

BIFUNCTIONAL MOLECULES TARGETING PD-L1 AND TGF-BETA**BACKGROUND**

[0001] The exciting advances in cancer immunotherapy in recent years have led to paradigm shifts in oncology. The most noticeable results have been with T-cell-based therapies including immune checkpoint inhibitors (ICI), genetically engineered T-cells and bispecific antibodies (BsAb). T-cells represent a major class of immunosurveillance and tumor eradication with exquisite specificity and long-term memory. However, in the tumor microenvironment, T-cells can become exhausted or tolerized to tumor cells. T-cell exhaustion is commonly associated with overexpression of inhibitory receptors, including programmed death receptor-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene-3 (LAG-3), T-cell immunoglobulin domain and mucin domain-3 (TIM-3), IL-10 receptor, and killer immunoglobulin receptors.

[0002] Monoclonal antibody (mAb) based therapies to counteract these checkpoint molecules can remove the brake that restrains tumor-infiltrating T-cells, thereby achieving significant clinical benefits in different malignancies. For instance, blocking PD-1/PD-L1 interactions can enhance immune normalization and reinforce anticancer responses. However, the noticeable deficiency of PD-1/PD-L1 blockades is inconsistency across a homogeneous study population with similar tumor characteristics. Also, PD-1/PD-L1 blockade treatments may also cause certain inflammatory side effects in some patients. The limitations of monotherapy with PD-1/PD-L1 blockades and the lack of promising alternatives has made it necessary to seek combination treatment methods which can activate antitumor immunity and enhance treatment efficacy.

[0003] M7824 (bintrafusp alfa) is a bifunctional protein composed of a monoclonal antibody against programmed death ligand 1 (PD-L1) fused to the extracellular domain of human transforming growth factor- β (TGF- β) receptor II, which functions as a “trap” for all three TGF- β isoforms. The PD-L1 portion is based on avelumab, which has been approved for the treatment of Merkel cell carcinoma and urothelial cancer. Current clinical data show, however, that the use of M7824 is associated with undesired skin growth and the overall response rate was only about 35% to 40% in a phase II trial for patients with HPV-positive malignancies. Improved therapies are needed, therefore.

SUMMARY

[0004] The present disclosure provides, in some embodiments, bifunctional molecules that target both the PD-L1 protein and TGF- β . The disclosed PD-L1 targeting unit, comprised of an anti-PD-L1 antibody, is fused to an extracellular domain of human transforming growth factor- β (TGF- β) receptor II which functions as a trap for TGF- β . Experimental data show that these new bifunctional molecules are more effective than M7824, the current lead candidate in clinical development.

[0005] In accordance with one embodiment of the present disclosure, therefore, provided is a multifunctional molecule, comprising an anti-PD-L1 (programmed death-ligand 1) antibody or fragment thereof and an extracellular domain of human TGF- β RII (TGF-beta receptor type-2), wherein the anti-PD-L1 antibody or fragment thereof has specificity to the human PD-L1 protein and comprises a heavy chain variable region (VH) comprising a VH CDR1, a VH CDR2 and a VH CDR3, and a light chain variable region (VL) comprising a VL CDR1, a VL CDR2, and a VL CDR3, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:7-12, or SEQ ID NO:13-18, or wherein the VH CDR1 comprises SEQ ID NO:19, the VH CDR2 comprises SEQ ID NO:20, 91 or 92, the VH CDR3 comprises SEQ ID NO:21, the VL CDR1 comprises SEQ ID NO:22, the VL CDR2 comprises SEQ ID NO:23, and the VL CDR3 comprises SEQ ID NO:24 or 93, wherein the human TGF- β RII extracellular domain comprises the amino acid sequence of SEQ ID NO:72 and is fused to the anti-PD-L1 antibody or fragment thereof.

[0006] In one embodiment, provided is an anti-PD-L1 (programmed death-ligand 1) antibody or fragment thereof, which has specificity to the human PD-L1 protein and comprises a heavy chain variable region (VH) comprising a VH CDR1, a VH CDR2 and a VH CDR3, and a light chain variable region (VL) comprising a VL CDR1, a VL CDR2, and a VL CDR3, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:7-12, or SEQ ID NO:13-18, or wherein the VH CDR1 comprises SEQ ID NO:19, the VH CDR2 comprises SEQ ID NO:20, 91 or 92, the VH CDR3 comprises SEQ ID NO:21, the VL CDR1 comprises SEQ ID NO:22, the VL CDR2 comprises SEQ ID NO:23, and the VL CDR3 comprises SEQ ID NO:24 or 93.

[0007] Also provided is a multifunctional molecule, comprising an antibody or antigen-binding fragment thereof fused, through a peptide linker, to the N-terminus of the amino acid sequence of SEQ ID NO:72, wherein the peptide linker (a) is at least 30 amino acid residues in length, or (b) is at least 25 amino acid residues in length and comprises an alpha helix motif.

[0008] Also provided are uses and methods for treating cancer with any of the molecules of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] **FIG. 1** shows that 47C6A3, 67F3G7 and 89C10H8 can bind to human PD-L1 with high affinity.

[0010] **FIG. 2** shows the 47C6A3, 67F3G7 and 89C10H8 antibodies can potently bind to PD-L1 expressed on mammalian cells.

[0011] FIG. 3 shows that 47C6A3, 67F3G7 and 89C10H8 antibodies can bind to cynomolgus PD-L1 with high affinity but cannot bind to rat or mouse PD-L1.

[0012] FIG. 4 shows that 47C6A3, 67F3G7 and 89C10H8 can efficiently inhibit the binding of human PD-L1 to human PD1.

[0013] FIG. 5 shows the binding kinetics of 47C6A3, 67F3G7 and 89C10H8 to recombinant PD-L1.

[0014] FIG. 6A-C show that all tested humanized antibodies have comparable binding efficiency to human PD-L1 as chimeric antibody.

[0015] FIG. 7 shows that tested humanized antibodies can high efficiently bind to PD-L1 expressed on mammalian cells, comparable with chimeric antibody.

[0016] FIG. 8A-C show that some humanized antibodies can efficiently inhibit the binding of human PD-L1 to human PD1.

[0017] FIG. 9A-C show that some humanized antibodies can efficiently inhibit the binding of human PD-L1 to human CD80.

[0018] FIG. 10 shows the binding kinetics of LP008-06, LP008-06a, LP008-06a-DA and LP008-06a-ES to recombinant human PD-L1.

[0019] FIG. 11 shows the binding kinetics of LP008-02 to human PD-L1 and human TGF- β 1.

[0020] FIG. 12 shows that LP008-02 and LP008-06a-ES can blockade PD1 and PD-L1 interaction with higher affinity than M7824.

[0021] FIG. 13 shows the M7824, LP008-02 and LP008-06a-ES can effectively blocked TGF- β canonical signaling.

[0022] FIG. 14 shows that LP008-02 and LP008-06a-ES bind to human PD-L1 with high affinity.

[0023] FIG. 15 shows that LP008-02 and LP008-06a-ES can bind to cynomolgus PD-L1 with higher affinity but cannot bind to rat PD-L1 or mouse PD-L1.

[0024] FIG. 16 shows that LP008-02 and LP008-06a-ES have comparable binding efficiency to human TGF- β as M7824.

[0025] FIG. 17 shows that LP008-02 and LP008-06a-ES have comparable binding efficiency to cynomolgus TGF- β , mouse TGF- β , and rat TGF- β as M7824.

[0026] FIG. 18A-B show the drug effects of LP008-02 and LP008-06a-ES in animal models.

[0027] FIG. 19 shows that all tested modified bifunctional molecules had comparable binding efficiency to human TGF- β as LP008-02-1.

[0028] FIG. 20 shows that all tested modified bifunctional molecules can effectively block TGF- β canonical signaling.

[0029] FIG. 21 shows that all tested modified bifunctional molecules had comparable binding efficiency to human TGF- β as LP008-02-1.

[0030] FIG. 22 shows that all tested modified bifunctional molecules can effectively block TGF- β canonical signaling.

[0031] FIG. 23 shows that antibodies MPDL3280A, 47C6A3, Hu67F3G7-22, and Hu89C10H8-7 can blockade PD1 and PD-L1 interaction with high affinity.

DETAILED DESCRIPTION

Definitions

[0032] It is to be noted that the term “a” or “an” entity refers to one or more of that entity; for example, “an antibody,” is understood to represent one or more antibodies. As such, the terms “a” (or “an”), “one or more,” and “at least one” can be used interchangeably herein.

[0033] As used herein, an “antibody” or “antigen-binding polypeptide” refers to a polypeptide or a polypeptide complex that specifically recognizes and binds to an antigen. An antibody can be a whole antibody and any antigen binding fragment or a single chain thereof. Thus the term “antibody” includes any protein or peptide containing molecule that comprises at least a portion of an immunoglobulin molecule having biological activity of binding to the antigen. Examples of such include, but are not limited to a complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework (FR) region, or any portion thereof, or at least one portion of a binding protein.

[0034] The terms “antibody fragment” or “antigen-binding fragment”, as used herein, is a portion of an antibody such as F(ab')₂, F(ab)₂, Fab', Fab, Fv, scFv and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. The term “antibody fragment” includes aptamers, spiegelmers, and diabodies. The term “antibody fragment” also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex.

[0035] 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 gamma, mu, alpha, delta, or epsilon (γ , μ , α , δ , ϵ) with some subclasses among them (*e.g.*, γ 1- γ 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, IgG₅,

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 clearly within the scope of the present disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, 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 wherein the light chains bracket the heavy chains starting at the mouth of the “Y” and continuing through the variable region.

[0036] By “specifically binds” or “has specificity to,” it is generally meant that an 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, an 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.”

[0037] As used herein, the terms “treat” or “treatment” refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the progression of cancer. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

[0038] By “subject” or “individual” or “animal” or “patient” or “mammal,” is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sport, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, cows, and so on.

[0039] As used herein, phrases such as “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 an antibody or composition of the present disclosure used, *e.g.*, for detection, for a diagnostic procedure and/or for treatment.

Multifunctional Molecules

[0040] As demonstrated in the appended experimental examples, the instant inventors were able to identify a number of bifunctional fusion proteins that include an anti-PD-L1 unit and a TGF- β -targeting unit. As shown in Example 14, for instance, both of the tested bifunctional proteins, LP008-02 and LP008-06a-ES, exhibited greater efficacy than M7824 in a MC38 mouse model. M7824 is a PD-L1/TGF- β dual targeting fusion protein currently in phase II clinical trial for patients with HPV-positive malignancies. M7824's anti-PD-L1 unit is based on avelumab, which is a leading PD-L1 antibody and has been approved for the treatment of Merkel cell carcinoma and urothelial cancer. The superior performance of the newly disclosed bifunctional proteins, as compared to M7824, is therefore surprising.

[0041] Further, as shown in Example 12, the instantly disclosed bifunctional proteins have better species specificity. Unlike M7824 which reacts with mouse and rat PD-L1 as well, the new bifunctional proteins bind to only human and cynomolgus PD-L1, in addition to its superior PD-L1 binding activity.

[0042] In one embodiment, therefore, the present disclosure provides a multifunctional molecule having at least an anti-PD-L1 unit and a TGF- β -targeting unit. The anti-PD-L1 unit can include an anti-PD-L1 antibody or fragment of the present disclosure. The TGF- β -targeting unit is preferably an extracellular domain of human transforming growth factor- β (TGF- β) receptor II (TGF- β RII or TGFBR2).

[0043] TGF- β RII has two isoforms. Isoform A (NP_001020018.1; SEQ ID NO:70) has a longer extracellular fragment than Isoform B (NP_003233.4; SEQ ID NO:71), but they share the same core ectodomain (SEQ ID NO:72). Their sequences are provided in Table A below.

Table A. Sequences related to TGF- β RII (underlined and bold: core ectodomain; underlined and italic: different residues between isoforms; underlined only: mutations)

Name	Sequence	SEQ ID NO:
TGFBR2 isoform A	MGRGLLRGLWPLHIVLWTRIASTIPPHVQKSDVEMEAQKDEITCPSCNRT <u>AHPLRH</u> <u>INND</u> MI ~~VT~~ DNNGAVKFPQ <u>LCKFC</u> <u>DVRFSTCDNQSCMSNCSITS</u> <u>ICEKPQ</u> <u>EV</u> <u>CVAVWRKNDENIT</u> <u>LET</u> <u>VCHDPKLPYHDF</u> <u>ILED</u> <u>AASPKCIMKE</u> <u>KKKPGETFFMCSSSDECNDNIIF</u> <u>SE</u> EYNTSNPDLLLIVFQVTGISLLPP LGVAISVIIIFYCYRVNRQKLSSTWETGKTRKLMFSEHCAIILED DISSTCANNINHNTLLPIELDTLVGKGRFAEVYKALKQNTSEQFETVA VKIFPYEYASWKTEKDI ~~FSD~~ INLKHENILQFLTAEERKTELKGQYWLIT AFHAKGNLQEYLTRHVISWEDLRKLGSSLARGIAHLHSDHTPCGRPKMPI VHRDLKSSNILVKNDLTCCLCDFGLSLRLDPTLSVDDLANSQVGTARYM APEVLESRMNLENVESFKQTDVYSMALVLWEMTSRCNAVGEVKDYEPFPG	70

