sequence of at least about 85%, 90%, or 95% sequence homology to the amino acid sequence as above mentioned. The light chain variable domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of amino acid 1-111 of SEQ ID NO. 3 and 1-110 of SEQ ID NO. 5. In some examples, the light chain variable region comprises an amino acid sequence of at least about 85%, 90%, or 95% sequence homology to the amino acid sequence as above mentioned.

[0063] Antibody generation from OmniMab library

[0064] For the generation of therapeutic antibodies against PD-L1, selections with the OmniMab phagemid library were carried out. The phagemid library is generated by AP Biosciences Inc. (APBio Inc.) from a collection of over hundred health donors B cells. Phages for the 1st round of pannings were prepared by Hyperphage (M13K07ΔpIII, Progen, Heidelberg, Germany). Solid phase

panning and cell panning against PD-L1 were applied for PD-L1 specific binder selection and isolation from OmniMab library. Solid phase panning was performed using recombinant human PD-L1-Fc (APBio Inc.) in the first round selection and then HEK293 cells expressed PD-L1 were used for additional two rounds enrichment. After three rounds selection, the specific PD-L1 binders were screened and isolated by direct ELISA with corresponding recombinant protein (Figs. 2A and 2B). Pre-coated PD-L1-Fc recombinant proteins were blotted with supernatant containing rescued phages for 1 hour and washed with PBS containing 0.1 % Tween-20 for three times. Bound phages were detected by HRP conjugated anti-M13 antibody (Roche) and TMB substrate was used for signal development. The OD₄₅₀ readings were recorded. The positive binders were isolated and sent for sequencing to confirm the sequence and diversity of heavy chain and light chain. The variable region of heavy chain and light chain specific to PD-L1 were described from the SEQ ID NO. 3 to SEQ ID NO. 6: SEQ ID NO. 3 is the light chain of PD-L1 clone 6, SEQ ID NO. 4 is the variable region of heavy chain of PD-L1 clone 6, SEQ ID NO. 5 is the light chain of PD-L1 clone 32, SEQ ID NO. 6 is the variable region of heavy chain of PD-L1 clone 32. As shown in the Figs. 2A and 2B, several clones were isolated and known to be recognized specifically for corresponding antigen as comparing with negative control.

[0065] Subcloning and expression/purification of selected PD-L1 specific binder as IgG format

[0066] To facilitate the quick screening of specific binder with functionality in T cell activation, the heavy chains and light chains of positive binders against

PD-L1 by ELISA were then amplified, digested and sub-clone into APBio specialized IgG expression vector carrying IgG4 constant region (SEQ ID NO. 7). After sequence validation, the plasmids were then prepared and transfected into HEK293 cells for antibody expression with 293fectin transfection reagent (Invitrogen). After 4 days culture, the antibody secreted into serum-free medium is affinity purified from culture supernatant by Protein G chromatography. Purified antibody is then concentrated, followed by dialysis in PBS buffer. The final concentration of dialyzed protein is determined by NanoDrop2000 spectrophotometer and the purity and integrity are determined by SDS-PAGE with or without reducing reagent as shown in the Fig 3. The integrity of various purified antibody leads is normal in the HEK293 cells as well as reference antibody, MPDL3280A.

[0067] Binding activity determination for PD-L1 specific IgG leads by direct ELISA

[0068] Purified antibody leads against PD-L1 (anti-PD-L1 antibody leads) were then applied for ELISA binding characterization on human PD-L1-Fc in a direct coated setup. Fig. 4 showed the ELISA binding result for anti-PD-L1 antibodies. For PD-L1 specific antibodies, most leads showed a similar or better binding activity with reference antibody (Ref Ab, MPDL3280A, Roche).

[0069] Purified human PD-L1 IgG1 Fc chimera (PD-L1-Fc, APBio) was dialyzed in Phosphate Buffered Saline (PBS), adjusted to 1 mg/mL and then diluted with PBS to a final concentration of 1 µg/mL. Nunc-Immuno Maxisorp 96 well plates were coated with 0.1 mL per well of recombinant PD-L1-Fc chimera leaving empty wells for nonspecific binding controls and incubated at 4°C

overnight. The PD-L1-Fc chimera solution was removed and the plates were washed three times with 0.4 mL wash buffer (0.1 % Tween-20 in PBS). 0.4 mL blocking buffer (5% low-fat milk powder in PBS) was added to all wells and incubated at room temperature for 1 hour with mixing. The blocking buffer was removed and plates washed three times with 0.4 mL wash buffer. Serial dilutions of the PD-L1 test antibodies were prepared in PBS and 0.1 mL diluted Ab was added per well. Plates were incubated 1 hour at room temperature. Antibody solution was removed and the plates washed three time with 0.4 mL wash buffer per well. Horseradish peroxidase labeled goat anti-human IgG, F(ab')₂ specific F(ab')₂ antibody (Jackson ImmunoResearch #109-036-097) was diluted 1:2000 with PBS and added 0.1 mL per well. The plates were incubated 1 hour at room temperature and washed with 0.4 mL per well wash buffer. 0.1 mL TMB reagent (Invitrogen) was added and incubated for 1 to 5 minutes at room temperature. The reaction was stopped by adding 0.05 mL 1N HCl and absorbance was read at 450 nm on a Bio-Tek Spectra. Calculated EC50 for anti-PD-L1 antibody leads to PD-L1 showed most leads possess good binding activity as well as MPDL3280A (Ref Ab) by direct ELISA (Fig. 4).

