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(54) Title: MODIFIED TYPE E MULTI-SPECIFIC ANTIBODIES

(57) Abstract: Provided are novel covalent multi-specific antibodies and uses thereof.

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MODIFIED TYPE E MULTI-SPECIFIC ANTIBODIES

FIELD OF THE INVENTION

[0001] The present invention relates to modified multi-specific antibodies and uses thereof.

BACKGROUND

[0002] In recent years, T cell engager (*i.e.*, TCE) therapy on the basis of CD3 has made significant progress in the treatment of hematological tumors. Currently, a CD3 × CD19 bi-specific antibody (*i.e.*, Blinatumomab), and a CD3 × CD20 bi-specific antibody (*i.e.*, Mosunetuzumab), have been approved for marketing in the treatment of B-cell malignant hematological tumors. However, several clinical trials using CD3 × EpCam, CD3 × Her2, CD3 × EGFR antibodies have failed.

[0003] In addition, when it comes to solid tumors, the TCE therapy still faces great challenges in providing sufficient therapeutic efficacy, potentially due to the complexity of the solid tumor surrounding tissue.

[0004] Regeneron has previously adopted a combination therapy using a first bi-specific antibody targeting tumor surface antigen (TSA) and CD3, and a second bi-specific antibody targeting TSA and CD28, to stimulate signal 1 and signal 2 pathways of T cells in order to counteract the inhibitory effect of local tumor microenvironment (*i.e.*, TME) on these T cells. Such combination therapy demonstrated advantages in tumor killing and inhibition compared to therapies stimulating CD3 alone.

[0005] In another clinical trial by Roche, the RG7802 (*i.e.*, a CEA × CD3 bi-specific antibody) monotherapy did not show significant efficacy in treating colorectal cancer, but it can be observed through immunohistochemistry that the tumor tissue was infiltrated by inflammatory cells after administration in preclinical study. When

combined with the therapy using PD-L1 monoclonal antibody (Atezolizumab), there was a significant improvement in efficacy compared to RG7802 monotherapy, indicating that the combination of the two therapies can to some extent effectively counteract the inhibitory effect of solid tumor TME on T cells on the basis of inflammatory cell infiltration.

[0006] In general, it is still difficult to achieve activation of T cells in solid tumors using CD3 based bispecific antibodies. On the other hand, T cell engager therapy involves T cell activation, accompanied by cytokine release, which clinically manifests as CRS (cytotoxic release syndrome). In severe cases, CRS can even lead to patient death.

[0007] Therefore, there exists great needs to develop novel therapies in order to overcome the deficiency of insufficient efficacy in treating cancers including solid tumors and also to control the safety of TCE.

SUMMARY OF THE INVENTION

[0008] Throughout the present disclosure, the articles "a," "an," and "the" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an antibody" means one antibody or more than one antibody.

[0009] In one aspect, the present disclosure provides a multi-specific polypeptide complex comprising a CD3-binding domain capable of specifically binding to CD3, a PD-L1-binding domain that is a low-affinity PD-L1-binding domain capable of specifically binding to PD-L1 at a Kd value of no less than 10^{-8} M as measured by Bio-Layer Interferometry(BLI), and a first disease antigen binding domain, wherein the CD3-binding domain, PD-L1 binding domain and the disease antigen binding domain are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1, CD3 and the disease antigen.

[0010] In another aspect, the present disclosure provides a method of modifying a parent multi-specific polypeptide complex to produce a modified multi-specific

polypeptide complex having improved half-life in a primate, wherein the multi-specific polypeptide complex comprises a CD3-binding domain capable of specifically binding to CD3, a PD-L1 binding domain capable of specifically binding to PD-L1, wherein the method comprises: introducing one or more mutations to the PD-L1 binding domain to produce a modified PD-L1 binding domain having reduced binding affinity to PD-L1 characterized in a Kd value of no less than 10^{-8} M as measured by Bio-Layer Interferometry(BLI).