	SKVREHPCVESMKDNVLRDRGRPEIPSWLHNHQIQMVCETLTECWDHDP EARLTAQCVARFSELEHLDRLSGRSCSEKIPEDGSLNTTK	
TGFR2 isoform B	MGRGLLRGLWPLHIVLWTRIASTIPPHVQKSVNNDMIVTDNNGAVKFPQ <u>CKFCDVRFSTCDNQKSCMSNCSTICEKPQEVCAVWRKNDENITLET</u> <u>CHDPKLPYHDFILEDAAAPKIMKEKKKPGETFFMCSSSDECNDNIIFS</u> EEYNTSNPDLLVIFQVTGISLLPPLGVAISVIIIFYCYRVNRQQLSST WETGKTRKLMFSEHCAILEDSDISSTCANNINHNTELLPIELDTLV GKGRFAEVYKAKLKQNTSEQFETVAVKIFPYEYASWKEKDFSDINLK HENILQFLTAEERKTELGKQYWLITAFHAKGNLQEYLTRHVISWEDLRKL GSSLARGIAHLSDHTPCGRPKMPIVHRDLKSSNILVKNDLTCCCLDFGL SLRLDPTLSVDDLANSQGQVGTARYMAPEVLESRMNLNVESEFKQTDVYSM ALVLWEMTSRCNAVEVKDYEPFGSKVREHPCVESMKDNVLRDRGRPEI PSFWLHNHQIQMVCETLTECWDHDPPEARLTAQCVARFSELEHLDRLSGR SCSEKIPEDGSLNTTK	71
Ectodomain	QLCKFCDVRFSTCDNQKSCMSNCSTICEKPQEVCAVWRKNDENITL TVCHDPKLPYHDFILEDAAAPKIMKEKKKPGETFFMCSSSDECNDNI IFS	72
Variant 1 (Longer extracellular fragment)	IPPHVQKSVNNDMIVTDNNGAVKFPQCKFCDVRFSTCDNQKSCMSNC <u>STICEKPQEVCAVWRKNDENITLET</u> TVCHDPKLPYHDFILEDAAAPKIM <u>KEKKKPGETFFMCSSSDECNDNIIFSEYNTSNPD</u>	61
Variant 2 (N-terminal 25 amino acid truncation from variant 1)	QLCKFCDVRFSTCDNQKSCMSNCSTICEKPQEVCAVWRKNDENITL TVCHDPKLPYHDFILEDAAAPKIMKEKKKPGETFFMCSSSDECNDNI <u>IFSEYNTSNPD</u>	73
Variant 3 (removal of potential glycosylation sites from variant 1)	IPPHVQXXVNNDMIVTDNNGAVKFPQCKFCDVRFSTCDNQKSCMSNC <u>STICEKPQEVCAVWRKNDENITLET</u> TVCHDPKLPYHDFILEDAAAPKIM <u>KEKKKPGETFFMCSSSDECNDNIIFSEYNTSNPD</u>	74
Variant 4 (Longer peptides substitute)	TAGHTQTSTGGGAITGTSGAGHGPQCKFCDVRFSTCDNQKSCMSNC <u>STICEKPQEVCAVWRKNDENITLET</u> TVCHDPKLPYHDFILEDAAAPKIM <u>KEKKKPGETFFMCSSSDECNDNIIFSEYNTSNPD</u>	75
Variant 5 (Longer peptides substitute and C terminal deletion)	TAGHTQTSTGGGAITGTSGAGHGPQCKFCDVRFSTCDNQKSCMSNC <u>STICEKPQEVCAVWRKNDENITLET</u> TVCHDPKLPYHDFILEDAAAPKIM <u>KEKKKPGETFFMCSSSDECNDNIIFS</u>	76
Variant 6 (Shorter peptides substitute and C terminal deletion)	HYPQLCKFCDVRFSTCDNQKSCMSNCSTICEKPQEVCAVWRKNDEN <u>ITLET</u> TVCHDPKLPYHDFILEDAAAPKIMKEKKKPGETFFMCSSSDECND <u>NIIFS</u>	77
Variant 7 (shorter peptides substitute)	HYPQLCKFCDVRFSTCDNQKSCMSNCSTICEKPQEVCAVWRKNDEN <u>ITLET</u> TVCHDPKLPYHDFILEDAAAPKIMKEKKKPGETFFMCSSSDECND <u>NIIFSEYNTSNPD</u>	78
Substitute N-terminal fragment of Variant 3	IPPHVQXXVNNDMIVTDNNGAVKFP	88

[0044] In some embodiments, the TGF- β RII extracellular domain includes the core ectodomain (SEQ ID NO:72) as well as some flanking residues. For instance, Variant 1 (SEQ ID NO:61) which was tested in Examples 8-16 includes additional 25 residues at the N-terminal side and nine on the C-terminal side. Another variant, Variant 2 (SEQ ID NO:73) only includes the nine C-terminal flanking residues. Other variants such as Variants 4-7 (SEQ ID NO:75-78) include alternative linkers replacing part of the N-terminal sequences of SEQ ID NO:61.

[0045] In some embodiments, the TGF- β RII extracellular domain does not include the first 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 amino acids of SEQ ID

NO:61. In some embodiments, the TGF- β RII extracellular domain does not include the last 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acids of SEQ ID NO:61.

[0046] Yet another variant, Variant 3, is based on Variant 1 but includes at least an amino acid substitution at the X positions within the N-terminal portion (SEQ ID NO:88). These X positions are potential glycosylation sites. The substitution, therefore, is with an amino acid other than K, S, and N. Examples of substitutions are R, A, G, Q, I, L, D, or E, without limitation.

[0047] The anti-PD-L1 unit, in some embodiments, is comprised of an anti-PD-L1 antibody or fragment thereof as further described below. The antibody or fragment may take any antibody format, such as the conventional full IgG format, a Fab fragment, a single chain fragment, or a single domain antibody, without limitation. When the antibody or fragment thereof has a light chain and a separate heavy chain, the TGF- β RII extracellular domain can be fused to either the light chain or the heavy chain. When the antibody or fragment thereof has a light chain and a heavy chain on a single protein chain (e.g., scFv), the TGF- β RII extracellular domain can be fused to be closer to either the light chain or the heavy chain.

[0048] In some embodiments, the TGF- β RII extracellular domain is fused to the N-terminus of a chain of the anti-PD-L1 unit. In some embodiments, the TGF- β RII extracellular domain is fused to the C-terminus of a chain of the anti-PD-L1 unit. In a preferred embodiment, the TGF- β RII extracellular domain is fused to the C-terminus of the heavy chain of the anti-PD-L1 unit, optionally through a peptide linker (e.g., SEQ ID NO:60, or one, two, or three GGGGS (SEQ ID NO:86) repeats).

[0049] In some embodiments, the anti-PD-L1 unit includes a VH (heavy chain variable region) and a VL (light chain variable region). The VH and VL regions include VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, such as those illustrated in Tables 1A-1C.

[0050] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of SDYAWN (SEQ ID NO:7), YIIYSGSTSYNPSLKS (SEQ ID NO:8), STMIATNWFAY (SEQ ID NO:9), KASQDVSLAVA (SEQ ID NO:10), WASTRHT (SEQ ID NO:11), and QQHYITPWT (SEQ ID NO:12), respectively. Examples of such VH sequences are provided in SEQ ID NO:25 (mouse) and 26-28 (humanized). Examples of such VL sequences are provided in SEQ ID NO:29 (mouse) and 30 (humanized). Example humanized antibodies include those that have a VH of SEQ ID NO:26, or 27, or 28 and a VL of SEQ ID NO:30.

[0051] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of DFWVS (SEQ ID NO:13), EIYPNSGVSRNEKFKG (SEQ ID NO:14), YFGYTYWFGY (SEQ ID NO:15), RASKSVSTYMH (SEQ ID NO:16), SASHLES (SEQ ID NO:17) and QQSNELPVT (SEQ ID NO:18), respectively. Examples of such VH sequences are

provided in SEQ ID NO:31 (mouse) and 32-37 (humanized). Examples of such VL sequences are provided in SEQ ID NO:38 (mouse) and 39-43 (humanized). Example humanized antibodies include those that have a VH of SEQ ID NO:34 and a VL of SEQ ID NO:39, 40, or 43, have a VH of SEQ ID NO:35 and a VL of SEQ ID NO:39, or have a VH of SEQ ID NO:37 and VL of SEQ ID NO:39. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:34 and a VL of SEQ ID NO:43.

[0052] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:20), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively. Alternatively, VH CDR2 can include SITNTGSSTFYPSVKG (SEQ ID NO:91) or SITNTGSSTFYPSVKG (SEQ ID NO:92). Alternatively, VL CDR3 can be SQYQSGNT (SEQ ID NO:93).

[0053] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:20), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:91), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:92), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively.

[0054] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:20), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYQSGNT (SEQ ID NO:93), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:91), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYQSGNT (SEQ ID NO:93), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVKG (SEQ ID NO:92), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYQSGNT (SEQ ID NO:93), respectively.

[0055] Examples of such VH sequences are provided in SEQ ID NO:44 (mouse) and 45-49 (humanized) and 57-58 (humanized). Examples of such VL sequences are provided in SEQ ID NO:50 (mouse) and 51-55 (humanized) and 56 (humanized).

[0056] Example humanized antibodies include those that have a VH of SEQ ID NO:49 and a VL of SEQ ID NO:52 or 54, or have a VH of SEQ ID NO:48 and a VL of SEQ ID NO:53 or 54. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:48 and a VL of SEQ ID NO:53. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:48 and a VL of SEQ ID NO:56. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:57 and a VL of SEQ ID NO:56. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:58 and a VL of SEQ ID NO:56.

[0057] In some embodiments, the antibody or fragment thereof further a heavy chain constant region (e.g., CH1, CH2 and/or CH3) and/or a light chain constant region (e.g., CL). An example heavy chain constant region is provided in SEQ ID NO:59, and an example light chain constant region is provided in SEQ ID NO:67 (residues 108-214).

TGF- β RII x Antibody Fusions

[0058] Testing with different fusion protein designs (e.g., Table 15) demonstrated that only the core ectodomain (SEQ ID NO:72) of TGF- β RII is required for activity. Further, the extracellular domain of TGF- β RII should not be directly fused to the antibody. There should be a sufficient distance, provided by a peptide linker.

[0059] With reference to the ectodomain, the peptide linker (which may be entirely an artificial linker, or include part of extracellular fragment N-terminal to the ectodomain, SEQ ID NO:89) should have a minimum length. If the distance is too short, the fusion protein has reduced stability or activity. The minimum length, in some embodiment, is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acid residents. In some embodiments, the linker is not longer than 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 170 or 200 amino acid residues.

[0060] Inclusion of a flexible linker, e.g., one or more G4S (SEQ ID NO:86) units and, in some embodiment, can be useful for the stability and/or activity of the multifunctional molecule. In some embodiments, the flexible linker includes at least 40%, 50%, 60%, 70% or 80% glycine. In some embodiments, the flexible linker includes one or more serine. In some embodiments, the flexible linker includes 1, 2, 3, 4, 5 or 6 G4S (SEQ ID NO:86) repeats.

[0061] It is shown that (e.g., Example 17), in some embodiments, the natural N-terminal fragment (IPPHVQKSVNNDMIVTDNNGAVKFP; SEQ ID NO:89) can be replaced with a substitute peptide to increase stability, without sacrifice or even with improvement of activity. In some

embodiments, the substitute peptide is different from SEQ ID NO:89 but has at least 30%, 40%, 50%, 60%, 70%, 80% or 90% sequence identity to SEQ ID NO:89.

[0062] An example substitute peptide is IPPHVQXXVNNDMIVTDNXGAVKFP (SEQ ID NO:88), wherein X is any amino acid except K, S, or N. In some embodiments, substitutions can be made to remove the rigid di-peptide PP, removal of potential cleavage sites QK, N and/or K, include multiple glycine residues to increase flexibility, and/or reduce hydrophobic residues. One such example is TAGHTQTSTGGGAITGTSGAGHGP (SEQ ID NO:87) or a variant having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:87. In some embodiments, the variant includes at least 4 G, no PP dipeptide, no more than 3 hydrophobic amino acid residues selected from the group consisting of I, L, M, F, V, W, Y and P. In some embodiments, the variant includes at least 5 G and no more than 1 hydrophobic amino acid residue selected from the group consisting of I, L, M, F, V, W, Y and P.

[0063] In some embodiments, the peptide linker between the antibody or fragment thereof and the ectodomain (SEQ ID NO:72) of TGF- β RII include a flexible linker. In some embodiments, the peptide linker includes a substitute peptide of SEQ ID NO:89. In some embodiments, the peptide linker includes both a flexible linker and a substitute peptide. In some embodiments, the flexible linker is N-terminal to the substitute peptide. In some embodiments, the flexible linker is C-terminal to the substitute peptide.

[0064] In some embodiments, the multifunctional molecule at least does not include the entire sequence of EEYNTSNPD (SEQ ID NO:90). The multifunctional molecule may have the entire SEQ ID NO:90 removed from the extracellular domain of TGF- β RII. In some embodiments, the multifunctional molecule does not include more than 1, 2, 3, 4, 5, 6, 7 or 8 amino acid residues of EEYNTSNPD (SEQ ID NO:90).

[0065] The antibody or antigen-binding fragment thereof of the multifunctional molecule may target any antigen. Non-limiting examples are PD-1, PD-L1, CTLA-4, LAG-3, CD28, CD122, 4-1BB, TIM3, OX-40, OX40L, CD40, CD40L, LIGHT, ICOS, ICOSL, GITR, GITRL, TIGIT, CD27, VISTA, B7H3, B7H4, BTLA, CD4, CD2, CD8, CD47 and CD73. They can also be any antibodies or fragments as disclosed herein.

[0066] The ectodomain of TGF- β RII may be fused to any part of the antibody or fragment. In some embodiments, the ectodomain is fused to the C-terminus of a heavy chain or a light chain of the antibody or fragment. In some embodiments, ectodomain is fused to the C-terminus of a Fc fragment of the antibody or fragment.

Anti-PD-L1 Antibodies and Fragments

[0067] Anti-PD-L1 antibodies and fragments are also provided, which can be used as an anti-PD-L1 unit in a multifunctional molecule, a bi- or multi-specific antibody, or alone in a monospecific antibody.

[0068] Example murine anti-PD-L1 antibodies and their humanized and improved ones have been prepared and tested in the accompanying experimental examples. All of the murine antibodies (47C6A3, 67F3G7, and 89C10H8) and their counterpart humanized versions exhibited superior binding affinities, cross-reactivities and effectiveness in inhibiting PD-1/PD-L1 binding.

[0069] Importantly, when compared to MPDL3280A (Atezolizumab), humanized 67F3G7, and 89C10H8 exhibited higher activity than MPDL3280A in blockading the interaction between PD-1 and PD-L1 (see, e.g., Example 18). Also, interestingly, all of the tested antibodies of the instant disclosure exhibited lower hydrophobicity and reduced viscosity than MPDL3280A. Higher hydrophobicity is known to reduce a protein's solubility. Likewise, high viscosity is also an obstacle to high concentration protein formulation development. Such data, therefore, demonstrate that the instant antibodies are better suited for preparation of high-concentration antibody formulations.

[0070] In addition, antigen-binding fragments of the instantly disclosed antibodies were included as a unit in bifunctional fusion proteins that also included a TGF- β -targeting unit. The resulting bifunctional fusion proteins exhibited greater efficacy than M7824 in a MC38 mouse model. M7824 is a PD-L1/TGF- β dual targeting fusion protein currently in phase II clinical trial for patients with HPV-positive malignancies. M7824's anti-PD-L1 unit is based on avelumab, which is a leading PD-L1 antibody and has been approved for the treatment of Merkel cell carcinoma and urothelial cancer. These data, therefore, demonstrate the unique advantages of the instantly disclosed antibodies in preparing bi- or multi-functional molecules.