[0070] Binding activity determination for PD-L1 specific IgG leads by FACS

[0071] Purified antibody leads (anti-PD-L1 antibody leads) were also applied for flow cytometry to determine and compare the binding activity with PD-L1 expressed HEK293 cells. Fig. 5 show the binding activity of corresponding antibody leads as indicated by FACS with stable expressed PD-L1 HEK293 cells.

[0072] FACS analysis of PD-L1 stable expression 293 cells stained with anti-PD-L1 antibody leads to examine the PD-L1 binding activity, stable expression cells were incubated with 1 µg/mL purified anti-PD-L1 antibody leads, reference antibody (Ref Ab MPDL3280A) or with isotype antibody as negative control on ice for 1 hr. The cells were washed three times with 1×PBS and then incubated with Alexa-488-conjugated goat anti-human IgG (H+L) (Invitrogen Inc.) on ice for additional 1 hr. After staining, the cells were washed three times with 1×PBS, resuspended in 1×PBS/2%FBS before analyzed by FACS Calibur (BD Biosciences, Inc.) and FlowJo (TreeStar, LLC). As shown in the Fig. 5, most anti-PD-L1 antibody leads possess a good binding activity as well as reference antibody. This indicated the phage clones selected from the OmniMab library indeed recognize the native PD-L1 in the cells.

[0073] Ligand competition binding (ELISA Assay)

[0074] Antibody leads were showed the binding selectivity and affinity assay used to evaluate the anti-PD-L1 antibody leads of present invention for their ability to block binding of PD-L1 to PD-1.

[0075] Antibodies were tested for their ability to block the binding of the human PD-L1-Fc chimera (PD-L1-Fc) to recombinant human PD-1/His (hPD-1/His) by ELISA. Purified recombinant hPD-1/His (APBio) was dialyzed to 1 mg/mL in PBS and then diluted to 5 µg/mL in PBS. Nunc Maxisorp 96 well pate was coated with 250 ng hPD-1/His per well in PBS overnight. The hPD-1/His solution was removed and the plates were washed three times with 0.4 mL wash buffer (0.1 % Tween-20 in PBS). 0.4 mL blocking buffer (5% low-fat milk

powder in PBS) was added to all wells and incubated at room temperature for 1 hour with mixing. During the blocking step the antibody stocks were diluted in a range from 200 nM to 0 nM in PBS with 2 folds serial dilution. Purified recombinant PD-L1-Fc chimera was diluted to 4 µg/mL in PBS. The PD-1/His coated plates were washed three times with 0.2 mL wash buffer (0.1% Tween 20 in PBS). 60 µL antibody dilutions (anti-PD-L1 antibody leads or Ref Ab MPDL3280A) were added alone with 60 µL PD-L1-Fc chimera and incubated at room temperature for 1 hour. Plates were washed as described previously. Horseradish peroxidase human IgG, Fc specific antibody was diluted 1:2000 in PBS, 100 µL of the resulting solution added to the wells of the washed plated, and incubated at room temperature for 1 hour. Plates were washed as previously described, 100 µL TMB substrate solution was added to each well and incubated for 10 minutes. The reaction was stopped with 50 µL 1N HCl and absorbance at 450 nm read using Bio-Tek reader and showed in Fig. 6. Partial antibody leads are showed to inhibit the interaction between PD-1-PD-L1 by competition ELISA. Most antibody leads revealed a similar blocking activity as comparing with reference antibody (Ref Ab MPDL3280A).

[0076] Enhanced stimulation of T cell activation by inhibition of PD-1:PD-L1 ligand interaction for anti-PD-L1 antibody

[0077] The PD-1 signaling pathway inhibits moderate TCR/CD28 costimulatory signals, with cytokine production being reduced first without a decrease in T cell proliferation. As the TCR/CD28 costimulatory signals weaken, the PD-1 pathway dominates, with a great reduction in cytokine production accompanied by a reduction in proliferation. Accordingly, in order to confirm that the

inhibition of the PD-1 via inhibition of the interaction with PD-L1, human antibodies of the invention enhances T cell activation, mixed lymphocyte reactions (MLRs) are performed.

[0078] Monocytes from human whole blood were enriched by RosetteSep™ Human Monocyte Enrichment Cocktail (Cat. No.15068) and cultured in differentiation medium, RPMI 1640 with 10% FBS, 100 ng/mL (1000 U/mL) GM-CSF, 100 ng/mL (500 U/mL) for 6 days. The differentiate dendritic cells (DC) from monocyte were checked by DC-SIGN-PE, anti-CD14 conjugated with FITC Ab, anti-CD83 conjugated with PE Ab, or anti-CD80 conjugated with FITC Ab to validate the differentiation and used to be APCs in MLRs.

