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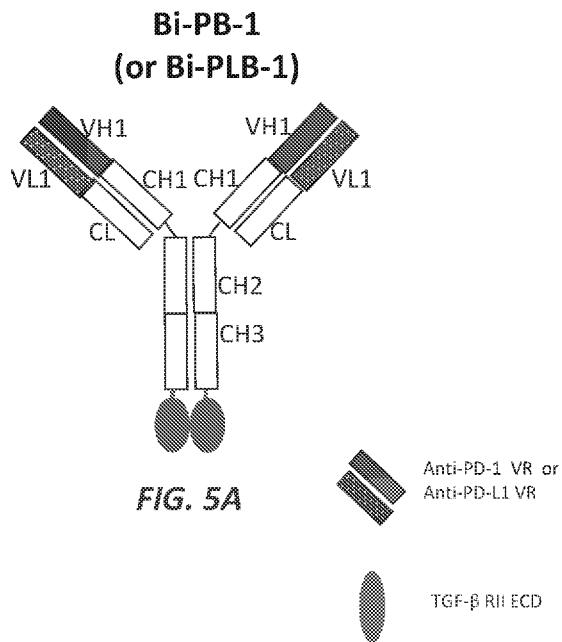
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## (54) Title: ANTITUMOR ANTAGONISTS



(57) **Abstract:** Antitumor antagonists that bind specifically to immune checkpoint regulators, angiogenesis pathway regulators and/or TGF pathway regulators are disclosed. Also disclosed are methods for treating proliferative disorders, infections, and immunological disorders with the antitumor antagonists described herein.

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**TITLE****ANTITUMOR ANTAGONISTS**

**[0001]** This application claims priority to U.S. Provisional Patent Application Serial No. 62/691,658, filed June 29, 2018 and U.S. Provisional Patent Application Serial No. 62/823,989, filed March 26, 2019, the contents of which are expressly incorporated herein by reference herein.

**FIELD**

**[0002]** The present application relates generally to cancer treatment and, in particular, to bispecific inhibitors capable of modulating pathways associated with tumorigenesis, tumor immunity, and angiogenesis.

**BACKGROUND**

**[0003]** The inability of the host to eliminate cancer cells remains a major problem. Although an increasing number of therapeutic monoclonal antibodies have been approved for treatment of various cancers, emergence of resistance to these antibodies is frequently observed, given the many different molecular pathways underlying cancer growth and progression to metastasis. Although the immune system is the principal mechanism of cancer prevention, cancer cells counteract immunosurveillance. Natural control mechanisms have been identified that limit T-cell activation so as to prevent collateral damage resulting from unrestrained T-cell activity. This process has been exploited by tumor cells to evade immune responses. Restoring the capacity of immune effector cells, especially T cells, to recognize and eliminate cancer is a major objective in immunotherapy. Both TGF $\beta$  and angiogenesis pathways have been implicated in the resistance to checkpoint inhibitors.

**[0004]** The need exists for improved therapeutic binding antagonists or antibodies and methods of treating cancer and chronic viral infections that can overcome resistance to checkpoint inhibitors that inhibit the TGF $\beta$  and VEGF pathways.

**SUMMARY**

**[0005]** One aspect of the present application relates to antitumor antagonists containing a first targeting domain specifically binding TGF $\beta$ 1 and a second targeting domain specifically binding PD-1 or PD-L1.

**[0006]** Another aspect of the present application relates to an antitumor antagonist containing a first targeting domain specifically binding TGF $\beta$ 1 and a second targeting domain specifically binding VEGF.

**[0007]** Another aspect of the present application relates to an antitumor antagonist containing an improved version of bevacizumab with increased expression in mammalian

cells.

**[0008]** Another aspect of the present application relates to methods for treating a cell proliferative disorder in a subject. The method comprises the step of administering to a subject in need of such treatment an effective amount the antitumor antagonist of the present application.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0009]** **FIG. 1** shows complementary determining region (CDR) sequences of certain anti-PD-1 mabs. The corresponding framework region (FR) sequences are listed in **FIG. 36** as **SEQ ID NOS:130-159**.

**[0010]** **FIGS. 2A-2B** show several embodiments of anti-PD-1 antibody variable domain sequences.

**[0011]** **FIG. 3** shows CDR sequences of certain anti-PD-L1 mabs. The corresponding FR sequences are listed in **FIG. 36** as **SEQ ID NOS:160-182**.

**[0012]** **FIGS. 4A-4C** show several embodiments of anti-PD-L1 antibody variable domain sequences.

**[0013]** **FIGS. 5A-5B** show four different bispecific antitumor antagonist configurations, Bi-PB-1, Bi-PLB-1, Bi-PB-2 and Bi-PLB-2, each comprising (1) anti-PD-1 or anti-PD-L1 variable regions and (2) a TGF- $\beta$  RII ECD domain.

**[0014]** **FIG. 6** shows exemplary functional domain sequences corresponding to the bispecific antibodies in **FIG. 5**.

**[0015]** **FIG. 7** shows exemplary heavy chain (HC) and light chain (LC) sequences corresponding to selected bispecific antibodies in **FIG. 5**.

**[0016]** **FIGS. 8A-8C** depict three different bispecific antagonists, Bi-AB-1, Bi-A1B-1 and Bi-ZB-1, respectively, each comprising a carboxy-terminal TGF- $\beta$ 1 RII extracellular domain (ECD) in a mutant IgG1 (K447A) scaffold. Bi-AB-1 and Bi-A1B-1 both contain amino-terminal anti-VEGF variable regions (VH1, VL1) from Avastin/bevacizumab; Bi-A1B-1 includes two amino acid substitutions in the VH region (E6Q, L11V). Bi-ZB-1 contains an amino terminal afibbercept domain upstream of an IgG1 Fc (K447A) region.

**[0017]** **FIGS. 9A and 9B** show the various functional domain sequences present in the bispecific antagonists depicted in **FIG. 8**.

**[0018]** **FIG. 10** shows the heavy chain (HC) and light chain (LC) amino acid sequences corresponding to the bispecific antagonists depicted in **FIG. 8**.

**[0019]** **FIG. 11** summarizes the arrangement of functional domains in the bispecific antagonists depicted in **FIG. 8**.

**[0020]** **FIG. 12** is a Coomassie blue stained polyacrylamide gel showing improved

expression levels of bispecific antibody antagonists Bi-AB-1 and Bi-A1B-1 containing E6Q and L11V mutations when transiently transfected into HEK293 cells as determined by non-reducing polyacrylamide gel electrophoresis (PAGE).

[0021] **FIG. 13A** shows size exclusion chromatography (SEC) profiles of Bi-AB-1 and Bi-A1B-1. **FIG. 13B** shows that protein A purified Bi-AB-1 and Bi-A1B-1 have low levels of high molecular weight (HMW, 1%, 1%, respectively) and low molecular weight (LMW, 0.4%, 0.4%, respectively) species in comparison to dimers (98.6%, 98.7%, respectively)

[0022] **FIGS. 14A** and **14B** show PAGE gels of transiently expressed Bi-ZB-1 under non-reducing (**FIG. 14A**) and reducing (**FIG. 14B**) conditions.

[0023] **FIG. 15** shows a size exclusion chromatography (SEC) profile showing that protein A purified Bi-ZB-1 has low levels of high molecular weight (HMW, 3.4%) and low molecular weight (LMW, 0.5%) species in comparison to dimers (96.1%).

[0024] **FIG. 16** shows the results of a cell-based assay in which recombinant HEK-293 cells expressing human VEGFR2 and firefly luciferase under the control of NFAT response elements were stimulated with huVEGF165 in the presence of serial dilutions of two anti-VEGF antagonists, Bi-A1B-1 and antibody A. Bioactivity was determined by measuring the decrease in the amount of luciferase-mediated luminescence.

[0025] **FIG. 17** is an ELISA analysis demonstrating simultaneous binding TGF- $\beta$ 1 and VEGF165 by Bi-A1B-1 in which huTGF- $\beta$ 1 coated 96 well plates incubated with serially diluted samples Bi-A1B-1, followed by biotinylated huVEGF165, whereby bound molecules were detected by Streptavidin-HRP using a TMB substrate.

[0026] **FIG. 18** shows the results of a cell-based assay in which recombinant HEK-293 cells expressing human TGF- $\beta$ 1 RII receptor and firefly luciferase under the control of a SMAD response element and stimulated with huTGF- $\beta$ 1 in the presence of serial dilutions of Bi-A1B-1, Bi-ZB-1, and a control, each containing a TGF- $\beta$ 1 RII extracellular domain (ECD) fusion. Bioactivity was determined by a decrease in the amount of luciferase-mediated luminescence.

[0027] **FIG. 19** is a pharmacokinetic profile showing the *in vivo* half-life ( $T_{1/2}$ ) of the bispecific antagonist Bi-A1B-1 following a tail vein injection into 6-10 week old female CD1 mice. The Bi-A1B-1 antagonist in serum was recovered at various times post-injection and subjected to analysis by ELISA.

[0028] **FIGS. 20A-20B** depict two bispecific antitumor antagonists, Bi-PB-1.2 (**FIG. 20A**) and Bi-PLB-1.2 (**FIG. 20B**), each comprising antibody backbone (IgG4 K447A or IgG1K447A) with variable region domains from anti-PD-1 and anti-PD-L1, respectively, and

additionally including a TGF- $\beta$ -RII ECD fused to the carboxy-terminal end of each heavy chain CH3 region.

[0029] **FIG. 21** shows functional domain sequences present in the bispecific antibodies in **FIGS. 20A** and **20B**.

[0030] **FIG. 22** show the heavy chain (HC) and light chain (LC) amino acid sequences corresponding to the bispecific antagonists depicted in **FIGS. 20A** and **20B**.

[0031] **FIG. 23** summarizes the arrangement of functional domains in the bispecific antagonists depicted in **FIGS. 20A** and **20B**.

[0032] **FIG. 24A** shows a non-denaturing polyacrylamide gel (PAGE) analysis showing increased expression of Bi-PB-1.2 and Bi-PLB-1.2 in a transient expression system following protein A-affinity purification in comparison to a parental control antibody (2P17). **FIG. 24B** depicts size exclusion chromatography (SEC) profiles showing of protein A purified Bi-PB-1.2, Bi-PLB-1.2 and a Benchmark antibody (2P17). **FIG. 24C** shows that Bi-PB-1.2, Bi-PLB-1.2 and the Benchmark antibody (2P17) have low levels of high molecular weight (HMW) low molecular weight (LMW) species in comparison to dimers.

[0033] **FIGS. 25A-25B** show that the Dimer, HMW and LMW forms of Bi-PB-1.2 (**FIG. 25A**) and Bi-PLB-1.2 (**FIG. 25B**) exhibit good stability for at least 4 weeks at 4° C.

[0034] **FIGS. 26A-26E** show the results of PD-1 and TGF- $\beta$ 1 binding to Bi-PB-1.2 (**FIGS. 26A, 26B**, respectively) and corresponding anti-PD-1 and anti-PDL1- TGF- $\beta$ 1 RII ECD benchmark molecules (**FIGS. 26C, 26D**, respectively), along with their resultant binding affinity constants (**FIG. 26E**).

[0035] **FIGS. 27A-27E** show the results of PD-L1 and TGF- $\beta$ 1 binding to Bi-PLB-1.2 (**FIGS. 27A, 27B**, respectively) and the anti-PDL1- TGF- $\beta$ 1 RII ECD benchmark molecule (**FIGS. 27C, 27D**), along with their resultant binding affinity constants (**FIG. 27E**).

[0036] **FIG. 28A** shows an ELISA assays demonstrating simultaneous binding of TGF- $\beta$ 1 and PD-1 by Bi-PB-1.2 in which huTGF- $\beta$ 1 coated 96 well plates were incubated with serially diluted samples of Bi-PB-1.2, followed by biotinylated hu PD-1, whereby bound molecules were detected by Streptavidin-HRP using a TMB substrate. **FIG. 28B** shows an ELISA assay demonstrating simultaneous binding of TGF- $\beta$ 1 and PD-L1 by Bi-PLB-1.2 in which huTGF- $\beta$ 1 coated 96 well plates were incubated with serially diluted samples of Bi-PLB-1.2, followed by biotinylated hu PD-L1, whereby bound molecules were detected by Streptavidin-HRP using a TMB substrate.

[0037] **FIG. 29A** shows the ability of Bi-PB-1.2 and anti-PD-1 benchmark antibody to block binding of PD-1 to PD-L1. **FIG. 29B** shows the results of a cell-based assay showing the ability of serial dilutions of Bi-PB-1.2 and antiPDL1- TGF- $\beta$ 1 RII ECD

benchmark molecule to block the ability of TGF- $\beta$ 1 to activate luciferase expression under the control of a SMAD response element. Bioactivity was determined by a decrease in the amount of luciferase-mediated luminescence.

[0038] **FIGS. 30A-30B** show binding of Bi-PB-1.2 and both anti-human PD-1 and anti-cyno PD-1 benchmark antibodies to human PD-1 (**FIG. 30A**) and cynomolgus PD-1 (**FIG. 30B**), along with their corresponding EC<sub>50</sub> values reflecting the half maximal effective concentrations (EC<sub>50</sub>) producing a response halfway between the baseline and maximum response with respect to binding human PD-1 and cyno PD-1, respectively.

[0039] **FIG. 31A** shows the ability of Bi-PLB-1.2 and anti-PD-L1 benchmark antibody to block binding of PD-L1 to PD-1. **FIG. 31B** shows the results of a cell-based assay showing the ability of serial dilutions of Bi-PLB-1.2 and anti-PDL1- TGF- $\beta$ 1 RII ECD benchmark molecule to block the ability of TGF- $\beta$ 1 to activate luciferase expression under the control of a SMAD response element. Bioactivity was determined by a decrease in the amount of luciferase-mediated luminescence.

[0040] **FIGS. 32A-32B** show binding of Bi-PLB-1.2 and both anti-human PD-L1 and anti-cyno PD-L1 benchmark antibodies to human PD-L1 (**FIG. 32A**) and cyno PD-L1 (**FIG. 32B**), along with their corresponding EC<sub>50</sub> values reflecting the half maximal effective concentrations (EC<sub>50</sub>) producing a response halfway between the baseline and maximum response with respect to binding human PD-L1 and cyno PD-L1, respectively.

[0041] **FIGS. 33A-33B** show increased IFN- $\gamma$  secretion from human PBMCs (Donor 1, **FIG. 33A**; Donor 2, **FIG. 33B**) with Bi-PB-1.2 relative to the negative control treatments. **FIGS. 33C-33D** show increased IL-2 secretion from human PBMCs (Donor 1, **FIG. 33C**; Donor 2, **FIG. 33D**) with Bi-PB-1.2 relative to the negative control treatments.

[0042] **FIGS. 34A-34B** show increased IFN- $\gamma$  secretion from human PBMCs (Donor 8, **FIG. 34A**; Donor 9, **FIG. 34B**) with Bi-PLB-1.2 relative to the parental anti-PD-L1 antibody and the negative control treatments. **FIGS. 34C-34D** show increased IL-2 secretion from human PBMCs (Donor 8, **FIG. 34C**; Donor 9, **FIG. 34D**) with Bi-PLB-1.2 relative to the parental anti-PD-L1 antibody and negative control treatments.

[0043] **FIGS. 35A-35F** show that Bi-PB-1.2 and Bi-PLB-1.2 exhibit improved pharmacokinetic profiles relative to a benchmark antibody following a tail vein injection into 6-10 week old female CD1 mice. The antibody antagonists, Bi-PB-1.2, Bi-PLB-1.2, and Benchmark were recovered from serum at various times post-injection and subjected to analysis by ELISA (**FIGS. 35A, 35C, 35E**, respectively) and Western blot (**FIGS. 35B, 35D, 35F**, respectively).

[0044] **FIG. 36** shows framework regions (FRs) corresponding to the anti-PD-1- and

anti-PD-L1 CDRs in **FIGS. 1 and 3**, respectively.

## **DETAILED DESCRIPTION**

### **Definitions**

**[0045]** Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed method and compositions belong. It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a peptide” includes “one or more” peptides or a “plurality” of such peptides. With respect to the teachings in the present application, any issued patent or patent application publication described in this application is expressly incorporated by reference herein.

**[0046]** As used herein, the term “PD-1” refers to any form of PD-1 and variants thereof that retain at least part of the activity of PD-1. Unless indicated differently, such as by specific reference to human PD-1, PD-1 includes all mammalian species of native sequence PD-1, e.g., human, canine, feline, equine, and bovine.

**[0047]** As used herein, the term “PD-L1” refers to any form of PD-L1 and variants thereof that retain at least part of the activity of PD-L1. Unless indicated differently, such as by specific reference to human PD-L1, PD-L1 includes all mammalian species of native sequence PD-L1, e.g., human, canine, feline, equine, and bovine.

**[0048]** The term “agonist” refers to a substance which promotes (i.e., induces, causes, enhances, or increases) the biological activity or effect of another molecule. The term agonist encompasses substances which bind receptor, such as antibody, and substances which promote receptor function without binding thereto (e.g., by activating an associated protein).

**[0049]** The term “antagonist” or “inhibitor” refers to a substance that prevents, blocks, inhibits, neutralizes, or reduces a biological activity or effect of another molecule, such as a receptor or ligand.

**[0050]** As used herein, the term “antibody” refers to a polypeptide or a polypeptide complex that specifically recognizes and binds to antigen through one or more immunoglobulin variable regions. Antibody can be a whole antibody, antigen binding fragment or a single chain thereof. The term “antibody” encompasses various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as alpha, delta, epsilon, gamma, and mu, or  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$  and  $\mu$  with some subclasses among them (e.g.,  $\gamma 1$ - $\gamma 4$ ). It is the nature of this chain that determines the “class” of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) e.g., IgG1, IgG2, IgG3, IgG4, etc. are well

characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant disclosure. All immunoglobulin classes are within the scope of the present disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules.

**[0051]** Antibodies or antibody antagonists of the present application may include, but are not limited to polyclonal, monoclonal, multispecific, bispecific, trispecific, human, humanized, primatized, chimeric and, single chain antibodies. Antibodies disclosed herein may be from any animal origin, including birds and mammals. Preferably, the antibodies are human, murine, rat, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In some embodiments, the variable region may be condriticoid in origin (e.g., from sharks).

**[0052]** The terms “antibody fragment” or “antigen-binding fragment” are used with reference to a portion of antibody, such as F(ab')2, F(ab)2, Fab', Fab, Fv, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, fragments produced by a Fab expression library and anti-idiotypic (anti-Id) antibodies. Regardless of structure, antibody fragment binds with the same antigen that is recognized by the intact antibody. The term “antibody fragment” includes DARTs and diabodies. The term “antibody fragment” also includes any synthetic or genetically engineered proteins comprising immunoglobulin variable regions that act like antibody by binding to a specific antigen to form a complex. A “single-chain fragment variable” or “scFv” refers to a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins. In some aspects, the regions are connected with a short linker peptide of ten to about 25 amino acids. The linker can be rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. With regard to IgGs, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000. The four chains are typically joined by disulfide bonds in a “Y” configuration where the light chains bracket the heavy chains starting at the mouth of the “Y” and continuing through the variable region.

**[0053]** Both the light and heavy chains are divided into regions of structural and functional homology. The terms “constant” and “variable” are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VL) and heavy (VH)

chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention, the numbering of the constant region domains in conventional antibodies increases as they become more distal from the antigen-binding site or amino-terminus of the antibody. In conventional antibodies, the N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

**[0054]** As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VL domain and VH domain, or subset of the complementarity determining regions (CDRs), of antibody combine to form the variable region that defines a three dimensional antigen-binding site. This quaternary antibody structure forms the antigen-binding site present at the end of each arm of the Y. More specifically, the antigen-binding site is defined by three CDRs on each of the VH and VL chains (i.e., HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3). In some instances, e.g., certain immunoglobulin molecules are derived from camelid species or engineered based on camelid immunoglobulins. Alternatively, an immunoglobulin molecule may consist of heavy chains only with no light chains or light chains only with no heavy chains.

**[0055]** In naturally occurring antibodies, the six CDRs present in each antigen-binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen-binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen-binding domains, referred to as “framework” regions, show less inter-molecular variability. The framework regions largely adopt a  $\beta$ -sheet conformation and the CDRs form loops which connect, and in some cases form part of, the  $\beta$ -sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been precisely defined.

**[0056]** As used herein, the terms “VH1” and “VH2” refer to immunoglobulin heavy

chain variable domains corresponding to two different binding specificities. Likewise, the terms “VL1” and “VL2” refer to light chain variable domains corresponding to two different binding specificities. When used together, it is to be understood that VH1 and VL1 regions define a common binding specificity and that VH2 and VL2 domains define a second binding specificity.

**[0057]** The term “framework region (FR)” as used herein refers to variable domain residues other than the CDR residues. Each variable domain typically has four FRs flanking the corresponding CDRs. For example, a VH domain typically has four HFRs: HFR1, HFR2, HFR3 and HFR4 flanking the three HCDRs in the configuration of HFR1-HCDR1-HFR2-HCDR2-HFR3-HCDR3-HFR4. Similarly, an LH domain typically has four LFRs flanking the three LCDRs in the configuration of: LFR1-LCDR1-LFR2-LCDR2-LFR3-LCDR3-LFR4. Exemplary FRs that may be utilized in the antagonists described herein are summarized in **FIGS. 36.**

**[0058]** Light chains are classified as either kappa or lambda (K,  $\lambda$ ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the “tail” portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

**[0059]** As used herein, the terms “light chain constant region” or “CL” are used interchangeably herein with reference to amino acid sequences derived from an antibody light chain. Preferably, the light chain constant region comprises at least one of a constant kappa domain or constant lambda domain.

**[0060]** As used herein, the term “heavy chain constant region” includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain constant region comprises at least one of: a CH1 domain, a hinge (e.g., upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. For example, antigen-binding polypeptide for use in the disclosure may comprise a polypeptide chain comprising a CH1 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain; a polypeptide chain comprising a CH1 domain and a CH3 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain, or a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, a CH2 domain, and a CH3 domain. In some embodiments, a polypeptide of the disclosure comprises a polypeptide chain comprising a

CH3 domain. Further, antibody for use in the disclosure may lack at least a portion of a CH2 domain (e.g., all or part of a CH2 domain). It should be understood that the heavy chain constant region may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

[0061] For example, as reflected in the disclosure herein below, Applicant has found that the CH3 domain can tolerate or accommodate significant insertions (e.g., greater than 100 aa) in the Fc loop of the CH3 domain (see e.g., Bi-PB-2, Bi-PLB-2 in FIG. 5B). Therefore, in the present application, any of the disclosed inhibitor domains, including but not limited to **SEQ ID NOS: 123-125**, may be similarly inserted in the Fc loop of the heavy chain in a manner analogous to the insertion of the TGF $\beta$ 1 RII ECD domain in the Fc loop.

[0062] The heavy chain constant region of antibody disclosed herein may be derived from different immunoglobulin molecules. For example, a heavy chain constant region of a polypeptide may comprise a CH1 domain derived from an IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, a heavy chain constant region can comprise a hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule.

[0063] A “light chain-heavy chain pair” refers to the collection of a light chain and heavy chain that can form a dimer through a disulfide bond between the CL domain of the light chain and the CH1 domain of the heavy chain.

[0064] The subunit structures and three dimensional configurations of the constant regions of the various immunoglobulin classes are well known. As used herein, the term “VH domain” includes the amino terminal variable domain of an immunoglobulin heavy chain and the term “CH1 domain” includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

[0065] As used herein the term “CH2 domain” includes the portion of a heavy chain molecule that extends, e.g., from about residue 244 to residue 360 of antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering system; and residues 231-340, EU numbering system). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. The CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 residues.

[0066] As used herein, the term “hinge region” includes the portion of a heavy chain

molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen-binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains.

**[0067]** As used herein the term “disulfide bond” includes a covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the CH1 and CL regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system).

**[0068]** As used herein, a “variant” of antibody, antibody fragment or antibody domain refers to antibody, antibody fragment or antibody domain that (1) shares a sequence identity of at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% with the original antibody, antibody fragment or antibody domain, and (2) binds specifically to the same target that the original antibody, antibody fragment or antibody domain binds specifically. It should be understood that where a measure of sequence identity is presented in the form of the phrase “at least x % identical” or “at least x % identity”, such an embodiment includes any and all whole number percentages equal to or above the lower limit. Further it should be understood that where an amino acid sequence is presented in the present application, it should be construed as additionally disclosing or embracing amino acid sequences having a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to that amino acid sequence.

**[0069]** As used herein, the phrase “humanized antibody” refers to antibody derived from a non-human antibody, typically a mouse monoclonal antibody. Alternatively, a humanized antibody may be derived from a chimeric antibody that retains or substantially retains the antigen binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans.

**[0070]** As used herein, the phrase “chimeric antibody,” refers to antibody where the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant disclosure) is obtained from a second species. In certain embodiments the target binding region or site will be from a non-human source (e.g., mouse or primate) and the constant region is human.

**[0071]** Included within the scope of the multispecific antibodies of the present application are various compositions and methodologies, including asymmetric IgG-like

antibodies (e.g., triomab/quadroma, Trion Pharma/Fresenius Biotech); knobs-into-holes antibodies (Genentech); Cross MAbs (Roche); electrostatically matched antibodies (AMGEN); LUZ-Y (Genentech); strand exchange engineered domain (SEED) body (EMD Serono; Biolonic, Merus); Fab-exchanged antibodies (Genmab), symmetric IgG-like antibodies (e.g. dual targeting (DT)-Ig (GSK/Domainis); two-in-one antibody (Genentech); crosslinked MAbs (Karmanos Cancer Center), mAb2 (F-star); Cov X-body (Cov X/Pfizer); dual variable domain (DVD)-Ig fusions (Abbott); IgG-like bispecific antibodies (Eli Lilly); Ts2Ab (Medimmune/AZ); BsAb (ZymoGenetics); HERCULES (Biogen Idec, TvAb, Roche); scFv/Fc fusions; SCORPION (Emergent BioSolutions/Trubion, ZymoGenetics/BMS); dual affinity retargeting technology (Fc-DART), MacroGenics; dual (scFv)2-Fabs (National Research Center for Antibody Medicine); F(ab)2 fusions (Medarex/AMGEN); dual-action or Bis-Fab (Genentech); Dock-and-Lock (DNL, ImmunoMedics); Fab-Fv (UCB-Celltech); scFv- and diabody-based antibodies (e.g., bispecific T cell engagers (BiTEs, Micromet); tandem diabodies (Tandab, Affimed); DARTs (MacroGenics); single-chain diabodies; TCR-like antibodies (AIT, Receptor Logics); human serum albumin scFv fusion (Merrimack); COMBODIES (Epigen Biotech); and IgG/non-IgG fusions (e.g., immunocytokines (EMDSerono, Philogen, ImmunGene, ImmunoMedics).

**[0072]** By “specifically binds” or “has specificity to”, it is generally meant that antibody binds to an epitope via its antigen-binding domain, and that the binding entails some complementarity between the antigen-binding domain and the epitope. According to this definition, antibody is said to “specifically bind” to an epitope when it binds to that epitope via its antigen-binding domain more readily than it would bind to a random, unrelated epitope. The term “specificity” is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody “A” may be deemed to have a higher specificity for a given epitope than antibody “B,” or antibody “A” may be said to bind to epitope “C” with a higher specificity than it has for related epitope “D.” In some embodiments, antibody or antibody fragment “has specificity to” antigen if the antibody or antibody fragment forms a complex with the antigen with a dissociation constant (Kd) of 10-6M or less, 10-7M or less, 10-8M or less, 10-9M or less, or 10-10M or less.

**[0073]** The phrase “immune checkpoint regulator” refers to a functional class of agents, which inhibit or stimulate signaling through an immune checkpoint. An “immune checkpoint regulator” includes cell surface receptors and their associated ligands, which together provide a means for inhibiting or stimulating signaling pathways associated with T-cell activation. Immune checkpoint regulators that are cell surface receptors are typically members of either the TNF receptor or B7 superfamilies that can induce checkpoint signaling

pathways to suppress immune responses, including agents which bind to negative co-stimulatory molecules including without limitation PD-1, TIGIT, LAG-3, TIM-3, BTLA, VISTA, CTLA-4, and their respective ligands.

[0074] The phrases “immune checkpoint regulator antagonist”, “immune checkpoint binding antagonist” and “immune checkpoint antagonist” are used interchangeably herein with reference to a class of agents that interfere with (or inhibit) the activity of an immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is blocked or inhibited. By inhibiting this signaling, immune-suppression can be reversed so that T cell immunity against cancer cells can be re-established or enhanced. Exemplary immune checkpoint antagonists include, but are not limited to PD-1 and its ligands, PD-L1 and PD-L2; TIGIT and its CD155 ligand, PVR; LAG-3 and its ligands, including liver sinusoidal endothelial cell lectin (LSECtin) and Galectin-3; CTLA-4 and its ligands, B7-1 and B7-2; TIM-3 and its ligand, Galectin-9; CD122 and its CD122R ligand; CD70, B7H3, B and T lymphocyte attenuator (BTLA), and VISTA. Immune checkpoint regulator antagonists may include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

[0075] The phrases “immune checkpoint binding agonist” and “immune checkpoint agonist” are used interchangeably herein with reference to a class of agents that stimulate the activity of an immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is stimulated. By stimulating this signaling, T cell immunity against cancer cells can be re-established or enhanced. Exemplary immune checkpoint regulator agonists include, but are not limited to members of the tumor necrosis factor (TNF) receptor superfamily, such as CD27, CD40, OX40 (CD 134), glucocorticoid-induced TNFR family-related protein (GITR), and 4-1BB (CD137) and their ligands. Additional checkpoint regulator agonists belong to the B7-CD28 superfamily, including CD28 and ICOS. Immune checkpoint regulator may include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

[0076] The term “antagonist antibody” refers to antibody that binds to a target and prevents or reduces the biological effect of that target. In some embodiments, the term can denote antibody that prevents the target, e.g., PD-1, to which it is bound from performing a biological function.

[0077] As used herein, an “anti-PD-1 antagonist antibody” refers to antibody that is able to inhibit PD-1 biological activity and/or downstream event(s) mediated by PD-1. Anti-

PD-1 antagonist antibodies encompass antibodies that block, antagonize, suppress or reduce (to any degree including significantly) PD-1 biological activity, including downstream events mediated by PD-1, such as PD-1 binding and downstream signaling, inhibition of T cell proliferation, inhibition of T cell activation, inhibition of IFN secretion, inhibition of IL-2 secretion, inhibition of TNF secretion, induction of IL-10, and inhibition of anti-tumor immune responses. For purposes of the present invention, it will be explicitly understood that the term “anti-PD-1 antagonist antibody” (interchangeably termed “antagonist PD-1 antibody”, “antagonist anti-PD-1 antibody” or “PD-1 antagonist antibody”) encompasses all the previously identified terms, titles, and functional states and characteristics whereby PD-1 itself, a PD-1 biological activity, or the consequences of the biological activity, are substantially nullified, decreased, or neutralized in any meaningful degree. In some embodiments, anti-PD-1 antagonist antibody binds PD-1 and upregulates anti-tumor immune response.

**[0078]** As used herein, an “anti-PD-L1 antagonist antibody” refers to antibody that is able to inhibit PD-L1 biological activity and/or downstream event(s) mediated by PD-L1. Anti-PD-L1 antagonist antibodies encompass antibodies that block, antagonize, suppress or reduce (to any degree including significantly) PD-L1 biological activity, including downstream events mediated by PD-L1, such as PD-L1 binding and downstream signaling, inhibition of T cell proliferation, inhibition of T cell activation, inhibition of IFN secretion, inhibition of IL-2 secretion, inhibition of TNF secretion, induction of IL-10, and inhibition of anti-tumor immune responses. For purposes of the present invention, it will be explicitly understood that the term “anti-PD-L1 antagonist antibody” (interchangeably termed “antagonist PD-L1 antibody”, “antagonist anti-PD-L1 antibody” or “PD-L1 antagonist antibody”) encompasses all the previously identified terms, titles, and functional states and characteristics whereby PD-L1 itself, a PD-L1 biological activity, or the consequences of the biological activity, are substantially nullified, decreased, or neutralized in any meaningful degree. The anti-PD-L1 antagonist is designed to bind PD-L1 and upregulate anti-tumor immune responses.

**[0079]** The phrases “dominant-negative protein” or “dominant-negative peptide” refer to a protein or peptide derived from a wild type protein that has been genetically modified by mutation and/or deletion so that the modified protein or peptide interferes with the function of the endogenous wild-type protein from which it is derived.

**[0080]** The phrase “VEGF binding antagonist” refers to a functional class of agents that bind to VEGF-A or its receptor, VEGFR-2, so that, as a result of the binding, activation of VEGFR-2 by VEGF-A is blocked or inhibited. As used herein, the term “VEGF binding

antagonists” include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

**[0081]** The phrase “Tie2 tyrosine kinase receptor binding antagonist” refers to a functional class of agents that bind to a Tie2 tyrosine kinase receptor or one of its ligands so that, as a result of the binding, activation of the Tie2 tyrosine kinase receptor by one or more of its ligands (i.e., Ang1, Ang2, Ang3 and Ang4) is blocked or inhibited. As used herein, the term “Tie2 tyrosine kinase receptor binding antagonist” include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate.

**[0082]** The phrase “small molecule drug” refers to a molecular entity, often organic or organometallic, that is not a polymer, that has medicinal activity, and that has a molecular weight less than about 2 kDa, less than about 1 kDa, less than about 900Da, less than about 800Da or less than about 700Da. The term encompasses most medicinal compounds termed “drugs” other than protein or nucleic acids, although a small peptide or nucleic acid analog can be considered a small molecule drug. Examples include chemotherapeutic anticancer drugs and enzymatic inhibitors. Small molecules drugs can be derived synthetically, semi-synthetically (i.e., from naturally occurring precursors), or biologically.

**[0083]** When describing polypeptide domain arrangements with hyphens between individual domains (e.g., CH2-CH3), it should be understood that the order of the listed domains is from the amino terminal end to the carboxy terminal end.

**[0084]** The term “immunoconjugate” refers to antibody which is fused by covalent linkage to an inhibitory peptide or small molecule drug. The peptide or small molecule drug can be linked to the C-terminus of a constant heavy chain or to the N-terminus of a variable light and/or heavy chain.

**[0085]** A “linker” may be used to link the peptide or small molecule drug, such as a maytansinoid, to the antitumor antagonists in a stable, covalent manner. Linkers can be susceptible to or be substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the compound or the antibody remains active. Suitable linkers are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Linkers also include GGGGS, charged linkers, and hydrophilic forms thereof as described herein and known in the art. The immunoconjugate may further include a flexible 3-15 amino acid peptide (or spacer) between an antitumor antagonist and the peptide and/or small molecule drug. In some embodiments, the linker comprises 3, 4, 5, 6, 7 or 8 repeats of GGGGS. In

some embodiments, the linker consists of 3 or 4 repeats of GGGGS.

**[0086]** As used herein, the term “immunoglobulin scaffold”, refers to any polymer of amino acids that exhibits properties desired to support the function of an antagonist, including addition of antibody specificity, enhancement of antibody function or support of antibody structure and stability. An immunoglobulin scaffold may have one or more immunoglobulin constant regions, including CH1, CH2, and/or CH3 regions from an immunoglobulin heavy chain and/or a CL region from an immunoglobulin light chain. The immunoglobulin scaffold can be grafted with binding domains of a donor polypeptide to confer the binding specificity of the donor polypeptide onto the scaffold.

**[0087]** As used herein, the phrase “multispecific inhibitor” refers to a molecule comprising at least two targeting domains with different binding specificities. In some embodiments, the multispecific inhibitor is a polypeptide comprising a scaffold and two or more immunoglobulin antigen binding domains targeting different antigens or epitopes. In certain embodiments, the multispecific inhibitor is a bispecific antibody or antagonist. In other embodiments, the multispecific inhibitor is a trispecific antibody or antagonist.

**[0088]** As used herein, the phrase “bispecific” refers to a molecule comprising at least two targeting domains with different binding specificities. Each targeting domain is capable of binding specifically to a target molecule and inhibiting a biological function of the target molecule upon binding to the target molecule. In some embodiments, the bispecific checkpoint regulator antagonist is a polymeric molecule having two or more peptides. In some embodiments, the targeting domain comprises antigen binding domain or a CDR of antibody. In some embodiments, the bispecific inhibitor is a bispecific antibody.

**[0089]** The terms “bispecific antibody,” and “bispecific antagonist” are used interchangeably herein with reference to antibody that can specifically bind two different antigens (or epitopes). In some embodiments, the bispecific antibody is a full-length antibody that binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). In these embodiments, the bispecific antibody has two distinct antigen binding arms (in both specificity and CDR sequences), and is monovalent for each antigen it binds to.

**[0090]** In other embodiments, the bispecific antibody is a full-length antibody that can bind two different antigens (or epitopes) in each of its two binding arms (two pairs of HC/LC) In these embodiments, the bispecific antibody has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen it binds to.

**[0091]** Exemplary bispecific antibodies may include asymmetric IgG-like antibodies

(e.g., triomab/quadroma, Trion Pharma/Fresenius Biotech); knobs-into-holes antibodies (Genentech); Cross MAbs (Roche); electrostatically matched antibodies (AMGEN); LUZ-Y (Genentech); strand exchange engineered domain (SEED) body (EMD Serono; biolonic, Merus); Fab-exchanged antibodies (Genmab), symmetric IgG-like antibodies (e.g. dual targeting (DT)-Ig (GSK/Domainis); two-in-one antibody (Genentech); crosslinked MAbs (Karmanos Cancer Center), mAb2 (F-star); Cov X-body (Cov X/Pfizer); dual variable domain (DVD)-Ig fusions (Abbott); IgG-like bispecific antibodies (Eli Lilly); Ts2Ab (Medimmune/AZ); BsAb (ZymoGenetics); HERCULES (Biogen Idec, TvAb, Roche); scFv/Fc fusions; SCORPION (Emergent BioSolutions/Trubion, ZymoGenetics/BMS); dual affinity retargeting technology (Fc-DART), MacroGenics; dual (scFv)2-Fabs (National Research Center for Antibody Medicine); F(ab)2 fusions (Medarex/AMGEN); dual-action or Bis-Fab (Genentech); Dock-and-Lock (DNL, ImmunoMedics); Fab-Fv (UCB-Celltech); scFv- and diabody-based antibodies (e.g., bispecific T cell engagers (BiTEs, Micromet); tandem diabodies (Tandab, Affimed); DARTs (MacroGenics); single-chain diabodies; TCR-like antibodies (AIT, Receptor Logics); human serum albumin scFv fusion (Merrimack); COMBODIES (Epigen Biotech); and IgG/non-IgG fusions (e.g., immunocytokines (EMDSerono, Philogen, ImmunGene, ImmunoMedics).