[0071] In some embodiments, the anti-PD-L1 antibody or fragment includes a VH (heavy chain variable region) and a VL (light chain variable region). The VH and VL regions include VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, such as those illustrated in Tables 1A-1C.

[0072] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of SDYAWN (SEQ ID NO:7), YIIYSGSTSYNPSLKS (SEQ ID NO:8), STMIATNWFAY (SEQ ID NO:9), KASQDVSLAVA (SEQ ID NO:10), WASTRHT (SEQ ID NO:11), and QQHYITPWT (SEQ ID NO:12), respectively. Examples of such VH sequences are provided in SEQ ID NO:25 (mouse) and 26-28 (humanized). Examples of such VL sequences are provided in SEQ ID NO:29 (mouse) and 30 (humanized). Example humanized antibodies include those that have a VH of SEQ ID NO:26, or 27, or 28 and a VL of SEQ ID NO:30.

[0073] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of DFWVS (SEQ ID NO:13), EIYPNSGVSRYNEKFKG (SEQ ID NO:14), YFGYTYWFGY (SEQ ID NO:15), RASKSVSTYMH (SEQ ID NO:16), SASHLES (SEQ ID NO:17) and QQSNELPVT (SEQ ID NO:18), respectively. Examples of such VH sequences are provided in SEQ ID NO:31 (mouse) and 32-37 (humanized). Examples of such VL sequences are provided in SEQ ID NO:38 (mouse) and 39-43 (humanized). Example humanized antibodies include those that have a VH of SEQ ID NO:34 and a VL of SEQ ID NO:39, 40, or 43, have a VH of SEQ ID NO:35 and a VL of SEQ ID NO:39, or have a VH of SEQ ID NO:37 and VL of SEQ ID NO:39. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:34 and a VL of SEQ ID NO:43.

[0074] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVK (SEQ ID NO:20), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively. Alternatively, VH CDR2 can include SITNTGSSTFYPSVK (SEQ ID NO:91) or SITNTGSSTFYPSVK (SEQ ID NO:92). Alternatively, VL CDR3 can be SQYQSGNT (SEQ ID NO:93).

[0075] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVK (SEQ ID NO:20), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVK (SEQ ID NO:91), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVK (SEQ ID NO:92), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYNSGNT (SEQ ID NO:24), respectively.

[0076] In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVK (SEQ ID NO:20), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYQSGNT (SEQ ID NO:93), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPSVK (SEQ ID NO:91), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYQSGNT (SEQ ID NO:93), respectively. In one embodiment, the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL

CDR2, and VL CDR3 comprise the sequences of NYWMT (SEQ ID NO:19), SITNTGSSTFYPESVKG (SEQ ID NO:92), DTTIAPFDY (SEQ ID NO:21), KASQNLNEYLN (SEQ ID NO:22), KTNTLQA (SEQ ID NO:23) and SQYQSGNT (SEQ ID NO:93), respectively.

[0077] Examples of such VH sequences are provided in SEQ ID NO:44 (mouse) and 45-49 (humanized) and 57-58 (humanized). Examples of such VL sequences are provided in SEQ ID NO:50 (mouse) and 51-55 (humanized) and 56 (humanized).

[0078] Example humanized antibodies include those that have a VH of SEQ ID NO:49 and a VL of SEQ ID NO:52 or 54, or have a VH of SEQ ID NO:48 and a VL of SEQ ID NO:53 or 54. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:48 and a VL of SEQ ID NO:53. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:48 and a VL of SEQ ID NO:56. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:57 and a VL of SEQ ID NO:56. In one embodiment, the humanized antibody includes a VH of SEQ ID NO:58 and a VL of SEQ ID NO:56.

[0079] In some embodiments, the antibody or fragment thereof further a heavy chain constant region (e.g., CH1, CH2 and/or CH3) and/or a light chain constant region (e.g., CL). An example heavy chain constant region is provided in SEQ ID NO:59, and an example light chain constant region is provided in SEQ ID NO:67 (residues 108-214).

[0080] It is contemplated that small changes (e.g., one amino acid addition, deletion or substitution) can be designed among these CDR sequences that can retain the antibodies' activities or even improve them. Such modified CDR sequences are referred to as CDR variants. It will also be understood by one of ordinary skill in the art that antibodies as disclosed herein may be modified such that they vary in amino acid sequence from the naturally occurring binding polypeptide from which they were derived. For example, a polypeptide or amino acid sequence derived from a designated protein may be similar, e.g., have a certain percent identity to the starting sequence, e.g., it may be 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% identical to the starting sequence. In some embodiments, the modified antibody or fragment retains the designate CDR sequences.

[0081] In certain embodiments, the antibody comprises an amino acid sequence or one or more moieties not normally associated with an antibody. Exemplary modifications are described in more detail below. For example, an antibody of the disclosure may comprise a flexible linker sequence, or may be modified to add a functional moiety (e.g., PEG, a drug, a toxin, or a label).

Polynucleotides Encoding the Proteins and Methods of Preparing the Proteins

[0082] The present disclosure also provides isolated polynucleotides or nucleic acid molecules encoding the multifunctional proteins, antibodies, variants or derivatives thereof of the disclosure. The polynucleotides of the present disclosure may encode the entire heavy and light chain variable

regions of the antigen-binding polypeptides, variants or derivatives thereof on the same polynucleotide molecule or on separate polynucleotide molecules. Additionally, the polynucleotides of the present disclosure may encode portions of the heavy and light chain variable regions of the antigen-binding polypeptides, variants or derivatives thereof on the same polynucleotide molecule or on separate polynucleotide molecules.

[0083] Methods of making antibodies are well known in the art and described herein. In certain embodiments, both the variable and constant regions of the antigen-binding polypeptides of the present disclosure are fully human. Fully human antibodies can be made using techniques described in the art and as described herein. For example, fully human antibodies against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Exemplary techniques that can be used to make such antibodies are described in U.S. patents: 6,150,584; 6,458,592; 6,420,140 which are incorporated by reference in their entireties.

Cancer Treatment

[0084] As described herein, the antibodies, variants or derivatives of the present disclosure may be used in certain treatment and diagnostic methods.

[0085] The present disclosure is further directed to multifunctional molecule- or antibody-based therapies which involve administering the multifunctional molecules or the antibodies of the disclosure to a patient such as an animal, a mammal, and a human for treating one or more of the disorders or conditions described herein. Therapeutic compounds of the disclosure include, but are not limited to, antibodies of the disclosure (including variants and derivatives thereof as described herein) and nucleic acids or polynucleotides encoding antibodies of the disclosure (including variants and derivatives thereof as described herein).

[0086] The antibodies of the disclosure can also be used to treat or inhibit cancer. PD-L1 can be overexpressed in tumor cells. Tumor-derived PD-L1 can bind to PD-1 on immune cells thereby limiting antitumor T-cell immunity. Results with small molecule inhibitors, or monoclonal antibodies targeting PD-L1 in murine tumor models, indicate that targeted PD-L1 therapy is an important alternative and realistic approach to effective control of tumor growth. As demonstrated in the experimental examples, the anti-PD-L1 antibodies activated the adaptive immune response machinery, which can lead to improved survival in cancer patients.

[0087] Accordingly, in some embodiments, provided are methods for treating a cancer in a patient in need thereof. The method, in one embodiment, entails administering to the patient an effective amount of a multifunctional molecule or an antibody of the present disclosure. In some embodiments, at least one of the cancer cells (e.g., stromal cells) in the patient expresses, over-

express, or is induced to express PD-L1. Induction of PD-L1 expression, for instance, can be done by administration of a tumor vaccine or radiotherapy.

[0088] Tumors that express the PD-L1 protein include those of bladder cancer, non-small cell lung cancer, renal cancer, breast cancer, urethral cancer, colorectal cancer, head and neck cancer, squamous cell cancer, Merkel cell carcinoma, gastrointestinal cancer, stomach cancer, oesophageal cancer, ovarian cancer, renal cancer, and small cell lung cancer. Accordingly, the presently disclosed antibodies can be used for treating any one or more such cancers.

[0089] Cellular therapies, such as chimeric antigen receptor (CAR) T-cell therapies, are also provided in the present disclosure. A suitable cell can be used, that is put in contact with an anti-PD-L1 antibody of the present disclosure (or alternatively engineered to express an anti-PD-L1 antibody of the present disclosure). Upon such contact or engineering, the cell can then be introduced to a cancer patient in need of a treatment. The cancer patient may have a cancer of any of the types as disclosed herein. The cell (e.g., T cell) can be, for instance, a tumor-infiltrating T lymphocyte, a CD4⁺ T cell, a CD8⁺ T cell, or the combination thereof, without limitation.

[0090] In some embodiments, the cell was isolated from the cancer patient him- or her-self. In some embodiments, the cell was provided by a donor or from a cell bank. When the cell is isolated from the cancer patient, undesired immune reactions can be minimized.

[0091] Additional diseases or conditions associated with increased cell survival, that may be treated, prevented, diagnosed and/or prognosed with the antibodies or variants, or derivatives thereof of the disclosure include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (*e.g.*, acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (*e.g.*, chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (*e.g.*, Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyo sarcoma, colon carcinoma, pancreatic cancer, breast cancer, thyroid cancer, endometrial cancer, melanoma, prostate cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma,

epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma and retinoblastoma.

Compositions

[0092] The present disclosure also provides pharmaceutical compositions. Such compositions comprise an effective amount of an antibody, and an acceptable carrier. In some embodiments, the composition further includes a second anticancer agent (e.g., an immune checkpoint inhibitor).

[0093] In a specific embodiment, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Further, a “pharmaceutically acceptable carrier” will generally be a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

[0094] The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents such as acetates, citrates or phosphates. Antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid, and agents for the adjustment of tonicity such as sodium chloride or dextrose are also envisioned. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin, incorporated herein by reference. Such compositions will contain a therapeutically effective amount of the antigen-binding polypeptide, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of

administration. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0095] In an embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

EXAMPLES

Example 1: Generation of Mouse Monoclonal Antibodies against Human PD-L1

[0096] This example describes the generation of anti-human-PD-L1 mouse monoclonal antibodies using the hybridoma technology.

[0097] *Antigen*: human PDL1-Fc protein and human PD-L1 highly expressed on the CHOK1 cell line (PDL1- CHOK1 cell line).

[0098] *Immunization*: To generate mouse monoclonal antibodies targeting human PD-L1, Balb/c mice and Wistar Rat were firstly immunized with PD-L1-Fc protein. The immunized mice and Rat were subsequently boosted with the PD-L1-Fc protein and CHO-K1/PD-L1 stable cells respectively. To select mice or rat producing antibodies that bound to PD-L1 protein, the serum of immunized mice or rat was subjected to the antibody titer evaluation by ELISA. Briefly, microtiter plates were coated with human PD-L1 protein at 0.5 µg/ml in ELISA coating buffer, 100 µl/well at 4°C overnight, then blocked with 150 µl/well of 1% BSA. Dilutions of serum from immunized mice were added to each well and incubated for 1-2 hours at 37°C. The plates were washed with PBS/Tween and then incubate with anti-mouse IgG antibody conjugated with Horse Radish Peroxidase (HRP) or anti-rat IgG antibody conjugated with HRP for 1 hour at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. After 3 rounds of immunization, immune responses were also tested by serum ELISA against rhPD-L1 protein and FACS against CHO-K1/PDL-1 stable cell line with CHO-K1 parental cell line served as negative control. Mice displayed with sufficient titers of anti-PDL1 IgG were boosted with 25 µg

human PDL1-Fc protein after 3 rounds of immunization. The resulting mice were used for fusions. The hybridoma supernatants were tested for anti-PD-L1 IgG by ELISA.

[0099] *Cell fusion*: Fusion was performed by electro fusion. Fused cells were plated into 50 96-well plates for each fusion.

[0100] *Screening*: The supernatants were screened by ELISA against recombinant human (rh) PD-L1-Fc protein and counter screening antigen. Then, positive supernatants underwent confirmative screening from primary screening with receptor blocking FACS against CHO-K1/PD-L1 stable cell line and rhPD-1-Fc protein.

[0101] *Subcloning and screening*: positive primary clones from each fusion were subcloned by limiting dilution to ensure that the subclones were derived from a single parental cell. Subcloning were screened in the same approach as primary clones and culture supernatant of positive clones underwent additional confirmative screening by affinity ranking.

[0102] Hybridoma clones 47C6A3, 67F3G7 and 89C10H8 were selected for further analysis. The amino acid sequences of the variable regions of 47C6A3, 67F3G7 and 89C10H8 are listed in **Table 1** below.

Table 1. Sequences of the variable regions of 47C6A3, 67F3G7 and 89C10H8

Name	Sequence	SEQ ID NO:
47C6A3 VH	DVQLQESGPGLVKPSQSLSTCTVTGYSITSDYAWNWIHQFPGNKLWGMGYIIYSGSTS YNPSLKSRIISITRDTSKNQFFLQLNSVTTEDTATYYCARSTMIATNWFAYWGQGLTVT SA	1
47C6A3 VL	DIIVMTQSHKFMSTSVDRVSITCKASQDVSLAVAWYQKPGQSPKLLIYWASTRHTGVP DRFTGSGSGTDFALTISSVQAEDLALYYCQOHYITPWTFGGGTKLEIK	2
67F3G7 VH	QVKLLQSGAALVKPGASVKMSCKASGYIFTDFWVSWVKQSHEKSLEWIGEIYPNSGVSR YNEKFKGRATMTVDKSTSTAYLELSRLTSEDSAIYYCTKYFGYTYWFGYWQGLTVTS S	3
67F3G7 VL	DTVLTQSPALAVSLGQRITISCRASKSVSTYMHWYQQRSGLPKLLIYSASHLESGVPS RFGSGSGTDFLTIDPVEADDIANYYCQSNELPVTFGSGTKLEIK	4
89C10H8 VH	EVQLVESGGGLVQPGRLTLSCVASGFTFSNYWMTWIRQAPGKLEWVASITNTGSSTF YPDSSVKGRFTISRDNTRSTLFLQINSLRSEDATYYCTRDTTIAPFDYWGQGMVTVSS	5
89C10H8 VL	DIQMTQSPSFLSASVGDRTITCKASONLNEYLNWYQKLGAPKRLIYKTNTLQAGIP SRFSGSGSGIDYTLTISSLQPEDVATYFCQYNSGNTFGAGTKLEIK	6

Table 1A. CDR sequences of 47C6A3

47C6A3	Sequence	SEQ ID NO:
CDRH1	SDYAWN	7
CDRH2	YIIYSGSTSYNPSLKS	8
CDRH3	STMIATNWFAY	9
CDRL1	KASQDVSLAVA	10
CDRL2	WASTRHT	11
CDRL3	QOHYITPWT	12

Table 1B. CDR sequences of 67F3G7

67F3G7	Sequence	SEQ ID NO:
CDRH1	DFWVS	13
CDRH2	EIYPNSGVSRVNEKFKG	14
CDRH3	YFGYTYWFGY	15
CDRL1	RASKSVSTYMH	16
CDRL2	SASHLES	17
CDRL3	QQSNELPVT	18

Table 1C. CDR sequences of 89C10H8

89C10H8	Sequence	SEQ ID NO:
CDRH1	NYWMT	19
CDRH2 ver DA ver ES	SITNTGSSTFYPD ^{SV} KG SITNTGSSTFYPD ^{AV} KG SITNTGSSTFYPE ^{SV} KG	20 91 92
CDRH3	DTTIAPFDY	21
CDRL1	KASQNLNEYLN	22
CDRL2	KTNTLQA	23
CDRL3 ver Q	SQYNSGNT SQY ^Q SGNT	24 93

Example 2: The binding activity to PD-L1 antigen**ELISA Testing**

[0103] To evaluate the binding activity of hybridoma clones 47C6A3, 67F3G7 and 89C10H8, the chimeric mAb from these clones were subjected to ELISA test.