[0079] Allogenic CD4⁺ T cells from human whole blood were isolated by RosetteSep™ Human CD4⁺ T Cell Enrichment Cocktail (Cat. NO. 15062). The purity of CD4⁺ T cells were checked with anti-CD4 conjugated APC Ab to make sure the purity is above 95% and then labeled with 1 μ M CFSE (CellTrace™ CFSE cell proliferation kit, Life technologies, Cat. NO. C34554) for T cells proliferation assay. Labeled CD4⁺ T cells were used to co-culture with immature DC with different antibody leads as indicated for 3 and 5 days to see whether the antibody leads could restore the T cell activation through blocking the interaction between PD-1 and PD-L1. After 3 and 5 days incubation, the supernatant were collected for cytokine, such as IL-2 and IFN- γ quantitation by ELISA. The addition of anti-PD-L1 antibody leads (clones 6, 32, 28, 51, 64, 27, and 37) to cultures of immature dendritic cells plus allogeneic T cells is predicted to result in an increase in T cell proliferation and cytokine production, as compared to isotype IgG (iso#1, #2) treated cultures and showed

in the Fig. 7A and 7B. The IL-2 and IFN- γ production increase significantly in the MLRs as comparing with isotype antibody treatment after 3 days (Fig. 7A) or 5 days (Fig. 7B) antibody treatment, especially for anti-PD-L1 antibody clone 6. The cytokine increment is still obviously after 5 days antibody treatment and similar to reference antibody (ref), MPDL3280A. This indicated the anti-PD-L1 antibody clone 6 should be one of the potential leads for bispecific antibody composite.

[0080] Construction, Expression and Purification of Anti-PD-L1-VID antibody

[0081] Since the bispecific is designed as IgG based fused with VEGF inhibition domain, the anti-PD-L1 antibody clone 6 is assigned to be IgG form, on the other hand, the VEGF binding domain, D1 and D2, in VEGF receptor is fused at C-terminal of Fc region in anti-PD-L1 clone 6 antibody. Since Fc isotype or engineered Fc is important for improving the effectiveness or production of the bispecific antibodies in mammalian cells; therefore, two different Fc isotype, IgG4 (SEQ ID NO. 7) and engineered IgG1 (eIgG1, reduction of antibody-dependent cell-mediated cytotoxicity (ADCC), SEQ ID NO. 8) were used to bispecific construction. Construction of bispecific anti-PD-L1 antibody Fc fused with VID (SEQ ID NO. 9) was depicted in Fig. 8. A short flexible peptide linker, (GGGGS)₃ (SEQ ID NO. 10) was placed between, for example, anti-PD-L1 antibody heavy chain C-terminal of Fc region (SEQ ID NO. 4) and N-terminal module of VID to ensure correct folding and minimize steric hindrance. The coding sequences of anti-PD-L1-VID heavy chain for IgG4 and eIgG1 were shown in SEQ ID NO. 12 and NO. 13. The constructed antibody

Fc fusion proteins were leaded by a signal peptide (SEQ ID NO. 11) and expressed by mammalian cells, and purified from the transfected cell culture supernatant via 1-step Protein G chromatography. As shown in Fig. 9, greater than 95% purity can be obtained in a single step purification process and shows that purified fusion proteins have correct molecular weight ($M_w = 220$ kDa). Since the low purity or recovery rate is the main issue for the bispecific antibody or fusion protein production in the chemistry, manufacturing, and controls (CMC) production; therefore, both purified bispecific antibodies were also applied for size elution column (SEC) with high -performance liquid chromatography (HPLC) to evaluate the purity of bispecific antibodies (Figs. 10A and 10B). Both bispecific antibodies, either anti-PD-L1-VID/IgG4 (Fig. 10A) or anti-PD-L1-VID/eIgG1 (Fig. 10B), revealed the purity is higher than 99% purity in the SEC-HPLC analysis. It implicated the format could be processed easily in the CMC development and provide the successful rate in the further development.

[0082] As shown in Fig. 8, the structure of anti-immune checkpoint antibody Fc-terminally fused with VID. In some embodiments, antibody can be inhibitory anti-immune checkpoint antibodies, such as anti-PD-L1, anti-PD-1, anti-CTLA4, anti-LAG3, etc., or stimulatory antibodies, such as anti-CD28, anti-CD137, anti-CD27, anti-ICOS, etc. or cell surface receptor/antigen, such as HER2, EGFR etc. A linker is placed between antibody Fc and VID to generate the bispecific antibody.