**[0092]** The terms “treat” and “treatment” refer to the amelioration of one or more symptoms associated with a cell proliferative disorder; prevention or delay of the onset of one or more symptoms of a cell proliferative disorder; and/or lessening of the severity or frequency of one or more symptoms of cell proliferative disorder.

**[0093]** The phrases “to a patient in need thereof”, “to a patient in need of treatment” or “a subject in need of treatment” includes subjects, such as mammalian subjects, that would benefit from administration of the antitumor antagonist of the present disclosure for treatment of a cell proliferative disorder.

**[0094]** The terms “therapeutically effective amount”, “pharmacologically effective amount”, and “physiologically effective amount” are used interchangeably to mean the amount of antitumor antagonist that is needed to provide a threshold level of active antagonist agents in the bloodstream or in the target tissue. The precise amount will depend upon numerous factors, e.g., the particular active agent, the components and physical characteristics of the composition, intended patient population, patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein or otherwise available in the relevant literature.

**[0095]** The terms, “improve”, “increase” or “reduce”, as used in this context, indicate values or parameters relative to a baseline measurement, such as a measurement in the same

individual prior to initiation of the treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein.

**[0096]** A “control individual” is an individual afflicted with the same cell proliferative disorder as the individual being treated, who is about the same age as the individual being treated (to ensure that the stages of the disease in the treated individual and the control individual(s) are comparable). The individual (also referred to as “patient” or “subject”) being treated may be a fetus, infant, child, adolescent, or adult human with a cell proliferative disorder.

**[0097]** The term “cell proliferative disorder” refers to a disorder characterized by abnormal proliferation of cells. A proliferative disorder does not imply any limitation with respect to the rate of cell growth, but merely indicates loss of normal controls that affect growth and cell division. Thus, in some embodiments, cells of a proliferative disorder can have the same cell division rates as normal cells but do not respond to signals that limit such growth. Within the ambit of “cell proliferative disorder” is a neoplasm, cancer or tumor.

**[0098]** The term “cancer” or “tumor” refers to any one of a variety of malignant neoplasms characterized by the proliferation of cells that have the capability to invade surrounding tissue and/or metastasize to new colonization sites, and includes leukemia, lymphoma, carcinoma, melanoma, sarcoma, germ cell tumor and blastoma. Exemplary cancers for treatment with the methods of the instant disclosure include cancer of the brain, bladder, breast, cervix, colon, head and neck, kidney, lung, non-small cell lung, mesothelioma, ovary, prostate, stomach and uterus, leukemia, and medulloblastoma.

**[0099]** The term “leukemia” refers to progressive, malignant diseases of the blood-forming organs and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Exemplary leukemias include, for example, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocytemic leukemia, basophilic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic

leukemia, promyelocytic leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, and undifferentiated cell leukemia.

**[0100]** The term “carcinoma” refers to the malignant growth of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Exemplary carcinomas include, for example, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatous, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiennoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypemephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, naspharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, and carcinoma villosum.

**[0101]** The term “sarcoma” refers to a tumor made up of a substance like the embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar or homogeneous substance. Exemplary sarcomas include, for example,

chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilns' tumor sarcoma, endometrial sarcoma, stromal sarcoma, Ewing's sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphomas (e.g., Non-Hodgkin Lymphoma), immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectatic sarcoma.

**[0102]** The term “melanoma” refers to a tumor arising from the melanocytic system of the skin and other organs. Melanomas include, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma subungual melanoma, and superficial spreading melanoma.

**[0103]** Additional cancers include, for example, Hodgkin's Disease, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insulanoma, malignant carcinoid, premalignant skin lesions, testicular cancer, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, and adrenal cortical cancer.

### **I. Bispecific Antagonists Targeting the TGF- $\beta$ pathway**

**[0104]** TGF- $\beta$  is considered the primary cytokine mediating immunosuppression through the induction and maintenance of T regulatory cells, as well as the direct suppression of innate and adaptive immune cells such as NK, DC, and T cells. TGF- $\beta$  also plays roles in neoangiogenesis and stabilizing tumor vessels, as well as directly impacting tumors through epithelial mesenchymal transition leading to cell migration and invasion. As a potent inducer of angiogenesis, TGF- $\beta$ 1 provides a critical support system for solid tumors, and plays an important role in tumor cell dissemination. Accordingly, TGF- $\beta$  status is a strong predictor of anti-PD1/PD-L1 resistance and overall survival for a variety of cancers.

**[0105]** Many cells synthesize TGF- $\beta$  and almost all of them have specific receptors for these peptides. TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 all function through the same receptor signaling system. The active form of TGF- $\beta$  is a dimer that signals through the formation of a membrane bound heterotetramer composed of the serine threonine type 1 and type 2

receptors, TGF- $\beta$  RI and TGF $\beta$  RII, respectively. Recently, TGF- $\beta$  pathway inhibitors have been developed in the form of *e.g.*, antibodies or binding fragments directed against TGF- $\beta$ 1 or TGF- $\beta$ 1 RII, such as dominant negative fusion protein fragments containing the extracellular domain (ECD) of TGF- $\beta$ 1 RII.

[0106] In one aspect, the present application provides a bispecific antagonist inhibiting both the TGF $\beta$  pathway and the PD-1/PD-L1 checkpoint regulator pathway.

[0107] In another aspect, the present application provides a bispecific antagonist inhibiting both the TGF $\beta$  pathway and the vascular endothelial growth factor (VEGF) pathway, vascular endothelial growth factor receptor (VEGFR), or both.

#### **A. Bispecific Antagonists Targeting the TGF- $\beta$ and PD-1/PD-L1 Pathways**

[0108] In some embodiments, the bispecific antagonists include a first targeting domain specifically inhibiting the TGF- $\beta$  pathway and a second targeting domain specifically binding PD-1 or PD-L1. In some embodiments, the first targeting domain comprises a TGF- $\beta$  pathway inhibitor.

##### ***TGF- $\beta$ pathway inhibitors***

[0109] The TGF- $\beta$  pathway involves a multifunctional set of peptides that control cell proliferation and differentiation, migration and adhesion, extracellular matrix modification including tumor stroma and immunosuppression, angiogenesis and desmoplasia, apoptosis, and other functions in many cell types. TGF- $\beta$  is considered the primary cytokine mediating immunosuppression through the induction and maintenance of T regulatory cells, as well as the direct suppression of innate and adaptive immune cells such as NK, DC, and T cells. TGFB also plays roles in neoangiogenesis and stabilizing tumor vessels, as well as directly impacting tumors through epithelial mesenchymal transition leading to cell migration and invasion. As a potent inducer of angiogenesis, TGF- $\beta$ 1 provides a critical support system for solid tumors, as well as a mechanism for tumor cell dissemination.

[0110] TGF- $\beta$  status is a strong predictor of anti-PD1/PD-L1 resistance and overall survival for a variety of cancers. For example, high TGF- $\beta$ 1 levels are associated with poor response to PD1/PD-L1 inhibition in metastatic urothelial cancer patients (Nature (2018) 554(7693):544-548), and a high TGF $\beta$  signature is associated with poor prognosis across 33 different cancer types (Immunity (2018) 48:812-830). Inhibition of TGF $\beta$  with anti-PD1/PD-L1 further releases CD8 effector cells to kill tumor cells, as well as stimulate other cell types to increase tumor killing capacity.

[0111] Many cells synthesize TGF- $\beta$  and almost all of them have specific receptors for these peptides. TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 all function through the same receptor signaling system. The active form of TGF- $\beta$  is a dimer that signals through the formation of a membrane

bound heterotetramer composed of the serine threonine type 1 and type 2 receptors, TGF- $\beta$  RI and TGF $\beta$  RII, respectively.

[0112] As used herein, a TGF- $\beta$  pathway inhibitor may be in the form of *e.g.*, antibodies or variable domain fragments directed against TGF- $\beta$ 1 or a TGF- $\beta$ 1 RII, a TGF binding peptide, or dominant negative fusion protein fragment, such as the extracellular domain (ECD) of TGF- $\beta$ 1 RII.

TGF- $\beta$ 1 RII ECD

[0113] In some embodiments, the TGF- $\beta$  pathway inhibitor comprises a TGF- $\beta$ 1 RII ECD. An exemplary human TGF- $\beta$ 1 RII ECD (wild-type) has the amino acid sequence set forth in **SEQ ID NO:89**.

[0114] In some embodiments, the TGF- $\beta$  pathway inhibitor is fused to the carboxy-terminus of an IgG in a bispecific antitumor antagonist, as depicted in *e.g.*, **FIGs. 5A-5B** and **8A-8C**. Alternatively, the TGF- $\beta$ 1 RII ECD may be fused to the amino-terminus of an IgG in a bispecific antitumor antagonist. In yet other embodiments, the TGF- $\beta$ 1 RII ECD is inserted within the IgG Fc receptor (*i.e.*, CH2 or CH3 regions) of an IgG as depicted in *e.g.*, **FIG. 5B**.

[0115] In some embodiments, anti-TGF- $\beta$ 1, anti-TGF- $\beta$ 1 RII antibodies or variable region fragments thereof may be used in place of a TGF- $\beta$ 1 RII ECD. Exemplary anti-TGF- $\beta$ 1 antibodies are described in U.S. Patent Nos. 7,067,637, 7,494,651, 7,527,791, and 7,619,069. Exemplary anti-TGF- $\beta$ 1 RII antibodies are described in U.S. Patent No. 7,579,186. Alternatively, a TGF- $\beta$ 1 RII ECD may be substituted with one or more anti-TGF- $\beta$ 1 and/or anti-TGF- $\beta$ 1 RII peptide inhibitors. An exemplary TGF- $\beta$ 1 peptide inhibitor is KRIWFIPRSSWYERA (**SEQ ID NO: 111**).

***Anti-PD-1 antibody and anti-PD-1 antibody fragments***

[0116] In some embodiments, the bispecific TGF $\beta$  pathway antagonist includes an anti-PD-1 antibody or antibody fragment. In one embodiment, the PD-1 inhibitor for use in the present application is antibody, or antigen-binding portion thereof, comprising: an immunoglobulin heavy chain complementarity determining region 1 (CDR1) sequence selected from the group consisting of **SEQ ID NOS: 1, 4, 7, 9, and 12**; an immunoglobulin heavy chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 2, 5, 10, and 13**; an immunoglobulin heavy chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 3, 6, 8, 11, and 14**; an immunoglobulin light chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 15, 18, 21-23, and 26**; an immunoglobulin light chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 16, 19, 24, and 27**; or an immunoglobulin light chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 17, 20, 25, and 28**.

[0117] In another embodiment, the PD-1 inhibitor for use in the present application (as e.g., the second targeting domain) is antibody, or antigen-binding portion thereof, comprising: an immunoglobulin heavy chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 1, 4, 7, 9, and 12**; an immunoglobulin heavy chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 2, 5, 10, and 13**; an immunoglobulin heavy chain CDR3 sequence from the group consisting of **SEQ ID NOS: 3, 6, 8, 11, and 14**; an immunoglobulin light chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 15, 18, 21-23, and 26**; an immunoglobulin light chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 16, 19, 24, and 27**; and an immunoglobulin light chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 17, 20, 25, and 28**.

[0118] In another embodiment, the PD-1 inhibitor for use in the present application includes: an immunoglobulin heavy chain variable region (HCVR) having at least 80%, 85%, 90%, 95%, or 99% identity to an HCVR amino acid sequence selected from the group consisting of **SEQ ID NOS: 29, 31, 33, 35, 37, and 39**; an immunoglobulin light chain variable region (LCVR) having at least 80%, 85%, 90%, 95%, or 99% identity to an LCVR amino acid sequence selected from the group consisting of **SEQ ID NOS: 30, 32, 34, 36, 38, and 40**; or both.

[0119] In another embodiment, the PD-1 inhibitor for use in the present application includes: an HCVR amino acid sequence selected from the group consisting of **SEQ ID NOS: 29, 31, 33, 35, 37, and 39**; an LCVR amino acid sequence selected from the group consisting of **SEQ ID NOS: 30, 32, 34, 36, 38, and 40**; or both.

[0120] In one embodiment, the PD-1 inhibitor includes: an HCVR having at least 80%, 85%, 90%, 95%, or 99% identity to the HCVR amino acid sequence of **SEQ ID NO:39**; an LCVR having at least 80%, 85%, 90%, 95%, or 99% identity to the LCVR amino acid sequence of **SEQ ID NO:40**; or both. In a more particular embodiment, the PD-1 inhibitor has an HCVR sequence of **SEQ ID NO:39**; an LCVR amino acid sequence of **SEQ ID NO:40**; or both.

[0121] In another embodiment, the PD-1 inhibitor has an immunoglobulin HCVR that comprises: (1) a heavy chain CDR1 of **SEQ ID NO:12**, a heavy chain CDR2 of **SEQ ID NO:13**, and a heavy chain CDR3 of **SEQ ID NO:14**; and (2) a heavy chain framework region (FR)1 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:150**, a heavy chain FR2 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:144**, an HFR3 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:155**, an HFR4 having at least 80%, 85% or 90% identity to the amino acid sequence of **SEQ ID NO:141**, and an immunoglobulin heavy chain variable region that comprises (1) a light chain CDR1 of **SEQ ID NO:26**, an

LCDR2 of **SEQ ID NO:27** and an LCDR 3 of **SEQ ID NO:28** and (2) an LFR1 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:156**, an LFR2 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:157**, an LFR3 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:158**, and an LFR4 having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:159**.

**[0122]** In another embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-1 inhibitor comprising: an immunoglobulin heavy chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:92** or **SEQ ID NO:106**; a light chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:93**; or both. In a more particular embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-1 inhibitor comprising: an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:92** or **SEQ ID NO:106**; an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:93**; or both.

**[0123]** In another embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-1 inhibitor comprising: an immunoglobulin heavy chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:94** or **SEQ ID NO:126**; an immunoglobulin light chain having at least 80%, 85%, 90%, 95%, or 99% identity to the LCVR amino acid sequence of **SEQ ID NO:95**; or both. In a more particular embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-1 inhibitor comprising: an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:94** or **SEQ ID NO:126**; an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:95**; or both. In another embodiment, the PD-1/TGF- $\beta$ 1 antitumor antagonist includes a TGF- $\beta$ 1 RII ECD inserted within a CH3 loop as depicted in **FIG. 5B**.

**[0124]** In one embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-1 inhibitor comprising: a heavy chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:107**; a light chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:108**; or both. In a more particular embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-1 inhibitor comprising: an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:107**; an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:108**; or both.

**[0125]** Other PD-1 targeted antagonists include anti-PD-1 antibodies, such as nivolumab (BMS-936558, MDX-1106, OPDIVO<sup>TM</sup>), a humanized immunoglobulin G4 (IgG4) mAb (Bristol-Myers Squibb); pembrolizumab (MK-3475, lambrolizumab, KEYTRUDA<sup>TM</sup>)(Merck); pidilizumab (CT-011)(Medivation); and AMP-224 (Merck). Anti-PD-1 antibodies are

commercially available, for example from ABCAM (AB137132), BIOLEGEND™ (EH12.2H7, RMP1-14) and AFFYMETRIX EBIOSCIENCE (J105, J116, MIH4). Anti-PD-1 targeted antitumor antagonists may include HCVRs, LCVR, and CDRs derived from any of the anti-PD-1 antibodies described herein, including those described in **FIGS. 1-2**.

***Anti-PD-L1 antibody and anti-PD-L1 antibody fragments***

**[0126]** In some embodiments, the bispecific TGF-β1 pathway antagonist further includes an anti-PD-L1 antibody or antibody fragment. In one embodiment, the PD-L1 inhibitor for use in the present application is antibody, or antigen-binding portion thereof, comprising: an immunoglobulin heavy chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 41, 44, 50, and 53**; an immunoglobulin heavy chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 42, 45, 47, 49, 51, and 54**; an immunoglobulin heavy chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 43, 46, 48, 52, and 55**; an immunoglobulin light chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 56, 59, 63, 66, and 69**; an immunoglobulin light chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 57, 60, 64, 67, and 70**; or an immunoglobulin light chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 58, 61, 62, 65, 68, and 71**.

**[0127]** In another embodiment, the PD-L1 inhibitor for use in the present application (as *e.g.*, the second targeting domain) is antibody, or antigen-binding portion thereof, comprising: an immunoglobulin heavy chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 41, 44, 50, and 53**; an immunoglobulin heavy chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 42, 45, 47, 49, 51, and 54**; an immunoglobulin heavy chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 43, 46, 48, 52, and 55**; an immunoglobulin light chain CDR1 sequence selected from the group consisting of **SEQ ID NOS: 56, 59, 63, 66, and 69**; an immunoglobulin light chain CDR2 sequence selected from the group consisting of **SEQ ID NOS: 57, 60, 64, 67, and 70**; and an immunoglobulin light chain CDR3 sequence selected from the group consisting of **SEQ ID NOS: 58, 61, 62, 65, 68, and 71**.

**[0128]** In another embodiment, the PD-L1 inhibitor for use in the present application includes: an immunoglobulin HCVR having at least 80%, 85%, 90%, 95%, or 99% identity to an HCVR amino acid sequence selected from the group consisting of **SEQ ID NOS: 72, 74, 76, 78, 80, 82, 84, and 86**; an immunoglobulin LCVR having at least 80%, 85%, 90%, 95%, or 99% identity to an LCVR amino acid sequence selected from the group consisting of **SEQ ID NOS: 73, 75, 77, 79, 81, 83, 85, and 87**; or both. In another embodiment, the PD-L1 inhibitor for use in the present application includes: an HCVR having an amino acid sequence selected from the group consisting of **SEQ ID NOS: 72, 74, 76, 78, 80, 82, 84, and 86**; an LCVR having an

amino acid sequence selected from the group consisting of **SEQ ID NOS: 73, 75, 77, 79, 81, 83, 85, and 87**; or both.

**[0129]** In one embodiment, the PD-L1 inhibitor includes: an HCVR having at least 80%, 85%, 90%, 95%, or 99% identity to the HCVR amino acid sequence of **SEQ ID NO:86**; an LCVR having at least 80%, 85%, 90%, 95%, or 99% identity to the LCVR amino acid sequence of **SEQ ID NO:87**; or both. In a more particular embodiment, the PD-L1 inhibitor includes: an HCVR having the amino acid sequence of **SEQ ID NO:86**; an LCVR having the amino acid sequence of **SEQ ID NO:87; or both**.

**[0130]** In another embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-L1 inhibitor comprising: an immunoglobulin heavy chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:94 or SQ ID NO:126**; an immunoglobulin light chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:95**; or both. In a more particular embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-L1 inhibitor comprising: an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:94 or SQ ID NO:126**; an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:95**; or both.

**[0131]** In another embodiment, the PD-L1/ TGF- $\beta$ 1 antitumor antagonist includes a TGF- $\beta$ 1 RII ECD inserted within a CH3 loop as depicted in **FIG. 5B**.

**[0132]** In a particular embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-L1 inhibitor comprising: an immunoglobulin heavy chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:109**; an immunoglobulin light chain having at least 80%, 85%, 90%, 95%, or 99% identity to the amino acid sequence of **SEQ ID NO:110**; or both. In a more particular embodiment, the TGF- $\beta$ 1 antitumor antagonist includes a PD-L1 inhibitor comprising: an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:109**; an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:110**, or both.

**[0133]** Other anti-PD-L1 targeted antagonists include anti-PD-L1 antibodies, such as atezolizumab (MPDL3280A, RG7446), a fully human IgG4 mAb Genentech/Roche); BMS-936559 (MDX-1105), a fully humanized IgG4 mAb (Bristol-Myers Squibb); MEDI4736, a humanized IgG antibody (Medimmune/AstraZeneca); and MSB0010718C, a fully human IgG4 monoclonal antibody (Merck, EMD Serono). Anti-PD-L1 targeted antitumor antagonists may include HCVRs, LCVR, and CDRs derived from any of the anti-PD-L1 antibodies described herein, including those described in **FIGs. 3-4**.

***B. Bispecific Antagonists Targeting TGF- $\beta$ 1 and Angiogenesis Pathway(s)***

[0134] In another aspect, a bispecific antitumor antagonist of the present application includes a first targeting domain specifically binding TGF $\beta$ 1 or TGF $\beta$ 1 RII, and a second targeting domain specifically binding VEGF-A, VEGFR, Ang1, Ang2, Tie2R, or a combination thereof. In these embodiments, any of the above described TGF $\beta$ 1- or TGF $\beta$ 1 RII-binding fragments may be used in combination with the second targeting domain.

#### *Angiogenesis Pathways*

[0135] Angiogenesis, the development of new blood vessels from pre-existing vessels, is essential for tumor growth and metastasis. Angiogenesis inhibition presents a potentially valuable strategy for treating diseases, such as cancer, in which progression (*e.g.*, metastasis) is dependent on neovascularization. Inhibition of angiogenesis leads to tumor cell death, which may feed tumor antigen into host antigen presentation pathways. Angiogenesis pathway inhibitors may be in the form of *e.g.*, antibodies, variable domain fragments, or dominant negative fusion protein fragments.

##### *1. VEGF/VEGFR pathway*

[0136] The principal VEGF pathway is mediated by the transmembrane tyrosine kinase VEGF-R2. Various isoforms of VEGF, particularly VEGF-A, bind to VEGFR-2, resulting in dimerization and activation through phosphorylation of various downstream tyrosine kinases.

[0137] In some embodiments, the antagonist of the present application include a VEGF pathway antagonist that binds to VEGF-A or its receptor VEGFR-2 so that, as a result of the binding, activation of VEGFR-2 by VEGF-A is blocked or inhibited.

[0138] In one embodiment, the TGF $\beta$  pathway antagonist further includes VEGF pathway antagonist in the form of a dominant negative VEGFR antagonist corresponding to the extracellular domain (ECD) of human VEGF receptor 1 or 2. In a particular embodiment, the TGF $\beta$  pathway antagonist includes afibbercept (also known as Zaltrap), a recombinant fusion protein containing VEGF-A binding portions from the extracellular domains of human VEGF receptors 1 and 2 fused to the human IgG1 Fc portion. VEGFR ECDs, such as afibbercept act as soluble receptor decoys for VEGF-A.

[0139] In one embodiment, the bispecific antitumor antagonist includes a first targeting domain comprising a TGF $\beta$  pathway inhibitor, and a second targeting domain specifically binding VEGF-A, wherein the second targeting domain comprises afibbercept. A suitable source of afibbercept comprises the amino acid sequence set forth in **SEQ ID NO:88**. In some embodiments, a bispecific antitumor antagonist includes a modified immunoglobulin heavy chain comprising an amino terminal afibbercept domain linked to a TGF- $\beta$ 1 RII ECD at the carboxy-terminal end via an IgG1 or IgG4 Fc receptor (Bi-ZB-1) as shown in **FIG. 8C**. In one embodiment, the modified immunoglobulin heavy chain has the amino acid sequence of **SEQ**

**ID NO:105.** Alternatively, the TGF- $\beta$ 1 RII ECD may be positioned at the amino terminal end and the afibbercept domain may be positioned at the carboxy-terminal end.

**[0140]** In another embodiment, the TGF $\beta$  pathway antagonist further includes VEGF pathway antagonist comprising anti-VEGFA or anti-VEGFR-2 variable region sequences, such as those derived from bevacizumab. Bevacizumab (AVASTIN<sup>TM</sup>) is a humanized antibody comprising mutated human IgG1 framework regions (FRs) and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF-A to VEGFR-2. Approximately 93% of the amino acid sequence of bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 Daltons and is glycosylated. In one embodiment, an HCVR of bevacizumab for use in the present application has the amino acid sequence set forth in **SEQ ID NO:90**, and an LCVR of bevacizumab has the amino acid sequence set forth in **SEQ ID NO:91**.

**[0141]** In some embodiments, amino acid substitutions may be included in a bevacizumab/AVASTIN antibody or fragment thereof as described in U.S. Patent No. 7,575,893. Exemplary amino acid substitutions include, but are not limited to E1Q, E6Q, L11V, Q13K, L18V, R19K, A23K, or combinations thereof. In one embodiment, a mutant HCVR of bevacizumab for use in the present application has the amino acid sequence set forth in **SEQ ID NO:96**, which may be used in conjunction with the LCVR in **SEQ ID NO:91**.

**[0142]** In one embodiment, the bispecific antitumor antagonist includes a first targeting domain specifically binding TGF $\beta$ 1, such as a TGF $\beta$ 1 RII ECD (or containing anti-TGF  $\beta$ 1- or anti- TGF $\beta$ 1 RII variable domains), and a second targeting domain specifically binding VEGFA or VEGFR2, such as bevacizumab or any other anti-VEGF or anti-VEGFR2 antibodies, variable region fragments thereof, or functionally active mutant fragments thereof.

**[0143]** In a particular embodiment, a bispecific antitumor TGF- $\beta$ /VEGF-VEGFR2 antagonist includes an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:102**, an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:103**, or both, such as Bi-AB-1 (**FIG. 8A**), which contains wild-type bevacizumab variable region sequences.

**[0144]** In another embodiment, a bispecific antitumor TGF- $\beta$  antagonist further includes an immunoglobulin heavy chain having the amino acid sequence of **SEQ ID NO:104**, an immunoglobulin light chain having the amino acid sequence of **SEQ ID NO:103**, or both, such as Bi-A1B-1 (**FIG. 8B**), which contains a mutant bevacizumab variable region sequence.

**[0145]** Additional anti-VEGF or anti-VEGFR antibodies or fragments thereof may be based on ranibizumab (trade name Lucentis<sup>TM</sup>), a monoclonal antibody fragment derived from the same parent murine antibody as bevacizumab; the G6 or B20 series antibodies (*e.g.*, G6-23, G6-31, B20-4.1) described in U.S. Publication No. 2006/0280747, 2007/0141065 and/or 2007/0020267, as well as the antibodies described in U.S. Patent Nos. 7,297,334, 7,060,269, 6,884,879, 6,582,959, 6,703,020; 6,054,297; U.S. Patent Application Publication Nos. U.S. 2007/059312, U.S. 2006/009360, U.S. 2005/0186208, U.S. 2003/0206899, U.S. 2003/0190317, and U.S. 2003/0203409.

**[0146]** An exemplary anti-VEGFR-2 antibody antagonist is the humanized IgG1 monoclonal antibody, Ramucirumab, which binds to the extracellular domain of VEGFR-2, thereby blocking its interaction with VEGF-A. Additional anti-VEGFR-2 antibodies are described in U.S. Patent Nos. 7,498,414, 6,448,077 and 6,365,157.

**[0147]** In some embodiments, the antitumor antagonists may further include one or more small molecule antagonists of the VEGF pathway, such as multikinase inhibitors of VEGFR-2, including sunitinib, sorafenib, cediranib, pazonpanib and nintedanib.

## **2. Ang-Tie2R pathway**

**[0148]** In some embodiments, the bispecific antagonist includes a targeting domain containing at least one Tie2 receptor binding antagonist. A Tie2 tyrosine kinase receptor binding antagonist binds to the Tie2 tyrosine kinase receptor or one of its ligands (*i.e.*, Ang1, Ang2, Ang3 and Ang4) so that, as a result of the binding, activation of the Tie2 tyrosine kinase receptor by one or more of its ligands is blocked or inhibited. Like VEGF, angiopoietin 2 (Ang2) is a critical player in tumor angiogenesis. ANG2 and VEGF also work together to prevent antigen presentation in dendritic cells and macrophages, enhance Treg accumulation, and suppress Teff accumulation. It has been previously shown that inhibiting both VEGF and Ang2 improves survival in a mouse breast cancer model, Tg MMTV-PyMT. Moreover, the addition of anti-PD1 further improves responses.

**[0149]** Tie2 tyrosine kinase receptor binding antagonists for use in the present application may include antibody fragments, peptide inhibitors, dominant negative peptides and small molecule drugs, either in isolated forms or as part of a fusion protein or conjugate. In one embodiment, the Tie2 receptor binding antagonist is an inhibitory peptide from trebananib, TBN-P. In a specific embodiment, the inhibitory peptide comprises the amino acid sequence:

AQQEECEWDPWTCEHMGSGSATGGSGSTASSGSGSATHQEECEWDPWTCEHMLE  
**(SEQ ID NO:113)**. In another embodiment, the Tie2 receptor binding antagonist comprises **SEQ ID NO:114**.

**[0150]** Other peptide inhibitors of Tie2 activation (including Ang-2 inhibitors) for use in the present application include A-11 (Compugen), which comprises the amino acid sequence ETFLSTNKLENQ (**SEQ ID NO:123**); the CVX-060 peptide QK(Ac)YQPLDEK(Ac)DK(0P)TLYDQFMLQQG (**SEQ ID NO:124**, Pfizer); the CVX-037 peptide (DFB)TNFMPMDDLEK(0P)RLYEQFILQQG (**SEQ ID NO:125**, Pfizer); and CGEN-25017 (Compugen). Additional peptide inhibitors of Tie2 activation are described in U.S. Patent No. 7,138,370.

**[0151]** Antibody inhibitors of Tie2 activation (and/or angiopoietin-2) for use in the present application include AMG-780 (Amgen), MEDI-3617 (MedImmune/AstraZeneca), DX-2240 (Dyax/Sanofi-Aventis), REGN-910 (Sanofi/Regeneron), RG7594 (Roche), LC06 (Roche), TAvi6 (Roche), AT-006 (Roche/Affitech). Additional Tie2 receptor binding antibody antagonists and antibody binding sequences therefrom are described in U.S. Patent Nos. 7,521,053, 7,658,924, and 8,030,025, as well as U.S. Patent Application Publication Nos. 2013/0078248, 2013/0259859, and 2015/0197578.

**[0152]** Tie2 binding antagonists for use in the present application may further include the small molecule inhibitors, CGI-1842 (CGI Pharmaceuticals), LP-590 (Locus Pharmaceuticals), ACTB-1003 (Act Biotech/Bayer AG), CEP-11981 (Cephalon/Teva), MGCD265 (MethylGene), Regorafenib (Bayer), Cabozantinib/XL-184/BMS-907351 (Exelixis), Foretnib (Exelixis), MGCD-265 (MethylGene Inc.)

**[0153]** In certain particular embodiments, the bispecific checkpoint regulator antagonist is a full-length antibody that binds human PD-1 or PD-L1 on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). In these embodiments, the bispecific antibody has two distinct antigen binding arms (in both specificity and CDR sequences), and is monovalent for each antigen it binds to.

**[0154]** In some embodiments, the bispecific checkpoint regulator antagonist is a full-length antibody that can bind human PD-1 and/or PD-L1 in each of its two binding arms (a pair of HC/LC). In these embodiments, the bispecific checkpoint regulator antagonist has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen it binds to.

#### ***Immunoglobulin and non-immunoglobulin scaffolds***

**[0155]** The bispecific antitumor antagonists of the present application may be constructed with an immunoglobulin backbone or non-immunoglobulin scaffold as described herein. In some embodiments, the bispecific antagonists of the present application are constructed with an immunoglobulin scaffold, such as an IgG1, IgG2 or IgG4 scaffold

comprising CH1, CH2 and/or CH3 domains. Use of an IgG1 backbone is preferable for cancer treatment where a target is present on antigen presenting cells that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Use of an IgG4 backbone allows targeting of the antigen where antigen binding alone is sufficient to generate the desired therapeutic benefits. IgG4-based antagonists preclude undesirable effector functions associated with *e.g.*, IgG1 antibodies, including Fc $\gamma$ R binding and complement activation.

**[0156]** Preferably, the first and second targeting domains are presented in a humanized IgG1 or IgG4 scaffold. Further, the second targeting domain may be fused to the carboxy-terminal end of an IgG1 or IgG4 scaffold. Additionally, the IgG1 or IgG4 scaffold may have a N297A or K447A amino acid substitution. In some embodiments, the first targeting domain may comprise one or more framework regions comprising one or more amino acid substitutions selected from the group consisting of E1Q, E6Q, L11V, Q13K, L18V, R19K, A23K, or any combination thereof. In other embodiments, one or more amino acid residues in the IgG1 or IgG4 scaffold are deglycosylated or mutated to produce aglycosylated variants thereof. Exemplary immunoglobulin scaffolds for use in the bispecific molecules described herein may be selected from the group consisting of **SEQ ID NOS: 103, 104, 105, 115-122, and 127-129**.

**[0157]** Any one of the antibodies or antagonists can be configured in the form of a monoclonal antibody, chimeric antibody, humanized antibody, scFv, or multi-specific antibody. In addition, any of the antibody antagonists described herein may include multiple binding specificities targeting PD-1, PD-L1, VEGF, VEGFR, Angiopoietin, and/or Tie2R. Moreover, any of the antibody antagonists may be engineered to target multiple epitopes in a given target. Furthermore, in some embodiments, the checkpoint antagonist and/or angiogenesis specificity may be included in the form of a dominant negative fusion protein, such as an extracellular domain (ECD) from a corresponding receptor.

**[0158]** The HCVRs and LCVRs described herein may be linked to a naturally-occurring Fc region or a non-naturally occurring or mutated Fc region, *e.g.*, an effectorless or mostly effectorless Fc (*e.g.*, human IgG2 or IgG4) or, alternatively, an Fc with enhanced binding to one or more activating Fc receptors (Fc $\gamma$ RI, Fc $\gamma$ RIIa or Fc $\gamma$ RIIIa) so as to enhance T<sub>reg</sub> depletion in the tumor environment. Accordingly, in certain embodiments the anti-PD-1, anti-PD-L1, and/or anti-VEGF HCVRs and LCVRs described herein may be linked to an Fc comprising one or more modifications, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, antibody described herein may be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or

it may be modified to alter its glycosylation, to alter one or more functional properties of the antibody. More specifically, in certain embodiments, the antibodies in the present application may include modifications in the Fc region in order to generate an Fc variant with (a) increased or decreased antibody-dependent cell-mediated cytotoxicity (ADCC), (b) increased or decreased complement mediated cytotoxicity (CDC), (c) increased or decreased affinity for C1q and/or (d) increased or decreased affinity for a Fc receptor relative to the parent Fc. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. Combining amino acid modifications is thought to be particularly desirable. For example, the variant Fc region may include two, three, four, five, etc. substitutions therein, *e.g.*, of the specific Fc region positions identified herein.

**[0159]** For uses where effector function is to be avoided altogether, *e.g.*, when antigen binding alone is sufficient to generate the desired therapeutic benefit, and effector function only leads to (or increases the risk of) undesired side effects, IgG4 antibodies may be used, or antibodies or fragments lacking the Fc region or a substantial portion thereof can be devised, or the Fc may be mutated to eliminate glycosylation altogether (*e.g.*, N297A). Alternatively, a hybrid construct of human IgG2 (CH1 domain and hinge region) and human IgG4 (CH2 and CH3 domains) may be generated that is devoid of effector function, lacking the ability to bind Fc $\gamma$ Rs (like IgG2) and activate complement (like IgG4). When using an IgG4 constant domain, it is usually preferable to include the substitution S228P, which mimics the hinge sequence in IgG1 and thereby stabilizes IgG4 molecules, reducing Fab-arm exchange between the therapeutic antibody and endogenous IgG4 in the patient being treated.

**[0160]** In certain embodiments, the anti-PD-1-, anti-PD-L1-, anti-VEGF-, anti-angiopoietin-, and/or or anti-Tie2R antibodies or fragments thereof may be modified to increase its biological half-life. Various approaches may be employed, including *e.g.*, those that increase the binding affinity of the Fc region for FcRn. In one embodiment, the antibody is altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Pat. Nos. 5,869,046 and 6,121,022. The numbering of residues in the Fc region is that of the EU index. Sequence variants disclosed herein are provided with reference to the residue number followed by the amino acid that is substituted in place of the naturally occurring amino acid, optionally preceded by the naturally occurring residue at that position. Where multiple amino acids may be present at a given position, *e.g.*, if sequences differ between naturally occurring isotypes, or if multiple mutations may be substituted at the position, they are separated by slashes (*e.g.*, “X/Y/Z”).

**[0161]** Exemplary Fc variants that increase binding to FcRn and/or improve pharmacokinetic properties include substitutions at positions 259, 308, and 434, including for example 259I, 308F, 428L, 428M, 434S, 434H, 434F, 434Y, and 434M. Other variants that increase Fc binding to FcRn include: 250E, 250Q, 428L, 428F, 250Q/428L (Hinton et al., 2004, *J. Biol. Chem.* 279(8): 6213-6216, Hinton et al. 2006 *Journal of Immunology* 176:346-356), 256A, 272A, 305A, 307A, 311A, 312A, 378Q, 380A, 382A, 434A (Shields et al. (2001) *J. Biol. Chem.*, 276(9):6591-6604), 252F, 252Y, 252W, 254T, 256Q, 256E, 256D, 433R, 434F, 434Y, 252Y/254T/256E, 433K/434F/436H (Dall'Acqua et al. (2002) *J. Immunol.*, 169:5171-5180, Dall'Acqua et al. (2006) *J. Biol. Chem.*, 281:23514-23524, and U.S. Pat. No. 8,367,805.