[0104] Briefly, microtiter plates were coated with human PD-L1-Fc protein at 0.5 µg/ml in PBS, 100 µl/well at 4°C overnight, then blocked with 150 µl/well of 1% BSA. Three-fold dilutions of 47C6A3, 67F3G7 and 89C10H8 antibodies starting from 10 µg/ml were added to each well and incubated for 1 hour at 37°C. The plates were washed with PBS/Tween and then incubated with Mouse-anti-human IgG Fab antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. As shown in **FIG. 1**, 47C6A3, 67F3G7 and 89C10H8 bound to human PD-L1 with high affinity (EC_{50} =10.24ng/ml for 47C6A3, EC_{50} =10.76ng/ml for 67F3G7, EC_{50} =8.112ng/ml for 89C10H8).

[0105] Cell-based binding: FACS was used to evaluate the binding activity of 47C6A3, 67F3G7 and 89C10H8 chimeric mAbs on human PD-L1 over-expressed CHOK1 cells.

[0106] Briefly, PDL1-CHOK1 cells were firstly incubated with 3-fold serially diluted 47C6A3, 67F3G7 and 89C10H8 chimeric mAbs starting at 100nM at 4°C for 40 mins. After washing by PBS, Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG (H+L) was added to each well and incubated

at 4°C for 30 mins. Samples were washed twice with FACS buffer. The mean fluorescence intensity (MFI) of Alexa Fluor® 647 was evaluated by FACSCanto. As shown in **FIG. 2**, 47C6A3, 67F3G7 and 89C10H8 bound to PDL1-CHOK1 cells with high affinity ($EC_{50}=0.1476$ nM for 47C6A3, $EC_{50}=0.1035$ nM for 67F3G7, $EC_{50}=0.1696$ nM for 89C10H8).

Cross species activity

[0107] ELISA testing was carried out to evaluate the binding of chimeric antibodies to human, mouse, rat, and cynomolgus PD-L1, respectively.

[0108] Briefly, microtiter plates were coated with human, mouse, rat and cynomolgus PD-L1 proteins at 0.5 µg/ml in PBS, 100 µl/well at 4°C overnight, then blocked with 150 µl/well of 1% BSA. Three-fold dilutions of chimeric antibodies starting from 10 µg/ml were added to each well and incubated for 1 hour at 37 °C. The plates were washed with PBS/Tween and then incubate with mouse-anti-human IgG Fab antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37 °C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. 47C6A3, 67F3G7 and 89C10H8 antibodies bound to human and cynomolgus PD-L1 but did not bind to rat and mouse PD-L1 (**FIG. 3** and **Table 2**).

Table 2. Cross species activity of 47C6A3, 67F3G7 and 89C10H8

	Human	Cynomolgus	Rat	Mouse
EC50 of 47C6A3	10.24 ng/ml	6.336 ng/ml	No binding	No binding
EC50 of 67F3G7	10.76 ng/ml	6.797 ng/ml	No binding	No binding
EC50 of 89C10H8	8.112 ng/ml	6.774 ng/ml	No binding	No binding

Example 3. Blockade of PD-L1 binding to PD-1 by antibodies

[0109] To evaluate the blocking effects of 47C6A3, 67F3G7 and 89C10H8 chimeric mAbs on recombinant human PD-L1 binding to its receptor PD-1, the ELISA based receptor blocking assay was employed.

[0110] Briefly, microtiter plates were coated with human PD-L1-Fc protein at 0.5 µg/ml in PBS, 100 µl/well at 4°C overnight, then blocked with 150 µl/well of 1% BSA. 50 µl biotin-labeled human PD-1-Fc protein and 3-fold dilutions of 47C6A3, 67F3G7 and 89C10H8 antibodies starting from 10 µg/ml at 50 µl were added to each well and incubated for 1 hour at 37°C. The plates were washed with PBS/Tween and then incubated with Streptavidin-HRP for 10 mins at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. As shown in **FIG. 4**, 47C6A3, 67F3G7 and 89C10H8 efficiently inhibited the binding of human PD-L1 to human PD1 at IC_{50} detected at 91.18 ng/ml, 139.8 ng/ml, 129.8 ng/ml, respectively.

Example 4: Binding affinity of mAbs

[0111] The binding of the 47C6A3, 67F3G7 and 89C10H8 antibodies to recombinant PD-L1 protein (human PD-L1-his tag) was tested with Biacore using a capture method. The 47C6A3, 67F3G7 and 89C10H8 mAbs were captured using Protein A chip. A serial dilution of human PD-L1-his tag protein was injected over captured antibody for 2mins at a flow rate of 30 μ l/min. The antigen was allowed to dissociate for 480-1500s. All the experiment were carried out on a Biacore T200. Data analysis was carried out using Biacore T200 evaluation software. The results are shown in **FIG. 5** and **Table 3** below.

Table 3. Affinity measured by Biacore

Abs	PDL1-His		
	ka (1/Ms)	kd (1/s)	KD (M)
47C6A3	1.851E+6	6.785E-4	3.665E-10
67F3G7	2.538E+6	6.193E-5	2.441E-11
89C10H8	1.597E+6	6.636E-5	4.154E-11

Example 5. Humanization of the Mouse Antibodies

[0112] The 47C6A3, 67F3G7, and 89C10H8 variable region genes were employed to create a humanized mAb. In the first step of this process, the amino acid sequences of the VH and VL or VK of 47C6A3, 67F3G7, and 89C10H8 were compared against the available database of human Ig gene sequences to find the overall best-matching human germline Ig gene sequences. For the light chain of 47C6A3, human Vk1-4 is the best fit germline, and for the heavy chain, human VH1-2 was chosen as the backbone. For the light chain of 67F3G7, the closest human match is the Vk1-39/JK4 gene, and for the heavy chain the closest human match is the VH1-2/JH4-FW4 gene. For the light chain of 89C10H8, the closest human match is the Vk1-17/JK2 gene, and for the heavy chain the closest human match is the VH3-21/JH3 gene.

[0113] For the VL of 47C6A3, human Vk1-4 is the best fit germline, and for VH of 47C6A3, human VH1-2 was chosen as the backbone. Humanized variable domain sequences of 47C6A3 were then designed where the CDRL1, L2, and L3 were grafted onto framework sequences of the Vk1-4 gene, and the CDRH1, H2, and H3 onto framework sequences of the VH1-2 gene. A 3D model was then generated to determine if there were any framework positions where replacing the mouse amino acids with the human amino acids could affect binding and/or CDR conformation. In the case of the heavy chain, R, M, and I in the framework were involved in back-mutations.

[0114] Humanized variable domain sequences of 67F3G7 were then designed where the CDRL1, L2 and L3 were grafted onto framework sequences of the Vk1-39/JK4 gene, and the CDRH1, H2, and H3 onto framework sequences of the VH1-2/JH4-FW4 gene. A 3D model was then generated to determine if there were any framework positions where replacing the mouse amino acids with the

human amino acids could affect binding and/or CDR conformation. In the case of the heavy chain, V, K, T, and I in the framework were involved in back-mutations. In the case of the light chain, T, V, L, and Q in the framework were involved in back-mutations.

[0115] Humanized variable domain sequences of 89C10H8 were then designed where the CDRL1, L2 and L3 were grafted onto framework sequences of the Vk1-17/JK2 gene, and the CDRH1, H2, and H3 onto framework sequences of the VH3-21/JH3 gene. A 3D model was then generated to determine if there were any framework positions where replacing the mouse amino acids to the human amino acids could affect binding and/or CDR conformation. In the case of the heavy chain, A, T, I, and S in the framework were involved in back-mutations. In the case of the light chain, Y, I, E, and F in the framework were involved in back-mutations.

[0116] The amino acid and nucleotide sequences of some of the humanized antibody are listed in Table 4 below.

Table 4. Humanized antibody sequences (underlining indicates CDR; bold/italic indicates back mutations)

47C6A3	Sequence	SEQ ID NO:
VH mouse	DVQLQESGPGLVKPSQSLSTCTVTGYSITSDYAWNWRQFPGNKLEWMGYIIYSGST SYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYCARSTMIATNWFAYWGQGLTV TVSA	25
VH-V0	QVQLQESGPGLVKPSSETLSLTCTVSGYSITSDYAWNWRQPPGKLEWIGYIIYSGST SYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARSTMIATNWFAYWGQGLTV TVSS	26
VH-V1	QVQLQESGPGLVKPSSETLSLTCTVSGYSITSDYAWNWRQPPGKLEWIGYIIYSGST SYNPSLKSRVTISRDTSKNQFSLKLSSVTAADTAVYYCARSTMIATNWFAYWGQGLTV TVSS	27
VH-V2	QVQLQESGPGLVKPSSETLSLTCTVSGYSITSDYAWNWRQPPGKLEWMGYIIYSGST SYNPSLKSRITISRDTSKNQFSLKLSSVTAADTAVYYCARSTMIATNWFAYWGQGLTV TVSS	28
VL mouse	DIVMTQSHKFMSTSVDRVSITCKASQDVSLAVAWYQQKPGQSPKLLIYWASTRHTGV PDRFTGSGSGTDFALTISVQAEDLALYYCQOHYITPWTFGGGTKLEIK	29
VL V0	DIQMTQSPSSLSASVGDRVTITCKASQDVSLAVAWYQQKPGKAPKLLIYWASTRHTGV PSRFSGSGSGTDFLTISLQPEDFATYYCQOHYITPWTFGGGTKVEIK	30

67F3G7	Sequence	SEQ ID NO:
VH mouse	QVKLLQSGAALVKPGASVKMSCKASGYIFTDFWVSWVKQSHEKSLEWIGETIYPNSGVS RYNEKFKGRATMTVDKSTSTAYLELSRLTSEDSATYYCTKYFGYTYWFGYWGQGLTVT VSS	31
VH V0	EVQLVQSGAEVKKPGASVKVSKASGYFTDFWVSWVRQAPGQGLEWMGETIYPNSGVS RYNEKFKGRVTMTDRDTSISTAYMELSRLSDDTAVYYCARYFGYTYWFGYWGQGLTVT VSS	32
VH V1	EVQLVQSGAEVKKPGASVKVSKASGYFTDFWVSWVRQAPGQGLEWMGETIYPNSGVS RYNEKFKGRVTMTVDKSTSTAYMELSRLSDDTAVYYCARYFGYTYWFGYWGQGLTVT VSS	33
VH V2	EVQLVQSGAEVKKPGASVKVSKASGYFTDFWVSWVRQAPGQGLEWMGETIYPNSGVS RYNEKFKGRVTMTVDKSTSTAYMELSRLSDDTAVYYCTKYFGYTYWFGYWGQGLTVT VSS	34

VH V3	EVQLVQSGAEVKKPGASVKVSCKASGYIFTFWVSWVRQAPGQGLEWIGEIYPNSGVS RYNEKFKGRVTMTVDKSIISTAYMELSRRLSDDTAVYYCTKYFGYTYWFGYWGQGTLLVT VSS	35
VH V4	EVQLVQSGAEVKKPGASVKVSCKASGYIFTFWVSWVRQAPGQGLEWIGEIYPNSGVS RYNEKFKGRVTMTVDKSIISTAYMELSRRLSDDTAVYYCARYFGYTYWFGYWGQGTLLVT VSS	36
VH V5	EVQLVQSGAEVKKPGASVKVSCKASGYIFTFWVSWVRQAPGQGLEWIGEIYPNSGVS RYNEKFKGRVTMTVDKSIISTAYMELSRRLSDDTAVYYCTKYFGYTYWFGYWGQGTLLVT VSS	37
VL mouse	DTVLTQSPALAVSLGQRITISCRASKSVSTYMHYQQRSGLQPKLLIYSASHLESGV PSRFSGSGSGTDFTLTIDPVEADDIANYCQOSNELPVTFGGGTKLEIK	38
VL V0	DIQMTQSPSSLASVGDRTITCRASKSVSTYMHYQQKPGKAPKLLIYSASHLESGV PSRFSGSGSGTDFTLTISLQPEDFATYYCQOSNELPVTFGGGTKVEIK	39
VL V1	DTVLTQSPSSLASVGDRTITCRASKSVSTYMHYQQKPGKAPKLLIYSASHLESGV PSRFSGSGSGTDFTLTISLQPEDFATYYCQOSNELPVTFGGGTKVEIK	40
VL V2	DTVLTQSPSSLASVGDRTITCRASKSVSTYMHYQQKPGKAPKLLIYSASHLESGV PSRFSGSGSGTDFTLTISLQPEDFATYYCQOSNELPVTFGGGTKVEIK	41
VL V3	DTVLTQSPSSLASVGDRTITCRASKSVSTYMHYQQKPGKAPKLLIYSASHLESGV PSRFSGSGSGTDFTLTISLQPEDFATYYCQOSNELPVTFGGGTKVEIK	42
VL V4	DIQMTQSPSSLASVGDRTITCRASKSVSTYMHYQQKPGKAPKLLIYSASHLESGV PSRFSGSGSGTDFTLTISLQPEDFATYYCQOSNELPVTFGGGTKVEIK	43