[0083] **Binding Affinity of the Fusion Proteins to PD-L1 and VEGF₁₆₅**

[0084] A direct binding enzyme-linked immunosorbent assay (ELISA) was used to measure the binding affinity of the bispecific antibodies to PD-L1 or VEGF₁₆₅, a splice variant of VEGF-A. A recombinant VEGF trapping protein (VEGFR-Fc) and parental anti-PD-L1 antibody clone 6 was used as positive control 1 for PD-L1 and VEGF, respectively. VEGFR-Fc, aflibercept, is a soluble VEGF receptor that was engineered for therapeutic use and is currently approved by U.S. food and drug administration (FDA) to treat age-related macular degeneration (AMD). VEGFR-Fc contains the second Ig-like domain (D2) of VEGFR1 fused to the third Ig-like domain (D3) of VEGFR2 fused to the Fc region of human IgG1 (Holash et al., 2002).

[0085] 100 μ L of a coating solution (1 μ g/mL VEGF₁₆₅ in phosphate buffered saline (PBS), pH 7.2) were added to each well of a 96-well ELISA plate, and the plate was incubated overnight at 4°C. The wells were washed twice with 400 μ L PBS buffer, and excess liquid was carefully removed with a paper towel. 400 μ L of a blocking solution (5% non-fat skim milk in PBS) was added to each well, and the plate was incubated at room temperature for 1 hour. The wells were washed twice with PBS buffer. Bispecific antibody and control samples were serially diluted three-fold in blocking solution, with the highest protein concentration of 100 nM. 100 μ L of the serially diluted samples were added to each well. The plate was covered and incubated on a plate shaker (about 100 rpm) for 1 hour at room temperature. The wells were washed three times with wash buffer (0.05% Tween-20 in PBS). 100 μ L of 1:2500 diluted horseradish peroxidase-conjugated goat anti-human IgG Fc specific antibodies in blocking solution were added to each well. The plates were sealed and incubated on a plate shaker for 1 hour at room temperature. The plates were washed three

times with wash buffer. 100 μ L TMB substrate was added to each well, and the plates were incubated for 3 to 5 minutes to allow for the reaction to take place. To stop the reaction, 100 μ L of stop solution (1N HCl) was added to each well. The optical density (OD) of each well was determined using an ELISA plate reader (Bio-Tek) at an absorbance wavelength of 450 nm. The absorbance was plotted against the protein concentration of the fusion protein or the control, and the concentration at which the signal was half the maximal effective concentration (EC50) was determined. Meanwhile, the binding activity of PD-L1 for both bispecific antibodies was also performed as a similar scenario as described above, except the bound bispecific antibodies were detected by horseradish peroxidase-conjugated goat anti-human IgG, F(ab')₂ specific F(ab')₂ antibody.

[0086] As the data shown in the Fig. 11A, the binding affinity, expressed as the EC50 value, was 0.075 for both anti-PD-L1-VID/IgG4 and anti-PD-L1-VID/eIgG1 antibodies for recombinant PD-L1 protein. Both bispecific antibodies possess a comparison binding activity as well as positive control, anti-PD-L1 clone 6 antibody (0.1 nM). Meanwhile, the result was also recorded for the VEGF binding activity test. As the data shown in the Fig. 11B, both bispecific antibodies showed a similar VEGF binding activity as compared with the positive control, VEGFR-Fc (aflibercept). The binding activity is not affected in both bispecific antibodies either for PD-L1 or for VEGF binding activity.

[0087] Inhibition of HUVEC Proliferation by the anti-PD-L1-VID bispecific antibodies

[0088] A human umbilical vein endothelial cell (HUVEC) proliferation assay was carried out to test the functionality of the bispecific antibody in the invention. VEGFR-Fc was used as a positive control as described above. 100 μ L of a coating solution (1% gelatin in double distilled water) were added to each well of a 96-well ELISA plate, and the plate was incubated for 2 hours or overnight at 37°C. The wells were washed twice with PBS buffer. 3500 cells of HUVEC cells in endothelial cell growth medium were added to each well, and the plate was incubated overnight at 37°C. Sample as indicated were diluted in assay buffer (Medium-199 1 $\times$ Earle's Salts, 10% fetal bovine serum, 10 mM HEPES, 1 $\times$ antibiotic/antimycotic), with a highest protein concentration of 30 nM. The samples were mixed with VEGF₁₆₅ (8 ng/mL), and the mixtures were incubated overnight at room temperature. The wells were then washed with 200 μ L PBS. 100 μ L VEGF₁₆₅/sample mixture were added to each well, and the plates were incubated for 72 hours at 37°C with 5% CO₂. Following incubation, 10 μ L MTS detection reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)+phenazine methosulfate in distilled PBS) was added to each well, and the plates were incubated at 37°C for 2.5 hours. The OD of each well was determined using an ELISA plate reader (Bio-Tek) at an absorbance wavelength of 490 nm. The absorbance was plotted against the protein concentration of the test sample, and the concentration at which the cell proliferation was inhibited by 50% (IC₅₀) was determined. The inhibition of cell proliferation (IC₅₀) was determined to be between 0.1070 and 0.1233 nM for the tested fusion proteins of the invention. One of the bispecific antibodies, anti-PD-L1-VID/eIgG1, revealed a better inhibition than another bispecific antibody, anti-PD-L1-VID/IgG4 (Fig. 12,

0.1070 nM vs. 0.1233 nM). The IC₅₀ of anti-PD-L1-VID/elgG1 is good as well as positive control, VEGFR-Fc (0.1072 nM).