**[0162]** Modification of certain conserved residues in IgG Fc (1253, H310, Q311, H433, N434), such as the N434A variant (Yeung et al. (2009) *J. Immunol.* 182:7663), have been proposed as a way to increase FcRn affinity, thus increasing the half-life of the antibody in circulation (WO 98/023289). The combination Fc variant comprising M428L and N434S has been shown to increase FcRn binding and increase serum half-life up to five-fold (Zalevsky et al. (2010) *Nat. Biotechnol.* 28:157). The combination Fc variant comprising T307A, E380A and N434A modifications also extends half-life of IgG1 antibodies (Petkova et al. (2006) *Int. Immunol.* 18:1759). In addition, combination Fc variants comprising M252Y-M428L, M428L-N434H, M428L-N434F, M428L-N434Y, M428L-N434A, M428L-N434M, and M428L-N434S variants have also been shown to extend half-life (U.S. 2006/173170). Further, a combination Fc variant comprising M252Y, S254T and T256E was reported to increase half-life-nearly 4-fold. Dall'Acqua et al. (2006) *J. Biol. Chem.* 281:23514.

#### *Homodimers and heterodimers*

**[0163]** One of the challenges for efficiently producing bispecific antibody preparations concerns mispairing of heavy and light chains, when co-expressing chains of different binding specificities. **Table 1** lists several amino acid substitution options for overcoming mispairing between heavy chains of different binding specificities, which "enforce" or preferentially promote correct association between desired heavy chains. Any approach to prevent or reduce mispairing between heavy chains may be used to make the bispecific antitumor antagonists according to the present disclosure.

**[0164]** The "knobs-into-hole" (KiH) approach relies on modifications of the interface between the two CH3 domains where most interactions occur. Typically, a bulky residue is introduced into the CH3 domain of one antibody heavy chain and acts similarly to a key. In the other heavy chain, a "hole" is formed that is able to accommodate this bulky residue,

mimicking a lock. The resulting heterodimeric Fc-part can be further stabilized by artificial disulfide bridges.

**[0165]** An alternative approach is based on charged residues with ionic interactions or steric complementarity. This includes altering the charge polarity in the CH3 interface so that co-expression of electrostatically matched Fc domains support favorable attractive interactions and heterodimer formation while retaining the hydrophobic core, whereas unfavorable repulsive charge interactions suppress homodimerization. See **Table 1**. The amino acid numbering in **Table 1** follows the Kabat numbering scheme and can be applied to heavy chain amino acid sequences of the antibodies described herein.

**[0166]** In a further approach, the bispecific molecules of the present application may be constructed with a non-immunoglobulin scaffold containing leucine zipper (LZ) domains. A leucine zipper is a common three-dimensional structural motif in proteins, typically as part of a DNA-binding domain in various transcription factors. A single LZ typically contains 4-5 leucine residues at approximately 7-residue intervals, which forms an amphipathic alpha helix with a hydrophobic region running along one side. In a particular embodiment, a heterodimeric protein scaffold comprises a LZ from the c-jun transcription factor associated with a LZ from the c-fos transcription factor. Although c-jun is known to form jun-jun homodimers and c-fos does not form homodimers, the formation of jun-fos heterodimers is greatly favored over jun-jun homodimers.

**[0167]** A leucine zipper domain may be incorporated in place of CH2-CH3 sequences in the protein scaffold or it may be placed at the carboxy terminal end of the two heavy chains in the bispecific antitumor antagonist. In the case of the latter, a furin cleavage site may be introduced between the carboxy terminal end of CH3 and the amino terminal end of the leucine zipper. This can facilitate furin-mediated cleavage of the leucine zipper following the heterodimerization step when co-expressing the heavy and light chains of the bispecific antitumor antagonist in an appropriate mammalian cell expression system (see Wranik et al., *J. Biol. Chem.*, 287(5):43331-43339, 2012).

**Table 1**

Type	HC1	HC2
Knobs-into-holes	Y349C, T366S, L368A, Y407V	S354C, T366W
Ionic, electrostatic	S183E, E356K, E357K, D399K	S183K, K370E, K409D, K439E
Ionic, electrostatic	K392D, K409D	E356K, D399K
HA-TF substitutions	S364H, F405A	Y349T, T394F
HF-TA substitutions	S364H, T394F	Y349T, F405A
Leucine zipper heterodimer	human c-Jun leucine zipper	human c-fos leucine zipper

[0168] The amino acid numbering in **Table 1** follows the Kabat numbering scheme and can be applied to heavy chain amino acid sequences of the antibodies described herein. The mutations described in Table 1 may be applied to the sequence (published or otherwise) of any immunoglobulin IgG1 heavy chain, as well as other immunoglobulin classes, and subclasses (or isotypes) therein.

[0169] When co-expressing heavy and light chains of bispecific antibodies, the light chains of one binding specificity can also mispair with heavy chains of a different binding specificity. Therefore, in certain embodiments, portions of the heavy chain, light chain or both may be modified relative to the "wild-type" antibody chains from which they are derived to prevent or reduce mispairing of both heavy chain constant regions to one another, as well mispairing of light chain constant regions to their heavy chain counterparts.

[0170] The light chain mispairing problem can be addressed in several ways. In some embodiments, sterically complementary mutations and/or disulfide bridges may be incorporated into the two VL/VH interfaces. In other embodiments, mutations can be incorporated based on ionic or electrostatic interactions. In some embodiments, light chain mispairing may be prevented or reduced by employing a first arm with an S183E mutation in the CH1 domain of the heavy chain and an S176K mutation in the CL domain of the light chain. A second arm may include an S183K mutation in the CH1 domain of the heavy chain and an S176E mutation in the CL domain of the light chain. In other embodiments, a "CrossMab" approach is employed, where one arm in the bispecific antitumor antagonist (e.g., Fab) is left untouched, but in the other arm containing the other binding specificity, one or more domains in the light chain are swapped with one or more domains in the heavy chain at the heavy chain:light chain interface.

[0171] Methods, immunoglobulin domain sequences, including specific mutations for preventing mispairing of heavy and light chains as disclosed above are further described in U.S. Patent Application Publication Nos. 2014/0243505, 2013/0022601.

### ***Conjugates***

**[0172]** In certain embodiments, the antitumor antagonists of the present application are chemically conjugated to one or more peptides and/or small molecule drugs. The peptides or small molecule drug can be the same or different. The peptides or small molecule drugs can be attached, for example to reduced SH groups and/or to carbohydrate side chains. Methods for making covalent or non-covalent conjugates of peptides or small molecule drugs with antibodies are known in the art and any such known method may be utilized.

**[0173]** In some embodiments the peptide or small molecule drug is attached to the hinge region of a reduced antibody component via disulfide bond formation. Alternatively, such agents can be attached using a heterobifunctional cross-linkers, such as N-succinyl 3-(2-pyridyldithio)propionate (SPDP). General techniques for such conjugation are well-known in the art. In some embodiments, the peptide or small molecule drug is conjugated via a carbohydrate moiety in the Fc region of the antibody. The carbohydrate group can be used to increase the loading of the same agent that is bound to a thiol group, or the carbohydrate moiety can be used to bind a different therapeutic or diagnostic agent. Methods for conjugating peptide inhibitors or small molecule drugs to antibodies via antibody carbohydrate moieties are well-known to those of skill in the art. For example, in one embodiment, the method involves reacting antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate. Exemplary methods for conjugating small molecule drugs and peptides to antibodies are described in U.S. Patent Application Publication No. 2014/0356385.

**[0174]** Preferably, the antitumor antagonists in the present disclosure retain certain desirable characteristics and pharmacokinetic properties of antibodies, including a desirable *in vitro* and *in vivo* stability (e.g., lone half-life and shelf-life stability), efficient delivery into desired target cells, increased affinity for binding partners, desirable antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, and reduced renal clearance or excretion. Accordingly, careful attention to size and need for particular constant region effector functions may be considered in the design of the antitumor antagonists.

**[0175]** The bispecific antitumor antagonists described herein may range in size from 50 kD to 300 kD, from 50 kD to 250 kD, from 60 kD to 250 kD, from 80 kDa to 250 kD, from 100 kD to 250 kD, from 125 kD to 250 kD, from 150 kD to 250 kD, from 60 kD to 225 kD, from 75 kD to 225 kD, from 100 kD to 225 kD, from 125 kD to 225 kD, from 150 kD to 225 kD, from 60 kD to 200 kD, from 75 kD to 200 kD, from 100 kD to 125 kD to 200 kD,

from 150 kD to 200 kD, from 60 kD to 150 kD, from 75 kD to 150 kD, from 100 kD to 150 kD, from 60 kD to 125 kD, from 75 kD to 125 kD, from 75 kD to 100 kD, or any range encompassed by any combination of whole numbers listed in the above cited ranges or any ranges specified by any combination of whole numbers between any of the above cited ranges.

### ***Kits***

**[0176]** The present application further provides a kit comprising a checkpoint regulator antagonist or antitumor antagonist of the present application. In some embodiment, the kit comprises one or more bispecific immune checkpoint regulators containing at least one TGF $\beta$  pathway inhibitory domain. In some embodiments, the kit further contains additional reagents, including secondary antibodies for detection, and additional human antibodies described herein (e.g., a human antibody having a complementary activity, which binds to a different epitope in the same antigen, etc.). Kits typically include a label with instructions indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

## **II. Methods of Using the AntitumorAntagonists**

**[0177]** The antitumor antagonists of the present application have numerous *in vitro* and *in vivo* utilities including, for example, enhancement of immune responses and treatment of cancers, infectious diseases or autoimmune diseases.

**[0178]** The antitumor antagonists of the present application may be administered to cells in culture, *in vitro* or *ex vivo*, or to human subjects, e.g., *in vivo*, to enhance immunity in a variety of diseases. Accordingly, provided herein are methods of modifying an immune response in a subject comprising administering to the subject antibody or antigen-binding fragment thereof as described herein such that the immune response in the subject is enhanced, stimulated or up-regulated. Preferred subjects include human patients in whom enhancement of an immune response would be desirable. The methods are particularly suitable for treating human patients having a disorder that can be treated by augmenting an immune response (e.g., the T-cell mediated immune response). The methods are particularly suitable for treatment of cancer or chronic infections *in vivo*. For example, the antitumor antagonists may be administered together with antigen of interest or the antigen may already be present in the subject to be treated (e.g., a tumor-bearing or virus-bearing subject) to enhance antigen-specific immunity. When antitumor antagonists are administered together with another agent, the two can be administered separately or simultaneously.

**[0179]** In some embodiments, the checkpoint regulator antagonist in the above-described method is an anti-PD-1 antibody, an anti-PD-L1 antibody, a VEGF antibody, a VEGFR antibody, or any fragment thereof in combination with a TGF $\beta$ RII ECD, a VEGFR ECD, or both.

**[0180]** In any of the antibody embodiments described herein, the antibody is preferably a human or humanized antibody.

**[0181]** Also encompassed within the scope of the present application are methods for detecting and/or measuring the presence of target molecule in a sample comprising contacting the sample, and a control sample, with an antibody, antibody fragment, or bispecific antagonist of the present application, which specifically binds to the target molecule under conditions that allow for formation of a complex between the antagonist and the target molecule. The formation of a complex is then detected, wherein a difference in complex formation between the sample compared to the control sample is indicative the presence of target molecule in the sample.

**[0182]** Given the ability of the antitumor antagonist of the present application to block inhibition or co-inhibition of T cell responses, *e.g.*, antigen-specific T cell responses, provided herein are *in vitro* and *in vivo* methods of using the antibodies described herein to stimulate, enhance or upregulate antigen-specific T cell responses, *e.g.*, anti-tumor T cell responses. In certain embodiments, CD3 stimulation is also provided (*e.g.*, by co-incubation with a cell expressing membrane CD3), which stimulation can be provided at the same time, before, or after treatment with a checkpoint regulator antagonist. For example, provided herein are methods of enhancing antigen-specific T cell response comprising contacting said T cell with a checkpoint regulator antagonist described herein, and optionally with CD3, such that antigen-specific T cell response is enhanced, *e.g.*, by removal of a checkpoint regulator mediated inhibitory effect. Any suitable indicator of antigen-specific T cell response can be used to measure the antigen-specific T cell response. Non-limiting examples of such suitable indicators include increased T cell proliferation in the presence of the antibody and/or increase cytokine production in the presence of the antibody. In a preferred embodiment, interleukin-2 and/or interferon- $\gamma$  production by the antigen-specific T cell is enhanced.

**[0183]** Further encompassed in the present application is a method for enhancing an immune response (*e.g.*, antigen-specific T cell response) in a subject by administering a bispecific antitumor antagonist described herein to the subject such that an immune response (*e.g.*, antigen-specific T cell response) in the subject is enhanced. In a preferred embodiment, the subject is a tumor-bearing subject and an immune response against the tumor is enhanced. A tumor may be a solid tumor or a liquid tumor, *e.g.*, a hematological malignancy. In certain

embodiments, a tumor is an immunogenic tumor. In other embodiments, a tumor is non-immunogenic. In certain embodiments, a tumor is PD-L1 positive. In other embodiments a tumor is PD-L1 negative. A subject may also be a virus-bearing subject in which an immune response against the virus is enhanced as a consequence of administering the bispecific antitumor antagonist as described herein.

**[0184]** In one embodiment, a method for inhibiting the growth of tumor cells in a subject comprises administering to the subject a bispecific antitumor antagonist described herein such that growth of the tumor is inhibited in the subject. Also provided are methods of treating chronic viral infection in a subject comprising administering to the subject a bispecific antitumor antagonist as described herein such that the chronic viral infection is treated in the subject.

**[0185]** Also encompassed herein are methods for depleting  $T_{reg}$  cells from the tumor microenvironment of a subject with a tumor, *e.g.*, cancerous tumor, comprising administering to the subject a therapeutically effective amount of a bispecific antitumor antagonist described herein that comprises an Fc that stimulates depletion of  $T_{reg}$  cells in the tumor microenvironment. An Fc may, *e.g.*, be an Fc with effector function or enhanced effector function, such as binding or having enhanced binding to one or more activating Fc receptors.

**[0186]** In a preferred embodiment,  $T_{reg}$  depletion occurs without significant depletion or inhibition of  $T_{eff}$  in the tumor microenvironment, and without significant depletion or inhibition of  $T_{eff}$  cells and  $T_{reg}$  cells outside of the tumor microenvironment. In certain embodiments, the subject has higher levels of checkpoint regulator(s) on  $T_{reg}$  cells than on  $T_{eff}$  cells, *e.g.*, in the tumor microenvironment. In certain embodiments, the bispecific antagonists may deplete  $T_{reg}$  in tumors and/or  $T_{reg}$  in tumor infiltrating lymphocytes (TILs). For example, in a CT26 tumor model, an anti-mouse TIGIT antibody formatted as a mouse IgG2a (which exhibits effector function) was found to partially depleted both  $T_{reg}$  and  $CD8^+$  T cells, but did not deplete  $CD4^+$  T cells. An effectorless counterpart antibody or antagonist formatted as a mouse IgG1 D265A, did not deplete T cells.

**[0187]** In certain embodiments, the bispecific antitumor antagonist described herein is given to a subject as an adjunctive therapy. Treatment of cancer patient with a bispecific antitumor antagonist according to the present application may lead to a long-term durable response relative to the current standard of care; long term survival of at least 1, 2, 3, 4, 5, 10 or more years, recurrence free survival of at least 1, 2, 3, 4, 5, or 10 or more years. In certain embodiments, treatment of a cancer patient with bispecific antitumor antagonist prevents recurrence of cancer or delays recurrence of cancer by, *e.g.*, 1, 2, 3, 4, 5, or 10 or more years.

Thus, treatment with these antagonists can be used as a primary or secondary line of treatment.

**[0188]** In certain preferred embodiments, the subject has a cell proliferative disease or cancer. Provided herein are methods for treating a subject having cancer, comprising administering to the subject a bispecific antitumor antagonist described herein, such that the subject is treated, *e.g.*, such that growth of cancerous tumors is inhibited or reduced and/or that the tumors regress. The bispecific antitumor antagonists described herein can be used alone to inhibit the growth of cancerous tumors. Alternatively, any of these antitumor antagonists can be used in conjunction with another agent, *e.g.*, other anti-cancer targets, immunogenic agents, standard cancer treatments, or other antibodies, as described below.

**[0189]** Accordingly, provided herein are methods of treating cancer, *e.g.*, by inhibiting growth of tumor cells, in a subject, comprising administering to the subject a therapeutically effective amount of a bispecific antitumor antagonist described herein. Preferably, the antibody contains human or humanized immunoglobulin sequences.

**[0190]** Cancers whose growth may be inhibited using the antibodies of the invention include cancers typically responsive to immunotherapy. Non-limiting examples of cancers for treatment include squamous cell carcinoma, small-cell lung cancer, non-small cell lung cancer, squamous non-small cell lung cancer (NSCLC), non NSCLC, glioma, gastrointestinal cancer, renal cancer (*e.g.* clear cell carcinoma), ovarian cancer, liver cancer, colorectal cancer, endometrial cancer, kidney cancer (*e.g.*, renal cell carcinoma (RCC)), prostate cancer (*e.g.* hormone refractory prostate adenocarcinoma), thyroid cancer, neuroblastoma, pancreatic cancer, glioblastoma (glioblastoma multiforme), cervical cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer (or carcinoma), gastric cancer, germ cell tumor, pediatric sarcoma, sinonasal natural killer, melanoma (*e.g.*, metastatic malignant melanoma, such as cutaneous or intraocular malignant melanoma), bone cancer, skin cancer, uterine cancer, cancer of the anal region, testicular cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, solid tumors of childhood, cancer of the ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally-induced cancers including those induced by asbestos, virus-related cancers (*e.g.*, human papilloma virus (HPV)-related tumor), and hematologic

malignancies derived from either of the two major blood cell lineages, i.e., the myeloid cell line (which produces granulocytes, erythrocytes, thrombocytes, macrophages and mast cells) or lymphoid cell line (which produces B, T, NK and plasma cells), such as all types of leukemias, lymphomas, and myelomas, *e.g.*, acute, chronic, lymphocytic and/or myelogenous leukemias, such as acute leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myelogenous leukemia (CML), undifferentiated AML (MO), myeloblastic leukemia (M1), myeloblastic leukemia (M2; with cell maturation), promyelocytic leukemia (M3 or M3 variant [M3V]), myelomonocytic leukemia (M4 or M4 variant with eosinophilia [M4E]), monocytic leukemia (M5), erythroleukemia (M6), megakaryoblastic leukemia (M7), isolated granulocytic sarcoma, and chloroma; lymphomas, such as Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NEIL), B-cell lymphomas, T-cell lymphomas, lymphoplasmacytoid lymphoma, monocytoid B-cell lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma, anaplastic (*e.g.*, Ki 1+) large-cell lymphoma, adult T-cell lymphoma/leukemia, mantle cell lymphoma, angio immunoblastic T-cell lymphoma, angiocentric lymphoma, intestinal T-cell lymphoma, primary mediastinal B-cell lymphoma, precursor T-lymphoblastic lymphoma, T-lymphoblastic; and lymphoma/leukemia (T-Lbly/T-ALL), peripheral T-cell lymphoma, lymphoblastic lymphoma, post-transplantation lymphoproliferative disorder, true histiocytic lymphoma, primary central nervous system lymphoma, primary effusion lymphoma, lymphoblastic lymphoma (LBL), hematopoietic tumors of lymphoid lineage, acute lymphoblastic leukemia, diffuse large B-cell lymphoma, Burkitt's lymphoma, follicular lymphoma, diffuse histiocytic lymphoma (DHL), immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, cutaneous T-cell lymphoma (CTLC) (also called mycosis fungoides or Sezary syndrome), and lymphoplasmacytoid lymphoma (LPL) with Waldenstrom's macroglobulinemia; myelomas, such as IgG myeloma, light chain myeloma, nonsecretory myeloma, smoldering myeloma (also called indolent myeloma), solitary plasmacytoma, and multiple myelomas, chronic lymphocytic leukemia (CLL), hairy cell lymphoma; hematopoietic tumors of myeloid lineage, tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; seminoma, teratocarcinoma, tumors of the central and peripheral nervous, including astrocytoma, schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoacanthoma, seminoma, thyroid follicular cancer and teratocarcinoma, hematopoietic tumors of lymphoid lineage, for example T-cell and B-cell tumors, including but not limited to T-cell disorders such as T-prolymphocytic leukemia (T-PLL), including of the small cell and cerebriform cell type; large granular lymphocyte leukemia (LGL)

preferably of the T-cell type; a/d T-NHL hepatosplenic lymphoma; peripheral/post-thymic T cell lymphoma (pleomorphic and immunoblastic subtypes); angiocentric (nasal) T-cell lymphoma; cancer of the head or neck, renal cancer, rectal cancer, cancer of the thyroid gland; acute myeloid lymphoma, as well as any combinations of said cancers. The methods described herein may also be used for treatment of metastatic cancers, refractory cancers (e.g., cancers refractory to previous immunotherapy, e.g., with a blocking CTLA-4 or PD-1 antibody), and recurrent cancers.

**[0191]** A bispecific antitumor antagonist of the present application can be administered alone, in combination with another antitumor antagonist, or concurrently with another antitumor antagonist. Alternatively, the bispecific antitumor antagonist can also be administered in combination, or concurrently with, an immunogenic agent, such as cancerous cells, tumor vaccines, purified tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), cells transfected with genes encoding immune stimulating cytokines, in a cancer vaccine strategy (He et al. (2004) *J. Immunol.* 173:4919-28), or an oncolytic virus.

**[0192]** Many experimental strategies for vaccination against tumors have been devised. In one of these strategies, a vaccine is prepared using autologous or allogeneic tumor cells. Some of these cellular vaccines have been shown to be most effective when the tumor cells are transduced to express GM-CSF. GM-CSF has been shown to be a potent activator of antigen presentation for tumor vaccination (Dranoff et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90: 3539-43). Cancer vaccines have been shown to enhance effector T-cell infiltration into the tumors in preclinical models. The major types of cancer vaccines include peptide vaccines, vector-based antigen specific vaccines, whole-cell vaccines, and dendritic cell vaccines. All vaccine-based therapies are designed to deliver either single or multiple antigenic epitopes or antigens from the whole cells to the patients and induce tumor-specific effector T cells. Thus, a vaccine-based therapy may be the most efficient way to induce T-cell infiltration into the tumor.

**[0193]** The study of gene expression and large scale gene expression patterns in various tumors has led to the definition of so called tumor specific antigens (Rosenberg, S A (1999) *Immunity* 10: 281-7). In many cases, these tumor specific antigens are differentiation antigens expressed in the tumors and in the cell from which the tumor arose, for example melanocyte antigens gp100, MAGE antigens, and Trp-2. More importantly, many of these antigens can be shown to be the targets of tumor specific T cells found in the host.

**[0194]** Inhibition of the checkpoint regulator pathway, TGF- $\beta$  pathway, and/or angiogenesis pathways may be used in conjunction with a collection of recombinant proteins

and/or peptides expressed in a tumor in order to generate an immune response to these proteins. Such proteins may be viewed by the immune system as self-antigens and are therefore tolerant to them. The tumor antigen can include the protein telomerase, which is required for the synthesis of telomeres of chromosomes and which is expressed in more than 85% of human cancers and in only a limited number of somatic tissues (Kim et al. (1994) *Science* 266: 2011-2013). Tumor antigens can also be “neo-antigens” expressed in cancer cells because of somatic mutations that alter protein sequence or create fusion proteins between two unrelated sequences (i.e., bcr-abl in the Philadelphia chromosome), or idiotype from B cell tumors.

**[0195]** Non-limiting examples of tumor vaccines include sipuleucel-T (Provenge®), an FDA-approved tumor vaccine for metastatic prostate cancer; tumor cells transfected to express the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF), such as the whole cell GM-CSF-secreting irradiated, allogeneic pancreatic cancer vaccine (GVAX; Johns Hopkins); a multi-peptide vaccine consisting of immunogenic peptides derived from breast cancer antigens, neu, legumain, and β-catenin, which prolonged the vaccine-induced progression-free survival of breast tumor-bearing mice when administered in combination with anti-PD-1 antibody (Karyampudi L. et al. (2014) *Cancer Res* 74:2974-2985); peptides of melanoma antigens, such as peptides of gp100, MAGE antigens, Trp-2, MART1 and/or tyrosinase, or. Other tumor vaccines include proteins from viruses implicated in human cancers such as human papilloma viruses (HPV)(e.g., Gardasil®, Gardasil 9®, and Cervarix®); hepatitis B virus (e.g., Engerix-B and Recombivax HB); hepatitis C virus (HCV), Kaposi’s sarcoma associated herpes sarcoma virus (KSHV). Another form of tumor specific antigen that can be used in conjunction with TIGIT inhibition is purified heat shock proteins (HSP) isolated from the tumor tissue itself. These heat shock proteins contain fragments of proteins from the tumor cells and these HSPs are highly efficient at delivery to antigen presenting cells for eliciting tumor immunity. Talimogene laherparepvec (T-VEC, or Imlygic®) is an FDA-approved oncolytic virus for the treatment of some patients with metastatic melanoma that cannot be surgically removed.

**[0196]** Dendritic cells (DC) are potent antigen presenting cells that can be used to prime antigen-specific responses. DC’s can be produced *ex vivo* and loaded with various protein and peptide antigens, as well as tumor cell extracts (Nestle et al. (1998) *Nature Medicine* 4: 328-332). DCs can also be transduced by genetic means to express these tumor antigens as well. DCs have also been fused directly to tumor cells for the purposes of immunization (Kugler et al. (2000) *Nature Medicine* 6:332-336). As a method of

vaccination, DC immunization may be effectively combined with checkpoint regulator blocking to activate (unleash) more potent anti-tumor responses.

**[0197]** Inhibition of the checkpoint regulator pathway, TGF- $\beta$  pathway, and/or angiogenesis pathways can also be combined with standard cancer treatments (e.g., surgery, radiation, and chemotherapy). In particular, checkpoint regulator inhibition can be effectively combined with chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered (Mokyr et al. (1998) *Cancer Research* 58: 5301-5304). An example of such a combination is antitumor antagonist in combination with decarbazine for the treatment of melanoma. Another example of such a combination is a checkpoint regulator antagonist or antitumor antagonist in combination with interleukin-2 (IL-2) for the treatment of melanoma. For example, the scientific rationale behind the combined use of checkpoint regulator inhibition, TGF- $\beta$ 1/TGF- $\beta$ 1 RII inhibition, and/or angiogenesis inhibition with chemotherapy can promote cell death as a consequence of the cytotoxic action of most chemotherapeutic compounds, thereby resulting in increased levels of tumor antigen in the antigen presentation pathway. Other combination therapies that may result in synergy with checkpoint regulator inhibition, TGF- $\beta$ 1/TGF- $\beta$ 1 RII inhibition, and/or angiogenesis inhibition through cell death are radiation, surgery, and hormone deprivation. Each of these protocols creates a source of tumor antigen in the host.

**[0198]** The bispecific antitumor antagonists described herein may also be constructed to target Fc $\alpha$  or Fc $\gamma$  receptor-expressing effector cells to tumor cells (see, e.g., U.S. Pat. Nos. 5,922,845 and 5,837,243). For example, anti-Fc receptor/antitumor antigen (e.g., Her-2/neu) bispecific antibodies have been used to target macrophages to sites of tumor. This type of targeting may be adapted to the present embodiments to more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by the inhibition of one or more checkpoint regulator antagonists described herein. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies that bind to tumor antigen and a dendritic cell specific cell surface marker.

**[0199]** Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of immunosuppressive proteins expressed by the tumors. These include among others TGF- $\beta$ , IL-10, and Fas ligand. Antibodies to each of these entities can be used in combination with the antitumor antagonists described herein to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

**[0200]** Other antibodies that activate host immune responsiveness can be used in combination with the antitumor antagonists described herein. These include molecules on the

surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity (Ridge et al. (1998) *Nature* 393: 474-478) and can be used in conjunction with the bispecific antagonists described herein. Activating antibodies to T cell costimulatory molecules, such as OX-40 (Weinberg et al. (2000) *Immunol* 164: 2160-2169), CD137/4-1BB (Melero et al. (1997) *Nature Medicine* 3: 682-685 (1997), and ICOS (Hutloff et al. (1999) *Nature* 397: 262-266) may also provide for increased levels of T cell activation. In addition, inhibitors of other immune checkpoint regulators may also be used in conjunction with other antitumor antagonists described herein, as further described below.

**[0201]** Bone marrow transplantation is currently being used to treat a variety of tumors of hematopoietic origin. While graft versus host disease is a consequence of this treatment, checkpoint regulator inhibition may be used to increase the effectiveness of the donor engrafted tumor specific T cells by reducing graft vs. tumor responses.

**[0202]** In certain embodiments, antitumor antagonist described herein may be administered to a subject with an infectious disease, especially chronic infections. In this case, similar to its application to cancer, antibody-mediated checkpoint regulator inhibition can be used alone, or as an adjuvant, in combination with vaccines, to enhance immune responsiveness to pathogens, toxins, and self-antigens. Exemplary pathogens for which this therapeutic approach can be applied include, but are not limited to, HIV, Hepatitis (A, B, & C), Influenza, Herpes, Giardia, Malaria, Leishmania, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Checkpoint regulator inhibition, TGF- $\beta$ 1/TGF- $\beta$ 1 RII inhibition, and/or angiogenesis inhibition is particularly useful against established infections by agents, such as HIV that present novel or altered antigens over the course of the infections. Administration of bispecific antitumor antagonists can allow for recognition of these antigens as foreign so as to provoke an appropriate T cell response.

**[0203]** Other pathogenic viruses causing infections treatable by the methods described herein include HIV, hepatitis (A, B, or C), herpesvirus infections (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), and infections caused by an adenovirus, influenza virus, flavivirus, echoviruses, rhinoviruses, coxsackie viruses, coronaviruses, respiratory syncytial viruses, mumps viruses, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus, arboviral encephalitis virus, or combination thereof.

**[0204]** Exemplary pathogenic bacteria or diseases caused therefrom which may be treatable by the methods described herein include *Chlamydia*, *Rickettsia*, *Mycobacteria*, *Staphylococci*, *Streptococci*, *Pneumonococci*, *Meningococci* and *Gonococci*, *Klebsiella*,

*Proteus, Serratia, Pseudomonas, Legionella, Diphtheria, Salmonella, Bacilli, Cholera, Leptospirosis* tetanus, botulism, anthrax, plague, and Lyme disease.

**[0205]** Exemplary pathogenic fungi causing infections treatable by the methods described herein include *Candida* (e.g., *albicans, krusei, glabrata, tropicalis*, etc.), *Cryptococcus neoformans*, *Aspergillus* (e.g., *fumigatus, niger*, etc.), *Mucorales* (e.g., *mucor, absidia, rhizopus*), *Sporothrix schenkii*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis* and *Histoplasma capsulatum*.

**[0206]** Exemplary pathogenic parasites causing infections treatable by the methods described herein include *Entamoeba histolytica*, *Balantidium coli*, *Naegleria fowleri*, *Acanthamoeba* sp., *Giardia Zambia*, *Cryptosporidium* sp., *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Nippostrongylus brasiliensis*.

**[0207]** In all of the above methods, checkpoint regulator inhibition, TGF- $\beta$ 1/TGF- $\beta$ 1 RII inhibition, and/or angiogenesis inhibition can be combined with other forms of immunotherapy such as cytokine treatment (e.g., interferons, GM-CSF, G-CSF, IL-2), or bispecific antibody therapy using two different binding specificities to provide enhanced presentation of tumor antigens.

**[0208]** The bispecific antitumor antagonists described herein can be used to enhance antigen-specific immune responses by co-administration of one or more of any of these antibodies with antigen of interest (e.g., a vaccine). Accordingly, provided herein are methods of enhancing an immune response to antigen in a subject, comprising administering to the subject: (i) the antigen; and (ii) the bispecific antitumor antagonist, such that an immune response to the antigen in the subject is enhanced. The antigen can be, for example, a tumor antigen, a viral antigen, a bacterial antigen or antigen from a pathogen. Non-limiting examples of such antigens include those discussed in the sections above, such as the tumor antigens (or tumor vaccines) discussed above, or antigens from the viruses, bacteria or other pathogens described above.

**[0209]** In certain embodiments, a peptide or fusion protein comprising the epitope to which the bispecific antitumor antagonist binds may be used as a vaccine instead of, or in addition to, the antitumor antagonist(s).

**[0210]** Suitable routes of administering the antibody compositions (e.g., human monoclonal antibodies, multi-specific antibodies or antagonists and immunoconjugates) described herein *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (e.g., intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age

and weight of the subject and the concentration and/or formulation of the antibody composition.

***Combination therapies***

**[0211]** In another aspect, the present application provides combination therapies for enhancing antigen-specific T cell response in a subject. In one embodiment, the method includes contacting a T cell with bispecific antitumor antagonist in combination with a second antibody, antibody fragment, antagonist or drug such that antigen-specific T cell response or apoptotic pathway is enhanced. For example, in some embodiments, the first bispecific antitumor antagonist specifically binds to a first checkpoint regulator, such PD-1 or PD-L1, and a second bispecific antitumor antagonist specifically binding to a different checkpoint regulator or to a different epitope. In some embodiments, the second antibody or antibody fragment comprises one or more different HCVRs, LCVR, or CDR(s) from PD-1 and/or PD-L1.

**[0212]** In a related aspect, a method of reducing or depleting regulatory T cells in a tumor of a subject in need thereof includes administering an effective amount of antibody or antibody fragment in combination with a second antibody, antibody fragment, antagonist or drug such that the number of regulatory T cells in the subject is reduced.

**[0213]** In some embodiments, the subject has a cell proliferative disease or cancer as described herein.

**[0214]** In other embodiments, the subject has a chronic viral infection, inflammatory disease or autoimmune disease as described herein.

**[0215]** The provision of two distinct signals to T-cells is a widely accepted model for lymphocyte activation of resting T lymphocytes by antigen-presenting cells (APCs). This model further provides for the discrimination of self from non-self and immune tolerance. 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). This induces T-cells to promote clonal expansion, cytokine secretion, and effector function. In the absence of co-stimulation, T-cells can become refractory to antigen stimulation, which results in a tolerogenic response to either foreign or endogenous antigens.

**[0216]** In the two-signal model, T-cells receive both positive co-stimulatory and negative co-inhibitory signals. The regulation of such positive and negative signals is critical to maximize the host's protective immune responses, while maintaining immune tolerance and preventing autoimmunity. Negative signals seem necessary for induction of T-cell

tolerance, while positive signals promote T-cell activation. Both co-stimulatory and co-inhibitory signals are provided to antigen-exposed T cells, and the interplay between co-stimulatory and co-inhibitory signals is essential to controlling the magnitude of an immune response. Further, the signals provided to the T cells change as an infection or immune provocation is cleared, worsens, or persists, and these changes powerfully affect the responding T cells and re-shape the immune response.

**[0217]** The mechanism of co-stimulation is of therapeutic interest because the manipulation of co-stimulatory signals has shown to provide a means to either enhance or terminate cell-based immune response. Recently, it has been discovered that T cell dysfunction or anergy can occur concurrently with an induced and sustained expression of immune checkpoint regulators, such as programmed death 1 polypeptide (PD-1) and its ligands, PD-L1 and PD-L2. PD-L1 is overexpressed in many cancers and is often associated with poor prognosis (Thompson R H et al., *Cancer Res* 2006, 66(7):3381). Further, the majority of tumor infiltrating T lymphocytes predominantly express PD-1, in contrast to T lymphocytes in normal tissues and peripheral blood T lymphocytes indicating that up-regulation of PD-1 on tumor-reactive T cells can contribute to impaired antitumor immune responses (Blood 2009 114(8):1537). This may be due to exploitation of PD-L1 signaling mediated by PD-L1 expressing tumor cells interacting with PD-1 expressing T cells to result in attenuation of T cell activation and evasion of immune surveillance. Inhibition of the PD-L1/PD-1 interaction provides a means to enhance T cell immunity, including CD8+ T cell-mediated killing of cancer cells and tumors. Similar enhancements to T cell immunity have been observed by inhibiting the binding of PD-L1 to the binding partner B7-1. Consequently, therapeutic targeting of PD-1 and other immune checkpoint regulators are an area of intense interest.

**[0218]** Combining inhibition of TGF- $\beta$ 1 signaling, checkpoint regulator signaling, and/or angiogenesis signaling with other signaling pathways deregulated in tumor cells can provide a means for enhance treatment efficacy. In recent years, a number of immune checkpoint regulators in the form of receptors and their ligands have been identified. One important family of membrane-bound ligands that bind to co-stimulatory or co-inhibitory receptors is the B7 family, which includes CTLA-4 and its ligands, B7-1 and B7-2; PD-1 and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC); B7-H2 (ICOS-L), B7-H3, B7-H4, B7-H5 (VISTA), and B7-H6. Additional immune checkpoint antagonists include, but are not limited to TIM-3 and its ligand, Galectin-9; LAG-3 and its ligands, including liver sinusoidal endothelial cell lectin (LSECtin) and Galectin-3; CD122 and its CD122R ligand; CD70, B7H3, B and T lymphocyte attenuator (BTLA), and VISTA (Le Mercier et al. (2015) *Front.*

Immunol., (6), Article 418). In addition, a number of checkpoint regulator antagonists have been identified and tested in various clinical and pre-clinical models and/or approved by the FDA (Kyi et al., FEBS Letters, 588:368-376 (2014). The concept of inhibitory receptor blockade, also known as immune checkpoint blockade, has been validated by virtue of *e.g.*, the FDA approval of the PD-1 inhibitors, nivolumab and pembrolizumab, as well as the anti-CTLA-4 antibody, ipilimumab for metastatic melanoma.