89C10H8	Sequence	SEQ ID NO:
VH mouse	EVQLVESGGGLVQPGRSLTSCVASGFTFSNYWMTWIRQAPGKLEWVASITNTGSST FYPDSVKGRFTISRDNTRSTLFLQINLSRSEDATYYCTRDTTIAPFDYWGQGMVTV SS	44
VH V0	EVQLVESGGGLVKPGGSLRLSCAASGFTFSNYWMTWVRQAPGKLEWVSSITNTGSST FYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDTTIAPFDYWGQGMVTV SS	45
VH V1	EVQLVESGGGLVKPGGSLRLSCAASGFTFSNYWMTWVRQAPGKLEWVASITNTGSST FYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDTTIAPFDYWGQGMVTV SS	46
VH V2	EVQLVESGGGLVKPGGSLRLSCAASGFTFSNYWMTWVRQAPGKLEWVASITNTGSST FYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRDTTIAPFDYWGQGMVTV SS	47
VH V3	EVQLVESGGGLVKPGGSLRLSCAASGFTFSNYWMTWIRQAPGKLEWVASITNTGSST FYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRDTTIAPFDYWGQGMVTV SS	48
VH V4	EVQLVESGGGLVKPGGSLRLSCAASGFTFSNYWMTWIRQAPGKLEWVASITNTGSST FYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRDTTIAPFDYWGQGMVTV SS	49
VL mouse	DIQMTQSPSFLASVGDRTITCKASONLNEYLNWYQQKLGAPKRLIYKTNLTQAGI PSRFSGSGSGIDYTLTISLQPEDVATYFCQYNSGNTFGGQTKLEIK	50
VL V0	DIQMTQSPSSLASVGDRTITCKASONLNEYLNWYQQKPGKAPKRLIYKTNLTQAGV PSRFSGSGSGTDFTLTISLQPEDFATYYCQYNSGNTFGGQTKLEIK	51
VL V1	DIQMTQSPSSLASVGDRTITCKASONLNEYLNWYQQKPGKAPKRLIYKTNLTQAGV PSRFSGSGSGTDYTLTISLQPEDFATYYCQYNSGNTFGGQTKLEIK	52
VL V2	DIQMTQSPSSLASVGDRTITCKASONLNEYLNWYQQKPGKAPKRLIYKTNLTQAGI PSRFSGSGSGTDYTLTISLQPEDFATYYCQYNSGNTFGGQTKLEIK	53
VL V3	DIQMTQSPSSLASVGDRTITCKASONLNEYLNWYQQKPGKAPKRLIYKTNLTQAGI PSRFSGSGSGIDYTLTISLQPEDFATYYCQYNSGNTFGGQTKLEIK	54
VL V4	DIQMTQSPSSLASVGDRTITCKASONLNEYLNWYQQKLGAPKRLIYKTNLTQAGI PSRFSGSGSGIDYTLTISLQPEDFATYFCQYNSGNTFGGQTKLEIK	55

[0117] The genes were cloned in pcDNA 3.4 vector and transfected into 293F cells. The antibodies were produced according to the following table.

[0118] The humanized VH and VL genes were produced synthetically and then respectively cloned into vectors containing the human gamma 1 and human kappa constant domains. The pairing of the human VH and the human VL created the 41 humanized antibodies (see Table 5).

Table 5. Humanized antibodies with their VH and VL regions

A. 47C6A3

VH	VL	47C6A3 VL	47C6A3 VL-V0
47C6A3 VH		47C6A3-chimera	
47C6A3 VH.V0			Hu47C6A3-1
47C6A3 VH.V1			Hu47C6A3-2
47C6A3 VH.V2			Hu47C6A3-3

B. 67F3G7

VH	VL	67F3G7 VL.V0	67F3G7 VL.V1	67F3G7 VL.V2	67F3G7 VL.V3	67F3G7 VL.V4	67F3G7 VL
67F3G7 VH.V1		Hu 67F3G7-1	Hu 67F3G7-6	Hu 67F3G7-11	Hu 67F3G7-16	Hu 67F3G7-21	
67F3G7 VH.V2		Hu 67F3G7-2	Hu 67F3G7-7	Hu 67F3G7-12	Hu 67F3G7-17	Hu 67F3G7-22	
67F3G7 VH.V3		Hu 67F3G7-3	Hu 67F3G7-8	Hu 67F3G7-13	Hu 67F3G7-18		
67F3G7 VH.V4		Hu 67F3G7-4	Hu 67F3G7-9	Hu 67F3G7-14	Hu 67F3G7-19		
67F3G7 VH.V5		Hu 67F3G7-5	Hu 67F3G7-10	Hu 67F3G7-15	Hu 67F3G7-20		
67F3G7 VH							67F3G7-chimera

C. 89C10H8

VH	VL	89C10H8 VL.V1	89C10H8 VL.V2	89C10H8 VL.V3	89C10H8 VL.V4	89C10H8 VL
89C10H8 VH.V1		Hu 89C10H8-1	Hu 89C10H8-5	Hu 89C10H8-9	Hu 89C10H8-13	
89C10H8 VH.V2		Hu 89C10H8-2	Hu 89C10H8-6	Hu 89C10H8-10	Hu 89C10H8-14	
89C10H8 VH.V3		Hu 89C10H8-3	Hu 89C10H8-7	Hu 89C10H8-11	Hu 89C10H8-15	
89C10H8 VH.V4		Hu 89C10H8-4	Hu 89C10H8-8	Hu 89C10H8-12	Hu 89C10H8-16	
89C10H8 VH						89C10H8-chimera

Example 6: Antigen binding properties of the Humanized Antibodies

Binding to recombinant human PD-L1

[0119] To evaluate antigen binding activity, the humanized antibodies were subjected to ELISA test. Briefly, microtiter plates were coated with human PD-L1-Fc protein at 0.5 µg/ml in PBS, 100 µl/well at 4°C overnight, then blocked with 200 µl/well of 1% BSA. Three-fold dilutions of humanized antibodies starting from 10 µg/ml were added to each well and incubated for 1 hour at 37°C. The plates were washed with PBS/Tween and then incubated with mouse-anti-human IgG Fab antibody conjugated with Horse Radish Peroxidase (HRP) for 1 hour at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. As shown in **FIG. 6**, all the humanized antibodies showed comparable binding efficiency to human PD-L1 as the chimeric antibody.

[0120] To explore the binding kinetics of the humanized antibodies, this example performed the affinity ranking by using Biacore. As shown in Table 6, Hu67F3G7-2, Hu67F3G7-3, Hu67F3G7-5, Hu67F3G7-7, Hu67F3G7-22, Hu89C10H8-4, Hu89C10H8-7, Hu89C10H8-11, and Hu89C10H8-12 showed high affinity, which are comparable to chimeric antibodies.

Table 6. Affinity ranking of humanized antibodies

Abs	PDL1-His		
	ka (1/Ms)	kd (1/s)	KD (M)
Hu67F3G7-1	1.855E+6	1.214E-4	6.548E-11
Hu67F3G7-2	2.569E+6	9.748E-5	3.795E-11
Hu67F3G7-3	2.803E+6	9.716E-5	3.466E-11
Hu67F3G7-4	1.883E+6	1.031E-4	5.477E-11
Hu67F3G7-5	2.621E+6	9.711E-5	3.704E-11
Hu67F3G7-6	2.006E+6	1.177E-4	5.867E-11
Hu67F3G7-7	2.687E+6	9.493E-5	3.533E-11
Hu67F3G7-8	2.765E+6	9.844E-5	3.560E-11
Hu67F3G7-9	2.214E+6	1.004E-4	4.535E-11
Hu67F3G7-10	2.383E+6	9.335E-5	3.918E-11
Hu67F3G7-11	5.114E+6	1.643E-4	3.213E-11
Hu67F3G7-12	2.565E+6	9.013E-5	3.513E-11
Hu67F3G7-13	2.798E+6	9.535E-5	3.408E-11
Hu67F3G7-14	2.092E+6	1.086E-4	5.191E-11
Hu67F3G7-15	2.610E+6	9.688E-5	3.712E-11
Hu67F3G7-16	1.478E+6	1.114E-4	7.537E-11
Hu67F3G7-17	2.741E+6	1.014E-4	3.698E-11
Hu67F3G7-18	2.876E+6	9.511E-5	3.307E-11
Hu67F3G7-19	1.970E+6	1.074E-4	5.451E-11
Hu67F3G7-20	2.698E+6	9.363E-5	3.471E-11
Hu67F3G7-21	5.399E+5	8.425E-5	1.560E-10
Hu67F3G7-22	1.669E+6	7.261E-5	4.351E-11

Abs	PDL1-His		
	ka (1/Ms)	kd (1/s)	KD (M)
Hu89C10H8-1	7.541E+5	4.654E-4	6.172E-10
Hu89C10H8-2	6.411E+5	8.506E-5	1.327E-10
Hu89C10H8-3	7.375E+5	5.793E-5	7.855E-11
Hu89C10H8-4	6.230E+5	5.944E-5	9.542E-11
Hu89C10H8-5	6.707E+5	4.939E-4	7.364E-10
Hu89C10H8-6	7.010E+5	8.560E-5	1.221E-10
Hu89C10H8-7	6.150E+5	6.613E-5	1.075E-10
Hu89C10H8-8	6.550E+5	6.663E-5	1.017E-10
Hu89C10H8-9	6.712E+5	4.784E-4	7.127E-10
Hu89C10H8-10	5.950E+5	8.988E-5	1.511E-10
Hu89C10H8-11	7.001E+5	6.732E-5	9.615E-11
Hu89C10H8-12	7.269E+5	7.036E-5	9.680E-11
Hu89C10H8-13	7.644E+5	4.327E-4	5.611E-10
Hu89C10H8-14	7.125E+5	9.752E-5	1.369E-10
Hu89C10H8-15	7.814E+5	7.170E-5	9.176E-11
Hu89C10H8-16	6.671E+5	6.537E-5	9.799E-11

Binding to human PD-L1 overexpressed on mammalian cells

[0121] To evaluate the antigen binding property, the humanized antibodies were analyzed for their binding to PD-L1 overexpressed on mammalian cells by FACS. Briefly, PDL1- CHOK1 cells were

firstly incubated with 3-fold serious diluted humanized antibodies starting at 15µg/ml at 4°C for 40 mins. After washed by PBS, the Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG (H+L) antibody was added to each well and incubated at 4°C for 30 mins. The MFI of Alexa Fluor® 647 was evaluated by FACSCanto. As shown in **FIG. 7**, all the humanized antibodies can high-efficiently bind to PD-L1 expressed on mammalian cells.

Full kinetic affinity of humanized antibodies by Biacore

[0122] The binding of the humanized antibodies to recombinant PD-L1 protein (human PD-L1-his tag) was tested by Biacore using a capture method. Hu47C6A3-1, Hu47C6A3-2, Hu47C6A3-3, Hu67F3G7-2, Hu67F3G7-3, Hu67F3G7-5, Hu67F3G7-7, Hu67F3G7-22, Hu89C10H8-4, Hu89C10H8-7, Hu89C10H8-11, and Hu89C10H8-12 mAbs were captured using Protein A chip. A serial dilution of human PD-L1-his tag protein was injected over captured antibody for 2 mins at a flow rate of 30 µl/min. The antigen was allowed to dissociate for 1500s. All the experiment were carried out on a Biacore T200. Data analysis was carried out using Biacore T200 evaluation software and the results are shown in Table 7 below.

Table 7 Affinity by Biacore

Abs	PDL1-His		
	ka (1/Ms)	kd (1/s)	KD (M)
HSP209-chi	1.851E+6	6.785E-4	3.665E-10
Hu47C6A3-1	1.235E+6	5.933E-4	4.805E-10
Hu47C6A3-2	2.451E+6	9.000E-4	3.672E-10
Hu47C6A3-3	2.176E+6	9.661E-4	4.440E-10

Abs	PDL1-His		
	ka (1/Ms)	kd (1/s)	KD (M)
Hu67F3G7-2	1.915E+6	7.032E-5	3.672E-11
Hu67F3G7-3	2.088E+6	7.737E-5	3.706E-11
Hu67F3G7-5	1.838E+6	7.920E-5	4.310E-11
Hu67F3G7-7	2.211E+6	7.252E-5	3.280E-11
Hu67F3G7-22	1.779E+6	5.791E-5	3.256E-11

Abs	PDL1-His		
	ka (1/Ms)	kd (1/s)	KD (M)
Hu89C10H8-4	5.623E+5	5.408E-5	9.618E-11
Hu89C10H8-7	5.685E+5	5.313E-5	9.346E-11
Hu89C10H8-11	5.640E+5	5.542E-5	9.826E-11
Hu89C10H8-12	5.867E+5	5.587E-5	9.522E-11

Example 7: Blockade of the binding of PDL1 to PD1 by humanized antibodies

Receptor blocking assay by using recombinant human PD-L1

[0123] There are two receptors, PD-1 and CD80, for human PD-L1. To explore the blocking property of humanized PD-L1 antibody to these two proteins, protein based receptor blocking assay was employed here.

[0124] Briefly, microtiter plates were coated with human PD-L1-Fc protein at 0.5 µg/ml in PBS, 100 µl/well at 4°C overnight, then blocked with 150 µl/well of 1% BSA at 37 °C for 2 hours. 50 µl biotin-labeled human PD-1-Fc or CD80-Fc protein and 3-fold dilutions of PD-L1 antibodies starting from 10 µg/ml at 50 µl were added to each well and incubated for 1 hour at 37°C. The plates were washed with PBS/Tween and then incubated with Streptavidin-HRP for 10 mins at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. As shown in **FIG. 8**, Hu47C6A3-1, Hu47C6A3-2, Hu47C6A3-3, Hu67F3G7-2, Hu67F3G7-3, Hu67F3G7-5, Hu67F3G7-7, Hu67F3G7-22, Hu89C10H8-4, Hu89C10H8-7, Hu89C10H8-11, and Hu89C10H8-12 efficiently inhibited the binding of human PD-L1 to human PD1. Moreover, Hu47C6A3-1, Hu47C6A3-2, Hu47C6A3-3, Hu67F3G7-2, Hu67F3G7-3, Hu67F3G7-5, Hu67F3G7-7, Hu67F3G7-22, Hu89C10H8-4, Hu89C10H8-7, Hu89C10H8-11, and Hu89C10H8-12 efficiently inhibited the binding of human PD-L1 to human CD80 in a dose-dependent manner (**FIG. 9**).

Example 8. Bifunctional Protein Targeting on Both PD-L1 and TGF-β pathways

[0125] Bifunctional recombinant anti-PD-L1 antibody and TGF-β RII fusion proteins were prepared and tested in this example.

[0126] The light chain of the molecule is the light chain of an anti-PDL1 mAb. The heavy chain is a fusion of the heavy chain of the anti-PDL1 mAb, via a flexible (Gly4Ser)₄Gly linker, to the N-terminus of the soluble extracellular domain of TGF-β RII. At the fusion junction, the C-terminal lysine residue of the antibody heavy chain was mutated to alanine to reduce the potential proteolytic cleavage.