[0089] Enhanced stimulation of T cell activation for anti-PD-L1-VID/elgG1 bispecific antibody leads in MLRs

[0090] To determine the antagonistic functionality of bispecific antibody in enhancing T cells activation through inhibition the interaction between PD-1 and PD-L1. The bispecific antibody leads, anti-PD-L1-VID/elgG1 antibody, were applied into MLRs as described above. IL-2 and IFN- γ production were then recorded after 3 or 5 days antibody treatment. Mono- or bispecific antibody was applied as an equal mole to compare the antagonistic functionality in T cell activation enhancement and isotype IgG was used a negative control. As the data shown in the Figs. 13A and 13B, the anti-PD-L1-VID/elgG1 antibody showed a significant IL2 induction after 3 days treatment and dropped down after 5 days treatment. The profile of cytokine production is highly similar with the reference antibody, MPDL3280A. Meanwhile, the IFN- γ production is also upregulated and accumulated in the bispecific antibody leads treatment after 3 or 5 days treatment. This indicated the anti-PD-L1-VID/elgG1 bispecific antibody also possess antagonistic functionality in T cell activation as well as reference antibody without loss any activities in the present invention.

[0091] In vitro serum stability of anti-PD-L1-VID/elgG1 bispecific antibody

[0092] Purified anti-PD-L1-VID/elgG1 were incubated with serum (15 μ g/mL) from different species as indicated at 37°C in water bath. Serum samples containing the purified bispecific antibodies were taken at different time points up to 14 days. Concentrations of the bispecific antibody in the serum samples

will be determined using a sandwiched ELISA assay as below. VEGF₁₆₅ (1 µg/mL) pre-coated wells were incubated with titrated concentrations of purified anti-PD-L1-VID/elgG1 bispecific antibody to be the standard curve to calculate the Abs concentration in the serum (fresh preparation, day 0). Collected samples from different time points were also applied to pre-coated VEGF₁₆₅ wells for detection. After washing with 0.1 % Tween-20 in PBS, the intact and bound Abs were detected by biotinylated PD-L1-Fc and HRP conjugated streptavidin before color developing. The concentration of Abs in the serum was calculated by interpolation method and then the half-life of Abs is plotted as shown in the Figs. 14A, 14B and 14C. The half-life of anti-PD-L1-VID/elgG1 is longer than 8 days either in cynomolgus (8.6 days), mouse (9.2 days) or human serum (11.9 days). The long half-life could provide usage flexibility of the bispecific antibody with less administration frequency in animal study or clinical trial in the future.

[0093] Anti-tumor activity of bispecific antibody (In Vivo model)

[0094] The lack of rodent cross-reactivity of the PD-L1 in bispecific antibodies prevented the use of standard murine syngeneic or human xenograft tumor models for the assessment of anti-human tumor efficacy of the antibodies. Accordingly, a novel huPBL-SCID-Bg xenogeneic tumor mouse model was generated using a SCID-Bg mouse (CB.17/Icr.Cg Pkrdc^{scid}Lyst^{tg}/CrI), which harbors the beige (Bg) mutation lack murine T and B lymphocytes and functional NK cells. The anti-human tumor efficacy of the bispecific antibodies was assessed using this model as described below.

[0095] The PC-3 human prostate was obtained from American Type Culture Collection and was cultured in RPMI-1640 (Invitrogen) with L-glutamine, sodium pyruvate, penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Gibco Cat. No. 10437). Cells were grown to confluency in T-150 Falcon flasks. Subsequently, cells were trypsinized (Trypsin 0.25%-EDTA; Invitrogen) and growth was scaled up to sufficient cell number for inoculation. Peripheral blood lymphocytes (PBMCs) were isolated from heparinized blood using Lymphoprep™ in accordance with the manufactures' protocol (STEMCELL Technologies Inc.). Counted cell suspensions were combine such that each mouse received an injection of 0.75×10^6 PBMCs and 3×10^6 tumor cells in a single bolus injection of 0.1 mL in PBS. In order to facilitate the tumor cells grown in the mouse, another 0.1 mL matrigel was then mixed with the combined cell suspension and then immediately injected into prepare mice.