**[0219]** An immune checkpoint antagonist modulates or interferes with the activity of the immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is blocked or inhibited. By inhibiting this signaling, immune-suppression can be reversed so that T cell immunity against cancer cells can be re-established or enhanced. In contrast, an immune checkpoint agonist (*of e.g.*, a costimulatory molecule) stimulates the activity of an immune checkpoint regulator so that, as a result of the binding to the checkpoint regulator or its ligand, signaling through the checkpoint regulator receptor is stimulated. By stimulating this signaling, T cell immunity against cancer cells can be re-established or enhanced.

**[0220]** Accordingly, in one embodiment, a method for stimulating an immune response in a subject comprises administering to the subject a bispecific antitumor antagonist described herein in combination with another immune checkpoint regulator described herein above, such that an immune response is stimulated in the subject, for example to inhibit tumor growth or to stimulate anti-viral response. In particular, the bispecific antitumor antagonist(s) can be administered as separate antagonists or as a multi-specific antagonist comprising binding specificities to multiple products.

**[0221]** In some embodiments, the bispecific antitumor antagonist of the present application can be combined to stimulate an immune response with (i) an antagonist of the IgSF family protein, B7 family or TNF family that inhibit T cell activation, or antagonist of a cytokine that inhibits T cell activation (*e.g.*, IL-6, IL-10, TGF- $\beta$ , VEGF, or other immunosuppressive cytokines) and/or (ii) an agonist of a stimulatory receptors of the IgSF family, B7 family or TNF family or of cytokines to stimulate T cell activation, for stimulating an immune response. In other embodiments, the subject is administered a bispecific antitumor antagonist in combination with anti-CTLA-4 antibody or CTLA-4 antagonist. Exemplary anti-CTLA-4 antibodies for use in accordance with the present application include ipilimumab, trevilizumab and tremelimumab.

**[0222]** In some embodiments, subjects with a cancer exhibiting high expression of a ligand for an immune checkpoint regulator may be administered a bispecific antagonist targeting a different checkpoint regulator antagonist. By way of example, in one

embodiment, a subject with a cancer exhibiting high expression of PVR (CD155) and/or Nectin-2 (CD112) and/or low expression PD-L1 may be selected for monotherapy with anti-TIGIT or anti-LAG-3 antibodies or fragments thereof alone, or in a combination therapy with a PD-1 antagonist or other immune checkpoint regulator antagonist. The bispecific antitumor antagonists of the present application may be co-administered with any additional agent(s), *e.g.*, antibodies, antagonists, or drugs in amount(s) effective in stimulating an immune response and/or apoptosis so as to further enhance, stimulate or upregulate an immune response and/or apoptosis in a subject.

**[0223]** In some embodiments, the bispecific antitumor antagonist is administered subsequent to treatment with a different antitumor antagonist. For example, in some embodiments, the bispecific antitumor antagonist of the present application may be administered only after treatment with a monospecific antitumor antagonist has failed, has led to incomplete therapeutic response, or there has been recurrence of the tumor or relapse (such as “PD-1 failure”). In some embodiments, cancers exhibiting such failures may be screened for expression of *e.g.*, PVR and/or Nectin-2 and only those having high level expression are treated with a bispecific antitumor antagonist of the present application.

**[0224]** In certain embodiments, the antitumor antagonist includes a dominant negative protein domain of the immune checkpoint regulator. In particular embodiments, the dominant negative protein comprises an extracellular domain derived from a member selected from the group consisting of PD-L1, PD-L2, PD-1, B7-1, B7-2, B7H3, CTLA-4, LAG-3, TIM-3, TIGIT, BTLA, VISTA, CD70, and combinations thereof. In certain particular embodiments, these extracellular domains are fused to an immunoglobulin constant region or Fc receptor in the presently described antibodies. Such mutants can bind to the endogenous receptor so as to form a complex that is deficient in signaling. In certain embodiments, the extracellular domain is fused to an immunoglobulin constant region or Fc fragment or to a monomer in the oligomeric protein complex.

**[0225]** In certain embodiments, a dominant negative PD-L1 antagonist comprises the extracellular domain of PD-L1, PD-L2, or PD-1. In another embodiment, a dominant-negative PD-1 antagonist is employed, which has a mutation so that it is no longer able to bind PD-L1. An exemplary dominant negative protein is AMP-224 (co-developed by Glaxo Smith Kline and Amplimmune), a recombinant fusion protein comprising the extracellular domain of PD-L2 and the Fc region of human IgG.

**[0226]** Exemplary immune checkpoint regulator agonists include, but are not limited to members of the tumor necrosis factor (TNF) receptor superfamily, such as CD27, CD40, OX40, GITR and 4-1BB (CD137) and their ligands, or members of the B7-CD28

superfamily, including CD28 and ICOS (CD278). Additional checkpoint regulator agonists include CD2, CDS, ICAM-1, LFA-1 (CD11a/CD18), CD30, BAFFR, HVEM, CD7, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3, CD83 ligand. Immune checkpoint agonists can include antibodies or soluble fusion protein agonists comprising one or more costimulatory domains. Agonist antibodies include, but are not limited to anti-CD40 mAbs, such as CP-870,893, lucatumumab, and dacetuzumab; anti-CD137 mAbs, such as BMS-663513 urelumab, and PF-05082566; anti-OX40 mAbs; anti-GITR mAbs, such as TRX518; anti-CD27 mAbs, such as CDX-1127; and anti-ICOS mAbs.

**[0227]** Exemplary GITR agonists include, *e.g.*, GITR fusion proteins and anti-GITR antibodies (*e.g.*, bivalent anti-GITR antibodies) such as, *e.g.*, a GITR fusion protein described in U.S. Pat. Nos. 6,111,090 and 8,586,023; European Patent No.: 090505B1, U.S. Pat. No. PCT Publication Nos.: WO 2010/003118 and 2011/090754. Anti-GITR antibodies are described in, *e.g.*, in U.S. Pat. Nos. 7,025,962, 7,618,632, 7,812,135, 8,388,967, and 8,591,886; European Patent Nos.: 1947183B1 and 1866339; PCT Publication Nos.: WO 2011/028683, WO 2013/039954, WO 2005/007190, WO 2007/133822, WO 2005/055808, WO 99/40196, WO 2001/03720, WO 99/20758, WO 2006/083289, WO 2005/115451, WO 2011/051726. An exemplary anti-GITR antibody is TRX518.

**[0228]** Another family of membrane bound ligands that bind to co-stimulatory or co-inhibitory receptors is the TNF family of molecules that bind to cognate TNF receptor family members, which include CD40 and CD40L, OX-40, OX-40L, CD70, CD27L, CD30, CD30L, 4-1BBL, CD137/4-1BB, TRAIL/Apo2-L, TRAILR1/DR4, TRAILR2/DR5, TRAILR3, TRAILR4, OPG, RANK, RANKL, TWEAKR/Fn14, TWEAK, BAFFR, EDAR, XEDAR, TACI, APRIL, BCMA, LT $\beta$ R, LIGHT, DcR3, HVEM, VEGI/TL1A, TRAMP/DR3, EDAR, EDA1, XEDAR, EDA2, TNFR1, Lymphotoxin  $\alpha$ /TNF  $\gamma$ , TNFR2, TNF $\alpha$ , LT $\beta$ R, Lymphotoxin  $\alpha$  1(32, FAS, FASL, RELT, DR6, TROY, NGFR (*see, e.g.*, Tansey, M.G. et al. (2009) Drug Discovery Today, 14(23-24):1082-1088).

**[0229]** Immune checkpoint agonists or costimulatory molecules include cell surface molecules other than antigen receptors or their ligands that are required for an efficient immune response, and include, but are not limited to MHC class I molecules, MHC class II molecules, TNF receptor proteins, immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha,

ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83.

**[0230]** In one aspect, T cell responses can be stimulated by a combination of the anti-PD-1 or anti-PD-L1 mAbs of the present invention and one or more of (i) antagonist of a protein that inhibits T cell activation (*e.g.*, immune checkpoint inhibitors), such as CTLA-4, PD-1, PD-L1, PD-L2, LAG-3, TIM-3, Galectin 9, CEACAM-1, BTLA, CD69, Galectin-1, CD113, GPR56, VISTA, 2B4, CD48, GARP, PD-1H, LAIR1, TIM-1, CD96 and TIM-4, and (ii) an agonist of a protein that stimulates T cell activation such as B7-1, B7-2, CD28, 4-1BB (CD137), 4-1BBL, ICOS, CD40, ICOS-L, OX40, OX40L, GITR, GITRL, CD70, CD27, CD40, DR3 and CD28H.

**[0231]** Exemplary agents modulating one of the above proteins may be combined with the anti-PD-1 antibodies or anti-PD-L1 antibodies of the present application for treating cancer, include *e.g.*, YERVOY™/ipilimumab or tremelimumab (to CTLA-4), galiximab (to B7.1), OPDIVO™/nivolumab/BMS-936558 (to PD-1), pidilizumab/CT-011 (to PD-1), KEYTRUDA™/pembrolizumab/MK-3475 (to PD-1), AMP224 (to B7-DC/PD-L2), BMS-936559 (to B7-H1), MPDL3280A (to B7-H1), MEDI-570 (to ICOS), AMG557 (to B7H2), MGA271 (to B7H3), IMP321 (to LAG-3), urelumab/BMS-663513 and PF-05082566 (to CD137/4-1BB), CDX-1127 (to CD27), MEDI-6383 and MEDI-6469 (to OX40), RG-7888 (to OX40L), Atacicept (to TACI), CP-870893 (to CD40), lucatumumab (to CD40), dacetuzumab (to CD40), and muromonab-CD3 (to CD3).

**[0232]** Other molecules that can be combined with the antitumor antagonists described herein for the treatment of cancer include antagonists of inhibitory receptors on NK cells or agonists of activating receptors on NK cells. For example, antagonist anti-PD-1, and/or anti-PD-L1 antibodies can be combined with antagonists of KIR (*e.g.*, lirilumab), CSF-1R antagonists, such as RG7155.

**[0233]** Tumors evade host immune surveillance by a large variety of mechanisms. Many of these mechanisms may be overcome by the inactivation of immunosuppressive proteins expressed by the tumors. These include among others TGF- $\beta$ , IL-10, and Fas ligand. Antibodies to each of these entities can be used in combination with the antitumor antagonists

described herein to counteract the effects of the immunosuppressive agent and favor tumor immune responses by the host.

**[0234]** Other antibodies that activate host immune responsiveness can be used in combination with the antitumor antagonists described herein. These include molecules on the surface of dendritic cells that activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity and can be used in conjunction with the antitumor antagonists described herein. Activating antibodies to T cell costimulatory molecules, such as OX-40, CD137/4-1BB, and ICOS may also provide for increased levels of T cell activation.

**[0235]** In certain embodiments, the antitumor antagonists described herein can be co-administered with one or other more therapeutic agents, *e.g.*, anti-cancer agents, radiotoxic agents or an immunosuppressive agent. Such co-administration can solve problems due to development of resistance to drugs, changes in the antigenicity of the tumor cells that would render them unreactive to the antagonist and toxicities (by administering lower doses of one or more agents).

**[0236]** The antitumor antagonists described herein can be linked to the agent (as an immuno-complex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.*, anti-cancer therapy, *e.g.*, radiation. The antitumor antagonists described herein may be co-administered with one or more anti-cancer agents so as to provide two anti-cancer agents operating synergistically via different mechanisms to yield a cytotoxic effect in human cancer cells.

**[0237]** The antitumor antagonists described herein may be combined with anti-cancer agent, such an alkylating agent; anthracycline antibiotic; anti-metabolite; a detoxifying agent; an interferon; a polyclonal or monoclonal antibody; an EGFR inhibitor; a HER2 inhibitor; a histone deacetylase inhibitor; a hormone; a mitotic inhibitor; a phosphatidylinositol-3-kinase (PI3K) inhibitor; an Akt inhibitor; a mammalian target of rapamycin (mTOR) inhibitor; a proteasomal inhibitor; a poly(ADP-ribose) polymerase (PARP) inhibitor; a Ras/MAPK pathway inhibitor; a centrosome declustering agent; a multi-kinase inhibitor; a serine/threonine kinase inhibitor; a tyrosine kinase inhibitor; a VEGF/VEGFR inhibitor; a taxane or taxane derivative, an aromatase inhibitor, anthracycline, a microtubule targeting drug, a topoisomerase poison drug, an inhibitor of a molecular target or enzyme (*e.g.*, a kinase or a protein methyltransferase), a cytidine analogue or combination thereof.

**[0238]** Exemplary alkylating agents include, but are not limited to, cyclophosphamide (Cytoxan; Neosar); chlorambucil (Leukeran); melphalan (Alkeran); carmustine (BiCNU);

busulfan (Busulfex); lomustine (CeeNU); dacarbazine (DTIC-Dome); oxaliplatin (Eloxatin); carmustine (Gliadel); ifosfamide (Ifex); mechlorethamine (Mustargen); busulfan (Myleran); carboplatin (Paraplatin); cisplatin (CDDP; Platinol); temozolomide (Temodar); thiotepa (Thioplex); bendamustine (Treanda); or streptozocin (Zanosar).

**[0239]** Exemplary anthracycline antibiotics include, but are not limited to, doxorubicin (Adriamycin); doxorubicin liposomal (Doxil); mitoxantrone (Novantrone); bleomycin (Blenoxane); daunorubicin (Cerubidine); daunorubicin liposomal (DaunoXome); dactinomycin (Cosmegen); epirubicin (Ellence); idarubicin (Idamycin); plicamycin (Mithracin); mitomycin (Mutamycin); pentostatin (Nipent); or valrubicin (Valstar).

**[0240]** Exemplary anti-metabolites include, but are not limited to, fluorouracil (Adrucil); capecitabine (Xeloda); hydroxyurea (Hydrea); mercaptopurine (Purinethol); pemetrexed (Alimta); fludarabine (Fludara); nelarabine (Arranon); cladribine (Cladribine Novaplus); clofarabine (Clolar); cytarabine (Cytosar-U); decitabine (Dacogen); cytarabine liposomal (DepoCyt); hydroxyurea (Droxia); pralatrexate (Folotyn); floxuridine (FUDR); gemcitabine (Gemzar); cladribine (Leustatin); fludarabine (Oforta); methotrexate (MTX; Rheumatrex); methotrexate (Trexall); thioguanine (Tabloid); TS-1 or cytarabine (Tarabine PFS).

**[0241]** Exemplary detoxifying agents include, but are not limited to, amifostine (Ethyol) or mesna (Mesnex).

**[0242]** Exemplary interferons include, but are not limited to, interferon alfa-2b (Intron A) or interferon alfa-2a (Roferon-A).

**[0243]** Exemplary polyclonal or monoclonal antibodies include, but are not limited to, trastuzumab (Herceptin); ofatumumab (Arzerra); bevacizumab (Avastin); rituximab (Rituxan); cetuximab (Erbitux); panitumumab (Vectibix); tositumomab/iodine131 tositumomab (Bexxar); alemtuzumab (Campath); ibritumomab (Zevalin; In-111; Y-90 Zevalin); gemtuzumab (Mylotarg); eculizumab (Soliris) ordenosumab.

**[0244]** Exemplary EGFR inhibitors include, but are not limited to, gefitinib (Iressa); lapatinib (Tykerb); cetuximab (Erbitux); erlotinib (Tarceva); panitumumab (Vectibix); PKI-166; canertinib (CI-1033); matuzumab (Emd7200) or EKB-569.

**[0245]** Exemplary HER2 inhibitors include, but are not limited to, trastuzumab (Herceptin); lapatinib (Tykerb) or AC-480.

**[0246]** Exemplary histone deacetylase inhibitors include, but are not limited to, vorinostat (Zolinza), valproic acid, romidepsin, entinostat abexinostat, givinostat, and mocetinostat.

**[0247]** Exemplary hormones include, but are not limited to, tamoxifen (Soltamox; Nolvadex); raloxifene (Evista); megestrol (Megace); leuprolide (Lupron; Lupron Depot; Eligard; Viadur); fulvestrant (Faslodex); letrozole (Femara); triptorelin (Trelstar LA; Trelstar Depot); exemestane (Aromasin); goserelin (Zoladex); bicalutamide (Casodex); anastrozole (Arimidex); fluoxymesterone (Androxy; Halotestin); medroxyprogesterone (Provera; Depo-Provera); estramustine (Emcyt); flutamide (Eulexin); toremifene (Fareston); degarelix (Firmagon); nilutamide (Nilandron); abarelix (Plenaxis); or testolactone (Teslac).

**[0248]** Exemplary mitotic inhibitors include, but are not limited to, paclitaxel (Taxol; Onxol; Abraxane); docetaxel (Taxotere); vincristine (Oncovin; Vincasar PFS); vinblastine (Velban); etoposide (Toposar; Etopophos; VePesid); teniposide (Vumon); ixabepilone (Ixempra); nocodazole; epothilone; vinorelbine (Navelbine); camptothecin (CPT); irinotecan (Camptosar); topotecan (Hycamtin); amsacrine or lamellarin D (LAM-D).

**[0249]** Exemplary phosphatidyl-inositol-3 kinase (PI3K) inhibitors include wortmannin an irreversible inhibitor of PI3K, demethoxyviridin a derivative of wortmannin, LY294002, a reversible inhibitor of PI3K; BKM120 (Buparlisib); Idelalisib (a PI3K Delta inhibitor); duvelisib (IPI-145, an inhibitor of PI3K delta and gamma); alpelisib (BYL719), an alpha-specific PI3K inhibitor; TGR 1202 (previously known as RP5264), an oral PI3K delta inhibitor; and copanlisib (BAY 80-6946), an inhibitor PI3K $\alpha, \delta$  isoforms predominantly.

**[0250]** Exemplary Akt inhibitors include, but are not limited to miltefosine, AZD5363, GDC-0068, MK2206, Perifosine, RX-0201, PBI-05204, GSK2141795, and SR13668.

**[0251]** Exemplary MTOR inhibitors include, but are not limited to, everolimus (Afinitor) or temsirolimus (Torisel); rapamune, ridaforolimus; deforolimus (AP23573), AZD8055 (AstraZeneca), OSI-027 (OSI), INK-128, BEZ235, PI-103, Torin1, PP242, PP30, Ku-0063794, WAY-600, WYE-687, WYE-354, and CC-223.

**[0252]** Exemplary proteasomal inhibitors include, but are not limited to, bortezomib (PS-341), ixazomib (MLN 2238), MLN 9708, delanzomib (CEP-18770), carfilzomib (PR-171), YU101, oprozomib (ONX-0912), marizomib (NPI-0052), and disulfiram.

**[0253]** Exemplary PARP inhibitors include, but are not limited to, olaparib, iniparib, velaparib, BMN-673, BSI-201, AG014699, ABT-888, GPI21016, MK4827, INO-1001, CEP-9722, PJ-34, Tiq-A, Phen, PF-01367338 and combinations thereof.

**[0254]** Exemplary Ras/MAPK pathway inhibitors include, but are not limited to, trametinib, selumetinib, cobimetinib, CI-1040, PD0325901, AS703026, RO4987655, RO5068760, AZD6244, GSK1120212, TAK-733, U0126, MEK162, and GDC-0973.

**[0255]** Exemplary centrosome declustering agents include, but are not limited to, griseofulvin; noscapine, noscapine derivatives, such as brominated noscapine (e.g., 9-bromonoscapine), reduced bromonoscapine (RBN), N-(3-brormobenzyl) noscapine, aminonoscapine and water-soluble derivatives thereof; CW069; the phenanthridene-derived poly(ADP-ribose) polymerase inhibitor, PJ-34; N2-(3-pyridylmethyl)-5-nitro-2-furamide, N2-(2-thienylmethyl)-5-nitro-2-furamide, and N2-benzyl-5-nitro-2-furamide.

**[0256]** Exemplary multi-kinase inhibitors include, but are not limited to, regorafenib; sorafenib (Nexavar); sunitinib (Sutent); BIBW 2992; E7080; Zd6474; PKC-412; motesanib; or AP24534.

**[0257]** Exemplary serine/threonine kinase inhibitors include, but are not limited to, ruboxistaurin; eril/easudil hydrochloride; flavopiridol; seliciclib (CYC202; Roscovitrine); SNS-032 (BMS-387032); Pkc412; bryostatin; KAI-9803; SF1126; VX-680; Azd1152; Arry-142886 (AZD-6244); SCIO-469; GW681323; CC-401; CEP-1347 or PD 332991.

**[0258]** Exemplary tyrosine kinase inhibitors include, but are not limited to, erlotinib (Tarceva); gefitinib (Iressa); imatinib (Gleevec); sorafenib (Nexavar); sunitinib (Sutent); trastuzumab (Herceptin); bevacizumab (Avastin); rituximab (Rituxan); lapatinib (Tykerb); cetuximab (Erbitux); panitumumab (Vectibix); everolimus (Afinitor); alemtuzumab (Campath); gemtuzumab (Mylotarg); temsirolimus (Torisel); pazopanib (Votrient); dasatinib (Sprycel); nilotinib (Tasigna); vatalanib (Ptk787; ZK222584); CEP-701; SU5614; MLN518; XL999; VX-322; Azd0530; BMS-354825; SKI-606 CP-690; AG-490; WHI-P154; WHI-P131; AC-220; or AMG888.

**[0259]** Exemplary VEGF/VEGFR inhibitors include, but are not limited to, bevacizumab (Avastin); sorafenib (Nexavar); sunitinib (Sutent); ranibizumab; pegaptanib; or vandetinib.

**[0260]** Exemplary microtubule targeting drugs include, but are not limited to, paclitaxel, docetaxel, vincristin, vinblastin, nocodazole, epothilones and navelbine.

**[0261]** Exemplary topoisomerase poison drugs include, but are not limited to, teniposide, etoposide, adriamycin, camptothecin, daunorubicin, dactinomycin, mitoxantrone, amsacrine, epirubicin and idarubicin.

**[0262]** Exemplary taxanes or taxane derivatives include, but are not limited to, paclitaxel and docetaxol.

**[0263]** Exemplary general chemotherapeutic, anti-neoplastic, anti-proliferative agents include, but are not limited to, altretamine (Hexalen); isotretinoin (Accutane; Amnesteem; Claravis; Sotret); tretinoin (Vesanoid); azacitidine (Vidaza); bortezomib (Velcade) asparaginase (Elspar); levamisole (Ergamisol); mitotane (Lysodren); procarbazine

(Matulane); pegaspargase (Oncaspar); denileukin diftitox (Ontak); porfimer (Photofrin); aldesleukin (Proleukin); lenalidomide (Revlimid); bexarotene (Targretin); thalidomide (Thalomid); temsirolimus (Torisel); arsenic trioxide (Trisenox); verteporfin (Visudyne); mimosine (Leucenol); (1M tegafur-0.4 M 5-chloro-2,4-dihydroxypyrimidine-1 M potassium oxonate) or lovastatin.

**[0264]** In certain embodiments, the antitumor antagonists described herein are administered at a subtherapeutic dose, another anti-immune checkpoint regulator antibody or antagonist is administered at a subtherapeutic dose, the angiogenesis antagonist is administered at a subtherapeutic dose, or any antagonist in a combination thereof is each administered at a subtherapeutic dose.

**[0265]** In certain embodiments, inhibition of the TGF $\beta$ /TGF $\beta$  RII, checkpoint regulator, and/or angiogenesis pathways may be combined with standard cancer treatments (e.g., surgery, radiation, and chemotherapy) in accordance with conventional chemotherapeutic regimes. In these instances, it may be possible to reduce the dose of chemotherapeutic reagent administered. An example of such a combination is a checkpoint regulator antagonist of the present application in combination with decarbazine for the treatment of melanoma. Another example of such a combination is a checkpoint regulator antagonist of the present application in combination with interleukin-2 (IL-2) for the treatment of melanoma. It is believed that the combined use of checkpoint regulator inhibition and chemotherapy can enhance apoptosis and increase tumor antigen presentation for cytotoxic immunity. Other synergistic combination therapies include checkpoint regulator inhibition through cell death when used in combination with radiation, surgery or hormone deprivation. Each of these protocols creates a source of tumor antigen in the host.

**[0266]** In certain embodiments, the checkpoint regulator antagonists described herein can be used in multi-specific antagonists or in combination with bispecific antibodies targeting Fc $\alpha$  or Fc $\gamma$  receptor-expressing effector cells to tumor cells (see, e.g., U.S. Pat. Nos. 5,922,845 and 5,837,243). Bispecific antibodies can be used to target two separate antigens. For example anti-Fc receptor/anti-tumor antigen (e.g., Her-2/neu) bispecific antibodies have been used to target macrophages to cancer cells or tumors. This targeting may more effectively activate tumor specific responses. The T cell arm of these responses would be augmented by checkpoint regulator inhibition. Alternatively, antigen may be delivered directly to DCs by the use of bispecific antibodies that bind to tumor antigen and a dendritic cell specific cell surface marker.

### **III. Nucleic Acids and Host Cells for Expressing the Antitumor Antagonists**

**[0267]** In another aspect, the present application provides nucleic acids encoding the antitumor antagonists of the present application, including the heavy and light chains, as well as expression vectors comprising such nucleic acids. In particular, the nucleic acids encode one or more HCDRs, LCDRs, HCVRs and/or LCVRs corresponding to any of the antibodies, antagonists or fragments described herein.

**[0268]** Thus, in one aspect, the present application provides one or more nucleic acids encoding any of the antitumor antagonists, antibodies or antigen-binding portions thereof as described herein.

**[0269]** In another aspect, the present application provides one or more expression vectors comprising the one or more nucleic acids encoding any of the antitumor antagonists, antibodies or antigen-binding portions thereof as described herein.

**[0270]** In another aspect, the present application provides a host cell transformed with the one or more expression vectors comprising the one or more nucleic acids encoding any of the antitumor antagonists, antibodies or antigen-binding portions thereof as described herein.

**[0271]** DNA(s) encoding antigen binding sites can be isolated and sequenced from a monoclonal antibody produced in hybridoma cells using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). Alternatively, amino acid sequences from immunoglobulins of interest may be determined by direct protein sequencing, and suitable encoding nucleotide sequences can be designed according to a universal codon table. In other cases, nucleotide and amino acid sequences of antigen binding sites or other immunoglobulin sequences, including constant regions, hinge regions and the like may be obtained from published sources well known in the art.

**[0272]** In one aspect, any of the binding antagonist fragments of the present application may include the use of codon optimized synthetic DNA fragments corresponding to e.g., antibody variable domains etc. For example, the cDNA sequences encoding immunoglobulin VH, VL, HC, LC, CH1, CH2, CH3, and/or framework region can be codon optimized for expression in various human, primate or mammalian cells, such as HEK or CHO cells. The invention also features a nucleic acid molecule that comprise one or both nucleotide sequences that encode heavy and light chain variable regions, CDRs, hypervariable loops, framework regions of the anti-PD-L1 antibody molecules, as described herein.

**[0273]** Expression vectors encoding a particular bispecific antitumor antagonist may be used to synthesize the antitumor antagonists of the present disclosure in cultured cells *in vitro* or they may be directly administered to a patient to express the antitumor antagonist *in*

*vivo* or *ex vivo*. As used herein, an “expression vector” refers to a viral or non-viral vector comprising a polynucleotide encoding one or more polypeptide chains corresponding to the bispecific antitumor antagonists of the present disclosure in a form suitable for expression from the polynucleotide(s) in a host cell for antibody preparation purposes or for direct administration as a therapeutic agent.

**[0274]** A nucleic acid sequence is “operably linked” to another nucleic acid sequence when the former is placed into a functional relationship with the latter. For example, a DNA for a presequence or signal peptide is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the DNA sequences being linked are contiguous and, in the case of a signal peptide, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers may be used in accordance with conventional practice.

**[0275]** Nucleic acid sequences for expressing the antitumor antagonists typically include an amino terminal signal peptide sequence, which is removed from the mature protein. Since the signal peptide sequences can affect the levels of expression, the polynucleotides may encode any one of a variety of different N-terminal signal peptide sequences. It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like.

**[0276]** The above described “regulatory sequences” refer to DNA sequences necessary for the expression of an operably linked coding sequence in one or more host organisms. The term “regulatory sequences” is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells or those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). Expression vectors generally contain sequences for transcriptional termination, and may additionally contain one or more elements positively affecting mRNA stability.

**[0277]** The expression vector contains one or more transcriptional regulatory elements, including promoters and/or enhancers, for directing the expression of antitumor antagonists. A promoter comprises a DNA sequence that functions to initiate transcription

from a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may operate in conjunction with other upstream elements and response elements.

**[0278]** As used herein, the term “promoter” is to be taken in its broadest context and includes transcriptional regulatory elements (TREs) from genomic genes or chimeric TREs therefrom, including the TATA box or initiator element for accurate transcription initiation, with or without additional TREs (*i.e.*, upstream activating sequences, transcription factor binding sites, enhancers, and silencers) which regulate activation or repression of genes operably linked thereto in response to developmental and/or external stimuli, and trans-acting regulatory proteins or nucleic acids. A promoter may contain a genomic fragment or it may contain a chimera of one or more TREs combined together.

**[0279]** Preferred promoters are those capable of directing high-level expression in a target cell of interest. The promoters may include constitutive promoters (*e.g.*, HCMV, SV40, elongation factor-1 $\alpha$  (EF-1 $\alpha$ )) or those exhibiting preferential expression in a particular cell type of interest. Enhancers generally refer to DNA sequences that function away from the transcription start site and can be either 5' or 3' to the transcription unit. Furthermore, enhancers can be within an intron as well as within the coding sequence. They are usually between 10 and 300 bp in length, and they function in *cis*. Enhancers function to increase and/or regulate transcription from nearby promoters. Preferred enhancers are those directing high-level expression in the antibody producing cell. Cell or tissue-specific transcriptional regulatory elements (TREs) can be incorporated into expression vectors to restrict expression to desired cell types. Pol III promoters (H1 or U6) are particularly useful for expressing shRNAs from which siRNAs are expressed. An expression vector may be designed to facilitate expression of the antitumor antagonist in one or more cell types.

**[0280]** In certain embodiments, one or more expression vectors may be engineered to express both the antitumor antagonist and one or more siRNA targeting the Tie2 pathway, the VEGF pathway or an immune checkpoint regulator.

**[0281]** An siRNA is a double-stranded RNA that can be engineered to induce sequence-specific post-transcriptional gene silencing of mRNAs. Synthetically produced siRNAs structurally mimic the types of siRNAs normally processed in cells by the enzyme Dicer. When expressed from an expression vector, the expression vector is engineered to transcribe a short double-stranded hairpin-like RNA (shRNA) that is processed into a targeted siRNA inside the cell. Synthetic siRNAs and shRNAs may be designed using well known algorithms and synthesized using a conventional DNA/RNA synthesizer.

**[0282]** To co-express the individual chains of the antitumor antagonist, a suitable splice donor and splice acceptor sequences may be incorporated for expressing both products. Alternatively, an internal ribosome binding sequence (IRES) or a 2A peptide sequence, may be employed for expressing multiple products from one promoter. An IRES provides a structure to which the ribosome can bind that does not need to be at the 5' end of the mRNA. It can therefore direct a ribosome to initiate translation at a second initiation codon within a mRNA, allowing more than one polypeptide to be produced from a single mRNA. A 2A peptide contains short sequences mediating co-translational self-cleavage of the peptides upstream and downstream from the 2A site, allowing production of two different proteins from a single transcript in equimolar amounts. CHYSEL is a non-limiting example of a 2A peptide, which causes a translating eukaryotic ribosome to release the growing polypeptide chain that it is synthesizing without dissociating from the mRNA. The ribosome continues translating, thereby producing a second polypeptide.

**[0283]** An expression vector may comprise a viral vector or a non-viral vector. A viral vectors may be derived from an adeno-associated virus (AAV), adenovirus, herpesvirus, vaccinia virus, poliovirus, poxvirus, a retrovirus (including a lentivirus, such as HIV-1 and HIV-2), Sindbis and other RNA viruses, alphavirus, astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, togaviruses and the like. A non-viral vector is simply a “naked” expression vector that is not packaged with virally derived components (e.g., capsids and/or envelopes).

**[0284]** In certain cases, these vectors may be engineered to target certain diseases or cell populations by using the targeting characteristics inherent to the virus vector or engineered into the virus vector. Specific cells may be “targeted” for delivery of polynucleotides, as well as expression. Thus, the term “targeting”, in this case, may be based on the use of endogenous or heterologous binding agents in the form of capsids, envelope proteins, antibodies for delivery to specific cells, the use of tissue-specific regulatory elements for restricting expression to specific subset(s) of cells, or both.

**[0285]** In some embodiments, expression of the antibody chains is under the control of the regulatory element such as a tissue specific or ubiquitous promoter. In some embodiments, a ubiquitous promoter such as a CMV promoter, CMV-chicken beta-actin hybrid (CAG) promoter, a tissue specific or tumor-specific promoter to control the expression of a particular antibody heavy or light chain or single-chain derivative therefrom.

**[0286]** Non-viral expression vectors can be utilized for non-viral gene transfer, either by direct injection of naked DNA or by encapsulating the antitumor antagonist-encoding polynucleotides in liposomes, microparticles, microcapsules, virus-like particles, or

erythrocyte ghosts. Such compositions can be further linked by chemical conjugation to targeting domains to facilitate targeted delivery and/or entry of nucleic acids into desired cells of interest. In addition, plasmid vectors may be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, and linked to cell targeting ligands such as asialoorosomucoid, insulin, galactose, lactose or transferrin.

[0287] Alternatively, naked DNA may be employed. Uptake efficiency of naked DNA may be improved by compaction or by using biodegradable latex beads. Such delivery may be improved further by treating the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

#### **IV. Methods for Producing the Bispecific Antagonists**

[0288] In another aspect, the present application provides host cells transformed with the nucleic acids or expression vectors encoding the bispecific antitumor antagonists of the present application. The host cells can be any eukaryotic or prokaryotic cell capable of expressing the bispecific antitumor antagonists of the present application, including immunoglobulin heavy and light chains thereof.

[0289] In a further aspect, a method of producing antitumor antagonist comprises culturing a host cell transformed with one or more nucleic acids or expression vectors encoding the bispecific antitumor antagonists of the present application under conditions that allow for production and purification of the antagonists, antibodies or fragments thereof expressed in suitable cells.

[0290] In a further aspect, the present application provides a method for producing antibody comprising culturing a cell transiently or stably expressing one or more constructs encoding one or more polypeptide chains in the antibody; and purifying the antibody from the cultured cells. Any cell capable of producing a functional antibody may be used. In preferred embodiments, the antibody-expressing cell is of eukaryotic or mammalian origin, preferably a human cell. Cells from various tissue cell types may be used to express the antibodies. In other embodiments, the cell is a yeast cell, an insect cell or a bacterial cell. Preferably, the antibody-producing cell is stably transformed with a vector expressing the antibody.

[0291] One or more expression vectors encoding the antibody heavy or light chains can be introduced into a cell by any conventional method, such as by naked DNA technique, cationic lipid-mediated transfection, polymer-mediated transfection, peptide-mediated transfection, virus-mediated infection, physical or chemical agents or treatments, electroporation, *etc.* In addition, cells may be transfected with one or more expression

vectors for expressing the antibody along with a selectable marker facilitating selection of stably transformed clones expressing the antibody. The antibodies produced by such cells may be collected and/or purified according to techniques known in the art, such as by centrifugation, chromatography, etc.

**[0292]** Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are CHO DHFR<sup>-</sup> cells and mouse LTK<sup>-</sup> cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

**[0293]** The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, mycophenolic acid, or hygromycin. The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puromycin.

**[0294]** Exemplary antibody-expressing cells include human Jurkat, human embryonic kidney (HEK) 293, Chinese hamster ovary (CHO) cells, mouse WEHI fibrosarcoma cells, as well as unicellular protozoan species, such as *Leishmania tarentolae*. In addition, stably transformed, antibody producing cell lines may be produced using primary cells immortalized with c-myc or other immortalizing agents.

**[0295]** In one embodiment, the cell line comprises a stably transformed *Leishmania* cell line, such as *Leishmania tarentolae*. *Leishmania* are known to provide a robust, fast-growing unicellular host for high level expression of eukaryotic proteins exhibiting

mammalian-type glycosylation patterns. A commercially available *Leishmania* eukaryotic expression kit is available (Jena Bioscience GmbH, Jena, Germany).

**[0296]** In some embodiments, the cell line expresses at least 1 mg, at least 2 mg, at least 5 mg, at least 10 mg, at least 20 mg, at least 50 mg, at least 100 mg, at least 200 mg, at least 300 mg, at least 400 mg, or at least 500 mg of the antibody/liter of culture.

**[0297]** The antibodies in the present application may be isolated from antibody expressing cells following culture and maintenance in any appropriate culture medium, such as RPMI, DMEM, and AIM V<sup>®</sup>. The antibodies can be purified using conventional protein purification methodologies (e.g., affinity purification, chromatography, etc.), including the use of Protein-A or Protein-G immunoaffinity purification. In some embodiments, antibodies are engineered for secretion into culture supernatants for isolation therefrom.

## **V. Pharmaceutical Compositions and Methods of Treatment**

**[0298]** Another aspect of the present application relates to pharmaceutical compositions and methods for treating a cell proliferative disorder, such as cancer, chronic infections, or immunologically compromised disease states. In one embodiment, the pharmaceutical composition comprises one or more antitumor antagonists of the present application. In some embodiments, the antitumor antagonist(s) comprise: one or more TGF- $\beta$ 1 inhibitors or TGF- $\beta$ 1 RII inhibitors in combination with: (1) a PD-1 inhibitor or a PD-L1 inhibitor; or (2) or one or more angiogenesis inhibitors, such as VEGF inhibitors, VEGFR2 inhibitors, angiopoietin-1/2 inhibitors, and Tie2R inhibitors. The antagonist(s) are formulated together with a pharmaceutically acceptable carrier. Pharmaceutical composition of the present application may include one or more different antibodies, one or more multispecific antibodies, one or more immunoconjugates, or a combination thereof as described herein.

**[0299]** As described above, methods for using the pharmaceutical compositions described herein comprise administering to a subject in need thereof an effective amount of the pharmaceutical composition according to the present disclosure.