[0127] In some examples, potential modification sites in the CDRs were mutated to similar amino acids. The sequences of the anti-PD-L1 portion are shown in Table 8 below.

Table 8. Sequences of the variable regions of antibody portion in the bifunctional molecules

Name	Sequence	SEQ ID NO:
02 VH (67F3G7 VH V2)	EVQLVQSGAEVKKPGASVKVSCKASGYTFT DFWWS WVRQAPGQGLEWMGE IYPNSGVSRYNEKF KGRVTMTVDKSISTAYMELSRSDDTAVYYCTKYF GYTYWFGYWGQ GLTVTVSS	34
02 VL (67F3G7 VL V4)	DIQMTQSPSSLSASVGDRTITC RASKSVSTYMH WYQQKPGKPKLLI YSASHLES GVPSRFSGSGSGTDFTLTISLQPEDFATYYC QQSNELPVT FGG GTKVEIK	43
06 VH (89C10H8 VH V3)	EVQLVESGGGLVKKPGGSLRLSCAASGFTFS NYWMT WIRQAPGKLEWVAS ITNTGSSTFYPDSVK GRFTISRDNKNSLYLQMNSLRAEDTAVYYCT TRDTTIAPFDY WGQGMVTVSS	48
06 VL (89C10H8 VL V2)	DIQMTQSPSSLSASVGDRTITC KASONLNEYLN WYQQKPGKAPKRLI YKINTLQAG IPSRFSGSGSGTDYTLTISLQPEDFATYYC SQYNSGNT FGQ GTKLEIK	53

06a VH (89C10H8 VH V3)	EVQLVESGGGLVQPKGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVAS <u>ITNTGSSTFYPD</u> SVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRD <u>TIAPFDY</u> WGQGMVTSS	48
06a VL	DIQMTQSPSSLSASVGRVTITCKASONLNEYLNWYQQKPGKAPKRLIYK <u>INTLOAG</u> IPSRFSGSGSDTYTLTISSLQPEDFATYYCSQYQSGNTFGQ GTKLEIK	56
06a-DA VH	EVQLVESGGGLVQPKGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVAS <u>ITNTGSSTFYPD</u> AVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRD <u>TIAPFDY</u> WGQGMVTSS	57
06a-ES VH	EVQLVESGGGLVQPKGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVAS <u>ITNTGSSTFYPD</u> SVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRD <u>TIAPFDY</u> WGQGMVTSS	58
89C10H8 CDRH2 DA	SITNTGSSTFYPDVAVK	91
89C10H8 CDRH2 ES	SITNTGSSTFYPDVAVK	92
89C10H8 CDRL3 ver Q	SQYQSGNT	93

Table 9. VH/VL of the bifunctional molecules

Bifunctional molecule	VH	VL
LP008-02	02 VH	02 VL
LP008-06	06 VH	06 VL
LP008-06a	06a VH	06a VL
LP008-06a-DA	06a-DA VH	06a VL
LP008-06a-ES	06a-ES VH	06a VL

[0128] Besides the VH, the heavy chain of the bifunctional molecule further includes constant regions (with C-terminal K mutated to A), the (Gly₄Ser)₄Gly linker, and the N-terminus of the soluble extracellular domain of TGF- β RII. Their sequences are shown in Table 10.

Table 10. Additional sequences of the heavy chain, and entire heavy/light chains

Name	Sequence	SEQ ID NO:
Heavy chain constant regions	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTPSSSLGTQTYICNVNHKPSNTKVDKRVPEKSCDKHTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGA	59
(Gly₄Ser)₄Gly linker	GGGGSGGGSGGGSGGGSGG	60
TGF-β RII extracellular domain	IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIKKEKKPGETFFMCSDECDNIIFSEEYNTSNPD	61
LP008-02 heavy chain	EVQLVQSGAEVKKPGASVKVSCKASGYTFDFWVSWVRQAPGQGLEWMGEIYPNSGVSRVNEKFKGRVTMTVDKSIATYMEISRLRSDDTAVYYCTKYFGYTYWFGYWGQGLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTPSSSLGTQTYICNVNHKPSNTKVDKRVPEKSCDKHTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYCKVSNKALPAPIEKTISKAKGQPREPQV	62

	YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLSLSPGAGGGSGGGGGGGGGGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD	
LP008-06 heavy chain	EVQLVESGGGLVLPKPGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVASITNTGSSTFYPD SVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCTRDTIAPFDYWGQGTMTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLSLSPGAGGGSGGGGGGGGGGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD	63
LP008-06a heavy chain	EVQLVESGGGLVLPKPGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVASITNTGSSTFYPD SVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCTRDTIAPFDYWGQGTMTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLSLSPGAGGGSGGGGGGGGGGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD	64
LP008-06a-DA heavy chain	EVQLVESGGGLVLPKPGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVASITNTGSSTFYPD AVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCTRDTIAPFDYWGQGTMTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLSLSPGAGGGSGGGGGGGGGGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD	65
LP008-06a-ES heavy chain	EVQLVESGGGLVLPKPGGSLRLSCAASGFTFSNYWMTWIRQAPGKGLEWVASITNTGSSTFYPD SVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCTRDTIAPFDYWGQGTMTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI SRTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLSLSPGAGGGSGGGGGGGGGGGGGGGGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD	66
LP008-02 light chain	DIQMTQSPSSLSASVGRVTITCRASKSVSTYMHWYQQKPGKPKLLIYSAHLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSNELPVTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV	67

	DNALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	
LP008-06 light chain	DIQMTQSPSSLSASVGDRVTITCKASQNLNEYLNWYQQKPGKAPKRLIYK TNTLQAGIPSRFSGSGSGTDYTLTISSLPEDFATYYCSQYNSGNTFGQ GTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	68
LP008-06a (& DA/ES) light chain	DIQMTQSPSSLSASVGDRVTITCKASQNLNEYLNWYQQKPGKAPKRLIYK TNTLQAGIPSRFSGSGSGTDYTLTISSLPEDFATYYCSQYQS-GNTFGQ GTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC	69

Example 9: The binding affinity of the bifunctional molecules

[0129] The bindings of the LP008-06, LP008-06a, LP008-06a-DA, and LP008-06a-ES bifunctional molecules to recombinant PD-L1 protein (human PD-L1-his tag) were tested with Biacore using a capture method.

[0130] The bifunctional molecules were captured using Protein A chip. A serial dilution of human PD-L1-his tag protein was injected over captured antibody for 2 mins at a flow rate of 30 μ l/min. The antigen was allowed to dissociate for 1500s. All the experiments were carried out on a Biacore T200. Data analysis was carried out using Biacore T200 evaluation software. The results are shown in FIG. 10 and Table 11 below.

Table 11. Affinity testing by Biacore

Abs	Human PD-L1		
	ka (1/Ms)	kd (1/s)	KD (M)
LP008-06	7.832E+5	5.918E-5	7.556E-11
LP008-06a	6.865E+5	5.276E-5	7.684E-11
LP008-06a-DA	6.613E+5	4.795E-5	7.252E-11
LP008-06a-ES	8.404E+5	5.300E-5	6.307E-11

[0131] The binding of the LP008-02 to recombinant PD-L1 protein and human TGF- β 1 was tested with Biacore using a capture method.

[0132] LP008-02 was captured using Protein A chip. A serial dilution of human PD-L1-his tag protein and human TGF- β 1 was injected over captured antibody for 2 mins at a flow rate of 30 μ l/min. PD-L1 was allowed to dissociate for 680s, and TGF- β 1 was allowed to dissociate for 1000s. All the experiments were carried out on a Biacore T200. Data analysis was carried out using Biacore T200 evaluation software. The results are shown in FIG. 11 and Table 12 below.

Table 12. Affinity testing by Biacore

	LP008-02		
	ka (1/Ms)	kd (1/s)	KD (M)
PD-L1	2.528E+6	1.044E-4	4.130E-11
TGF- β 1	1.294E+8	2.716E-4	2.099E-12

Example 10: Functional assay for PD-1/PD-L1 blockade

[0133] The activities of the bifunctional molecules in blocking PD1/PD-L1 interaction were measured by a bioluminescent cell-based assay in this example.

[0134] In this assay, when PD1 effector cells are co-cultured with PD-L1 target cells, the PD-1/PD-L1 interaction inhibits TCR signaling and NFAT-RE-mediated luminescence. Addition of either an anti-PD-1 or anti-PD-L1 antibody that blocks the PD-1/PD-L1 interaction that would release the inhibitory signal and result in TCR activation and NFAT-RE-mediated luminescence.

[0135] As shown in FIG. 12, LP008-02 and LP008-06a-ES blockaded PD1 and PD-L1 interaction with considerably higher activity than M7824 (M7824 EC_{50} = 0.8504nM, LP008-02 EC_{50} =0.3630nM, LP008-06a-ES EC_{50} =0.4553nM).

Example 11: Functional assay for TGF- β

[0136] This example used luciferase assay to evaluate the effect of LP008-02 and LP008-06a-ES on canonical TGF- β signaling.

[0137] Serial dilutions of M7824 (bifunctional anti-PD-L1/TGF β Trap fusion protein, see, e.g., Knudson et al., *Oncoimmunology*. 2018; 7(5): e1426519), LP008-02 or LP008-06a-ES were incubated with SBE luciferase reporter-transfected 293cells for about 20 hours in the presence of recombinant human TGF- β .

[0138] As shown in FIG. 13, M7824, LP008-02 and LP008-06a-ES blocked TGF- β canonical signaling (IC_{50} = 0.06687nM, IC_{50} =0.07352nM, IC_{50} =0.07167nM) in a TGF- β SBE luciferase reporter assay system constructed in 293 cells.

Example 12: Binding activity to human PD-L1***ELISA with recombinant human PD-L1***

[0139] To evaluate the binding activity of M7824, LP008-02, and LP008-06a-ES, the bifunctional molecules were subjected to ELISA test.

[0140] Briefly, microtiter plates were coated with human PD-L1-His protein at 0.5 μ g/ml in PBS, 100 μ l/well at 4°C overnight, then blocked with 150 μ l/well of 1% BSA. Three-fold dilutions of M7824, LP008-02, and LP008-06a-ES starting from 1 μ g/ml were added to each well and incubated for 1 hour at 37°C. The plates were washed with PBS/Tween and then incubated with Goat-anti-human IgG antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm.

[0141] As shown in FIG. 14, LP008-02 and LP008-06a-ES bound to human PD-L1 with considerably higher activity than M7824 ($EC_{50}=11.82\text{ng/ml}$, and $EC_{50}=14.36\text{ ng/ml}$ vs. $EC_{50}=23.68\text{ng/ml}$).

Cross species activity

[0142] To evaluate the binding of bispecific antibodies to Mouse PD-L1, Rat PD-L1, Cynomolgus PD-L1, the antibodies were tested with ELISA.

[0143] Briefly, microtiter plates were coated with mouse, rat and cynomolgus PD-L1 proteins at $0.5\text{ }\mu\text{g/ml}$ in PBS, $100\text{ }\mu\text{l/well}$ at 4°C overnight, then blocked with $150\text{ }\mu\text{l/well}$ of 1% BSA. Three-fold dilutions of bispecific antibodies starting from $1\text{ }\mu\text{g/ml}$ were added to each well and incubated for 1 hour at 37°C . The plates were washed with PBS/Tween and then incubated with Goat-anti-human IgG antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37°C . After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm.

[0144] LP008-02 and LP008-06a-ES were able to bind to cynomolgus PD-L1 with higher affinity than M7824, but only M7824 is capable of binding to rat and mouse PD-L1 (FIG. 15 and Table 13).

Table 13. Cross species activity of M7824, CZ010-02, and CZ010-06a-ES

EC50	Cynomolgus	Rat	Mouse
M7824	25.60 ng/ml	14.25 ng/ml	10.60 ng/ml
LP008-02	7.890 ng/ml	No binding	No binding
LP008-06a-ES	10.92 ng/ml	No binding	No binding

Example 13: Binding activity to human TGF- β

ELISA by using recombinant human TGF- β

[0145] To evaluate the binding activity of M7824, LP008-02, and LP008-06a-ES to human TGF- β , these bifunctional molecules were subjected to ELISA test.

[0146] Briefly, microtiter plates were coated with human TGF- β protein at $1\text{ }\mu\text{g/ml}$ in PBS, $100\text{ }\mu\text{l/well}$ at 4°C overnight, then blocked with $150\text{ }\mu\text{l/well}$ of 1% BSA. Three-fold dilutions of the M7824, LP008-02, and LP008-06a-ES bifunctional molecules starting from $10\text{ }\mu\text{g/ml}$ were added to each well and incubated for 1 hour at 37°C . The plates were washed with PBS/Tween and then incubated with Goat-anti-human IgG antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37°C . After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm.

[0147] As shown in FIG. 16, all of M7824, LP008-02, and LP008-06a-ES bound to human TGF- β with high activity ($EC_{50}=43.43\text{ ng/ml}$, $EC_{50}=28.58\text{ ng/ml}$, $EC_{50}=39.38\text{ ng/ml}$).

Cross species activity

[0148] To evaluate the binding of bispecific antibodies to Mouse, Rat, and Cynomolgus TGF- β , the bifunctional molecules were subjected for the ELISA test.

[0149] Briefly, microtiter plates were coated with mouse, rat and cynomolgus TGF- β protein at 1 μ g/ml in PBS, 100 μ l/well at 4°C overnight, then blocked with 150 μ l/well of 1% BSA. Three-fold dilutions of bispecific antibodies starting from 10 μ g/ml were added to each well and incubated for 1 hour at 37 °C. The plates were washed with PBS/Tween and then incubated with Goat-anti-human IgG antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37 °C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm.

[0150] All of the tested bifunctional molecules bound to cynomolgus, rat, and mouse TGF- β with high activity (FIG. 17 and Table 14).

Table 14. Cross species activity of M7824, LP008-02, and LP008-06a-ES

EC50	Cynomolgus	Rat	Mouse
M7824	39.66 ng/ml	123.9 ng/ml	46.17 ng/ml
LP008-02	34.41 ng/ml	77.84 ng/ml	34.70ng/ml
LP008-06a-ES	58.35 ng/ml	120.7 ng/ml	58.26 ng/ml

Example 14: Efficacy in a MC38 tumor mouse model

[0151] This example used a tumor mouse model to test the *in vivo* efficacy of the bifunctional molecules.