[0096] For each mouse, 0.2 mL volume of the combined cell suspension was injected subcutaneously into the right flank of the animal. After 14 days inoculation, the solid tumor is formed and reached around 250 to 300 mm³ and the bispecific antibody (10 mg/kg) or control antibody is challenged twice per week for three to four weeks with an intraperitoneal injection (i.p.). Tumor measurement was made via Pressier caliper twice per week as well as test sample administration for the duration of the experiments and body weights were also recorded. Tumor volume was calculated using the following calculation: $\text{length} \times \text{width}^2 \times 0.44 = \text{volume (mm}^3\text{)}$ and plotted in the Fig. 15A. Mice were removed from the study in the event that the tumor volume reached 2000 mm³ or animal lost 20% of body weight before termination of the experiment. Similar results were observed when tumors were measured on

day 7 post-inoculation, and the animals were randomized according to tumor volume. For animal study, each group contained 6 mice. As the data showed in the Fig, 15A, the bispecific antibody showed a significant anti-tumor efficiency in the PC-3 xenografted mouse model. The tumor size is smaller after 18 days post tumor inoculation as well as PD-L1 reference antibody and continued to reduce below 200 mm³. Fig. 15B shows that the tumor size bispecific antibody treatment is significant smaller than isotype or reference antibody treatment on day 35 post-inoculation. It indicated the anti-PD-L1-VID/elgG1 Abs has the synergic effect in anti-tumor activity in animal. The PC-3 xenografted mouse model is preliminarily demonstrated the anti-tumor of bispecific antibody and revealed its potential to be a therapeutic drug lead in the future.

[0097] Collectively, these results indicated bi-specific antibody sustain its immune checkpoint blocking in PD-1/PD-L1 signaling and neutralized the pro-angiogenic protein, VEGF. Studies are ongoing to further investigate the biological activity of these proteins using proper animal model, such as the PC-3 tumor in the humanized NOD.Cg-Prkdc^{scid} Il2rg^{tm1wjl}/SzJ (NSG) model.

[0098] The Fc region in the present invention could be from any immunoglobulin isotypes, subclasses, allotypes, or engineered mutants, such as knob and hole Fc fragment(s).

[0099] **EXAMPLES**

[0100] The example below describe the generation of monoclonal antibodies suitable for therapeutic purpose targeting human PD-L1 and VEGF. Composite, human anti- human PD-L1 and VEGF neutralized domains were generated from anti-PD-L1 antibody clone 6 and VEGF trapping domain from

human VEGF receptors, respectively. Segments of human V region sequence were sourced from unrelated human antibody (germline and non-germline) sequence databases.

[0101] Example 1 Generation of IgG antibodies that bind to PD-L1 and VEGF

[0102] Certain antibodies provided by present invention were originally generated from Fabs bind to human PD-L1. The Fabs were selected from a phage display library, the OmniMab phagemid library, following alternating panning on corresponding Fc fusion proteins (PD-L1-Fc) and cells expressing human corresponding protein (PD-L1). After direct ELISA screening, the positive clones were then sequenced for heavy chain and light chain. These Fabs included those that are designated as "OM-PD-L1-6", and "OM-PD-L1-32" etc. for PD-L1. PD-L1 antibodies PD-L1-Clone 6, and PD-L1-Clone 32 disclosed in this application were generated from "OM-PD-L1-6" and "OM-PD-L1-32" in HEK293 cell or CHO-S cells. And bispecific antibody targeting PD-L1 and VEGF simultaneously was designed as anti-PD-L1-VID (VEGF inhibition domain) antibody. The amino acid sequence of the light chain variable region and heavy chain variable region of a given Fab are identical to the amino acid sequence of the light chain variable region and heavy chain variable region, respectively.

[0103] Example 2 *In vitro* binding of anti-PD-L1-VID bispecific antibody to its corresponding target

[0104] Anti-PD-L1-VID bispecific antibody was constructed as shown in the Fig. 8 and expressed in the HEK293 cells or CHO-S cell. The medium containing

bispecific antibody was affinity purified from culture supernatant by Protein G chromatography. Purified antibody is then concentrated, followed by dialysis in PBS buffer and analyzed by SDS-PAGE as shown in the Fig 9. To test direct binding of purified fusion proteins to PD-L1 or VEGF₁₆₅ on ELISA, 100 ng/well recombinant PD-L1 was coated in a 96-well ELISA plate. Various concentrations of purified anti-PD-L1-VID Abs were then added to each well and incubated for 1hr. After washing, 1:5000 dilution of anti-Fab or anti-Fc HRP conjugate (Jackson Immunochemicals) was added to each well and incubated for another hour. After final washing, TMB substrate (Invitrogen Inc.) was added and OD absorbance at 450 nm was measured. The data analyzed by sigmoidal curve fitting using GraphPad Prism 5 and EC50 is calculated.

[0105] Example 3 Antigen binding specificity of anti-PD-L1-VID by FACS analysis

[0106] To test anti-PD-L1-VID Abs binding specificity, stable PD-L1 expression 293 cells, IFN- γ stimulated A549 or WiDr (colon colorectal adenocar) were stained with 1 μ g/mL anti-PD-L1-VID Abs antibody for 1hr on ice before wash three times with 1 $\times$ PBS. The bound antibody fusion proteins were detected with Alexa-488 conjugated goat anti-human IgG (H+L) followed by FACS analysis. Isotype antibody was used as negative control for the test. Results showed anti-PD-L1-VID Abs sustains its antigen binding specificity as compared with anti-PD-L1 alone.