[0011] In another aspect, the present disclosure provides a method of modifying a multi-specific polypeptide complex to reduce its ability to induce cytokine release in a subject, wherein the multi-specific polypeptide complex comprises a CD3-binding domain capable of specifically binding to CD3 and an Fc domain that re operably linked to allow the multi-specific polypeptide complex to bind to CD3, the method comprises: introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a.

[0012] In another aspect, the present disclosure provides a method of modifying a multi-specific polypeptide complex to improve its potency or improve its therapeutic window in a subject, wherein the multi-specific polypeptide complex comprises a low-affinity CD3-binding domain capable of specifically binding to CD3 at a Kd value of no less than 1E-8 M or no less than 5E-8M as measured by Surface Plasmon Resonance (SPR), and an Fc domain, that are operably linked to allow the multi-specific polypeptide complex to bind to CD3 and the disease antigen, the method comprises introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a.

[0013] In another aspect, the present disclosure provides a method of modifying a multi-specific polypeptide complex to improve its potency or improve its therapeutic window in a subject, wherein the multi-specific polypeptide complex comprises a low-affinity PD-L1-binding domain capable of specifically binding to PD-L1 at a Kd value of no less than 10^{-8} M as measured by Bio-Layer Interferometry(BLI), a CD3-binding domain, and an Fc domain, that are operably linked to allow the multi-

specific polypeptide complex to bind to PD-L1 and CD3, the method comprises introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a.

[0014] In another aspect, the present disclosure provides a PD-L1 binding polypeptide complex comprising an affinity variant of a parent anti-PD-L1 antibody, wherein the parent anti-PD-L1 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6, and wherein the affinity variant is capable of specifically binding to human PD-L1 at a Kd value ranging from 10^{-6} M to 10^{-8} M as measured by BLI.

[0015] In another aspect, the present disclosure provides a PD-L1 binding polypeptide complex comprising an affinity variant of a parent anti-PD-L1 antibody, wherein the parent anti-PD-L1 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134, and wherein the affinity variant is capable of specifically binding to human PD-L1 at a Kd value no more than $5*10^{-7}$M as measured by BLI.

[0016] In another aspect, the present disclosure provides a CD3 binding polypeptide complex comprising an affinity variant of a parent anti-CD3 antibody, wherein the parent anti-CD3 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the

amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14, and wherein the affinity variant is capable of specifically binding to human CD3 at a Kd value ranging from 10^{-6} M to 10^{-8} M (optionally from $5*10^{-7}$ M to $5*10^{-8}$ M) as measured by BLI or SPR.

BRIEF DESCFRIPTION OF THE DRAWINGS

[0017] **FIG 1** depicts diagram of the TRIAD structure of the 2:1 multi-specific polypeptide complex 12A22 and 12A40.

[0018] **FIG 2** depicts diagram of the Type E structure of the 2:1:1 asymmetric multi-specific polypeptide complex 12A39.

[0019] **FIG 3** shows the results of the level of IL-6 release and cytotoxicity of polypeptide complex 12A22.

[0020] **FIG 4** shows the results of the level of IL-6 release and cytotoxicity of polypeptide complex 12A39.

[0021] **FIG 5A** shows the effects of polypeptide complex 12A22 at a dose of 5mpk, 1mpk and 0.2mpk, respectively, and 12A39 at a dose of 5mpk on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model. **FIG 5B** shows the effects of polypeptide complex 12A39 at a dose of 5mpk, 1mpk, 0.2mpk, respectively, and 12A40 at a dose of 3.75mpk on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model.

[0022] **FIG 6** shows the results of the concentration of polypeptide complex 12A22, 12A39, 12A44, 12A65 and 12A73 at various time points after administration, respectively.

[0023] **FIG 7A** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A64, 12A65 and 12A66. **FIG 7B** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A67, 12A68 and 12A69.

[0024] **FIG 8** shows the effects of polypeptide complex 12A39, 12A44, 12A46, 12A58 and 12A65 on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model.