**[0300]** Any suitable route or mode of administration can be employed for providing the patient with a therapeutically or prophylactically effective dose of the antibody or antagonist. Exemplary routes or modes of administration include parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous, intratumoral), oral, topical (nasal, transdermal, intradermal or intraocular), mucosal (e.g., nasal, sublingual, buccal, rectal, vaginal), inhalation, intralymphatic, intraspinal, intracranial, intraperitoneal, intratracheal, intravesical, intrathecal, enteral, intrapulmonary, intralymphatic, intracavital, intraorbital, intracapsular and transurethral, as well as local delivery by catheter or stent.

**[0301]** A pharmaceutical composition comprising antibody or antagonist in accordance with the present disclosure may be formulated in any pharmaceutically acceptable carrier(s) or excipient(s). As used herein, the term “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Pharmaceutical compositions may comprise suitable solid or gel phase carriers or excipients. Exemplary carriers or excipients include but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols. Exemplary pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the therapeutic agents.

**[0302]** The antitumor antagonist can be incorporated into a pharmaceutical composition suitable for parenteral administration. Suitable buffers include but are not limited to, sodium succinate, sodium citrate, sodium phosphate or potassium phosphate. Sodium chloride can be used to modify the toxicity of the solution at a concentration of 0-300 mM (optimally 150 mM for a liquid dosage form). Cryoprotectants can be included for a lyophilized dosage form, principally 0-10% sucrose (optimally 0.5-1.0%). Other suitable cryoprotectants include trehalose and lactose. Bulking agents can be included for a lyophilized dosage form, principally 1-10% mannitol (optimally 2-4%). Stabilizers can be used in both liquid and lyophilized dosage forms, principally 1-50 mM L-Methionine (optimally 5-10 mM). Other suitable bulking agents include glycine, arginine, can be included as 0-0.05% polysorbate-80 (optimally 0.005-0.01%). Additional surfactants include but are not limited to polysorbate 20 and BRIJ surfactants.

**[0303]** Therapeutic antitumor antagonist preparations can be lyophilized and stored as sterile powders, preferably under vacuum, and then reconstituted in bacteriostatic water (containing, for example, benzyl alcohol preservative) or in sterile water prior to injection. Pharmaceutical composition may be formulated for parenteral administration by injection e.g., by bolus injection or continuous infusion.

**[0304]** The therapeutic agents in the pharmaceutical compositions may be formulated in a “therapeutically effective amount” or a “prophylactically effective amount”. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of

time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the recombinant vector may vary depending on the condition to be treated, the severity and course of the condition, the mode of administration, whether the antibody or agent is administered for preventive or therapeutic purposes, the bioavailability of the particular agent(s), the ability of the antitumor antagonist to elicit a desired response in the individual, previous therapy, the age, weight and sex of the patient, the patient's clinical history and response to the antibody, the type of the antitumor antagonist used, discretion of the attending physician, etc. A therapeutically effective amount is also one in which any toxic or detrimental effect of the recombinant vector is outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result.

**[0305]** Preferably, the polypeptide domains in the antitumor antagonist are derived from the same host in which they are to be administered in order to reduce inflammatory responses against the administered therapeutic agents.

**[0306]** The antitumor antagonist is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The antitumor antagonist may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

**[0307]** As a general proposition, a therapeutically effective amount or prophylactically effective amount of the antitumor antagonist will be administered in a range from about 1 ng/kg body weight/day to about 100 mg/kg body weight/day whether by one or more administrations. In a particular embodiment, each antitumor antagonist is administered in the range of from about 1 ng/kg body weight/day to about 10 mg/kg body weight/day, about 1 ng/kg body weight/day to about 1 mg/kg body weight/day, about 1 ng/kg body weight/day to about 100 µg/kg body weight/day, about 1 ng/kg body weight/day to about 10 µg/kg body weight/day, about 1 ng/kg body weight/day to about 1 µg/kg body weight/day, about 1 ng/kg body weight/day to about 100 ng/kg body weight/day, about 1 ng/kg body weight/day to about 10 ng/kg body weight/day, about 10 ng/kg body weight/day to about 1 mg/kg body weight/day, about 10 ng/kg body weight/day to about 100 ng/kg body weight/day, about 10 ng/kg body weight/day to about 10 µg/kg body weight/day, about 10 ng/kg body weight/day to about 1 µg/kg body weight/day, 10 ng/kg body weight/day to about 100 ng/kg body weight/day, about 100 ng/kg body weight/day to about 100 mg/kg body weight/day, about 100 ng/kg body weight/day to about 10 mg/kg body weight/day, about 100 ng/kg body weight/day to about 1 mg/kg body

weight/day, about 100 ng/kg body weight/day to about 100  $\mu$ g/kg body weight/day, about 100 ng/kg body weight/day to about 10  $\mu$ g/kg body weight/day, about 100 ng/kg body weight/day to about 1  $\mu$ g/kg body weight/day, about 1  $\mu$ g/kg body weight/day to about 100 mg/kg body weight/day, about 1  $\mu$ g/kg body weight/day to about 10 mg/kg body weight/day, about 1  $\mu$ g/kg body weight/day to about 1 mg/kg body weight/day, about 1  $\mu$ g/kg body weight/day to about 100  $\mu$ g/kg body weight/day, about 1  $\mu$ g/kg body weight/day to about 10  $\mu$ g/kg body weight/day, about 10  $\mu$ g/kg body weight/day to about 10 mg/kg body weight/day, about 10  $\mu$ g/kg body weight/day to about 1 mg/kg body weight/day, about 10  $\mu$ g/kg body weight/day to about 100 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 100 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 10 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 1 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 100 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 10 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 1 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 100 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 10 mg/kg body weight/day, about 100  $\mu$ g/kg body weight/day to about 1 mg/kg body weight/day.

**[0308]** In other embodiments, the antitumor antagonist is administered at a dose of 500  $\mu$ g to 20 g every three days, or 25 mg/kg body weight every three days.

**[0309]** In other embodiments, each antitumor antagonist is administered in the range of about 10 ng to about 100 ng per individual administration, about 10 ng to about 1  $\mu$ g per individual administration, about 10 ng to about 10  $\mu$ g per individual administration, about 10 ng to about 100  $\mu$ g per individual administration, about 10 ng to about 1 mg per individual administration, about 10 ng to about 10 mg per individual administration, about 10 ng to about 100 mg per individual administration, about 10 ng to about 1000 mg per injection, about 10 ng to about 10,000 mg per individual administration, about 100 ng to about 1  $\mu$ g per individual administration, about 100 ng to about 10  $\mu$ g per individual administration, about 100 ng to about 100  $\mu$ g per individual administration, about 100 ng to about 1 mg per individual administration, about 100 ng to about 10 mg per individual administration, about 100 ng to about 100  $\mu$ g per individual administration, about 100 ng to about 1000 mg per injection, about 100 ng to about 10,000 mg per individual administration, about 1  $\mu$ g to about 10  $\mu$ g per individual administration, about 1  $\mu$ g to about 100  $\mu$ g per individual administration, about 1  $\mu$ g to about 1 mg per individual administration, about 1  $\mu$ g to about 10 mg per individual administration, about 1  $\mu$ g to about 100 mg per individual administration, about 1  $\mu$ g to about 1000 mg per injection, about 1  $\mu$ g to about 10,000 mg per individual administration, about 10  $\mu$ g to about 100  $\mu$ g per individual administration, about 10  $\mu$ g to about 1 mg per individual administration, about 10  $\mu$ g to about 10 mg per individual administration, about 10  $\mu$ g to about 100 mg per individual administration, about 10  $\mu$ g to about 1000 mg per injection, about 10  $\mu$ g to about 10,000 mg per individual administration, about 100  $\mu$ g to about 1000 mg per injection, about 100  $\mu$ g to about 10,000 mg per individual administration, about 1000  $\mu$ g to about 10,000 mg per injection, about 1000  $\mu$ g to about 10,000 mg per individual administration, about 10000  $\mu$ g to about 10,000 mg per injection, about 10000  $\mu$ g to about 10,000 mg per individual administration.

administration, about 10 µg to about 100 mg per individual administration, about 10 µg to about 1000 mg per injection, about 10 µg to about 10,000 mg per individual administration, about 100 µg to about 1 mg per individual administration, about 100 µg to about 10 mg per individual administration, about 100 µg to about 100 mg per individual administration, about 100 µg to about 1000 mg per injection, about 100 µg to about 10,000 mg per individual administration, about 1 mg to about 10 mg per individual administration, about 1 mg to about 100 mg per individual administration, about 1 mg to about 1000 mg per injection, about 1 mg to about 10,000 mg per individual administration, about 10 mg to about 100 mg per individual administration, about 10 mg to about 1000 mg per injection, about 10 mg to about 10,000 mg per individual administration, about 100 mg to about 1000 mg per injection, about 100 mg to about 10,000 mg per individual administration and about 1000 mg to about 10,000 mg per individual administration. The antitumor antagonist may be administered daily, every 2, 3, 4, 5, 6 or 7 days, or every 1, 2, 3 or 4 weeks.

**[0310]** In other particular embodiments, the amount of the antitumor antagonist may be administered at a dose of about 0.0006 mg/day, 0.001 mg/day, 0.003 mg/day, 0.006 mg/day, 0.01 mg/day, 0.03 mg/day, 0.06 mg/day, 0.1 mg/day, 0.3 mg/day, 0.6 mg/day, 1 mg/day, 3 mg/day, 6 mg/day, 10 mg/day, 30 mg/day, 60 mg/day, 100 mg/day, 300 mg/day, 600 mg/day, 1000 mg/day, 2000 mg/day, 5000 mg/day or 10,000 mg/day. As expected, the dosage will be dependent on the condition, size, age and condition of the patient.

**[0311]** In certain embodiments, the coding sequences for a antitumor antagonist are incorporated into a suitable expression vector (*e.g.*, viral or non-viral vector) for expressing an effective amount of the antitumor antagonist in patient with a cell proliferative disorder. In certain embodiments comprising administration of *e.g.*, one or more recombinant AAV (rAAV) viruses, the pharmaceutical composition may comprise the rAAVs in an amount comprising at least  $10^{10}$ , at least  $10^{11}$ , at least  $10^{12}$ , at least  $10^{13}$ , or at least  $10^{14}$  genome copies (GC) or recombinant viral particles per kg, or any range thereof. In certain embodiments, the pharmaceutical composition comprises an effective amount of the recombinant virus, such as rAAV, in an amount comprising at least  $10^{10}$ , at least  $10^{11}$ , at least  $10^{12}$ , at least  $10^{13}$ , at least  $10^{14}$ , at least  $10^{15}$  genome copies or recombinant viral particles genome copies per subject, or any range thereof.

**[0312]** Dosages can be tested in several art-accepted animal models suitable for any particular cell proliferative disorder.

**[0313]** Delivery methodologies may also include the use of polycationic condensed DNA linked or unlinked to killed viruses, ligand linked DNA, liposomes, eukaryotic cell delivery vehicles cells, deposition of photopolymerized hydrogel materials, use of a handheld

gene transfer particle gun, ionizing radiation, nucleic charge neutralization or fusion with cell membranes, particle mediated gene transfer and the like.

[0314] The present invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and Tables are incorporated herein by reference.

## EXAMPLES

### Example 1: Generation of monoclonal antibodies

[0315] Monoclonal antibodies (mAbs) of the present application are generated and screened using techniques well known in the art, see, e.g., Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York. The antigen specific hybridoma mabs are cloned, sequenced and engineered using techniques well known in the art, see e.g., Lo. B.K.C Methods in Molecular Biology™. Volume 248 2004. Antibody Engineering.

### Example 2: Design of bispecific antitumor antagonists

[0316] FIGS. 5A and 5B show two bispecific antitumor antagonists, Bi-PB-1 (or Bi-PLB-1) and Bi-PB-1 (or Bi-PLB-2), respectively. These antagonists comprise a checkpoint regulator antibody backbone (anti-PD-1 or anti-PD-L1) along with a TGF- $\beta$  RII extracellular domain (TGF- $\beta$ -RII ECD): (i) fused to the carboxy-terminal end of the CH3 region in each of the two heavy chains (FIG. 5A) or (ii) inserted within the CH3 region of the Fc loop (FIG. 5B).

[0317] In the embodiments depicted in FIGS. 5A and 5B, the bispecific antibody can have an IgG1 or IgG4 backbone. Furthermore, any one or more of the antibody specificities may be substituted with any other checkpoint regulator antagonist specificity and/or any tumor targeting antibody specificity, such as CD20, EGFR, etc.

[0318] FIG. 6 shows exemplary functional domain sequences corresponding to the bispecific antibodies in FIGS. 5A and 5B.

[0319] FIGS. 7A-7B show exemplary heavy chain (HC) and light chain (LC) sequences corresponding to the bispecific antibodies depicted in FIGS. 5A and 5B.

### Example 3: Design of additional bispecific antagonists

[0320] FIGS. 8A-8C show the design of three different bispecific antagonists, Bi-AB-1, Bi-A1B-1 and Bi-ZB-1, respectively, each comprising a carboxy-terminal TGF- $\beta$ 1 RII extracellular domain (ECD) in a mutant IgG1 (K447A) scaffold. Bi-AB-1 and Bi-A1B-1 both contain amino-terminal anti-VEGF variable regions (VH1, VL1) from Avastin/bevacizumab; Bi-A1B-1 includes two amino acid substitutions in the VH region (E6Q, L11V). Bi-ZB-1

contains an amino terminal afibbercept domain upstream of an IgG1 Fc (K447A) region. In other embodiments, instead of a mutant IgG1(K447A) scaffold, the bispecific antagonists comprise a wild-type IgG1 scaffold, a different mutant IgG1 scaffold, an IgG2 scaffold, a mutant IgG2 scaffold, an IgG4 scaffold, or a mutant IgG4 scaffold.

[0321] FIGS. 9A and 9B show the various functional domain sequences present the bispecific antagonists depicted in FIGS. 8A-8C.

[0322] FIG. 10 shows the heavy chain (HC) and light chain (LC) amino acid sequences corresponding to the bispecific antagonists depicted in FIGS. 8A-8C.

[0323] FIG. 11 summarizes the arrangement of functional domains in the bispecific antagonists depicted in FIGS. 8A-8C.

**Example 4: Expression and purification of the antagonists in FIGS. 10-11**

[0324] FIG. 12 is a Coomassie blue stained polyacrylamide gel showing improved expression levels of antagonists containing wt VEGF-A or mutant E6Q and L11V mutations (i.e., Bi-AB-1, Bi-A1B-1, respectively) when transiently transfected into HEK293 cells as determined by non-reducing polyacrylamide gel electrophoresis (PAGE). The titers depicted in FIG. 12 were determined by quantifying the cell supernatant using a POROS A column (Applied Biosystems). The results of this analysis showed that the expression level of Bi-A1B-1 was increased by 167% relative to Bi-AB-1.

[0325] FIG. 13A depicts a size exclusion chromatography (SEC) profile showing relative levels of high molecular weight (HMW), low molecular weight (LMW) species, and dimers resulting from protein A purified Bi-AB-1 and Bi-A1B-1. FIG. 13B shows that the percentage of dimers (98.6% for Bi-AB-1; 98.7% for Bi-A1B-1) greatly outnumber the HMW (1%) and LMW (0.4%) species purified as determined by SE-UPLC using Tosoh TSKgel UP-G3000SWXL columns. These results show that TGF- $\beta$ 1 RII extracellular domain (ECD) fusions to Bi-AB-1 and Bi-A1B-1 did not result in appreciable levels of high molecular weight (HMW) or low molecular weight (LMW) species.

[0326] FIGS. 14A-14B depict Coomassie blue stained PAGE analyses of under non-reducing and reducing conditions, respectively, from supernatants obtained following transient transfection of Bi-ZB-1. The results of this analysis confirmed good transient expression levels of Bi-ZB-1 (220  $\mu$ g/ml) after 8 days of growth of cells *in vitro*.

[0327] FIG. 15 depicts a size exclusion chromatography (SEC) profile showing that protein A purified Bi-ZB-1 has low levels of high molecular weight (HMW, 3.4%) and low molecular weight (LMW, 0.5%) species in comparison to dimers (96.1%).

**[0328] Example 5: Functional characterization of the antitumor antagonists in FIGS. 8A-8C.**

**[0329]** To evaluate the ability of the anti-VEGF antagonist, Bi-A1B-1, to antagonize VEGF-mediated activation of VEGFR2 (i.e., the cognate receptor), a cell-based luciferase assay was conducted. In this experiment, a recombinant HEK-293 cell line expressing human VEGFR2 and a firefly luciferase construct under the control of NFAT response elements was stimulated with huVEGF165 in the presence of serial dilutions of anti-VEGF antagonist (Bi-A1B-1). The results of this assay are shown in **FIG. 16**. As expected, increasing levels of the anti-VEGF antagonist progressively neutralized the ability of VEGF165 to activate VEGFR2 and induce NFAT-mediated luciferase activity.

**[0330]** To evaluate the ability of the bispecific antagonist, Bi-A1B-1 to simultaneously bind TGF- $\beta$ 1 and VEGF165, a sandwich ELISA assay was performed. In this experiment, huTGF- $\beta$ 1 was coated onto 96 well plates and blocked with 5% BSA, followed by addition and incubation of serially diluted samples of Bi-A1B-1, followed by addition of biotinylated huVEGF165. Bound molecules were detected by Streptavidin-HRP using a TMB substrate. The results of this analysis in **FIG. 17** show that the bispecific antagonist, Bi-A1B-1 can simultaneously bind both TGF- $\beta$ 1 and VEGF165.

**[0331]** To evaluate the ability of the TGF- $\beta$ 1 RII ECD to antagonize TGF- $\beta$ 1-mediated activation of the human TGF- $\beta$ 1 RII (i.e., the cognate receptor), a cell-based luciferase assay was conducted. In this experiment, recombinant HEK-293 cells expressing human TGF- $\beta$ 1 RII receptor and a firefly luciferase construct under the control of a SMAD response element was stimulated with huTGF- $\beta$ 1 in the presence of serial dilutions of antibodies fused to a TGF- $\beta$ 1 RII ECD (Bi-A1B-1, Bi-ZB-1). The results of this analysis are shown in **FIG. 18**. As expected, increasing levels of antibodies containing a TGF- $\beta$ 1 RII ECD progressively neutralized the ability of TGF- $\beta$ 1 to activate the human TGF- $\beta$ 1 RII and induce NFAT-mediated luciferase activity. In this case, Bi-A1B-1 and Bi-ZB-1 exhibited similar antagonism ability, as reflected in an IC<sub>50</sub> of 0.32 nM for Bi-A1B-1 and an IC<sub>50</sub> of 0.31 for Bi-ZB-1. These IC<sub>50</sub>s were comparable to a control TGFB2 fusion product (IC<sub>50</sub>=0.20 nM).

**[0332]** To evaluate the pharmacokinetic properties of Bi-A1B-1 *in vivo*, pharmacokinetic profiles were generated. Briefly, 10 mg/kg of each antagonist was intravenously injected into the tail vein of 6-10 week old female CD1 mice (n=2 mice per molecule). Serum was harvested at 3 minutes, 3 hours, 1 day, 3 days, 7 days and 10 days post injection. To detect the antibodies in the serum, 96 well ELISA plates were coated with 5  $\mu$ g/ml goat anti-human IgG F(ab')2 fragment and then blocked with 5% milk in PBS. Serially diluted mouse serum in 5% milk and serially diluted purified protein molecule as standard were added to the plates. Following incubation with peroxidase conjugated mouse anti-human IgG and further washes, bispecific antagonist Bi-A1B-1 (**FIG. 19**) antibodies were detected following incubation with TMB-

ELISA substrate. The results of this analysis showed that the half-life ( $T_{1/2}$ ) of the bispecific antagonist Bi-A1B-1 is 7.4-16 days.

**Example 6: Design of bispecific PD-1/PD-L1 antagonists with TGF- $\beta$ 1 ECD**

[0333] FIGS. 20A-20B depict two bispecific antitumor antagonists, Bi-PB-1.2 (FIG. 20A) and Bi-PLB-1.2 (FIG. 20B), each comprising an antibody backbone (IgG4 K447A or IgG1 K447A) with variable region domains from anti-PD-1 and anti-PD-L1, respectively, and additionally including a TGF- $\beta$ -RII ECD fused to the carboxy-terminal end of each heavy chain CH3 region.

[0334] FIG. 19 shows functional domain sequences present in the bispecific antibodies in FIGS. 20A and 20B. FIG. 24 show the heavy chain (HC) and light chain (LC) amino acid sequences corresponding to the bispecific antagonists depicted in FIGS. 20A and 20B. FIG. 23 summarizes the arrangement of functional domains in the bispecific antagonists depicted in FIGS. 20A and 20B.

**Example 7: Expression and purification of bispecific antagonists in FIGS. 20A-20B**

[0335] FIG. 24A shows a non-denaturing polyacrylamide gel (PAGE) analysis showing increased expression of Bi-PB-1.2 and Bi-PLB-1.2 in a transient expression system following protein A-affinity purification in comparison to a parental control antibody (2P17). FIG. 24B depicts size exclusion chromatography (SEC) profiles showing that protein A purified Bi-PB-1.2, Bi-PLB-1.2 and 2P17 (Benchmark) from transiently transfected HEK293 cells have low levels of high molecular weight (HMW) low molecular weight (LMW) species in comparison to dimerized molecule (Dimer), as shown in FIG. 24C. FIGS. 25A-25B show that the Dimer, HMW and LMW forms of Bi-PB-1.2 and Bi-PLB-1.2 exhibit good stability for at least 4 weeks at 4° C.

**Example 8: Functional characterization of Bi-PB-1.2 and Bi-PLB-1.2 antagonists in FIGS. 20A-20B**

[0336] 1. *Binding of Bi-PB-1.2 to PD-1 and TGF- $\beta$ 1 by bio-layer interferometry*

[0337] Bio-light interferometry was carried out using the Octet RED96 system (ForteBio Octet RED96 System) to characterize the binding kinetics of antibodies against His tagged human PD-1 protein or His tagged TGF- $\beta$ 1. 20 nM of antibody was loaded onto the anti-human IgG capture biosensors. Association of analyte (His tagged human PD-1 or TGF- $\beta$ 1 protein) was observed by placing the biosensors in wells containing 2 or 3 fold serial dilution of analytes (72 nM being the highest concentration) for 5 mins. Dissociation was measured after transfer of the biosensors into kinetic buffer alone and monitoring of the interferometry signal for 10 minutes. The observed on and off rates (Ka and Kd) were fit using a 1:1 binding global fit

model comprising at least 5 concentrations tested, and the equilibrium binding constant  $K_D$  was then calculated.

**[0338]** FIGS. 26A-26E show the results of PD-1 and TGF- $\beta$ 1 binding to Bi-PB-1.2 (FIGS. 26A, 26B, respectively) and corresponding anti-PD-1 antibody and the benchmark (M7824 from Merck KGA) (FIGS. 26C, 26D, respectively), along with their resultant binding affinity constants (FIG. 26E). The results show that the binding affinities of Bi-PB-1.2 for PD-1 and TGF- $\beta$ 1 are better than the anti-PD-1 benchmark and the M7824 benchmark.

**[0339]** 2. *Binding of Bi-PLB-1.2 to PD-L1 and TGF- $\beta$ 1 by bio-layer interferometry*

**[0340]** Binding of Bi-PLB-1.2 to PD-L1 and TGF- $\beta$ 1 by bio-layer interferometry was carried out as described for Bi-PB-1.2 above. FIGS. 27A-27E show the results of PD-1 and TGF- $\beta$ 1 binding to Bi-PB-1.2 (FIGS. 27A, 27B, respectively) and corresponding anti-PD-1 and anti-TGF- $\beta$ 1 benchmark antibodies (FIGS. 27C, 27D, respectively), along with their resultant binding affinity constants (FIG. 27E). The results show that the binding affinity of Bi-PLB-1.2 for TGF- $\beta$ 1 is better than the TGF- $\beta$ 1 ECD benchmark, and the binding affinity of Bi-PLB-1.2 for PD-L1 is comparable to the anti-PD-L1 benchmark.

**[0341]** 3. *Simultaneous binding to PD-1/PD-L1 by Bi-PB-1.2 and Bi-PLB-1.2*

**[0342]** FIG. 28A shows an ELISA assay demonstrating simultaneous binding of TGF- $\beta$ 1 and PD-1 by Bi-PB-1.2 in which huTGF- $\beta$ 1 coated 96 well plates were incubated with serially diluted samples of Bi-PB-1.2, followed by his tagged huPD-1, whereby bound molecules were detected by anti-His tag-HRP using a TMB substrate. FIG. 28B shows an ELISA assay demonstrating simultaneous binding of TGF- $\beta$ 1 and PD-L1 by Bi-PLB-1.2 in which huTGF- $\beta$ 1 coated 96 well plates were incubated with serially diluted samples of Bi-PLB-1.2, followed by His-tagged huPD-L1, whereby bound molecules were detected by anti-his tag-HRP using a TMB substrate. The EC50 values reflect the half maximal effective concentrations producing a response halfway between the baseline and maximum response with respect to binding human PD-1 or PD-L1.

**[0343]** 4. *Bi-PB-1.2 potently blocks binding of both PD-L1 and TGF- $\beta$ 1 to their receptors*

**[0344]** A blocking assay was carried out to calculate IC50 values corresponding to binding between Bi-PB-1.2 and PD-L1 in comparison to an anti-PD-1 benchmark antibody. Briefly, 2 fold serial dilutions of Bi-PB-1.2 or the benchmark antibody (highest Ab concentration: 128 nM; Triplicates for each mAb) were prepared. Human PD-1 transfected CHO-K1 cells were washed with FACS buffer (0.5%BSA 2mM EDTA in PBS) and re-suspended at a concentration of  $10^6$  cells/ml. FITC labeled human PD-L1-Fc protein was added to the human PD-1 transfected CHO-K1 cells at a final concentration of 7  $\mu$ g/ml and mixed well.

Without incubation, 2,000 of these CHO-K1 cells (with PD-L1 Protein) in 20  $\mu$ l FACS buffer was immediately added to a 96-well round bottom plate and 20  $\mu$ l or 2 fold serial diluted antibodies were immediately added to the cells and incubated at 4°C for 30 mins. The cells were then washed and re-suspended in 30  $\mu$ l 7AAD solution; 35  $\mu$ l 10% neutral buffered formalin solution was then added and incubated for 15 min. before analysis using the iQue intellicyt system. The results of this analysis are shown in **FIG. 29A**. As expected, increasing levels of Bi-PB-1.2 progressively blocked the ability of PD-L1 to bind to its receptor. Further, the calculated IC50 of Bi-PB-1.2 for PD-L1 was found to be comparable to the IC50 of the anti-PD-1 benchmark for PD-L1.

**[0345]** To evaluate the ability of the TGF- $\beta$ 1 RII ECD to antagonize TGF- $\beta$ 1-mediated activation of the human TGF- $\beta$ 1 RII (i.e., the cognate receptor), a cell-based luciferase assay was conducted. In this experiment, recombinant HEK-293 cells expressing human TGF- $\beta$ 1 RII receptor and a firefly luciferase construct under the control of a SMAD response element was stimulated with huTGF- $\beta$ 1 in the presence of serial dilutions of Bi-PB-1.2 containing a TGF- $\beta$ 1 RII ECD. Bioactivity was determined by a decrease in the amount of luciferase-mediated luminescence.

**[0346]** The results of this assay are shown in **FIG. 29B**. As expected, increasing levels of antibodies containing a TGF- $\beta$ 1 RII ECD progressively neutralized the ability of TGF- $\beta$ 1 to activate the human TGF- $\beta$ 1 RII and induce NFAT-mediated luciferase activity. Further, the calculated IC50 Bi-PB-1.2 for TGF- $\beta$ 1 was found to be comparable to the IC50 of the anti-PD-1 benchmark for TGF- $\beta$ 1.

**[0347] 5. Bi-PB-1.2 binds both human and cynomolgus PD-1 with similar activity**

**[0348]** To evaluate the ability of Bi-PB-1.2 to bind both human and cyno PD-1, serial dilutions of Bi-PB-1.2 were added to CHO-K1 cells (20,000 cells/well) overexpressing human or cyno PD-1. The mixtures were incubated at 4°C for 20 min, washed 3 times, and stained with the secondary antibody, PE labeled F(ab')2-Goat anti-human IgG Fc (Thermo H10104) at 4°C for 20 min. Cells were washed and resuspended in 7AAD solution and fixed in 10% neutral buffered formalin solution for 15 minutes before analysis with the iQue Intellicyt system.

**FIGS. 30A-30B** show binding of Bi-PB-1.2 to both human PD-1 (**FIG. 30A**) and cyno PD-1 (**FIG. 30B**). As expected, the anti-human PD-1 benchmark antibody was similarly found to bind human PD-1 (**FIG. 30A**) and cynomolgus PD-1 (**FIG. 30B**). Corresponding EC50 values reflecting the half maximal effective concentrations (EC<sub>50</sub>) producing a response halfway between the baseline and maximum response with respect to binding human PD-1 and cyno PD-1, respectively, were also determined. In this case, the EC50 values show similar binding properties of Bi-PB-1.2 as the benchmark antibody.

**[0349] 6. Bi-PLB-1.2 potently blocks binding of both PD-1 and TGF- $\beta$ 1 to their receptors**

**[0350]** A blocking assay was carried out to calculate IC<sub>50</sub> values corresponding to the binding between Bi-PLB-1.2 and PD-L1 in comparison to anti-PD-L1 benchmark antibody, essentially as described in Example 8, section 4 above. **FIG. 31A** shows that increasing concentrations of Bi-PLB-1.2 or anti-PD-L1 benchmark antibody progressively blocked binding of PD-1 to PD-L1-TGFBR2. As reflected in the IC<sub>50</sub> values obtained, the bispecific are comparable to the anti-PD-L1 benchmark.

**[0351]** To calculate IC<sub>50</sub> values corresponding to the binding between Bi-PLB-1.2 and TGF- $\beta$ 1 in comparison to anti-TGF- $\beta$ 1 benchmark antibody, the cell-based assay described in Example 8, section 4 was carried out. **FIG. 31B** shows the ability of serial dilutions of Bi-PLB-1.2 and anti-PDL1-TGFBR2 benchmark antibody to block the ability of TGF- $\beta$ 1 to activate luciferase expression under the control of a SMAD response element. As reflected in the IC<sub>50</sub> values obtained, the bispecific antibody exhibited similar binding characteristics as the anti-TGF- $\beta$ 1 benchmark.

**[0352] 7. Bi-PLB-1.2 binds both humand cynomolgys PD-1 with similar activity**

**[0353]** To evaluate the ability of Bi-PLB-1.2 to bind both humand cynomulgus PD-L1, serial dilutions of Bi-PLB-1.2 were added to CHO-K1 cells (20,000 cells/well) overexpressing human or cyno PD-L1, essentially as described in Example 9, section 5. **FIGS. 32A-32B** show that Bi-PLB-1.2 bound both human PD-L1 (**FIG. 32A**) and cyno PD-L1 (**FIG. 32B**). The resulting EC<sub>50</sub> values reflect the half maximal effective concentrations (EC<sub>50</sub>) producing a response halfway between the baseline and maximum response with respect to binding human PD-1 and cyno PD-1, respectively, were also determined.

**[0354] 8. Bi-PB-1.2 enhances T cell activation**

**[0355]** Human T cell assays were performed to show the functionality of the Bi-PB-1.2 antibodies. Briefly, normal healthy human PBMC collected from Donors 1 and 2 were each activated with SEB (Toxin Technology, Cat#: BT202). To assay for IFN- $\gamma$  production, 100,000 cells were stimulated with 0.5ug/ml SEB in a 96 well plate. 25,000 tumor suppressing, SHP77 cells were added to provide inhibitory signals. 66 nM of Bi-PB-1.2 mAbs or isotype control Ab was added. 5 days later, the supernatant was examined for IFN- $\gamma$  and IL-2 by ELISA. **FIGS. 33A-33B** show increased IFN- $\gamma$  (Donor 1, 40A; Donor 2, **FIG. 33B**) and IL-2 (Donor 1, 40C; Donor 2, **FIG. 33D**) with Bi-PB-1.2 relative to the negative control treatments.

**[0356] 9. Bi-PLB-1.2 enhances T cell activation and overcomes T cell suppression by tumor cells better than the parental anti-PD-L1 antibody.**

[0357] FIGS. 34A-34D and show increased IFN- $\gamma$  secretion from human PBMCs (Donor 8, FIG. 34A; Donor 9, FIG. 34B) with Bi-PLB-1.2 relative to the negative control treatments and the parental anti-PD-L1 antibody. FIGS. 34C-34D show increased IL-2 secretion from human PBMCs (Donor 8, FIG. 34C; Donor 9, FIG. 34D) with Bi-PLB-1.2 relative to the negative control treatments and the parental anti-PD-L1 antibody. These results are consistent with the notion that Bi-PLB-1.2 overcomes T cell suppression by tumor cells better than the parental anti-PD-L1 antibody.

[0358] 11. Improved pharmacokinetic profile in mice for Bi-PB-1.2 and Bi-PLB-1.2 compared to a benchmark antibody

[0359] To evaluate the pharmacokinetic properties of Bi-PB-1.2, Bi-PLB-1.2 and a benchmark antibody *in vivo*, pharmacokinetic profiles were generated. Briefly, 10 mg/kg of each antagonist was intravenously injected into the tail vein of 6-10 week old female CD1 mice (n=2 mice per molecule). Serum was harvested at 3 minutes, 3 hours, 1 day, 3 days, 7 days and 10 days post injection. To detect the antibodies in the serum, 96 well ELISA plates were coated with 5  $\mu$ g/ml goat anti-human IgG F(ab')2 fragment and then blocked with 5% milk in PBS. Serially diluted mouse serum in 5% milk and serially diluted purified protein molecule as standard were added to the plates.

[0360] Following incubation with peroxidase conjugated mouse anti-human IgG and further washes, Bi-PB-1.2 (FIG. 35A), Bi-PLB-1.2 (FIG. 35C), and the benchmark (FIG. 35E) antibody (PDL1-TGFBR2, M8724) were detected following incubation with TMB-ELISA substrate. The integrity and clipping of the injected antibodies Bi-PB-1.2 (FIG. 35B), Bi-PLB-1.2 (FIG. 35D), and the benchmark (FIG. 35F) in the mouse serum were also analyzed by anti-human Fc Western blot. The results of this analysis show improved pharmacokinetic profiles in mice for Bi-PB-1.2 and Bi-PLB-1.2 compared to the benchmark antibody.

[0361] The above description is for the purpose of teaching a person of ordinary skill in the art how to practice the present invention and is not intended to detail all those obvious modifications and variations which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

**WHAT IS CLAIMED IS:**

1. An antitumor antagonist comprising:  
a first targeting domain comprising a TGF- $\beta$ 1 RII extracellular domain (ECD); and  
a second targeting domain that comprises a PD-1 binding antagonist, a PD-L1 binding antagonist, a VEGF binding antagonist, or a Tie2 tyrosine kinase receptor binding antagonist.
2. The antitumor antagonist of Claim 1, wherein the ECD comprises the amino acid sequence of SEQ ID NO: 89.
3. The antitumor antagonist of Claim 1 or Claim 2, wherein the second targeting domain specifically binds to PD-1.
4. The antitumor antagonist of any one of Claims 1-3, wherein the second targeting domain comprises:
  - (1) an immunoglobulin heavy chain variable region (HCVR) comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,  
wherein the HCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 4, 7, 9 and 12,
  - wherein the HCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 5, 10 and 13, and
  - wherein the HCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 3, 6, 8, 11 and 14; and
- (2) an immunoglobulin LCVR comprising three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,  
wherein the LCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 15, 18, 21, 21-23 and 26;
- wherein the LCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 16, 19, 24 and 27; and
- wherein the LCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 17, 20, 25 and 28.

5. The antitumor antagonist of any one of Claims 1-4, comprising:  
an immunoglobulin HCVR having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS: 29, 31, 33, 35, 37 and 39; and/or  
an immunoglobulin LCVR having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS: 30, 32, 34, 36, 38 and 40.
6. The antitumor antagonist of Claim 1-5, comprising:  
an immunoglobulin HCVR comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 29, 31, 33, 35, 37 and 39; and/or

an immunoglobulin LCVR comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 30, 32, 34, 36, 38 and 40.

7. The antitumor antagonist of any one of Claims 1-6, comprising:
  - an immunoglobulin HCVR having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 39; and/or
    - an immunoglobulin LCVR having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 40.
8. The antitumor antagonist of Claim 7, comprising:
  - an immunoglobulin HCVR comprising the amino acid sequence of SEQ ID NO: 39; and/or
    - an immunoglobulin LCVR comprising the amino acid sequence of SEQ ID NO: 40.
9. The antitumor antagonist of any one of Claims 1-3, comprising:
  - an immunoglobulin heavy chain having at least 90% sequence identity to SEQ ID NO: 92 or SEQ ID NO: 106; and/or
    - an immunoglobulin light chain having at least 90% sequence identity to SEQ ID NO: 93.
10. The antitumor antagonist of any one of Claims 1-3, comprising:
  - an immunoglobulin heavy chain having at least 90% sequence identity to SEQ ID NO: 107; and/or
    - an immunoglobulin light chain having at least 90% sequence identity to SEQ ID NO: 108.
11. The antitumor antagonist of Claim 1 or Claim 2, wherein the second targeting domain specifically binds PD-L1, and wherein the second targeting domain comprises:
  - (1) an immunoglobulin HCVR comprising three complementarity determining regions (HCDRs): HCDR1, HCDR2 and HCDR3,
    - wherein the HCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 41, 44, 50 and 53,
    - wherein the HCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 42, 45, 47, 49, 51 and 54, and
    - wherein the HCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 43, 46, 48, 52 and 55; and
  - (2) an immunoglobulin LCVR comprising three complementarity determining regions (LCDRs): LCDR1, LCDR2 and LCDR3,
    - wherein the LCDR1 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 56, 59, 63, 66 and 69,

wherein the LCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 57, 60, 64, 67 and 70, and

wherein the LCDR3 comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 58, 61, 62, 65, 68 and 71.