[0107] To test anti-PD-L1-VID Abs binding specificity, stable PD-L1 expression 293 cells (human embryonic kidney cells), IFN- γ stimulated A549 (lung

carcinoma) or NCI-H292 (mucoepidermoid pulmonary carcinoma) were stained with 1 μ g/mL anti-PD-L1-VID Abs antibody for 1hr on ice before wash three times with 1x PBS. The bound antibody fusion proteins were detected with Alexa-488 conjugated goat IgG (H+L) followed by FACS analysis. Isotype antibody was used as negative control for the test. Results showed anti-PD-L1-VID Abs sustains its antigen binding specificity as compared with anti-PD-L1 alone (Figs. 16A, 16B and 16C).

[0108] Example 4 In vitro immunomodulatory effect of anti-PD-L1-VID bi-specific antibody

[0109] To measure the ability of the anti-PD-L1-VID Abs to modulate T cell responsiveness purified T cells will be cultured with allogeneic dendritic cells, prepared by culturing monocytes in GM-CSF and IL-4 for few days. Parallel plates were set up to allow collection of supernatants at day 3 and day 5 to measure IL-2 and IFN- γ respectively using a commercial ELISA kit. Genentech/Roche's humanized anti-PD-L1, MPDL3280A, will be produced in-house and used as positive control. As the data shown in the Figs. 13A and 13B, the IL2 and IFN- γ production are highly upregulated in the bispecific antibody treatment as well as reference antibody after 3 or 5 days antibody treatment. It revealed the bispecific antibody still possess the ability to inhibit the PD-1/PD-L1 interaction between T cell and dendritic cells to activate the T cell activity.

[0110] Example 5 Human leukocyte expansion induced by bispecific antibodies in vivo

[0111] The lack of detectable cross-reactivity of the PD-L1 antibody with murine PD-L1 and the requirement for the presence of human immune cells required the development of models for the *in vivo* functional assessment of the bispecific antibodies. Mice with the NOD genetic background carrying the severe combined immunodeficient (SCID) mutation and deficiency in the IL-2 receptor common gamma chain (commonly termed NSG) are able to support the engraftment of large number of human peripheral blood leukocytes (huPBL) and maintain engraftment for at least 30 days (King et al., 2008). This mouse model, also known as huPBL-NSG model, was used to assess the functional effect of *in vivo* systemic administration of the antibodies on human immune cells.

[0112] Specifically, 6 million freshly isolated human PBMCs were adoptively transferred via intravenous injection into huPBL-NSG mice. Nine days post PBMC injections, the animals were administered a single 1 mg/kg of mono-antibody, bispecific antibody or isotype control antibody via intraperitoneal injection. At day 24 to 28 post PBMC engraftment, PBMC were stained with antibodies to human and murine CD45 assessed via flow cytometry. Forward and side scatter profiles were used to determine a lymphocyte gate. Bispecific antibodies were able to enhance expansion of human leukocytes as evidenced by increased proportion of human CD45⁺ cells in the peripheral blood of engrafted mice. For each group, n≥6 mice.

[0113] Example 6 Inhibition of PC-3 or A498 tumor cell growth in huPBL-NSG by anti-PD-L1-VID/elgG1 antibody

[0114] PD-L1 positive human prostate cancer cell line, PC-3 (ATCC# CRL-1435) or kidney cancer cell line, A498 (ATCC® HTB-44™) can be used to establish xenograft models in huPBL-NSG mice. For tumor formation, 3×10^6 PC-3 cells (or A498 cells) /mouse will be injected subcutaneously in huPBL-NSG mice as described above. In order to assess the inhibitory effects on the tumor growth, different concentrations of anti-PD-L1-VID/elgG1 antibody, reference antibody, or isotype antibody from 0.1 – 3 mg/kg will be administered intravenously twice weekly for 4 weeks in the mice after 14 days tumor cells implantation. The tumor growth will be measured twice per week up to 5 weeks as described in the Fox Chase SCID®Beige mice model.

[0115] Example 7 Pharmacokinetic assessment of anti-PD-L1-VID/elgG1 in mice and monkeys

[0116] 10 mg/kg to 40 mg/kg of bi-functional proteins, anti-PD-L1-VID/elgG1 will be administered into mice or monkeys via subcutaneous injection or intravenous injection. Serum samples will be taken at different time points after the injection up to 15 days. Concentrations of the Fc fusion protein in the serum samples will be determined using a sandwiched ELISA assay.

[0117] While the disclosure has been described by way of example(s) and in terms of the preferred embodiment(s), it is to be understood that the disclosure is not limited thereto. On the contrary, it is intended to cover various modifications and similar arrangements and procedures, and the scope of the appended claims therefore should be accorded the broadest interpretation so as to encompass all such modifications and similar arrangements and procedures.