[0152] MC38 cells expressing human PD-L1 resuspended in PBS were seeded subcutaneously into right skin of B-hPD-L1 humanized mice at a concentration of 5×10^5 cells in a volume of 0.2 mL. When the average tumor volume reached approximately 55 mm³, 24 mice with an appropriate individual tumor volume were selected for the group, and the animals were randomly assigned to 4 experimental groups according to the tumor volume, with 6 animals in each group. After anti-mCD20 mAbs injection, total human IgG, M7824, LP008-02 and LP008-06a-ES were administered 3 times a week by intraperitoneal injection. The dose was calculated based on the experimental animal's body weight at 10 μ g/g. Mice weight and tumor size were tested twice a week.

[0153] The results are shown in FIG. 18. Bifunctional molecules LP008-02 and LP008-06a-ES exhibited better efficacy than M7824 in these animal models in terms of tumor growth inhibition. Furthermore, animal deaths were observed in both the IgG and M7824 groups, but not in the LP008-02 and LP008-06a-ES groups, demonstrating better safety profiles of the new bifunctional molecules.

Example 15. Modification of bifunctional molecules

[0154] This example tested certain modified bifunctional molecules (Table 15) for their *in vitro* efficacy of functional assay for TGF- β . Some of them included a linker sequence of

TAGHTQTSTGGGAITGTSGAGHGP (SEQ ID NO:87), HYP, and/or G4S (SEQ ID NO:86) repeats. These molecules are referred to as LP008-02-1 to LP008-02-7, respectively.

Table 15. Modified sequences design of linker and TGF- β RII

Name	Sequences (not showing the antibody portion)	SEQ ID NO:
1	<u>GGGGSGGGSGGGSGGGSGIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRF</u> <u>STCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILE</u> <u>DAASPKCIMKEKKKPGETFFMCSSSDECNDNIIFSEYNTSNPD</u>	79
2	<u>GGGGSTAGHTQTSTGGGAITGTSGAGHGPQLCKFCDVRFSTCDNQKSCMSNCSIT</u> <u>SICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGE</u> <u>ETFFMCSSSDECNDNIIFSEYNTSNPD</u>	80
3	<u>GGGGSGGGSGGGSGGGSGGGSGTAGHTQTSTGGGAITGTSGAGHGPQLCKFC</u> <u>DVRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHD</u> <u>FILEDAASPKCIMKEKKKPGETFFMCSSSDECNDNIIFSEYNTSNPD</u>	81
4	<u>GGGGSGGGSGGGSGGGSGGGSGTAGHTQTSTGGGAITGTSGAGHGPQLCKFC</u> <u>DVRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHD</u> <u>FILEDAASPKCIMKEKKKPGETFFMCSSSDECNDNIIFS</u>	82
5	<u>SGGGSGGGSGGGSGGGSGGGSGHYPQLCKFCDVRFSTCDNQKSCMSNCSITS</u> <u>ICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGE</u> <u>TFFMCSSSDECNDNIIFS</u>	83
6	<u>SGGGSGGGSGGGSGGGSGGGSGHYPQLCKFCDVRFSTCDNQKSCMSNCSITS</u> <u>ICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGE</u> <u>TFFMCSSSDECNDNIIFSEYNTSNPD</u>	84
7	<u>GGGGSGGGSGGGSGHYPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCA</u> <u>VWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSSSDE</u> <u>CNDNIIFSEYNTSNPD</u>	85
8	<u>GGGGS</u>	86
9	<u>EYNTSNPD</u>	90

ELISA with recombinant human TGF- β 1

[0155] To evaluate the binding activity of modified LP008-02 bifunctional molecules, these bifunctional molecules were tested with ELISA.

[0156] Briefly, microtiter plates were coated with human TGF- β 1 protein (Acro, TG1-H4212) at 1 μ g/ml in PBS, 100 μ l/well at 4°C overnight, then blocked with 150 μ l/well of 1% BSA. Three-fold dilutions of modified LP008-02 bifunctional molecules starting from 30nM were added to each well and incubated for 1 hour at 37°C. The plates were washed with PBS/Tween and then incubated with Goat-anti-human IgG (H+L) antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins at 37°C. After washing, the plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm.

[0157] As shown in FIG. 19, all of modified LP008-02 bifunctional molecules bound to human TGF- β 1 with high activity, comparable to LP008-02-1.

TGF- β functional assay

[0158] Serial dilutions of modified LP008-02 bifunctional molecules were incubated with SBE luciferase reporter-transfected 293 cells for about 22 hours in the presence of recombinant human TGF- β 1.

[0159] As shown in FIG. 20, LP008-02-2, LP008-02-3, and LP008-02-4 effectively blocked TGF- β canonical signaling ($IC_{50} = 0.1435 \text{ nM}$, $IC_{50} = 0.1639 \text{ nM}$, $IC_{50} = 0.1882 \text{ nM}$) in a TGF- β SBE luciferase reporter assay system constructed in 293 cells, which was compared with LP008-02-1.

Example 16. Comparison of the Bifunctional Molecules

[0160] The molecules 1-7 of Table 15 included different sequences at the N- and C-terminal ends of the ectodomain (SEQ ID NO:72). They were tested for stability and activity to evaluate the impact of these sequences.

[0161] Molecule 1 (LP008-02-1) included the entire extracellular portion of the protein (SEQ ID NO:61), which contained 25 amino acids (IPPHVQKSVNNDMIVTDNNGAVKFP, SEQ ID NO:89, or amino acids 24-48 of isoform B, SEQ ID NO:71) from the N-terminus of the extracellular domain, and the C-terminal fragment (EEYNTSNPD, SEQ ID NO:90). In addition, this molecule added a few G4S (SEQ ID NO:86) repeats in the linker.

[0162] Molecule 2 (LP008-02-2), compared to Molecule 1, replaced the N-terminal portion (amino acids 24-48 of isoform B, SEQ ID NO:89) of the extracellular domain with an artificial linker TAGHTQTSTGGGAITGTSGAGHGP (SEQ ID NO:87). This linker was modeled based on SEQ ID NO:89. The changes included (i) removal of the rigid di-peptide PP, (ii) removal of potential cleavage sites QK, N and K, (iii) inclusion of multiple glycine residues to increase flexibility, (iv) partial removal of hydrophobic residues (e.g., retaining only one I). These changes are illustrated in Table 16 below. Molecule 2 also included a single G4S unit at the N-terminus.

Table 16. Artificial linker

Name	Sequence	SEQ ID NO:
Original	IPPHVQKSVNNDMIVTDNNGAVKFP	89
Modified	TAGHTQTSTGGGAITGTSGAGHGP	87

[0163] Molecule 3 (LP008-02-3) included a longer G4S linker than Molecule 2. On top of Molecule 3, Molecule 4 (LP008-02-4) had a deletion of the C-terminal fragment, EEYNTSNPD (SEQ ID NO:90). Molecule 5 (LP008-02-5) replaced the artificial linker, SEQ ID NO:87, with a short linker

HYP. Molecules 6 (LP008-02-6) and 7 (LP008-02-7) included different lengths of the G4S linker at the N-terminal side of the HYP linker.

Example 17. Binding Activity and Stability of the Bifunctional Molecules

[0164] This example used SEC-HPLC and CE-SDS to evaluate the stability of some of the modified bifunctional molecules, including LP008-02-1, and four further modified ones, LP008-02-2, LP008-02-3, LP008-02-6 and LP008-02-7.

[0165] The 5 sequences were expressed in CHO-K1 cells by polyethyleneimine (PEI) mediated transient transfection, and the supernatant was harvested 10 days later. The bifunctional molecules were purified from the culture supernatant by protein A, and thereafter purified by Superdex 200pg with the level of purity greater than 99% as detected by SEC-HPLC (Table 17).

Table 17. The results of SEC-HPLC and CE-SDS of test article on day 0

Sample ID	Buffer	SEC-HPLC			CE-SDS Non-Reduced			CE-SDS Reduced		
		HMW (%)	Mono (%)	LMW (%)	HMW (%)	Main (%)	LMW (%)	LC (%)	LMW (%)	HC (%)
LP008-02-1	A	0.32	99.56	0.12	ND	94.5	5.5	24.0	2.3	73.7
LP008-02-2		0.37	99.58	0.05	ND	97.7	2.3	24.3	0.5	75.2
LP008-02-3		0.31	99.64	0.05	ND	97.0	3.0	24.5	0.9	74.6
LP008-02-6		0.35	99.62	0.03	ND	97.2	2.8	24.0	1.1	74.9
LP008-02-7		0.41	99.54	0.05	ND	97.2	2.8	23.8	0.9	75.3
LP008-02-1	B	0.25	99.72	0.03	ND	94.2	5.8	23.8	2.4	73.8
LP008-02-2		0.32	99.65	0.03	ND	97.5	2.5	23.9	0.6	75.6
LP008-02-3		0.26	99.72	0.03	ND	97.8	2.2	23.9	1.7	74.4
LP008-02-6		0.31	99.68	0.02	ND	97.1	2.9	23.8	1.0	75.2
LP008-02-7		0.39	99.58	0.03	ND	96.7	3.3	24.1	0.9	75.0

[0166] To evaluate the binding activity of modified LP008-02 bifunctional molecules, these bifunctional molecules were tested by ELISA.

[0167] Briefly, the microtiter plate was coated with human TGF- β 1 protein (Acro, TG1-H4212) at 1 μ g/ml in PBS, 100 μ l/well at 4°C overnight, then blocked with 150 μ l/well of 1% BSA. Four-fold serial dilutions of modified LP008-02 bifunctional molecules starting from 30nM were added to each well and incubated for 1 hour. The plate was washed with PBS/Tween and then incubated with Goat-anti-human IgG Fc antibody conjugated with Horse Radish Peroxidase (HRP) for 30 mins. After washing, the plate was incubated with TMB substrate for color development and analyzed by spectrophotometer at OD 450nm.

[0168] As shown in FIG. 21, all the other modified LP008-02 bifunctional molecules bound to human TGF- β 1 with high activity, which is comparable to LP008-02-1.

[0169] To evaluate the effect of the modified LP008-02 bifunctional molecules on canonical TGF- β signaling, the modified bifunctional molecules were tested with luciferase assay. Serial dilutions of bifunctional molecules were incubated with SBE luciferase reporter-transfected 293 cells for 24 hours in the presence of recombinant human TGF- β 1. As shown in FIG. 22, like LP008-02-1, LP008-02-2, LP008-02-3, LP008-02-6 and LP008-02-7 efficiently blocked TGF- β canonical signaling (IC_{50} =0.04231nM, IC_{50} = 0.0527nM, IC_{50} =0.09616nM, and IC_{50} =0.1962nM) in a TGF- β SBE luciferase reporter assay system constructed in 293 cells.

[0170] The bifunctional molecules were separately dissolved in two buffers for antibody stability detection. Buffer information is listed as: Buffer A: 20mM Acetic acid-sodium acetate, 250mM sorbitol, 0.02% Polysorbate 80, pH 4.9; Buffer B: 20mM His/His-HCl, 250mM Trehalose, pH 5.4.

[0171] The prepared samples at 3.0 mg/ml were incubated at 40°C, and then detected by SEC-HPLC and CE-SDS on day 0 and day 14, respectively. As shown in Table 18, LP008-02-2, LP008-02-3, LP008-02-6, and LP008-02-7 formulated in both buffer A and buffer B have higher stability than LP008-02-1 in SEC-HPLC, Non-Reduced CE-SDS and Reduced CE-SDS.

Table 18. The results of SEC-HPLC and CE-SDS of test article on day 14

Sample ID	Buffer	SEC-HPLC			CE-SDS Non-Reduced			CE-SDS Reduced		
		HMW (%)	Mono (%)	LMW (%)	HMW (%)	Main (%)	LMW (%)	LC (%)	LMW (%)	HC (%)
LP008-02-1	A	0.77	43.74	55.49	ND	66.1	33.9	25.8	31.0	43.1
LP008-02-2		0.83	98.40	0.77	ND	95.0	5.0	25.2	1.1	73.7
LP008-02-3		0.67	97.99	1.34	0.2	89.5	10.3	25.5	7.2	67.3
LP008-02-6		0.89	98.39	0.72	ND	94.1	5.9	25.2	3.8	71.0
LP008-02-7		1.40	98.11	0.49	0.1	92.4	7.5	25.6	2.6	71.9
LP008-02-1	B	0.41	22.01	77.59	0.1	33.4	66.4	26.8	53.0	20.2
LP008-02-2		0.46	98.81	0.73	0.1	93.2	6.7	25.2	4.1	70.7
LP008-02-3		0.39	98.9	0.71	0.4	82.3	17.4	25.6	17.3	57.1
LP008-02-6		1.10	98.33	0.58	0.2	91.9	7.9	25.5	5.8	68.6
LP008-02-7		0.82	98.65	0.52	0.6	93.2	6.2	25.2	2.0	72.9

[0172] This example, therefore, shows that modified bifunctional molecules LP008-02-2, LP008-02-3, LP008-02-6, and LP008-02-7 exhibited similar activities to LP008-02-1, and yet were significantly higher stability than LP008-02-1. The replacement of the N-terminal portion (IPPHVQKSVNNDMIVTDNNGAVKFP, SEQ ID NO:89) of TGF- β RII in LP008-02-1 with an

artificial linker (e.g., TAGHTQTSTGGGAITTTGTSAGHGP (SEQ ID NO:87) or HYP) led to significantly improved stability.

Example 18. High Concentration Formulation of Anti-PD-L1 Antibodies

[0173] This example used HIC-HPLC, and viscosity tests to evaluate the potential and risk of development of high concentration anti-PD-L1 molecule formulations.

[0174] Four anti-PD-L1 molecules were expressed in CHO-K1 or 293F cells by transient transfection. The constant regions of heavy chain are Human IgG1(N297A)-Fc. Purified MPDL3280A (Atezolizumab), 47C6A3, Hu67F3G7-22, and Hu89C10H8-7 antibodies were tested by HIC-HPLC, and the concentration of ammonium sulfate corresponding to the hydrophobic elution time was obtained, which was used to predict the solubility range of these molecules. As shown in Table 19, the concentration of ammonium sulfate corresponding to the hydrophobic elution time of MPDL3280A, 47C6A3, Hu67F3G7-22, and Hu89C10H8-7 is 0.41M, 0.78M, 0.97M, and 1.10 M respectively. All of the newly developed antibodies have lower hydrophobicity than the reference antibody MPDL3280A.