[0025] **FIG 9** shows the results of the concentration of polypeptide complex 12A22, 12A39, 12A44, 12A65 and 12A73 at various time points after administration, respectively.

[0026] **FIG 10A** shows the results of the level of cytotoxicity of polypeptide complex 12A22, 12A39, 12A42, 12A43 and 12A44. **FIG 10B** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A45, 12A46 and 12A47. **FIG 10C** shows the results of the level of cytotoxicity of polypeptide complex 12A39 and 12A51.

[0027] **FIG 11** shows the results of the level of IL-6 release and cytotoxicity of polypeptide complex 12A46.

[0028] **FIG 12A** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A57, 12A58, 12A59 and 12A61. **FIG 12B** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A62, 12A64, 12A65 and 12A66. **FIG 12C** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A56, 12A58, 12A60, 12A63 and 12A65.

[0029] **FIG 13** shows the results of the level of IL-6 release and cytotoxicity of polypeptide complex 12A70.

[0030] **FIG 14** shows the effects of polypeptide complex 12A39, 12A44, 12A46, 12A58 and 12A65 on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model.

[0031] **FIG 15A** shows the affinity of polypeptide complex 12A39 for CD16a through BLI method. **FIG 15B** shows the affinity of polypeptide complex 12A39 for CD32a through BLI method. **FIG 15C** shows the affinity of polypeptide complex 12A39 for CD64 through BLI method. **FIG 15D** shows the affinity of polypeptide

complex 12A52 for CD16a through BLI method. **FIG 15E** shows the affinity of polypeptide complex 12A52 for CD32a through BLI method. **FIG 15F** shows the affinity of polypeptide complex 12A52 for CD64 through BLI method.

[0032] **FIG 16** shows the dose-response curves of polypeptide complex 12A22, 12A39 and 12A52 in the first test of reporter assay on LS174T cells.

[0033] **FIG 17** shows the results of the level of IL-6 release produced by PBMC in the presence of LS174T cells and the polypeptide complex 12A22, 12A39 and 12A52, respectively.

[0034] **FIG 18** shows the effects of polypeptide complex 12A39 and 12A52 on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model.

[0035] **FIG 19** shows the level of human CD45+ cell in mouse model caused by polypeptide complex 12A39 and 12A52.

[0036] **FIG 20** shows the level of human CD3+ cell in mouse model caused by polypeptide complex 12A39 and 12A52.

[0037] **FIG 21** shows the effects of polypeptide complex 12A39 and 12A74 on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model.

[0038] **FIG 22** shows the level of human CD45+ cell in mouse model caused by polypeptide complex 12A39 and 12A74;

[0039] **FIG 23** shows the level of human CD3+ cell in mouse model caused by polypeptide complex 12A39 and 12A74;

[0040] **FIG 24A** shows the affinity of polypeptide complex 12A73 for CD16a through BLI method. **FIG 24B** shows the affinity of polypeptide complex 12A73 for CD32a through BLI method. **FIG 24C** shows the affinity of polypeptide complex 12A73 for CD64 through BLI method.

[0041] **FIG 25** shows the dose-response curves of polypeptide complex 12A39, 12A70 and 12A73 in the first test of reporter assay on LS174T cells.

[0042] **FIG 26** shows the results of the level of IL-6 release and cytotoxicity of polypeptide complex 12A73.

[0043] **FIG 27** shows the effects of polypeptide complex 12A39 at a dose of 1mpk, and 12A73 at a dose of 1mpk and 3mpk, respectively, on the growth of subcutaneous transplanted tumors of LS174T cells.

[0044] **FIG 28A** shows the level of human CD45+ cell in mouse model caused by polypeptide complex 12A39 at a dose of 1mpk, and 12A73 at a dose of 1mpk and 3mpk, respectively. **FIG 28B** shows the level of human CD3+ cell in mouse model caused by polypeptide complex 12A39 at a dose of 1mpk, and 12A73 at a dose of 1mpk and 3mpk, respectively.