12. The antitumor antagonist of Claim 11, comprising:

an immunoglobulin HCVR having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS: 72, 74, 76, 78, 80, 82, 84 and 86; and/or

an immunoglobulin LCVR having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS: 73, 75, 77, 79, 81, 83, 85 and 87.

13. The antitumor antagonist of Claim 12, comprising:

an immunoglobulin HCVR comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 72, 74, 76, 78, 80, 82, 84 and 86; and/or

an immunoglobulin LCVR comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 73, 75, 77, 79, 81, 83, 85 and 87.

14. The antitumor antagonist of Claim 13, comprising:

an immunoglobulin HCVR comprising the amino acid sequence of SEQ ID NO: 86 and/or an immunoglobulin LCVR comprising the amino acid sequence of SEQ ID NO: 87.

15. The antitumor antagonist of Claim 11, comprising:

an immunoglobulin heavy chain having at least 90% sequence identity to SEQ ID NO: 94 or SEQ ID NO: 126; and/or

an immunoglobulin light chain having at least 90% sequence identity to SEQ ID NO: 95.

16. The antitumor antagonist of Claim 11, comprising:

an immunoglobulin heavy chain having at least 90% sequence identity to SEQ ID NO: 109; and/or

an immunoglobulin light chain having at least 90% sequence identity to SEQ ID NO: 110.

17. The antitumor antagonist of Claim 1 or Claim 2, wherein the second targeting domain specifically binds to VEGF.

18. The antitumor antagonist of Claim 17, comprising the amino acid sequence of SEQ ID NO: 88.

19. The antitumor antagonist of Claim 17, wherein the second targeting domain includes an immunoglobulin HCVR comprising the amino acid sequence of SEQ ID NO: 90 or SEQ ID NO: 96.

20. The antitumor antagonist of Claim 17 or Claim 18, comprising an immunoglobulin heavy chain having the amino acid sequence of SEQ ID NO: 105.
21. The antitumor antagonist of Claim 19, comprising:
  - an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO: 102; and/or
    - an immunoglobulin light chain comprising the amino acid sequence of SEQ ID NO: 103.
22. The antitumor antagonist of Claim 19, comprising:
  - an immunoglobulin heavy chain having the amino acid sequence of SEQ ID NO: 104; and/or
    - an immunoglobulin light chain having the amino acid sequence of SEQ ID NO: 103.
23. An antitumor antagonist that binds VEGF, wherein the antagonist comprises mutations that provide improved expression in mammalian cells.
24. The antitumor antagonist of Claim 23, wherein the antagonist includes an immunoglobulin heavy chain comprising the amino acid sequence of SEQ ID NO: 96.
25. The antitumor antagonist of Claim 23 or Claim 24, wherein the antagonist includes an immunoglobulin LCVR comprising the amino acid sequence of SEQ ID NO: 91.
26. The antitumor antagonist of any one of Claims 1-25, wherein the first targeting domain is linked to the immunoglobulin scaffold via a peptide linker.
27. The antitumor antagonist of Claim 26, wherein the peptide linker comprises the amino acid sequence of SEQ ID NO: 101 or SEQ ID NO: 112.
28. The antitumor antagonist of any one of Claims 1-27, comprising an immunoglobulin scaffold, wherein the immunoglobulin scaffold is an IgG1, IgG2 or IgG4 scaffold.
29. The antitumor antagonist of Claim 28, wherein the immunoglobulin scaffold is an IgG1 scaffold containing an N297A mutation and/or a K447A mutation.
30. The antitumor antagonist of Claim 28 or Claim 29, wherein the immunoglobulin scaffold comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 100, 115-122 and 127-129.
31. A method of treating a cell proliferative disorder in a subject, comprising:
  - administering to a subject in need thereof an effective amount of the antitumor antagonist of any one of Claims 1-30.
32. One or more nucleic acids encoding the antitumor antagonist of any one of Claims 1-30.
33. One or more expression vectors comprising the one or more nucleic acids of Claim 32.
34. A host cell transformed with the one or more expression vectors of Claim 33.

## Anti-PDI mab sequences

Mab	Heavy Chain CDR1	Heavy Chain CDR2	Heavy Chain CDR3
PD-01	NHLMS (SEQ ID:1)	TISGGGRDIFYYVDSVKG (SEQ ID:2)	RTIVSMBY (SEQ ID:3)
PD-02	NSVLY (SEQ ID:4)	GINPSNNGGINENIKFKTI (SEQ ID:5)	RDVNYBGGFDS (SEQ ID:6)
PD-03	NSYIV (SEQ ID:7)	GINPSNNGGINENIKFKTI (5)	RRDYRYDGGFDS (SEQ ID:8)
PD-04	NSYIV (7)	GINPSNNGGINENIKFKTI (5)	RDVNYBGGFDS (6)
PD-05	TYYIV (SEQ ID:9)	GINPGNGGGINENIKFKTI (SEQ ID:10)	RYHGYDGGGLDY (SEQ ID:11)
PD-06	SYYIH (SEQ ID:12)	WIFPGSGNNSKYNENFKG (SEQ ID:13)	SETYYDYGDY (SEQ ID:14)
Mab	Light Chain CDR1	Light Chain CDR2	Light Chain CDR3
PD-01	LASQQTIGTIWLA (SEQ ID:15)	AATSLAD (SEQ ID:16)	QQFYSSIPWT (SEQ ID:17)
PD-02	RASSTLYSNYLH (SEQ ID:18)	RASFLAS (SEQ ID:19)	QQGSSSPLT (SEQ ID:20)
PD-03	SASSLYSSYLH (SEQ ID:21)	RASFLAS (19)	QQGSSSPLT (20)
PD-04	RASSLYSNYLH (SEQ ID:22)	RASFLAS (19)	QQGSSSPLT (20)
PD-05	RASKSYSTSQESYIH (SEQ ID:23)	IASNLLES (SEQ ID:24)	QHTWKLPLNT (SEQ ID:25)
PD-06	KASQNVGTNVA (SEQ ID:26)	SASYRYS (SEQ ID:27)	QQYYSSYPVT (SEQ ID:28)

FIG. 1

**anti-PD-1 Antibody Variable Domain Sequences**

PD-01

VH (SEQ ID NO: 29):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNFLMSWVRQAPGKGLEWVSTISGGGR  
DTYYVDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRTTYSMDYWGQGT  
SVTVSS

VL (SEQ ID NO: 30):

DIQMTQSPSSVSASVGDRVTITCLASQTIGTWLAWYQQKPGKAPKLLIYAAATSLADG  
VPSRFSGSGSGTDFLTISLQPEDFATYYCQQFYSIPWTFGGGTKLEIK

PD-02

VH (SEQ ID NO: 31):

QVQLVQSGAEVKKPGASVKVSCKASDYTFTNSYLYWLRQAPGQGLEWMGGINPSN  
GGTNFNEKFKTRTTSTRDTISIAYMELSRLRSDDTVVYYCTRRDYNYDGGFDSWG  
QGTLTVSS

VL (SEQ ID NO: 32):

DIQMTQSPSSLSASVGDRVTFTCRASSTLYSNYLHWYQQKPGKAPKLLIYRASFLAS  
GVPSRFSGSGSGTDFLTISLQPEDFATYYCQQSSIPLTFGGGTKEIK

PD-03

VH (SEQ ID NO: 33)

QVQLVQSGAEVKKPGASVKVSCKASDYTFTNSYIYWVRQAPGQGLEWMGGINPSN  
GGTNFNEKFKTRVTSTRDTISIAYMELSRLRSDDTVVYYCARRDYRDGGFDSWG  
QGTTLTVSS

VL (SEQ ID NO: 34)

DIQMTQSPSSLSASVGDRVTITCSASSSLYSSYLHWYQQKPGKAPKLLIYRASFLASG  
VPSRFSGSGSGTDFLTISLQPEDFATYYCQQGSSIPLTFGAGTKLDLK***FIG. 2A***

PD-04

VH (SEQ ID NO: 35):

QVQLVQSGAEVKKPGASVKVSCKASDYTFTNSYIYWVRQAPGQGLEWMGGINPSN  
GGTNFNEKFKTRVTSTRDTISIAYMELSRLRSDDTVVYYCARRDYNYDGGFDSWG  
QGTLTVSS

VL (SEQ ID NO: 36):

DIQMTQSPSSLASAVGDRVTFTCRASSSLYSNYLHWYQQKPGKAPKLLIYRASFLAS  
GVPSRFGSGSGTDFLTISLQPEDFATYYCQQGSSIPLTFGGGTKEIK

PD-05 (2P16)

VH (SEQ ID NO: 37):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTIYYIYWVRQAPGQGLEWMGGINPGN  
GGTNFNEKFKIRVTMTRDTISIAYMELSSLRSEDTAVYYCARRYHGYDGGLDYWG  
QGTLTVSS

VL (SEQ ID NO: 38):

DIVLTQSPASLA VSPGQRATITCRASKVSTSGFSYIHWYQQKPGQPPKLLIYLASNLE  
SGVPARFSGSGSGTDFLTINPVEANDTANYYCQHTWELPNTFGGGTKVEIK

PD-06 (2P17)

VH (SEQ ID NO: 39):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIHWVRQAPGQGLEWMGWIFPGS  
GNSKYNNENFKGRVTLTADTSTSTVYMELSSLRSEDTAVYYCASETYDYGDYWGQGT  
LTVSS

VL (SEQ ID NO: 40):

DIQMTQSPSFLASAVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYSG  
VPSRFGSGSGTEFTLTISLQPEDFATYYCQQYYSYPYTFQGQGTKEIK

## ***FIG. 2B***

## Anti-PD-L1 mab sequences

Mab	Heavy Chain CDR1	Heavy Chain CDR2	Heavy Chain CDR3
PL-01	NYWMH (SEQ ID:41)	MHHPTNTNNYNNYNEKFKS (SEQ ID:42)	SDYGSSSPYYVFDY (SEQ ID:43)
PL-02	SYWMH (SEQ ID:44)	MHHPTNGSINYNEKFKS (SEQ ID:45)	SRYGSSSPYYVFDY (SEQ ID:46)
PL-03	SYWMH (44)	MHHPTNGGNNYNNYNEKFKS (SEQ ID:46)	SWYGSSSPYYVFDY (SEQ ID:48)
PL-04	SYWMH (44)	MHHPTGVSTDYNEKFKS (SEQ ID:47)	SDYGSSSPYYVFDY (43)
PL-05	SDYAWN (SEQ ID:50)	YISDSGTSYNSPLSKS (SEQ ID:49)	SFLRLRSYEDH (SEQ ID:52)
PL-06	SYGIN (SEQ ID:53)	CIVIGNDYTINYNEKFKG (SEQ ID:51)	AYYGSRVVDY (SEQ ID:55)
PL-07	SYGIN (53)	CIVIGNDYTINYNEKFKG (54)	AYYGSRVVDY (55)
PL-08	SYWMH (44)	MHHPTNGGNNYNNYNEKFKS (47)	SWYGSSSPYYVFDY (48)
Light Chain CDR1			
PL-01	RASQBDIDNYLN (SEQ ID:56)	YTSLRHS (SEQ ID:57)	QQGYTLPWWT (SEQ ID:58)
PL-02	RASQBDISNYLN (SEQ ID:59)	YTSLRQS (SEQ ID:60)	QQGNTLPWWT (SEQ ID:61)
PL-03	RASQBDISNYLN (59)	YTSLRHS (57)	QQGNTLPWWT (61)
PL-04	RASQBDISNYLN (59)	YTSLRHS (57)	QQGDTLPWWT (SEQ ID:62)
PL-05	KASQDWNVAVA (SEQ ID:63)	WASTRHI (SEQ ID:64)	QQHYSIPYT (SEQ ID:65)
PL-06	KASQBDINKYIA (SEQ ID:66)	YISTLQP (SEQ ID:67)	LQYDNLYT (SEQ ID:68)
PL-07	QSIISDYLH (SEQ ID:69)	CASQSIISG (SEQ ID:70)	QNGHSEPYT (SEQ ID:71)
PL-08	RASQBDIDNYLN (56)	YTSLRHS (57)	QQGYTLPWWT (58)
Light Chain CDR2			
Light Chain CDR3			

FIG. 3

**anti-PD-L1 Antibody Variable Domain Sequences**

PL-01

VH (SEQ ID NO: 72):

QVQLVQSGAEVKPGASVKVSCKASGYTFTNYWMHWMKQAPGQGLEWMGMIHPNT  
NNYNNEKFKSRVTSTRDTSISTAYMELSRLRSDDTVVYYCARSDYGSSPYYFDYWGQ  
GTLTVSS

VL (SEQ ID NO: 73):

DIQMTQSPSSLSASVGDRVTISCRASQDIDNYLNWYQQKPGKAPKLLIKYTSRLHSGVPS  
RFSGSGSGTDFTLTISSLQPEDFATYFCQQGYTLPWTFGGGTKVEIK

PL-02

VH (SEQ ID NO: 74):

QVQLVQSGAEVKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGMIHPNVG  
STNYNEKFKSKATMTRDKSSSTVYMESSLRSEDTAVYYCARSRYGSSPYYFDYWGQG  
TLTVSS

VL (SEQ ID NO: 75):

DIQMTQSPSSLSASVGDRVTISCRASQDISNYLNWYQQKPGKAPKLLIYYTSRLQSGVPS  
RFSGSGSGTDFTFTISSLQPEDIATYFCQQGNTLPWTFGQGTKVEIK

PL-03

VH (SEQ ID NO: 76):

QVQLVQSGAEVKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGMIHPNSG  
GNNYNEKFKSRVTMTRDTSISTAYMELSRLRSDDTAVYYCARSWYGSSPYYFDYWGQ  
GTLTVSS

VL (SEQ ID NO: 77):

DIQMTQSPSSLSASVGDRVTISCRASQDISNYLNWYQQKPGKAPKLLIYYTSRLHSGVPS  
RFSGSGSGTDFTFTISSLQPEDIATYFCQQGNTLPWTFGQGTKVEIK

**FIG. 4A**

PL-04

VH (SEQ ID NO: 78):

QVQLVQSGAEVKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGMIHPTGV  
STDYNEKFKSRVTMTRDTSTVYMESSLRSEDTAVYYCARSDYGSSPYYFDYWGQG  
TLVTVSS

VL (SEQ ID NO: 79):

DIQMTQSPSSLSASVGDRVTISCRASQDISNYLNWYQQKPGKAPKLLIKYTSLHSGVPS  
RFSGSGSGTDFLTISSLQPEDFATYFCQQGDTLPWTFGGGTKVEIK

PL-05

VH (SEQ ID NO: 80):

DVQLQESGPGLVKPSQLSLTCTVTGYSITSDYAWNWIRQFPGNKLEWMGYISDSGSTS  
YNPSLKSRSISITRDTSKNQFFLQLNSVTTEDTATYYCANSFLRLRSYFDHWGQGTTLTV  
S

VL (SEQ ID NO: 81):

DIVMTQSHKFMSTSVGDRVSITCKASQDVNVAVA  
WYQQKPGQSPKLLIFWASTRHIGV  
PDRFTGSGSGTDYTLTISSVQAEDLALYYCQQHYSTPYTFGGGTKEIK

PL-06

VH (SEQ ID NO: 82):

QVQLVQSGAEVKPGASVKVSCKASGYTFTSYGINWVRQAPGQRLEWMGWC  
IYIGND  
YTNYNEKFKGRVTITRDT  
SASTAYME  
SSLRSEDTAVYYCARAYYGS  
RV  
DYGQG  
TLV  
TVSS

VL (SEQ ID NO: 83):

DIQMTQSPSSLSAFVGDRVTITCKASQDINKYIA  
WYQQKPGKAPKLLIHYT  
STLQPGVPS  
RFSGSGSGRDF  
FTISSLQ  
PEDIATYYCLQYDNL  
YTFGGGTKEIK

PL-07

VH (SEQ ID NO: 84):

QVQLVQSGAEVKPGASVKVSCKASGYTFTSYGINWVRQAPGQRLEWMGWC  
IYIGND  
YTNYNEKFKGRVTITRDT  
SASTAYME  
SSLRSEDTAVYYCARAYYGS  
RV  
DYGQG  
TLV  
TVSS

VL (SEQ ID NO: 85):

EIVLTQSPVTL  
SLSPGERATL  
SCQSISDYLHWY  
LQKPGQAPRLLIK  
CASQSISGIPAR  
FSGS  
GSGSDFTL  
TISSLE  
PEDFAVYYC  
QNGHS  
FPYTFGG  
GTKEIK

**FIG. 4B**

7/40

PL-08

VH (SEQ ID NO: 86):

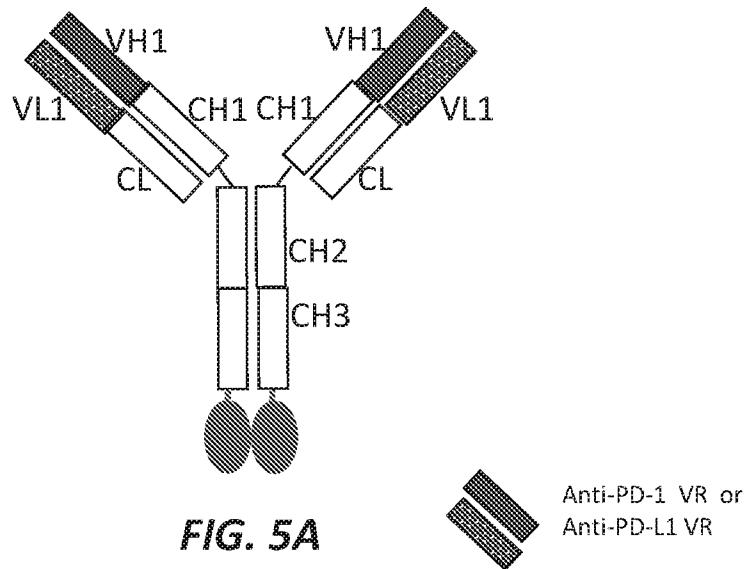
QVQLVQSGAEVKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGMIHPNSGGNNYNEKFKSRVTMTRDTSISTAYMELSRLRSDDTAVYYCARSWYGSSPYYFDYWGQGTLTVSS

VL (SEQ ID NO: 87):

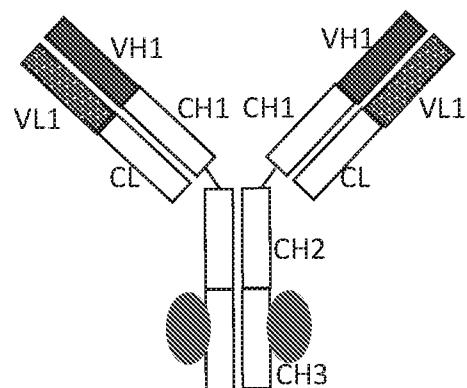
DIQMTQSPSSLSASVGDRVTISCRASQDIDNYLNWYQQKPGKAPKLLIKYTSRLHSGVPSRFSGSGSGTDFLTISSSLQPEDFATYFCQQGYTLPWTGGGTKEIK

***FIG. 4C***

**Bi-PB-1  
(or Bi-PLB-1)**



**Bi-PB-2  
(or Bi-PLB-2)**



## Exemplary Functional Domain Sequences in FIG. 5

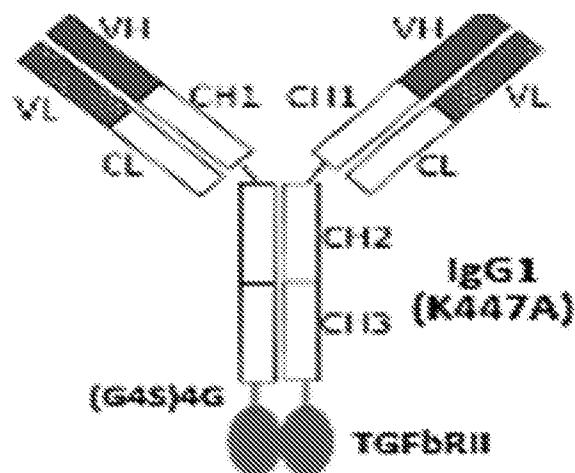
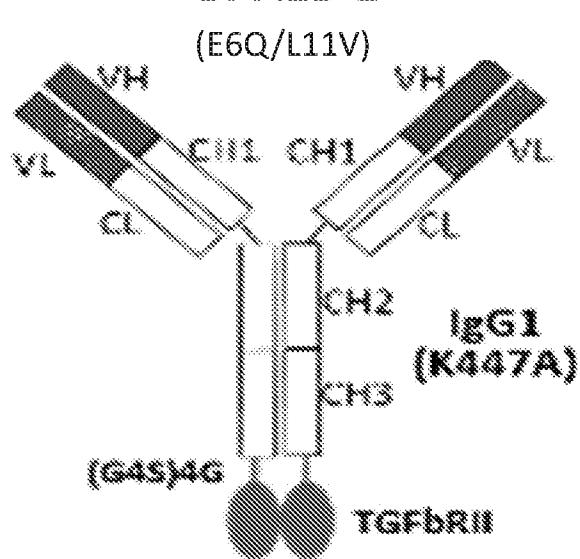
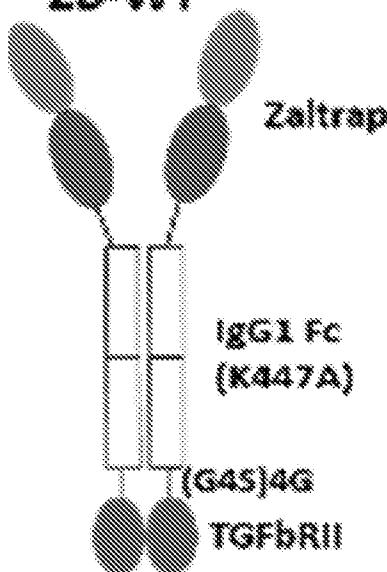
VH/VL or Fusion Protein Domain	Amino Acid Sequences of Functional Domains
Anti-PD-1 HCVR (2P17)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYIHWRQAPGQGLEWMGWIFP GSGNSKYNENFKGRVTLTADTSTSTVYMESSLRSEDTAVYYCASETYDYGDYWG QGTLTVSS (SEQ ID NO: 39)
Anti-PD-1 LCVR (2P17)	DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYS GVPSRFSGSGSGTEFTLTISLQPEDFATYYCQQYYSPYTFGQGTKLEIK (SEQ ID NO: 40)
Anti-PD-L1 HCVR (PL-08)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGMI HPNSGGNNYNEKFKSRVTMTRDTSISTAYMELSLRSDDTAVYYCARSWYGSSPY YFDYWGQGTQGTLTVSS (SEQ ID NO: 86)
Anti-PD-L1 LCVR (PL-08)	DIQMTQSPSSLSASVGDRVTISCRASQDIDNYLNWYQQKPGKAPKLLIKYTSRLHS GVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQGYTLWPWTGGGTKVEIK (SEQ ID NO: 87)
TGF- $\beta$ RII-ECD	IPPHVQKSVNNDMIVTDNNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCITSICEK PQEVCVAVWRKNDENITLETVC HDPKLPYHDFILEDAAASP KCIMKEKKPGETFFM CSCSSDECNDNIIIFSEEEYNTSNPD (SEQ ID NO: 89)

**FIG. 6**

## Exemplary heavy chain (HC) and light chain (LC) sequences in FIG. 5

Bispecific Antagonist Name	Amino acid sequence	Functional Domains
Bi-PB-1.1 HC	QVQLVQSGAEVKPGASVKVSKASGYFTTSYYIHWWVRQAPGQGLEWMGWFPG SGNSKYNNENFKGRVTLADTSTSTVYMESSLRSEDTAVYYCASETYDYGDYWGQG TLTVSSASTKGPSVFLAPCSRSTSESTAALGCLVKDYLPEPVTVSWNSGALTSGVH TFPAVLQSSGLYLSLSSVTVPSSSLGTKTYTCNVVDHKPSNTKVDKRVESKGPPCPC PAPEFLGGPSVFLPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVH NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ PREPVQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPNENYKTPPVVL DSDGSFFLYSRLTVDKSRWQEGNVFSCSVVMHEALHNHYTQKSLSLGKGGGGSG GGGGGGGGSIPPHVQKSVNNDMIVTDNNNGAVKFPQLCKFCDFRSTCDNQKSCM SNCSITSICEKPQEVCVAVWRKNDENITLETVCCHDPKLHYDFILEDAAASPCKIMKEK KKPGETFFMCSCSSDECNDNIIIFSEYNTSNPD (SEQ ID NO: 92)	anti-PD-1 TGF- $\beta$ -RII ECD
Bi-PB-1 .1LC	DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYS GVPSRSGSGSGTEFTLTISLQPEDFATYYCQQYYSYPTFGQGKLEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLNNFYPREAKVQWVKVDNALQSGNSQESVTEQDSKDS TYSLSSTTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 93)	
Bi-PLB-1.1 HC	QVQLVQSGAEVKPGASVKVSKASGYFTTSYWMHWVRQAPGQGLEWMGMIH PNSGGNNYNEKFKSRVTMTRDTISAYMELSRLRSDDTAVYYCARSWYGSSPYF DYWGQGTIVTSSASTKGPSVFLAPSSKSTSGGTAAALGCLVKDYLPEPVTVSWNS GALTSGVHTFPALQSSGLYLSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPK SCDKTHTCPPCPAPELLGGPSVFLPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWYVDGVEVHNNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPVQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVVMHEALHNHYTQKSL SLSPGAGGGGGGGGGGGGGGGGGGGGGGIPPHVQKSVNNDMIVTDNNNGAVKFPQ LCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCCHDPKL HYDFILEDAAASPCKIMKEKKPGETFFMCSCSSDECNDNIIIFSEYNTSNPD (SEQ ID NO: 94)	anti-PD-L1 TGF- $\beta$ -RII ECD
Bi-PLB-1 .1LC	DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSASYRYS GVPSRSGSGSGTEFTLTISLQPEDFATYYCQQYYSYPTFGQGKLEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLNNFYPREAKVQWVKVDNALQSGNSQESVTEQDSKDS TYSLSSTTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 95)	

**FIG. 7**

**Bi-AB-1****FIG. 8A****Bi-A1B-1****FIG. 8B****Bi-ZB-1****ZB-WT****FIG. 8C**

Avastin VH wide type (A) or Avastin VH mutant (A1)

Aflibercept (Zaltrap)

TGF- $\beta$  RII ECD

## Functional Domain Sequences in FIGs. 8A-8C

Functional Domain	Amino Acid Sequences of Functional Domains	Antagonist(s)
Bevacizumab (Avastin) VH (wt)	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGL EWVGWINTYTGEPTYAADFKRRTFTSLDTSKSTAYLQMNSLRAEDT AVYYCAKYPHYYGSSHWWYFDVVGQGTLTVSS (SEQ ID NO: 90)	Bi-AB-1
Bevacizumab (Avastin) VH (mt)	EVQLV <u>QSGGGV</u> QPGGSLRLSCAASGYTFTNYGMNWVRQAPGKG LEWVGWINTYTGEPTYAADFKRRTFTSLDTSKSTAYLQMNSLRAEDT AVYYCAKYPHYYGSSHWWYFDVVGQGTLTVSS (SEQ ID NO: 96)	Bi-A1B-1
Bevacizumab (Avastin) VL	DIQMTQSPSSLSASVGDRVITCSASQDISNYLNWYQQKPGKAPKVL IYFTSSLHSGVPSRFSGGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPW TTFGQGTLKVEIK (SEQ ID NO: 91)	Bi-AB-1 Bi-A1B-1
lgG1 CH1-CH2-CH3 (N297A HC)	ASTKGPSVFP LAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALT GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK KVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVFKFNWYVDGVEVHNAKTPREEQYASTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPVYTLPP REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQOPENNYKTPPVLD DGSFFLYSKLTVDKSRWQQGNVFCSVVMHEALHNHYTQKSLSLPG K (SEQ ID NO:97)	
lgG1 CH1-CH2-CH3 (K447A HC)	ASTKGPSVFP LAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALT GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK RVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVFKFNWYVDGVEVHNAKTPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPVYTLPP SREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQOPENNYKTPPVLD SDGSFFLYSKLTVDKSRWQQGNVFCSVVMHEALHNHYTQKSLSLPG GA (SEQ ID NO:98)	Bi-AB-1 Bi-A1B-1
lgG1 Fc (CH2-CH3) (K447A HC)	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVFKFNWYVDGVEVHNAKTPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQOPENNYKTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFCSVVMHEALHNHYTQKSLSLPGA (SEQ ID NO:99)	Bi-ZB-1

**FIG. 9A**

## Functional Domain Sequences in FIGs. 8A-8C (CONT)

Functional Domain	Amino Acid Sequences of Functional Domains	Antagonist(s)
CL (LC)	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLSSTTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:100)	Bi-AB-1 Bi-A1B-1
(G4S) <sub>4</sub> G	GGGGSGGGGSGGGGSGGGGSG (SEQ ID NO: 101)	Bi-AB-1 Bi-A1B-1 Bi-ZB-1
TGF- $\beta$ RII-ECD	IPPHVQKSVNNNDMIVTDNNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCCHDPKLPYHDFILEDAAASPKCIMKEKKPGETFFMCSCSSDECNDNIIIFSEEVNTSNPD (SEQ ID NO: 89)	Bi-AB-1 Bi-A1B-1 Bi-ZB-1
VEGF binding domain from Aflibercept	SDTGRPFVEMYSEIPEIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQNTIIDVVVLSPSHGIELSVGEKVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEK (SEQ ID NO: 88)	Bi-ZB-1

## FIG. 9B

## Heavy chain (HC) and light chain (LC) sequences in FIG. 8

Antibody (HC/LC) in FIGs. 12-13	HC or LC amino acid sequence	Functional Domains
Bi-AB-1 HC (FIG. 8A)	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVG WINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYG SSHWYFDVVGQGTLTVSSASTKGPSVFPLAPSSKSTSGTAALGCLVKDYFP EPVTWSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHK PSNTKVDKRVEPKSCDKTHTCPCPAPELLGGPSVFLFPPKPKDLMISRTPEVT CVVVDVSHEDPEVKFNWYVGVEVHNAKTPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVS LTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFCSVMHEALHNHYTQKSLSPGAGGGGGGGGGGGGGGGGGGG GIPPHVQKSVNNDMIVTDNGAVKFPQLCKFCDFRFSTCDNQKSCMSNCITS SICEKPQEVCVAVWRKNDENITLETVCVHDPKLPYHDFILEDAAASPCKCIMKEKKK PGETFFMCSCSSDECNDNIIFSEYNTSNPD (SEQ ID NO: 107)	Anti-VEGF (bevacizumab) TGF $\beta$ RII ECD
Bi-AB-1 LC (FIG. 8A)	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPVKLIYFTSSL HSGVPSRFSGSGSGTDFITLTISSLQPEDFATYYCQQYSTVPWTFGQGTKEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLNNFYPREAKVQWVVDNALQSGNSQE SVTEQDSKDSTYSLSTTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGEC (SEQ ID NO: 91)	
Bi-A1B-1 HC (FIG. 8B)	EVQLVQSGGGVVPQGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVG WINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYG SSHWYFDVVGQGTLTVSSASTKGPSVFPLAPSSKSTSGTAALGCLVKDYFP EPVTWSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHK PSNTKVDKRVEPKSCDKTHTCPCPAPELLGGPSVFLFPPKPKDLMISRTPE VTCVVDVSHEDPEVKFNWYVGVEVHNAKTPREEQYNSTYRVVSVLTVLHQ QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVS SLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFCSVMHEALHNHYTQKSLSPGAGGGGGGGGGGGGGGGGG GIPPHVQKSVNNDMIVTDNGAVKFPQLCKFCDFRFSTCDNQKSCMSNCITS SICEKPQEVCVAVWRKNDENITLETVCVHDPKLPYHDFILEDAAASPCKCIMKEKKK PGETFFMCSCSSDECNDNIIFSEYNTSNPD (SEQ ID NO: 104)	Anti-VEGF (mutant E6Q/L11V bevacizumab) TGF $\beta$ RII ECD
Bi-A1B-1 LC (FIG. 8B)	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPVKLIYFTSSL HSGVPSRFSGSGSGTDFITLTISSLQPEDFATYYCQQYSTVPWTFGQGTKEIKR TVAAPSVFIFPPSDEQLKSGTASVVCLNNFYPREAKVQWVVDNALQSGNSQE SVTEQDSKDSTYSLSTTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGEC (SEQ ID NO: 91)	
Bi-ZB-1 HC (FIG. 8C)	SDTGRPFVEMYSEIPEIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRII WDSRKGFIIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQNTNIIDVVLSPSHGIE LSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKF LSTLTIDGVTRSDQGLYTCASSGLMTKKNSTFVRVHEKDKHTCPCPAPELL GGPSVFLFPPKPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVGVEVHNA KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAG QPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSVMHEALHNHYTQKSLSPG AGGG GIPPHVQKSVNNDMIVTDNGAVKFPQLCKFCDFRFSTCDNQKSCMSNCITS SICEKPQEVCVAVWRKNDENITLETVCVHDP KLHYHDFILEDAAASPCKCIMKEKKKGETFFMCSCSSDECNDNIIFSEYNTSNPD (SEQ ID NO: 105)	VEGFR-Fc (Zaltrap; afibbercept) TGF $\beta$ RII ECD

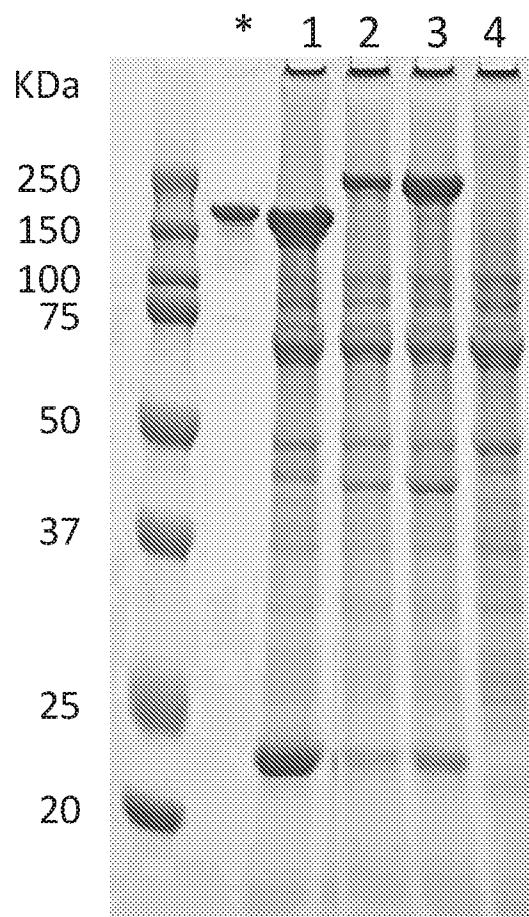
**FIG. 10**

## Antagonist Configurations in FIG. 10

Antagonist construct	IgG	Binding moieties	Antagonist polypeptide chain	Arrangement of functional domains (NH <sub>2</sub> --COOH)
Bi-AB-1	IgG1 (K447A) IgG2 or IgG4	Avastin (wt) TGF $\beta$ RII ECD	Bi-AB HC	VH-CH1-CH2-CH3-TGF $\beta$ RII ECD
			Bi-AB LC	VL-CL
Bi-A1B-1	IgG1 (K447A) IgG2 or IgG4	Avastin (mt) TGF $\beta$ RII ECD	Bi-A1B HC	VH-CH1-CH2-CH3-TGF $\beta$ RII ECD
			Bi-A1B LC	VL-CL
Bi-ZB-1	IgG1 (K447A) IgG2 or IgG4	Aflibercept (Zaltrap) TGF $\beta$ RII ECD	Bi-ZB HC	VEGFR-Fc-CH2-CH3-TGF $\beta$ RII ECD

*FIG. 11*

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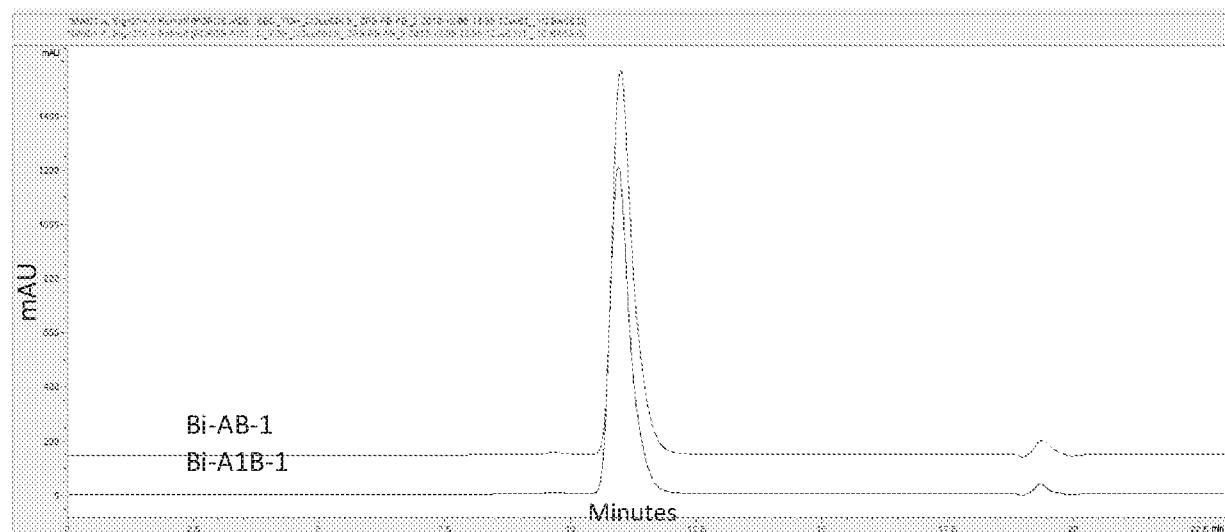
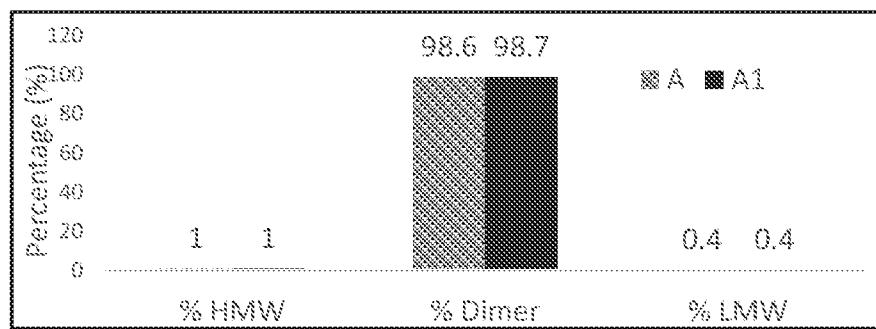
\* STD: 1.0ug