Table 19. Antibody Hydrophobicity from HIC-HPLC test

Sample	HIC(M)
MPDL3280A	0.41
47C6A3	0.78
Hu67F3G7-22	0.97
Hu89C10H8-7	1.10

[0175] The activities of the anti-PD-L1 antibodies in blocking PD1/PD-L1 interaction were then measured with a bioluminescent cell-based assay. In this assay, when PD1 effector cells are co-cultured with PD-L1 target cells, the PD-1/PD-L1 interaction inhibits TCR signaling and NFAT-RE-mediated luminescence. Addition of either an anti-PD-1 or anti-PD-L1 antibody that blocks the PD-1/PD-L1 interaction that would release the inhibitory signal and result in TCR activation and NFAT-RE-mediated luminescence. As shown in FIG. 23, MPDL3280A, 47C6A3, Hu67F3G7-22, and Hu89C10H8-7 blockaded PD1 and PD-L1 interaction with considerably high activity (MPDL3280A EC_{50} = 0.1327nM, 47C6A3 EC_{50} =0.1501nM, Hu67F3G7-22 EC_{50} =0.1034nM, Hu89C10H8-7 EC_{50} =0.2138nM).

[0176] MPDL3280A and Hu67F3G7-22, with human IgG1 Fc, were expressed in CHO-K1 cells by transient transfection. Purified MPDL3280A-hIgG1 Fc and Hu67F3G7-22-hIgG1 Fc antibodies were tested by HIC-HPLC, and the concentration of ammonium sulfate corresponding to the hydrophobic elution time was obtained, which was used to predict the solubility range of the two molecules. As shown in Table 20, the concentration of ammonium sulfate corresponding to the hydrophobic elution time of MPDL3280A-hIgG1 Fc and Hu67F3G7-22-hIgG1 Fc is 0.42M and

0.99M respectively. Again, with the same Fc fragment, Hu67F3G7-22 exhibited lower hydrophobicity than MPDL3280A.

Table 20. Results of HIC-HPLC test

Sample	Temp	HIC
		(NH ₄) ₂ SO ₄ -M
MPDL3280A-hIgG1 Fc	25°C	0.42
Hu67F3G7-22-hIgG1 Fc		0.99

[0177] To further confirm the solubility and viscosity property of the antibodies, the two purified candidates were concentrated directly in phosphate buffer (including 60mM NaCl) by ultrafiltration. During the ultrafiltration process, concentration, SEC-HPLC, and viscosity property were measured at different stages. As shown in Table 21, the viscosity of MPDL3280A -hIgG1 Fc is much higher than that of Hu 67F3G7-22-hIgG1 Fc at similar concentrations. And for high concentration formulation, generally antibodies with lower viscosity are preferred over those having higher viscosity. Accordingly, as a therapeutic protein, Hu67F3G7-22 antibody has higher potential than MPDL3280A.

Table 21. The results of solubility test

Sample	Buffer	Temp	Conc.			SEC (%)			Viscosity mPa•S-1
			mg/ml	A260/280	A320	HMW	Mono	LMW	
MPDL3280A -hIgG1 Fc	PB+60m M NaCl	25°C	118.41	0.52	0.159	0.97	99.03	0	7.01
			141.37	0.52	0.222	1.31	98.69	0	16.1
			183.42	0.52	0.239	1.35	98.65	0	60.01
Hu67F3G7- 22-hIgG1 Fc	PB+60m M NaCl		119	0.5	0.189	0.88	99.12	0	2.96
			139.34	0.5	0.183	1.35	98.65	0	4.995
			180.32	0.5	0.25	1.43	98.57	0	14.56

[0178] The present disclosure is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the disclosure, and any compositions or methods which are functionally equivalent are within the scope of this disclosure. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present disclosure without departing from the spirit or scope of the disclosure. Thus, it is intended that the present disclosure cover the modifications and variations of this disclosure provided they come within the scope of the appended claims and their equivalents.

[0179] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A multifunctional molecule, comprising an anti-PD-L1 (programmed death-ligand 1) antibody or fragment thereof and an extracellular domain of human TGF- β RII (TGF-beta receptor type-2),

wherein the anti-PD-L1 antibody or fragment thereof has specificity to the human PD-L1 protein and comprises a heavy chain variable region (VH) comprising a VH CDR1, a VH CDR2 and a VH CDR3, and a light chain variable region (VL) comprising a VL CDR1, a VL CDR2, and a VL CDR3,

wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:7-12, or SEQ ID NO:13-18, or wherein the VH CDR1 comprises SEQ ID NO:19, the VH CDR2 comprises SEQ ID NO:20, 91 or 92, the VH CDR3 comprises SEQ ID NO:21, the VL CDR1 comprises SEQ ID NO:22, the VL CDR2 comprises SEQ ID NO:23, and the VL CDR3 comprises SEQ ID NO:24 or 93,

wherein the human TGF- β RII extracellular domain comprises the amino acid sequence of SEQ ID NO:72 and is fused to the anti-PD-L1 antibody or fragment thereof.

2. The multifunctional molecule of claim 1, wherein anti-PD-L1 antibody or fragment thereof comprises a heavy chain that comprises the VH and a separate light chain comprising the VL.

3. The multifunctional molecule of claim 2, wherein the TGF- β RII extracellular domain is fused to the heavy chain of the anti-PD-L1 antibody or fragment thereof.

4. The multifunctional molecule of claim 3, wherein the TGF- β RII extracellular domain is fused to the C-terminus of the heavy chain of the anti-PD-L1 antibody or fragment thereof.

5. The multifunctional molecule of claim 3 or 4, wherein the TGF- β RII extracellular domain is fused to the heavy chain of the anti-PD-L1 antibody or fragment thereof through a peptide linker.

6. The multifunctional molecule of any one of claims 1-5, wherein the TGF- β RII extracellular domain comprises SEQ ID NO:72 and includes at least a partial deletion of amino acid residues 24-48 of SEQ ID NO:71.

7. The multifunctional molecule of any one of claims 1-5, wherein the TGF- β RII extracellular domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO:61, and 73-78, wherein for SEQ ID NO:74, X is any amino acid except K, S, or N.

8. The multifunctional molecule of any one of claims 1-5, which includes at least 30 amino acid residues between SEQ ID NO:72 and the anti-PD-L1 antibody or fragment thereof.
9. The multifunctional molecule of any one of claims 1-5, which includes an alpha helix motif between SEQ ID NO:72 and the anti-PD-L1 antibody or fragment thereof.
10. The multifunctional molecule of any one of claims 1-9, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:13-18.
11. The multifunctional molecule of claim 10, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NO:31-37, and the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NO:38-43.
12. The multifunctional molecule of claim 11, wherein the VH comprises the amino acid sequence of SEQ ID NO:34, and the VL comprises the amino acid sequence of SEQ ID NO:43.
13. The multifunctional molecule of any one of claims 1-9, wherein the VH CDR1 comprises SEQ ID NO:19, the VH CDR2 comprises SEQ ID NO:20, 91 or 92, the VH CDR3 comprises SEQ ID NO:21, the VL CDR1 comprises SEQ ID NO:22, the VL CDR2 comprises SEQ ID NO:23, and the VL CDR3 comprises SEQ ID NO:24 or 93.
14. The multifunctional molecule of claim 13, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NO:44-49 and 57-58, and the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NO:50-56.
15. The multifunctional molecule of claim 13, wherein the VH comprises the amino acid sequence of SEQ ID NO:48, 57 or 58, and the VL comprises the amino acid sequence of SEQ ID NO:53 or 56.
16. The multifunctional molecule of any one of claims 1-9, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:19, 92, 21, 22, 23 and 93.
17. The multifunctional molecule of claim 16, wherein the VH comprises the amino acid sequence of SEQ ID NO:58, and the VL comprises the amino acid sequence of SEQ ID NO:56.
18. The multifunctional molecule of any one of claims 1-17, which comprises a light chain comprising the VL and a light chain constant region, and a heavy chain comprising the VH, a heavy chain constant region, a peptide linker, and the TGF- β RII extracellular domain.

19. The multifunctional molecule of claim 18, wherein the heavy chain constant region comprises the amino acid sequence of SEQ ID NO:59.
20. An anti-PD-L1 (programmed death-ligand 1) antibody or fragment thereof, which has specificity to the human PD-L1 protein and comprises a heavy chain variable region (VH) comprising a VH CDR1, a VH CDR2 and a VH CDR3, and a light chain variable region (VL) comprising a VL CDR1, a VL CDR2, and a VL CDR3, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:7-12, or SEQ ID NO:13-18 or wherein the VH CDR1 comprises SEQ ID NO:19, the VH CDR2 comprises SEQ ID NO:20, 91 or 92, the VH CDR3 comprises SEQ ID NO:21, the VL CDR1 comprises SEQ ID NO:22, the VL CDR2 comprises SEQ ID NO:23, and the VL CDR3 comprises SEQ ID NO:24 or 93.
21. The anti-PD-L1 antibody or fragment thereof of claim 20, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:13-18.
22. The anti-PD-L1 antibody or fragment thereof of claim 21, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NO:31-37, and the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NO:38-43.
23. The anti-PD-L1 antibody or fragment thereof of claim 21, wherein the VH comprises the amino acid sequence of SEQ ID NO:34, and the VL comprises the amino acid sequence of SEQ ID NO:43.
24. The anti-PD-L1 antibody or fragment thereof of claim 20, wherein the VH CDR1 comprises SEQ ID NO:19, the VH CDR2 comprises SEQ ID NO:20, 91 or 92, the VH CDR3 comprises SEQ ID NO:21, the VL CDR1 comprises SEQ ID NO:22, the VL CDR2 comprises SEQ ID NO:23, and the VL CDR3 comprises SEQ ID NO:24 or 93.
25. The anti-PD-L1 antibody or fragment thereof of claim 24, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NO:44-49 and 57-58, and the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NO:50-56.
26. The anti-PD-L1 antibody or fragment thereof of claim 24, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:19, 92, 21, 22, 23 and 93.

27. The anti-PD-L1 antibody or fragment thereof of claim 26, wherein the VH comprises the amino acid sequence of SEQ ID NO:58, and the VL comprises the amino acid sequence of SEQ ID NO:56.
28. The anti-PD-L1 antibody or fragment thereof of claim 20, wherein the VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3, respectively, comprise the amino acid sequences of SEQ ID NO:7-12.
29. The anti-PD-L1 antibody or fragment thereof of claim 28, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NO:25-28, and the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NO:29-30.
30. The anti-PD-L1 antibody or fragment thereof of claim 28, wherein the VH comprises the amino acid sequence of SEQ ID NO:26, 27 or 28, and the VL comprises the amino acid sequence of SEQ ID NO:30.
31. A multifunctional molecule, comprising an antibody or antigen-binding fragment thereof fused, through a peptide linker, to the N-terminus of the amino acid sequence of SEQ ID NO:72.
32. The multifunctional molecule of claim 31, wherein the peptide linker comprises a flexible linker and/or a substitute peptide of IPPHVQKSVNNDMIVTDNNGAVKFP (SEQ ID NO:89), wherein the substitute peptide is different from SEQ ID NO:89.
33. The multifunctional molecule of claim 32, wherein the substitute peptide comprises the amino acid sequence of IPPHVQXXVNNDMIVTDNXGAVKFP (SEQ ID NO:88), wherein X is any amino acid except K, S, or N.
34. The multifunctional molecule of claim 33, wherein the substitute peptide has at least 50% sequence identity to SEQ ID NO:88.
35. The multifunctional molecule of claim 32, wherein the substitute peptide comprises the amino acid sequence of TAGHTQTSTGGGAITGTSGAGHGP (SEQ ID NO:87) or a variant having at least 75% sequence identity to SEQ ID NO:87, wherein the variant includes at least 4 G, no PP dipeptide, no more than 3 hydrophobic amino acid residues selected from the group consisting of I, L, M, F, V, W, Y and P.
36. The multifunctional molecule of claim 35, wherein the variant includes at least 5 G and no more than 1 hydrophobic amino acid residue selected from the group consisting of I, L, M, F, V, W, Y and P.

37. The multifunctional molecule of any one of claims 32-36, wherein the flexible linker comprises S and at least 50% G.
38. The multifunctional molecule of claim 37, wherein the flexible linker comprises one or more GGGGS (SEQ ID NO:86) units.
39. The multifunctional molecule of any one of claims 31-38, wherein the C-terminus of the antibody or antigen-binding fragment thereof is at least 20 amino acid residues from the N-terminus of the amino acid sequence of SEQ ID NO:72.
40. The multifunctional molecule of any one of claims 31-39, which at least does not include the entire sequence of EEYNTSNPD (SEQ ID NO:90).
41. The multifunctional molecule of any one of claims 31-40, wherein the antibody or fragment thereof is specific to an antigen selected from the group consisting of PD-1, PD-L1, CTLA-4, LAG-3, CD28, CD122, 4-1BB, TIM3, OX-40, OX40L, CD40, CD40L, LIGHT, ICOS, ICOSL, GITR, GITRL, TIGIT, CD27, VISTA, B7H3, B7H4, BTLA, CD4, CD2, CD8, CD47 and CD73.
42. The multifunctional molecule of any one of claims 31-41, wherein the antibody or fragment thereof comprises a Fc fragment.
43. The multifunctional molecule of claim 42, wherein the peptide linker is fused to the C-terminus of the Fc fragment.
44. A cell comprising one or more polynucleotides encoding for the multifunctional molecule of any one of claims 1-19, and 31-43 or the anti-PD-L1 antibody or fragment thereof of any one of claims 20-30.
45. One or more polynucleotides encoding for the multifunctional molecule of any one of claims 1-19, and 31-43 or the anti-PD-L1 antibody or fragment thereof of any one of claims 20-30.
46. A composition comprising the multifunctional molecule of any one of claims 1-19, and 31-43 or the anti-PD-L1 antibody or fragment thereof of any one of claims 20-30 and a pharmaceutically suitable carrier.
47. Use of the multifunctional molecule of any one of claims 1-19, and 31-43 or the anti-PD-L1 antibody or fragment thereof of any one of claims 20-30 for the manufacturing of a medicament for the treatment of cancer.

48. A method for treating cancer in a patient in need thereof, comprising administering to the patient an effective amount of the multifunctional molecule of any one of claims 1-19, and 31-43 or the anti-PD-L1 antibody or fragment thereof of any one of claims 20-30.

49. The use of claim 47 or the method of claim 48, wherein the cancer is a solid tumor.

50. The use of claim 47 or 49 or the method of claim 48 or 49, wherein the cancer is selected from the group consisting of bladder cancer, liver cancer, colon cancer, rectal cancer, endometrial cancer, leukemia, lymphoma, pancreatic cancer, small cell lung cancer, non-small cell lung cancer, breast cancer, urethral cancer, head and neck cancer, gastrointestinal cancer, stomach cancer, oesophageal cancer, ovarian cancer, renal cancer, melanoma, prostate cancer and thyroid cancer.