WHAT IS CLAIMED IS:

1. A bispecific antibody or antigen-binding portion thereof comprising at least one of polypeptide chain, wherein the polypeptide chain comprising:

a binding domain binding cell surface protein; and

a vascular endothelial growth factor (VEGF) inhibiting domain.

2. The bispecific antibody or antigen-binding portion thereof of claim 1, wherein the cell surface protein comprising programmed cell death protein 1 ligand (PD-L1), programmed cell death protein 1 (PD-1), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG3), B- and T-lymphocyte attenuator (BTLA), OX40 (cluster of differentiation 134, CD134), CD27, CD28, tumor necrosis factor receptor superfamily member 9 (TNFRSF9 or CD137), inducible T cell costimulator (ICOS or CD278), CD40, or a combination thereof.

3. The bispecific antibody or antigen-binding portion thereof of claim 2, wherein the binding domain binds the PD-L1, and the binding domain comprises:

a heavy chain variable domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of SEQ ID NOs. 4 and 6; and

a light chain variable domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from

the group consisting of amino acid 1-111 of SEQ ID NO. 3 and 1-110 of SEQ ID NO. 5.

4. The bispecific antibody or antigen-binding portion thereof of claim 1, wherein the VEGF inhibiting domain is from human VEGF receptor 1 (VEGFR-1) or human VEGF receptor 2 (VEGFR-2).

5. The bispecific antibody or antigen-binding portion thereof of claim 1, wherein the VEGF inhibiting domain comprising an amino acid sequence of at least about 80% sequence homology to the amino acid sequence selected from the group consisting of SEQ ID NOs. 1, 2, 9, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, and a combination thereof.

6. The bispecific antibody or antigen-binding portion thereof of claim 1, wherein the polypeptide chain further comprising:

a Fc domain; and

a Fab fragment connected to the N-terminus of the Fc domain, and the Fab fragment comprising the binding domain,

wherein the VEGF inhibiting domain is connected to the C-terminus of the Fc domain.

7. The bispecific antibody or antigen-binding portion thereof of claim 6, further comprising a linker between the Fc domain and the VEGF inhibiting domain.

8. The bispecific antibody or antigen-binding portion thereof of claim 7, wherein the bispecific antibody comprises an amino acid sequence set forth in SEQ ID NO. 12 or 13.

9. The bispecific antibody or antigen-binding portion thereof of claim 1, wherein the bispecific antibody or antigen-binding portion thereof comprises one pairs of polypeptide chains.

10. The bispecific antibody or antigen-binding portion thereof of claim 9, wherein the bispecific antibody is an IgG, IgE, IgM, IgD, IgA, or IgY antibody.

11. The bispecific antibody or antigen-binding portion thereof of claim 10, wherein the bispecific antibody is an IgG antibody.

12. The bispecific antibody or antigen-binding portion thereof of claim 11, wherein the IgG antibody is an IgG1, IgG2, IgG3, or IgG4 antibody.

13. The bispecific antibody or antigen-binding portion thereof of claim 12, wherein the IgG1 antibody is a reduction of antibody-dependent cell-mediated cytotoxicity of IgG1 antibody.

14. The bispecific antibody or antigen-binding portion thereof of claim 1, wherein the bispecific antibody is a human antibody.

15. A pharmaceutical composition, comprising:

a bispecific antibody or an antigen-binding fragment thereof of claim 1, and at least one pharmaceutically acceptable carrier.

16. An antibody-drug conjugate comprising:

a therapeutic agent; and

a bispecific antibody or an antigen-binding fragment binding PD-L1 and/or a VEGF inhibiting domain,

wherein the therapeutic agent is covalently conjugated to the antibody or the antigen-binding fragment by a linker.

17. The antibody-drug conjugate of claim 16, wherein the bispecific antibody or the antigen-binding fragment is selected from claim 3.

18. A method of treating cancer, the method comprising administering to the subject in need thereof an effective amount of the bi-specific antibody or antigen-binding portion thereof of claim 1.

19. The method of claim 18, wherein the cancer is selected from the group consisting of prostate cancer, lung cancer, Non-Small Cell Lung Cancer (NSCLC), melanoma, lymphoma, breast cancer, head and neck cancer, renal cell carcinoma (RCC), and ovarian cancer.

20. The method of claim 18, wherein the effective amount is from 0.001 $\mu\text{g/kg}$ to 250 mg/kg .

21. A nucleic acid molecule encoding the antibody or the antigen-binding portion of claim 3.

22. A method for cancer diagnosis in a subject, comprising:

(a) obtaining a body fluid sample or a cell sample from a subject;

(b) contacting the sample with one or more antibodies that can detect expression of a panel of cancer markers selected from the group consisting PD-L1 and VEGF;

(c) assaying the binding of the one or more antibodies to the fluid sample or the cell sample; and

(d) assessing the cancer status of the subject in an assay by measuring and comparing the level of antibody binding with a normal control to determine the presence of the cancer in the subject.