1. Transfection control
2. Bi-AB-1
3. Bi-A1B-1
4. Mock

Molecule	Titer
Bi-AB-1	90ug/ml
Bi-A1B-1	240ug/ml

*FIG. 12*

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**FIG. 13A****FIG. 13B**

## Transient Expression of Bi-ZB-1

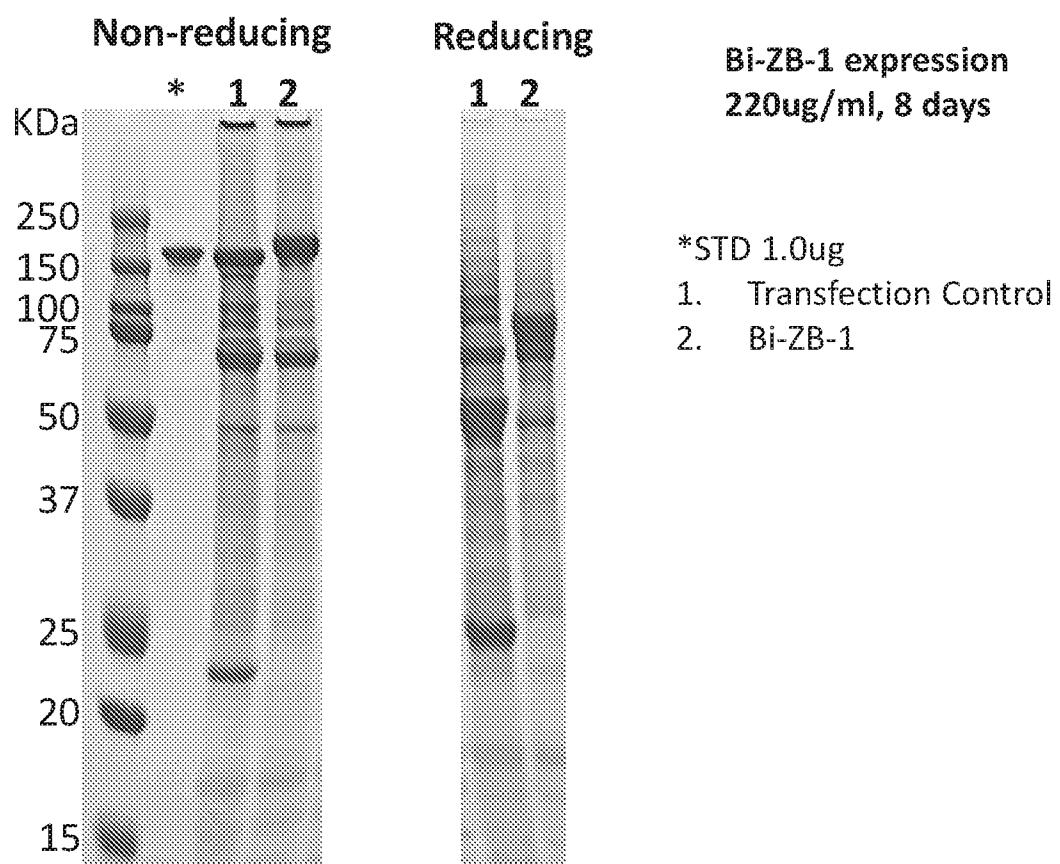
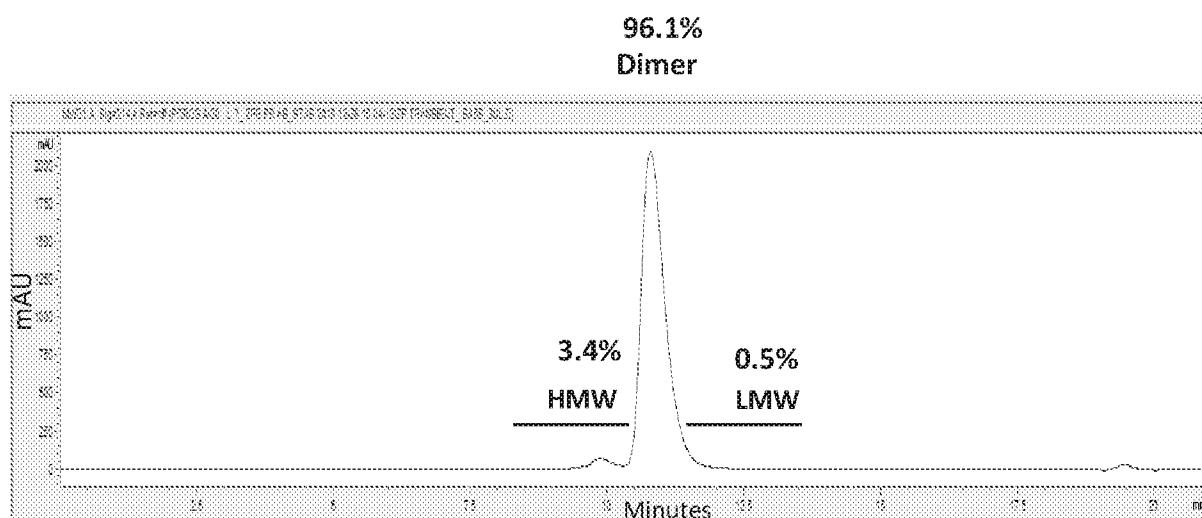


FIG. 14A

FIG. 14B

**Protein A purified Bi-ZB-1 has low levels of high molecular weight (HMW) and low molecular weight (LMW) species in comparison to dimers**



**FIG. 15**

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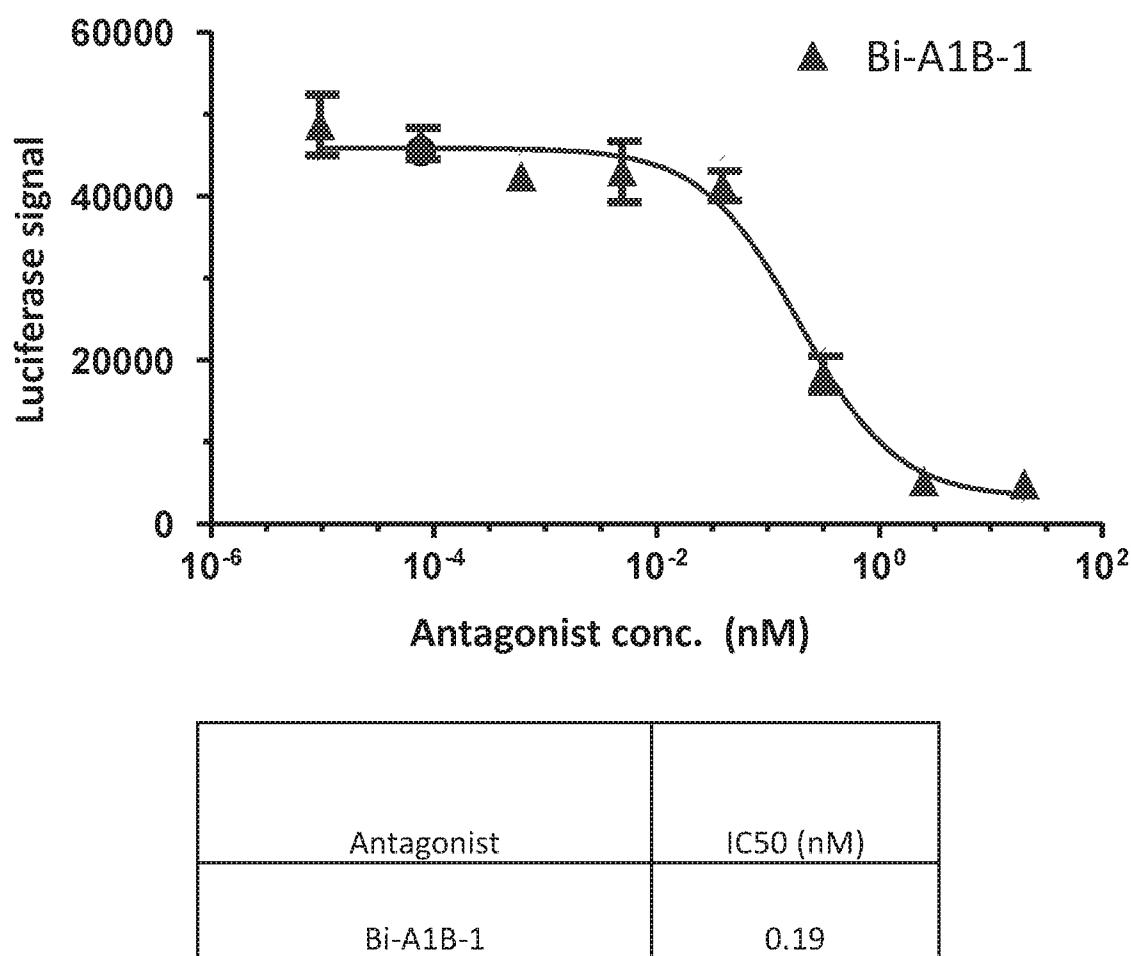
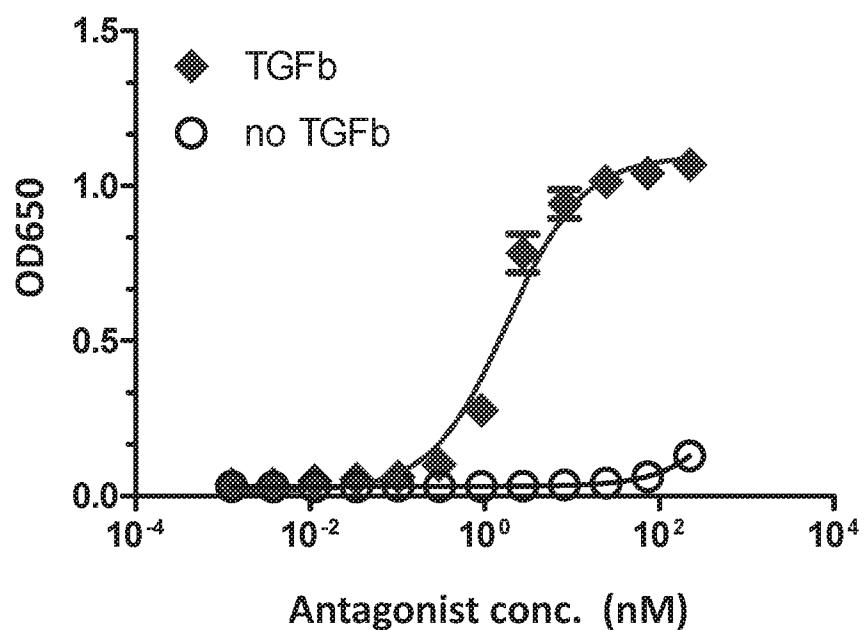


FIG. 16

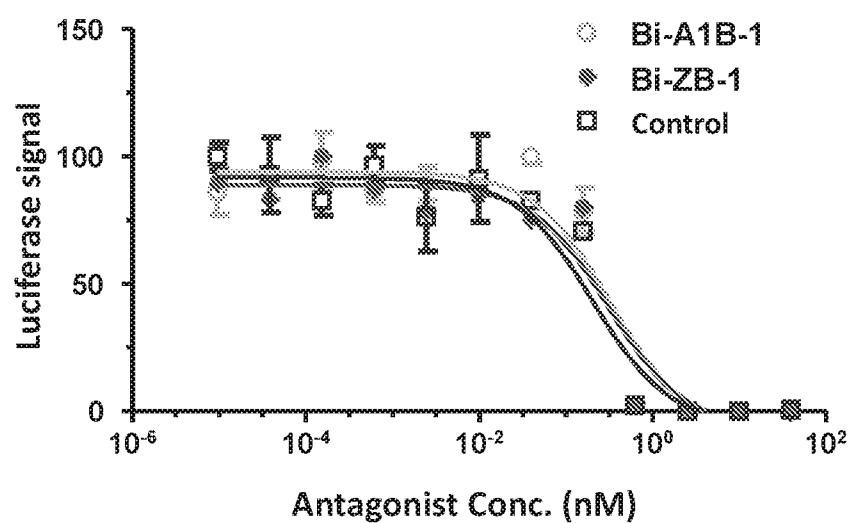
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Antagonist	EC50 (ug/ml)	EC50 (nM)
Bi-A1B-1	0.09	1.80

**FIG. 17**

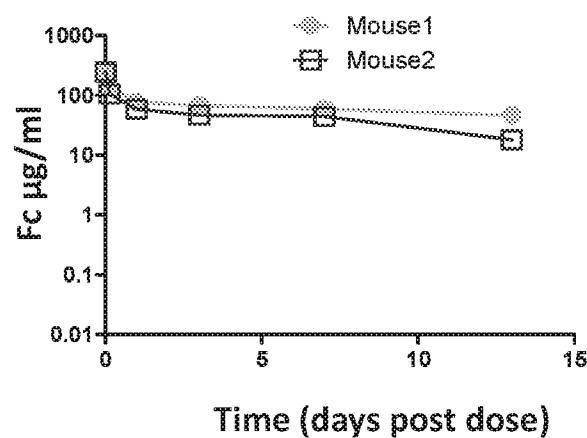
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Antagonist	IC50 (nM)
Bi-A1B-1	0.32
Bi-ZB-1	0.31
Control TGFBR2 fusion	0.20

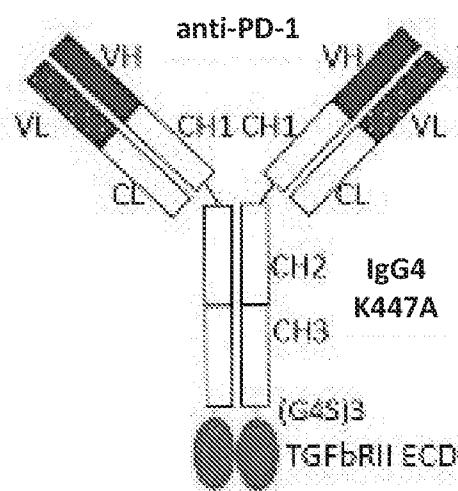
FIG. 18

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**Bi-A1B-1** $T_{1/2} = 7.4 - 16$  days***FIG. 19***

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Bi-PB-1.2



Bi-PLB-1.2

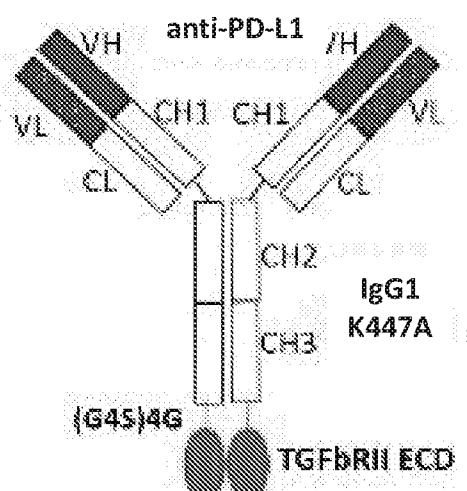


FIG. 20A

FIG. 20B

## Functional Domain Sequences in FIGs. 20A, 20B

Functional Domain	Amino Acid Sequences of Functional Domains	Antagonist(s)
Anti-PD-1 VH (2P17)	QVQLVQSGAEVKPGASVKVSKCASGYTFTSYYIHWVRQAPGQ GLEWMGWIFPGSGNSKYNENFKGRVTLTADTSTSTVYMEMLSLR SEDTAVYYCASETYDYGDYWGQGTLTVSS (SEQ ID NO: 39)	Bi-PB-1.1, -1.2
Anti-PD-L1 VH	QVQLVQSGAEVKPGASVKVSKCASGYTFTSYWMHWVRQAPG QGLEWMGMIHPNSGGNNYNEKFKSRVTMTRDTSISTAYMELS RLRSDDTAVYYCARSWYGSSPYYFDYWGQGTLTVSS (SEQ ID NO: 86)	Bi-PLB-1.1, -1.2
IgG4 CH1-CH2-CH3 (K447A) HC	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSVVTVPSQLGTKYTCNVDHKPSNT KVDKRVESKYGPPCPCCPAPEFLGGPSVFLFPPKPKDTLMISRTPE VTCVVDVSVQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQP QVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP YKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHN HYTQKSLSLG (SEQ ID NO: 130)	Bi-PB-1.2
IgG1 CH1-CH2-CH3 (K447A) HC	ASTKGPSVFPLAPSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSVVTVPSQLGTQTYICNVNHKPSN TKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPVQVTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLPGA (SEQ ID NO: 129)	Bi-PLB-1.2
(G4S) <sub>3</sub>	GGGGSGGGGGGGGG (SEQ ID NO: 114)	Bi-PB-1.1, -1.2
(G4S) <sub>4</sub> G	GGGGSGGGGGGGGGGGGG (SEQ ID NO: 101)	Bi-PLB-1.1, -1.2
TGF-β RII-ECD	IPPHVQKSVNNDMIVTDNNNGAVKFPQLCKFCDFVRSTCDNQKS CMSNCITSICEPKPQEVCVAVWRKNDENITLETVCNDPKLPYHDF ILEDAASPKCIMKEKKPGETFFMCSCSSDECNDNIIFSEYNTSN PD (SEQ ID NO: 89)	Bi-PB-1.1, -1.2 Bi-PLB-1.1, -1.2
Anti-PD-1 VL (2P17)	DIQMTQSPSFLSASVGDRVITCKASQNVGTNVAWYQQKPGKA PKALIYSASYRYSGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQ YYSYPYTFGQGKLEIK (SEQ ID NO: 40)	Bi-PB-1.1, -1.2
Anti-PD-L1 VL	DIQMTQSPSSLSASVGDRVITCRASQDIDNYLNWYQQKPGKAP KLLIKYTSRLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYFCQQG YTLPTWFGGGTKVEIK (SEQ ID NO: 87)	Bi-PLB-1.1, -1.2
CL (LC)	RTVAAPSVFIFPPSDEQLKSGTASVVCNNFYPREAKVQWKVD NALQSGNSQESVTEQDSKDSTYSLSTLTKADYEKHKVYACEV THQGLSSPVTKSFNRGEC (SEQ ID NO: 100)	Bi-PB-1.1, -1.2 Bi-PLB-1.1, -1.2

**FIG. 21**

**Heavy chain (HC) and Light chain (LC) Sequences  
of Antagonists in FIGs. 20A, 20B**

Antibody (HC/LC) in FIG. 27A/B	Amino acid sequence	Functional Domains
Bi-PB-1.2 HC	<p>QVQLVQSGAEVKKPGASVKVSKASGYTFTSYIHWVRQAPGQGLEWMGW IFPGSGNSKYNENFKGRVTLTADTSTSTVYMEPLLSEDTAVYYCASETYDYG DYWGQGTIVTSSASTKGPSVFLAPCSRSTSESTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPALQSSGLYSLSSVTVPSLSSGTKTYTCNVVDHKSNTK VDKRVESKYGPPCPCCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKGLPSSIEKTISKAKGQPREPVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVF SCSVMHEALHNHYTQKSLSLGAGGGGSGGGGSGGGGSIYPHVQKSNN MIVTDNNNGAVKFPQLCKFCDFRFSTCDNQKSCMSNCITSICEKPQEVC WRKNDENITLETVCVHDPKLPYHDFILEDAAASPCKIMKEKKPGETFFMC DECNDNIIIFSEYNTSNPD (SEQ ID NO: 108)</p>	Anti-PD-1 TGF $\beta$ RII ECD
Bi-PB-1.2 LC	<p>DIQMTQSPSFLSASVGDRVTITCKASQNVGTNVAWYQQKPGKAPKALIYSAS YRYSGVPSRFSGSGSGTEFTLTISLQPEDFATYYCQQYYSYPTFGQGTKLEIK RTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQW/KVDNALQSGNS QESVTEQDSKDKSTYSLSTLTKADYEHKVVYACEVTHQGLSSPVTKSFNRGE C (SEQ ID NO: 93)</p>	
Bi-PLB-1.2 HC	<p>QVQLVQSGAEVKKPGASVKVSKASGYTFTSYWMHWVRQAPGQGLEWM GMIHPNSGGNNYNEKFKSRVTMTRDTISIAYMELSRLRSDDTAVYYCARS YGSSPYYFDYWGQGTIVTSSASTKGPSVFLAPSSKSTSGGTAA LGCCLVKDYFPEPVTVSWNSGALTSGVHTFPALQSSGLYSLSSV TVPSLSSGTQTYICNVN HKPSNTKVDKRVEPKSCDKTHTCPCPAPELLGGPSVFLFPPKPKDTLM ISRTPEVTCVVVDVSHEDPEVKFNWYVGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPVYTLPPSREEMTKN QVSLTCLVKGFPYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFCSV MHEALHNHYTQKSLSPGAGGGGSGGGGSGGGGSG GGGSGIPPHVQKSNN D MIVTDNNNGAVKFPQLCKFCDFRFSTCDNQKSC MSNCITSICEKPQEVC AVWRKNDENITLETVCVHDPKLPYHDFILEDAAASP CKIMKEKKPGETFFMC CSSDECNDNIIIFSEYNTSNPD (SEQ ID NO: 128)</p>	Anti-PD-1 TGF $\beta$ RII ECD
Bi-PLB-1.2 LC	<p>DIQMTQSPSLLSASVGDRVTISCRASQDIDNYLNWYQQKPGKAPKLLIKY TSRLHSGVPSRFSGSGSGTDFLTISLQPEDFATYFCQQGY TLFWGGT KLEIK RTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQW/KVDNALQSGNS QESVTEQDSKDKSTYSLSTLTKADYEHKVVYACEVTHQGLSSPV TKSFNRGE C (SEQ ID NO: 95)</p>	

**FIG. 22**

## Antagonist Configurations in FIGs. 20A, 20B

Antagonist construct	IgG	Binding moieties	Antagonist polypeptide chain	Arrangement of functional domains (NH <sub>2</sub> --COOH)
Bi-PB-1.2	IgG4 (K447A)	Anti-PD-1 (2P17) TGF $\beta$ RII ECD	Bi-PB-1.2 HC TGF $\beta$ RII ECD	VH-CH1-CH2-CH3-linker- TGF $\beta$ RII ECD
			Bi-PB-1.2 LC	VL-CL
Bi-PLB-1.2	IgG1 (K447A)	Anti-PD-L1 (PL-03) TGF $\beta$ RII ECD	Bi-PLB-1.2 HC TGF $\beta$ RII ECD	VH-CH1-CH2-CH3-linker- TGF $\beta$ RII ECD
			Bi-PLB-1.2 LC	VL-CL

**FIG. 23**

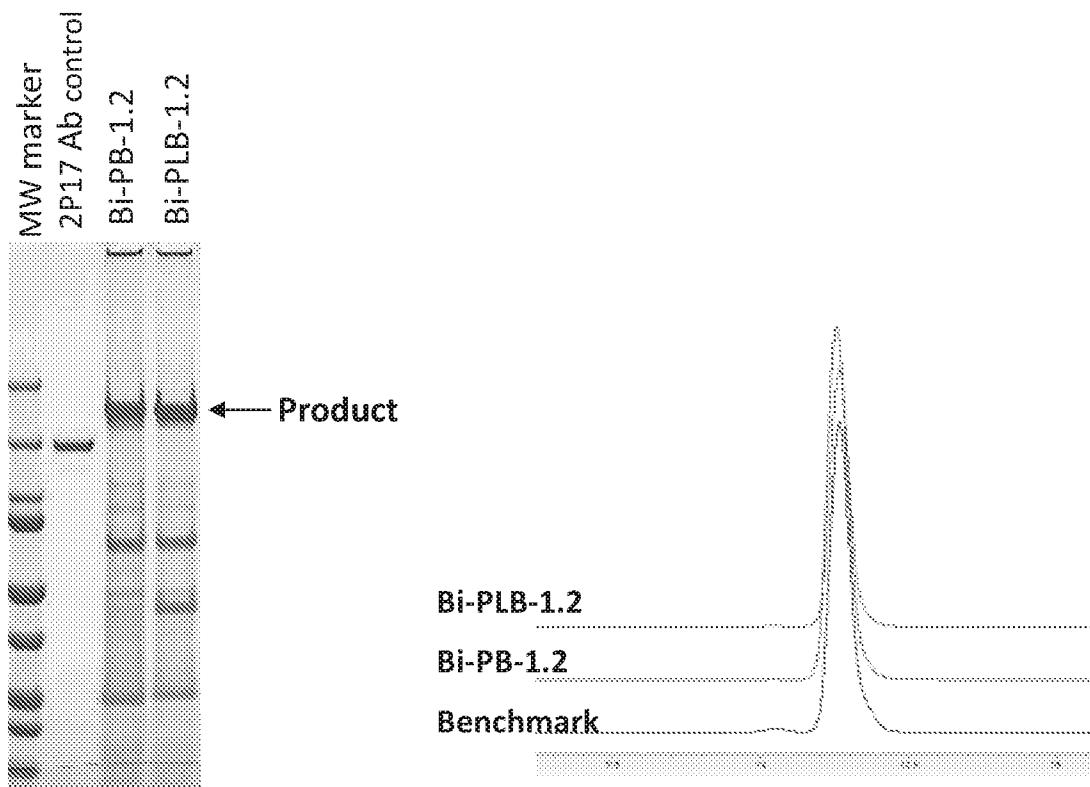


FIG. 24A

FIG. 24B

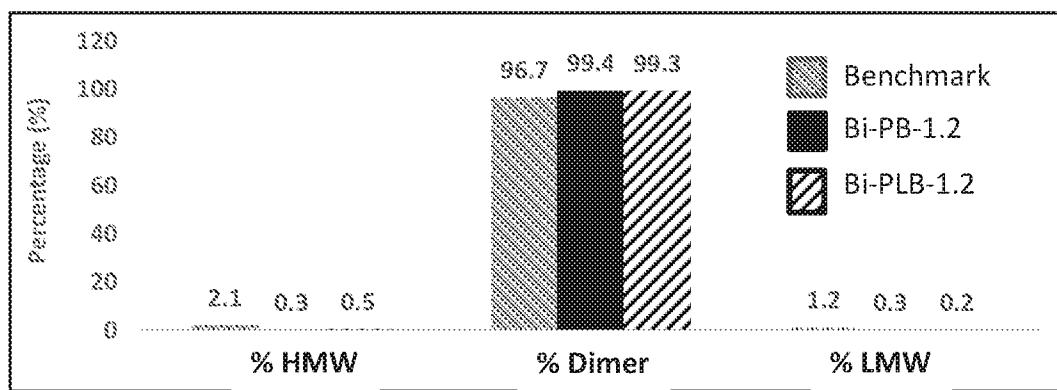
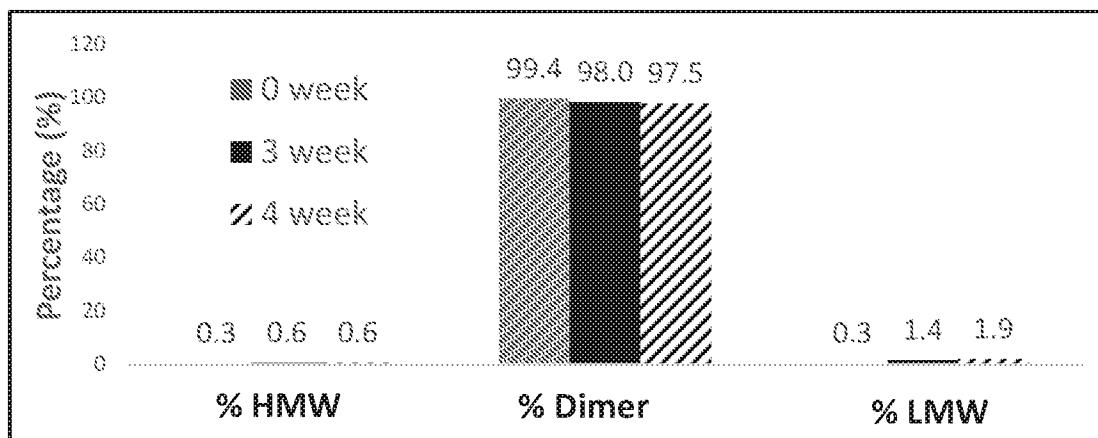
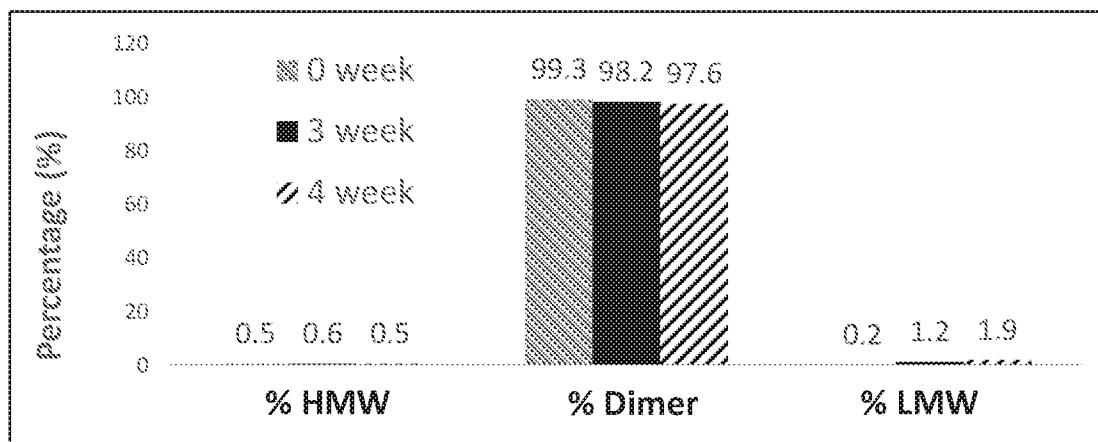


FIG. 24C

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**Bi-PB-1.2****FIG. 25A****Bi-PLB-1.2****FIG. 25B**

Bi-PB-1.2

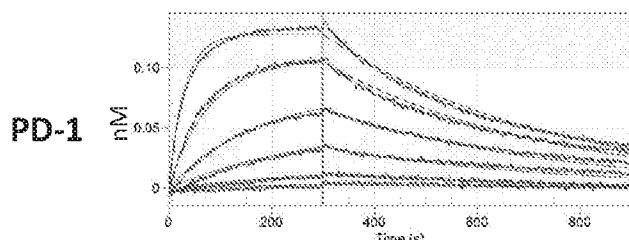


FIG. 26A

Anti-PD-1 Benchmark

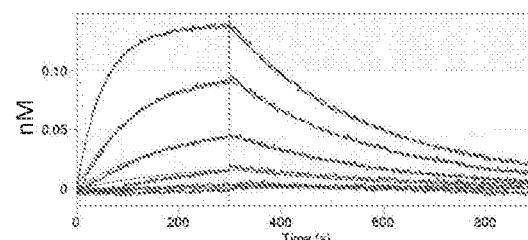


FIG. 26C

Bi-PB-1.2

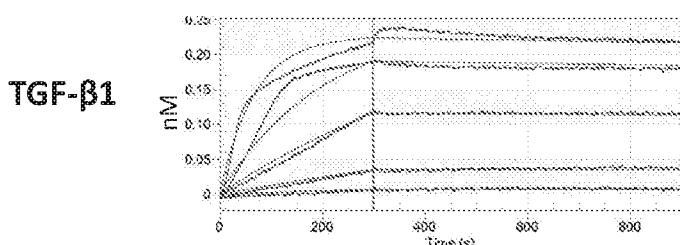


FIG. 26B

Anti-TGF-β1 Benchmark

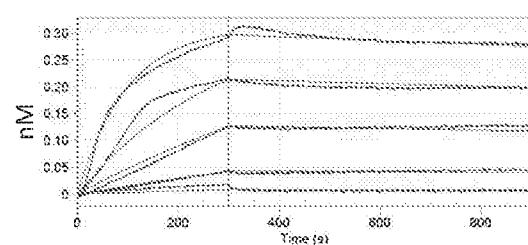
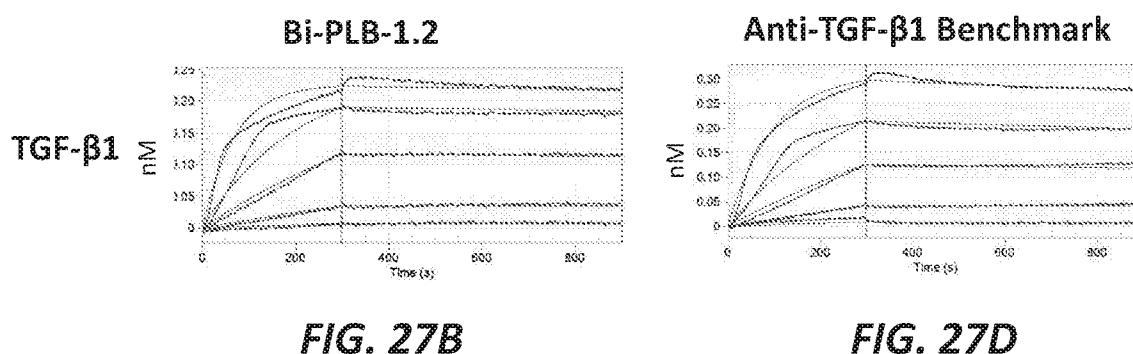
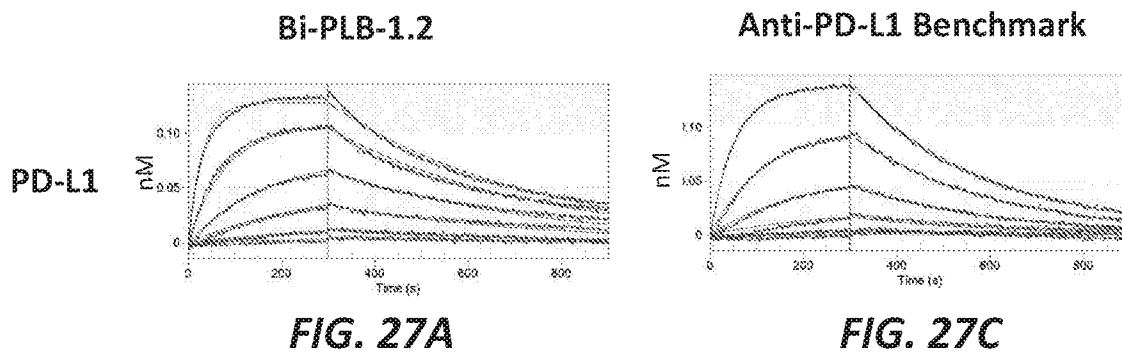


FIG. 26D

Soluble Human PD-1			
Antagonist	$K_D$ (nM)	$K_a$ ( $M^{-1}s^{-1}$ )	$K_d$ ( $s^{-1}$ )
Bi-PB-1.2	3.0	3.84E+05	1.16E-03
Anti-PD-1 Benchmark	8.8	1.88E+05	1.66E-03
TGF-β1 Benchmark		NR	
Soluble Human TGF-β1			
Bi-PB-1.2	0.14	3.76E+05	5.10E-05
Anti-PD-1 Benchmark		NR	
TGF-β1 Benchmark	0.48	2.60E+05	1.25E-04

FIG. 26E



Soluble Human PD-L1			
Antagonist	$K_D$ (nM)	$K_a$ ( $M^{-1}s^{-1}$ )	$K_d$ ( $s^{-1}$ )
Bi-PLB-1.2	0.83	9.04E+05	7.50E-04
Anti-PD-L1 benchmark	0.75	1.71E+06	1.28E-03
Soluble Human TGF- $\beta$ 1			
Bi-PLB-1.2	0.28	2.78E+05	7.78E-05
Anti-TGF- $\beta$ 1 benchmark	0.48	2.60E+05	1.25E-04

FIG. 27E

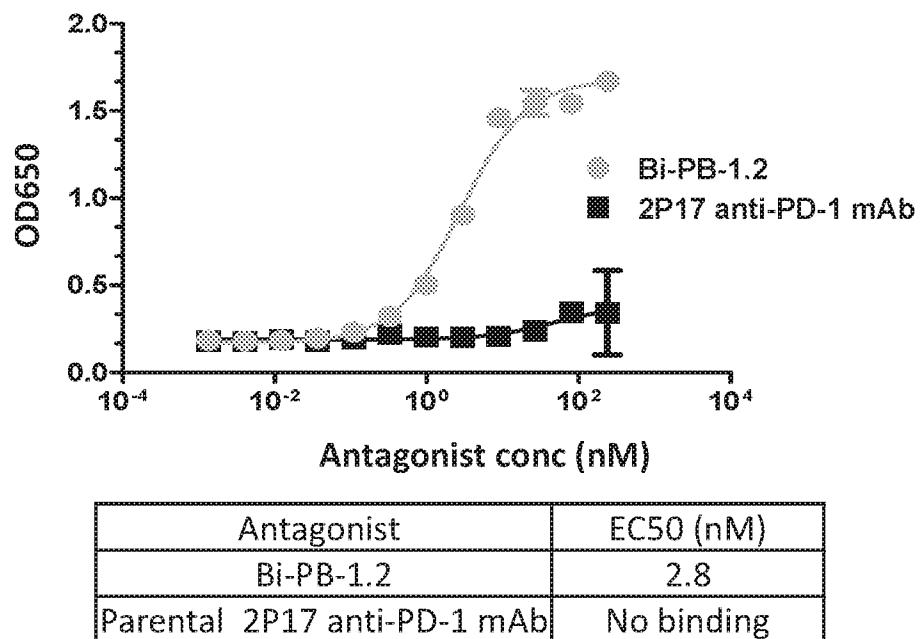


FIG. 28A

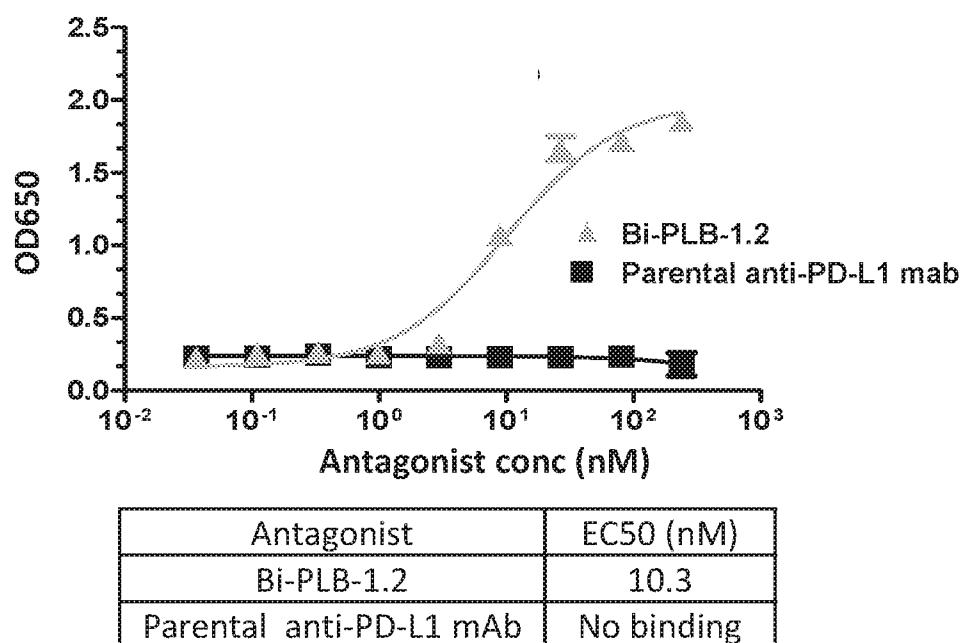
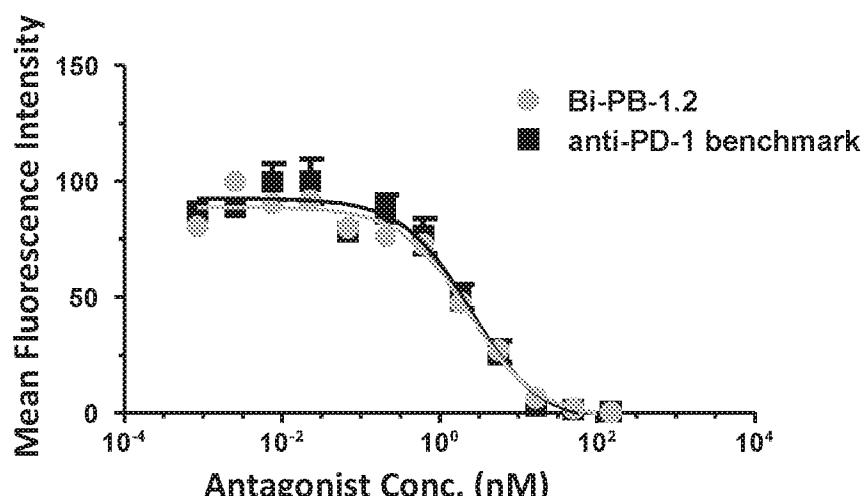
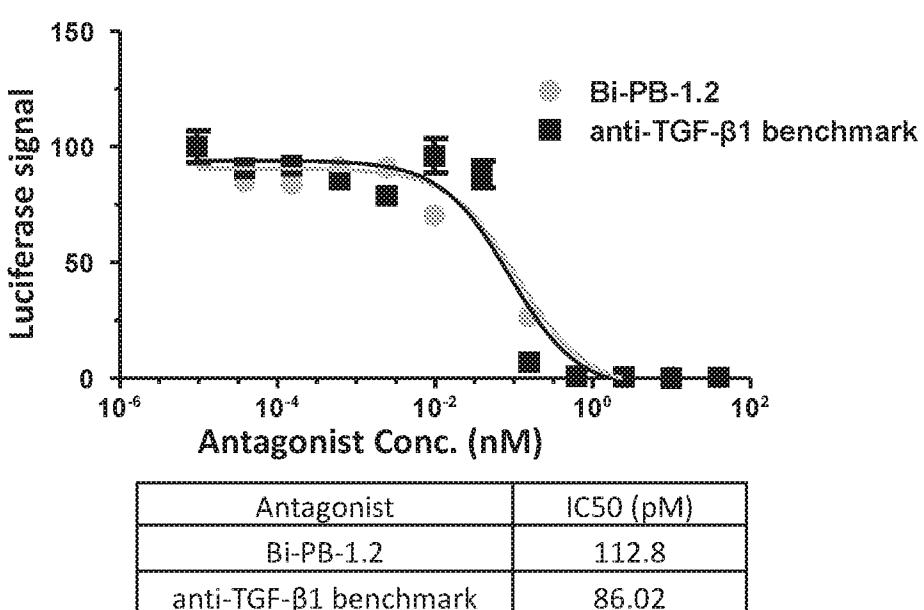


FIG. 28B

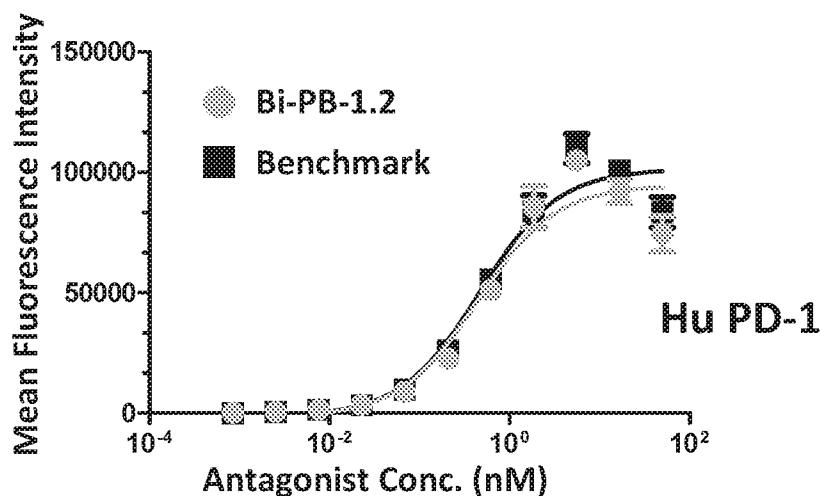


*FIG. 29A*



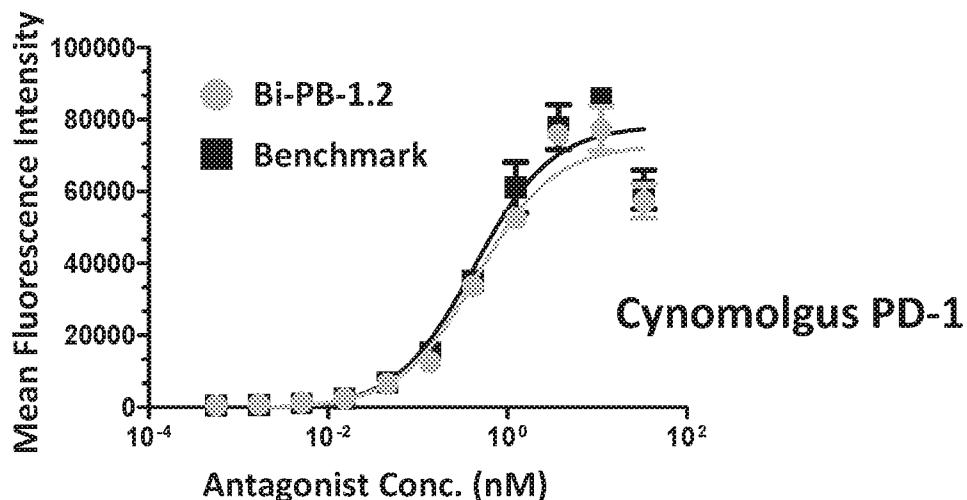
*FIG. 29B*

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Antagonist	Hu PD-1 EC50 (nM)
Bi-PB-1.2	0.44
anti-PD-1 benchmark	0.46

FIG. 30A



Antagonist	Cyno PD-1 EC50 (nM)
Bi-PB-1.2	0.42
anti-PD-1 benchmark	0.45

FIG. 30B

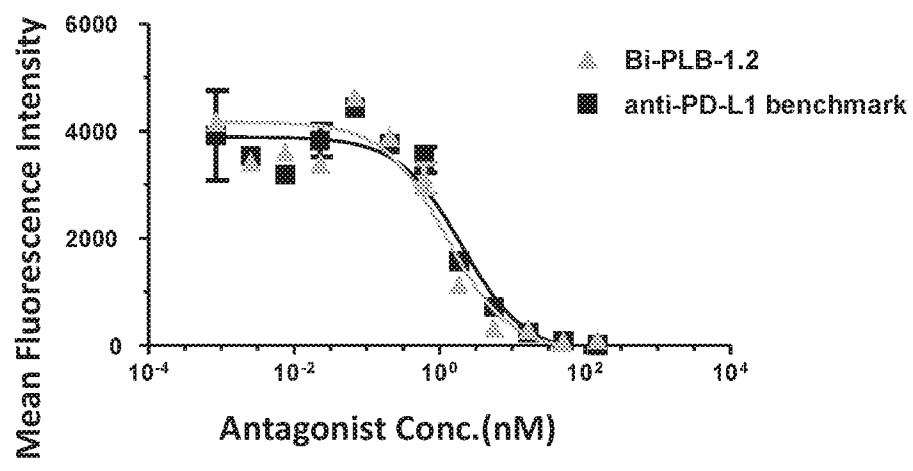


FIG. 31A

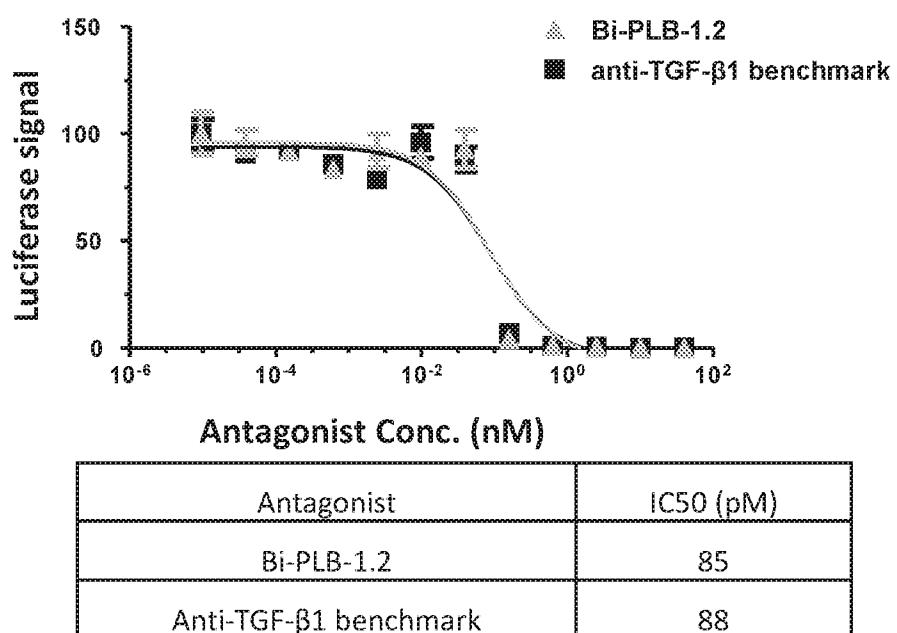


FIG. 31B

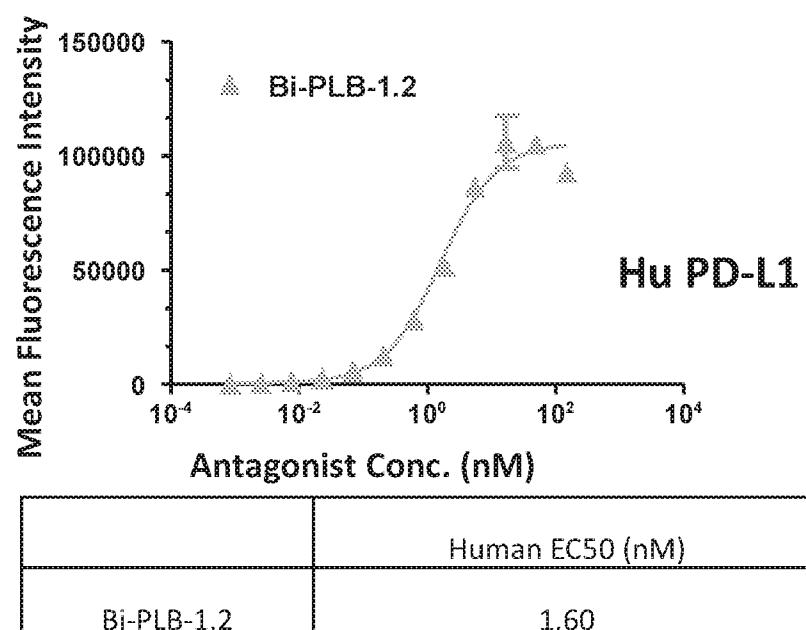


FIG. 32A

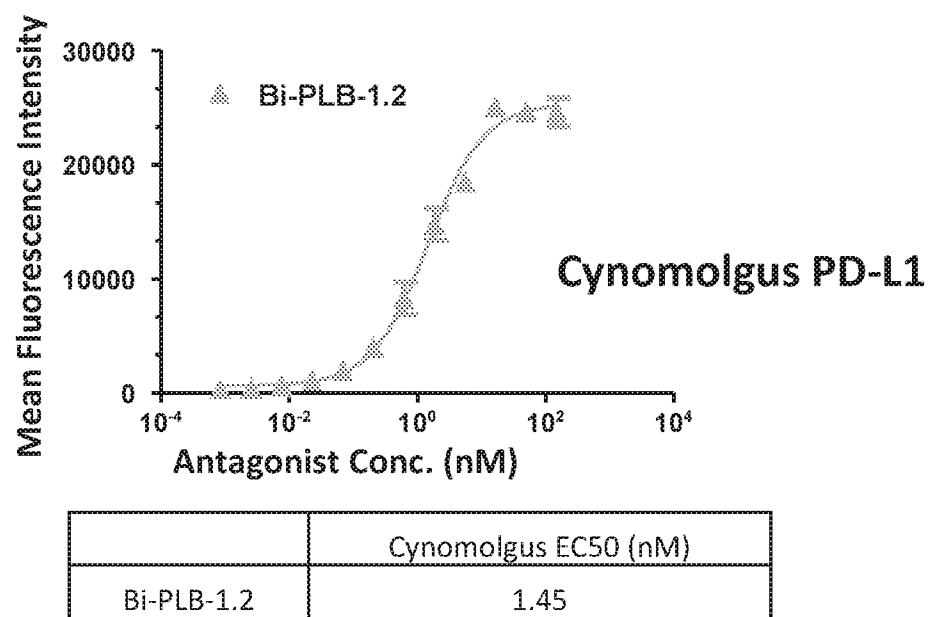
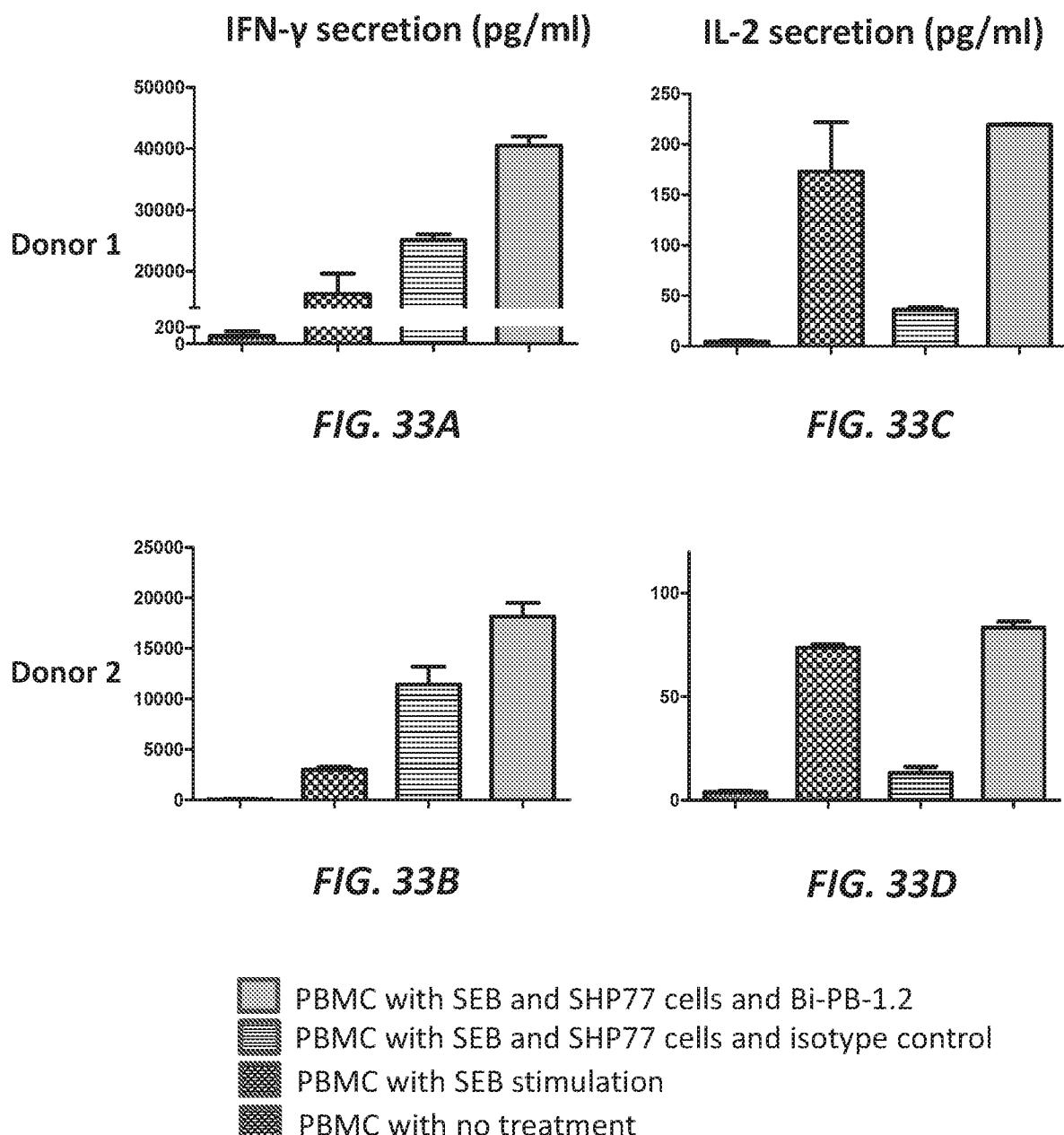
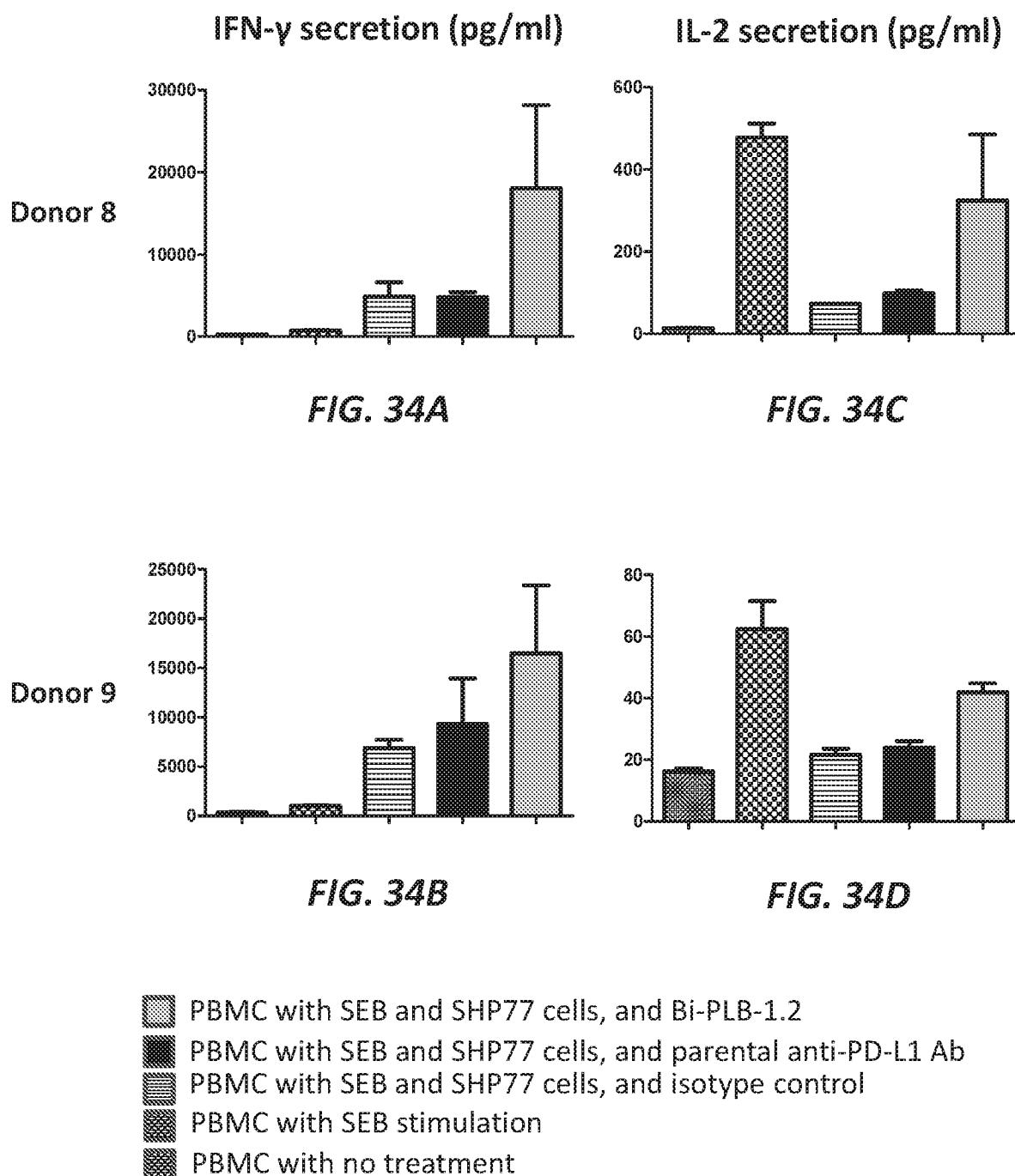
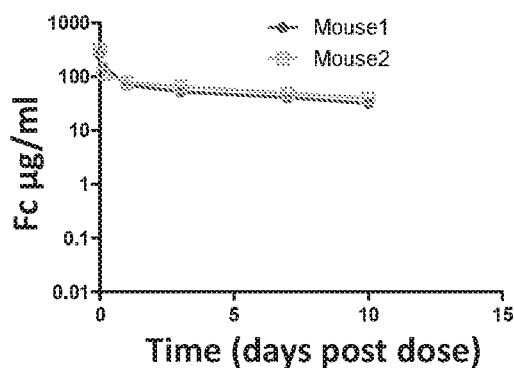
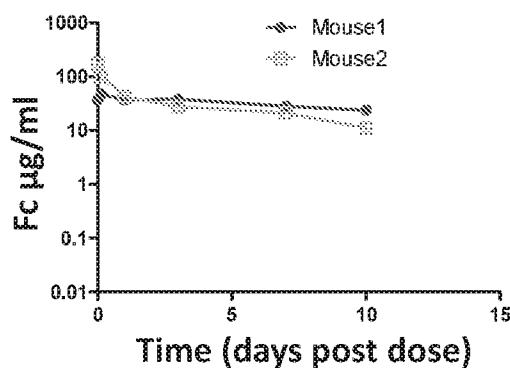
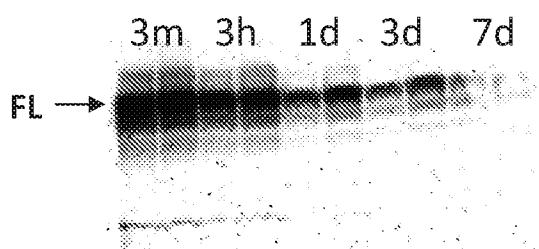
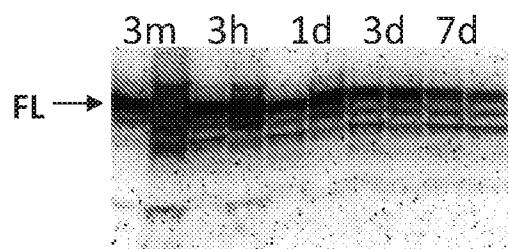
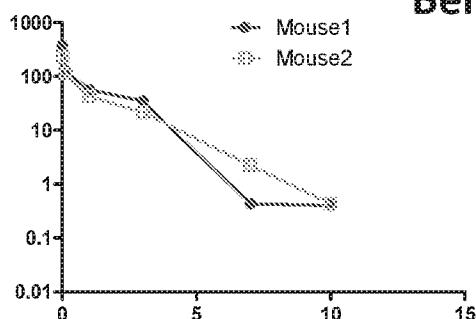
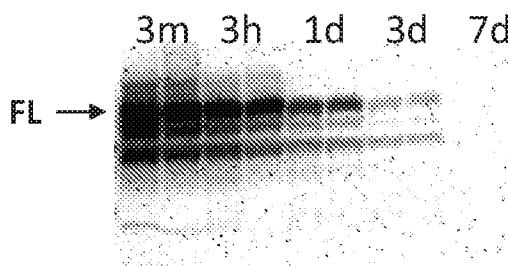


FIG. 32B





**Bi-PB-1.2****Bi-PLB-1.2****FIG. 35A****FIG. 35C****FIG. 35B****FIG. 35D****Benchmark Ab****FIG. 35E****FIG. 35F**

SEQ ID NO	Sequence	Description
130	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	PD-01 HFR1
131	WVRQAPGKGLEWVS	PD-01 HFR2
132	RIFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	PD-01 HFR3
133	WGQGTSVTVSS	PD-01 HFR4
134	DIQMTQSPSSVSASVGDRVITTC	PD-01 LFR1
135	WYQQKPGKAPKLIY	PD-01, 02, 03, 04; PL-02, 03 LFR2
136	GVPNSRFGSGSGTDFLTISLQPEDFATYYC	PD-01, 02, 03, 04 LFR3
137	FGGGTKEIK	PD-01; PL-05 LFR4
138	QVQLVQSGAEVKPGASVKVSCKASDYTFT	PD-02, 03, 04 HFR1
139	WLRQAPGQGLEWMG	PD-02 HFR2
140	RTTSTRDTSISTAYMELSLRSLRSDDTVVYCTR	PD-02 HFR3
141	WGQGTLTVSS	PD-02, 04, 05, 06; PL-01, 02, 03, 04, 06, 07, 08 HFR4
142	DIQMTQSPSSLSASVGDRVITTC	PD-02, 04 LFR1
143	FGGGTKEIK	PD-02, 04, 05; PL-01, 04, 06, 07, 08 LFR4
144	WVRQAPGQGLEWMG	PD-03, 04, 05, 06; PL-02, 03, 04, 08 HFR2
145	RVTSTRDTSISTAYMELSLRSLRSDDTVVYCA	PD-03, 04; PL-01 HFR3
146	WGQGTTLTVSS	PD-03; PL-05 HFR4
147	DIQMTQSPSSLSASVGDRVITTC	PD-03 LFR1
148	FGAGTKLDLK	PD-03 LFR4
149	FGGGTKEIK	PD-04, 05; PL-01, 04, 06, 07, 08 LFR4
150	QVQLVQSGAEVKPGASVKVSCKASGTYFT	PD-05, 06; PL-01, 02, 03, 04, 06, 07, 08 HFR1
151	RVTMTRDTSISTAYMELSLRSEDTAVYYCAR	PD-05 HFR3
152	DIVLTQSPASLAQSPGQRATITC	PD-05 LFR1
153	WYQQKPGQPPKLIY	PD-05 LFR2
154	GVPARFSGSGSGTDFLTINPVEANDTANYC	PD-05 LFR3
155	RVTLTADTSTSTVYMELOSSRLSEDATAVYYCA	PD-06 HFR3
156	DIQMTQSPSFLSASVGDRVITTC	PD-06 LFR1
157	WYQQKPGKAPKALIY	PD-06 LFR2
158	GVPNSRFGSGSGTEFTLTISLQPEDFATYYC	PD-06 LFR3
159	FGGGTKEIK	PD-06 LFR4
160	WVRLQAPGQGLEWMG	PL-01 HFR2
161	DIQMTQSPSSLSASVGDRVITSC	PL-01, 02, 03, 04, 08 LFR1
162	WYQQKPGKAPKLIK	PL-01, 04, 08 LFR2
163	GVPNSRFGSGSGTDFLTISLQPEDFATYFC	PL-01, 04, 08 LFR3
164	KATMTRDKSSTVYMELOSSRLSEDATAVYYCAR	PL-02 HFR3
165	GVPNSRFGSGSGTDFLTISLQPEDIATYFC	PL-02, 03 LFR3
166	FGQGTTKEIK	PL-02, 03 LFR4
167	RVTMTRDTSISTAYMELSLRSLRSDDATAVYYCAR	PL-03, 08 HFR3
168	RVTMTRDTSISTVYMELOSSRLSEDATAVYYCAR	PL-04 HFR3
169	QVQLQESGPGLVKPSQSLSLTCTVTGYSIT	PL-05 HFR1
170	WVROFGPNKLEWMG	PL-05 HFR2
171	RISITRDTSKNQFFLQLNSVTTEDATATYYCAN	PL-05 HFR3
172	DIVMTQSHKFMSTSVGDRVTSITC	PL-05 LFR1
173	WYQQKPGQSPKLIY	PL-05 LFR2
174	GVPDRFTGSGSGTDFLTISLQPEDFATYYC	PL-05 LFR3
175	WVRQAPGQGLEWMG	PL-06, 07 HFR2
176	RVTITRDTSASTAYMELSLRSEDTAVYYCAR	PL-06, 07 HFR3
177	DIQMTQSPSSLSAFVGDRVITTC	PL-06 LFR1
178	WYQQKPGKAPKLIH	PL-06 LFR2
179	GVPNSRFGSGSGRDFTLTISLQPEDIATYYC	PL-06 LFR3
180	EIVLTQSPVTLSPGERATLSC	PL-07 LFR1
181	WYIQLQKPGQGAPRLLIK	PL-07 LFR2
182	GPARFSGSGSGSDFTLTISLQPEDFAVYYC	PL-07 LFR3

FIG. 36

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/39979

## A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/16, 38/17, 39/395; A61P 35/00 (2019.01)

CPC - A61K 38/17, 38/1764, 38/1774, 39/39558, 2039/507; A61P 35/00; C07K 16/2803, 16/2818

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- A	US 2015/0225483 A1 (MERCK PATENT GMBH) 13 August 2015; abstract; paragraphs [0007], [0037], [0224]	1-2 --- 11/1-2, 12/11/1-2, 13/12/11/1-2, 15/11/1-2 11/1-2, 12/11/1-2,
A	WO 2014/062659 A4 (ONCOMED PHARMACEUTICALS, INC.) 24 April 2014; claim 5	13/12/11/1-2, 15/11/1-2
A	US 2011/0217318 A1 (TAKAYAMA et al.) 08 September 2011; paragraph [0243]	11/1-2, 12/11/1-2, 13/12/11/1-2, 15/11/1-2
A	WO 2017/161976 A1 (MABSPACE BIOSCIENCES (SUZHOU) CO., LTD) 28 September 2017; paragraph [0039]	11/1-2, 12/11/1-2, 13/12/11/1-2, 15/11/1-2

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 October 2019 (28.10.2019)

Date of mailing of the international search report

12 NOV 2019

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US19/39979

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: 4-10, 20, 26-34 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

-\*\*\*-Please See Supplemental Page-\*\*\*-

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

-\*\*\*-Please See Supplemental Page-\*\*\*-

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT  
Information on patent family members

International application No.  
PCT/US19/39979

-\*\*\*-Continued from Box No. III Observations where unity of invention is lacking: -\*\*\*-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, Claims 1-3, 11-19, 21-22; PD-L1 binding antagonist (binding antagonist) encompassing SEQ ID NO: 41 (HCDR1); SEQ ID NO: 42 (HCDR2); SEQ ID NO: 43 (HCDR3); SEQ ID NO: 56 (LCDR1); SEQ ID NO: 57 (LCDR2); SEQ ID NO: 58 (LCDR3); SEQ ID NO: 72 (HCVR); SEQ ID NO: 73 (LCVR); SEQ ID NO: 94 (heavy chain); SEQ ID NO: 95 (light chain) are directed toward antitumor antagonists that bind specifically to immune checkpoint regulators, angiogenesis pathway regulators and/or TGF pathway regulators.

The antitumor antagonists will be searched to the extent that they encompass a PD-L1 binding antagonist (first exemplary binding antagonist) encompassing SEQ ID NO: 41 (first exemplary HCDR1); SEQ ID NO: 42 (first exemplary HCDR2); SEQ ID NO: 43 (first exemplary HCDR3); SEQ ID NO: 56 (first exemplary LCDR1); SEQ ID NO: 57 (first exemplary LCDR2); SEQ ID NO: 58 (first exemplary LCDR3); SEQ ID NO: 72 (first exemplary HCVR); SEQ ID NO: 73 (first exemplary LCVR); SEQ ID NO: 94 (first exemplary heavy chain); and SEQ ID NO: 95 (first exemplary light chain). Applicant is invited to elect additional binding antagonist(s), with specified associated VL and VH, and associated CDR(s) thereof, with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), and available as an option within at least one searchable claim, to be searched. Additional binding antagonist(s) and associated set(s) of sequences will be searched upon the payment of additional fees. It is believed that claims 1-2 (each in-part) and 11-13(each in-part), and 15 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 41 (HCDR1); SEQ ID NO: 42 (HCDR2); SEQ ID NO: 43 (HCDR3); SEQ ID NO: 56 (LCDR1); SEQ ID NO: 57 (LCDR2); SEQ ID NO: 58 (LCDR3); SEQ ID NO: 72 (HCVR); SEQ ID NO: 73 (LCVR); SEQ ID NO: 94 (heavy chain); and SEQ ID NO: 95 (light chain). Applicants must specify the searchable claims that encompass any additionally elected sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a PD-L1 binding antagonist (binding antagonist) encompassing SEQ ID NO: 44 (HCDR1); SEQ ID NO: 45 (HCDR2); SEQ ID NO: 46 (HCDR3); SEQ ID NO: 59 (LCDR1); SEQ ID NO: 60 (LCDR2); SEQ ID NO: 61 (LCDR3); SEQ ID NO: 74 (HCVR); SEQ ID NO: 75 (LCVR); SEQ ID NO: 126 (heavy chain).

Group II, Claims 23-25 are directed toward an antitumor antagonist that binds VEGF, wherein the antagonist comprises mutations that provide improved expression in mammalian cells.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I+ include a first and second targeting domain and first targeting domain comprising a TGF-B1 RII extracellular domain (ECD), not present in any of Groups II; the special technical features of Groups II include a VEGF antagonist comprising mutations that provide improved expression in mammalian cells, not present in any of Groups I+.

However, these shared technical features are previously disclosed by the publication entitled 'M7824, a Novel Bifunctional Anti-PD-L1/TGF $\beta$  Trap Fusion Protein, Promotes Anti-tumor Efficacy as Monotherapy and in Combination with Vaccine' by Knudson, et al. (hereinafter 'Knudson').

Knudson discloses an antitumor antagonist (abstract; page e1426519-8, column 2, first paragraph).

No technical features are shared between the binding antagonists and sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: an antitumor antagonist comprising: a first targeting domain comprising a TGF-B1 RII extracellular domain (ECD); and a second targeting domain that comprises a PD-1 binding antagonist, a PD-L1 binding antagonist, a VEGF binding antagonist, or a Tie2 tyrosine kinase receptor binding antagonist; these shared technical features are previously disclosed by Kundson, as above.

Knudson discloses an antitumor antagonist (abstract; page e1426519-8, column 2, first paragraph) comprising: a first targeting domain comprising a TGF-B1 RII extracellular domain (ECD) (abstract; page e1426519-8, column 2, first paragraph); and a second targeting domain that comprises a PD-L1 binding antagonist (abstract; page e1426519-8, column 2, first paragraph).

Since none of the special technical features of the Groups I+ and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Knudson reference, unity of invention is lacking.

No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3, 11-19, 21-22; PD-L1 binding antagonist (binding antagonist) encompassing SEQ ID NO: 41 (HCDR1); SEQ ID NO: 42 (HCDR2); SEQ ID NO: 43 (HCDR3); SEQ ID NO: 56 (LCDR1); SEQ ID NO: 57 (LCDR2); SEQ ID NO: 58 (LCDR3); SEQ ID NO: 72 (HCVR); SEQ ID NO: 73 (LCVR); SEQ ID NO: 94 (heavy chain); SEQ ID NO: 95 (light chain); Exemplary election: PD-L1 binding antagonist (binding antagonist) encompassing SEQ ID NO: 44 (HCDR1); SEQ ID NO: 45 (HCDR2); SEQ ID NO: 46 (HCDR3); SEQ ID NO: 59 (LCDR1); SEQ ID NO: 60 (LCDR2); SEQ ID NO: 61 (LCDR3); SEQ ID NO: 74 (HCVR); SEQ ID NO: 75 (LCVR); SEQ ID NO: 126 (heavy chain)