[0045] **FIG 29** shows the results of the concentration of polypeptide complex 12A22, 12A39, 12A44, 12A65 and 12A73 at various time points after administration, respectively.

[0046] **FIG 30** shows the results of the level of cytotoxicity of polypeptide complex 12A39, 12A58 and 12A78.

[0047] **FIG 31A** shows the effects of polypeptide complex 12A78 at a dose of 10mpk on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model. **FIG 31B** shows the effects of polypeptide complex 12A22 at a dose of 3mpk, 12A39 at a dose of 1mpk, and 12A58 at a dose of 3mpk and 9mpk, respectively, on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing LS174T tumor xenograft model.

[0048] **FIG 32A** shows the level of human CD45+ cell in mouse model caused by polypeptide complex 12A39 at a dose of 1mpk, 12A58 at a dose of 3mpk and 9mpk, respectively. **FIG 32B** shows the level of human CD3+ cell in mouse model caused

by polypeptide complex 12A39 at a dose of 1mpk, 12A58 at a dose of 3mpk and 9mpk, respectively.

[0049] **FIG 33A** shows the level of human CD45+ cell in mouse model caused by polypeptide complex 12A78 at a dose of 10mpk. **FIG 33B** shows the level of human CD3+ cell in mouse model caused by polypeptide complex 12A78 at a dose of 10mpk.

[0050] **FIGs 34A-34D** show the results of the level of cytotoxicity of polypeptide complex 5A52 against A431 Kato3, HT29 and LS174T cell line, respectively.

[0051] **FIG 35** shows the effects of polypeptide complex 5A52 at a dose of 1mpk on the growth of subcutaneous transplanted tumors of LS174T cells.

[0052] **FIG 36** shows the results of the level of cytotoxicity of polypeptide complex 2A42 against Raji cell line.

[0053] **FIG 37** shows the effects of polypeptide complex 2A42 at a dose of 0.5mpk on the growth of subcutaneous transplanted tumors of PBMC-NCG mice bearing Jeko1 tumor xenograft model.

[0054] **FIG 38** shows the results of the level of cytotoxicity of polypeptide complex 24A3 against OV90 cell line.

[0055] **FIG 39** shows the results of the level of cytotoxicity of polypeptide complex 22A8 and 22A18 against H929 cell line.

[0056] **FIG 40** shows the variable region sequences of PD-L1 and CD3 binding domains with low affinity mutations.

[0057] **FIG 41** shows the sequences of CH1 region, CL region, linkers and hinge regions.

[0058] **FIG 42** shows the sequences of Fc regions comprised in exemplary antibodies.

[0059] **FIG 43** shows the sequences comprised in 12A39, 12A73, 12A94 and 12A106 without Fc regions.

[0060] **FIGs 44A-44B** show the results of the level of cytotoxicity of polypeptide complex 12A39, 12A94, 12A95, 12A96, 12A97, 12A98, 12A99, 12A106, 12A107 and 12A108.

[0061] **FIGs 45A-45F** show the results of the level of IL-6 release and cytotoxicity of polypeptide complex 12A94, 12A98, 12A99, 12A106, 12A107 and 12A108.

DETAILED DESCRIPTION OF THE INVENTION

[0062] Several aspects of the invention are described below with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of the invention. One having ordinary skill in the relevant art, however, will readily recognize that the invention can be practiced without one or more of the specific details or with other methods. The present invention is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events.

[0063] Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with the present invention.

I. Definitions and Abbreviations

[0064] Before the detailed description of the inventions is provided, the following are noted and defined.

[0065] All the description provided herein is merely intended to illustrate various embodiments of the inventions provided in the present disclosure. As such, the specific modifications discussed are not to be construed as limitations on the scope of the disclosure. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the

disclosure, and it is understood that such equivalent embodiments are to be included herein.

[0066] All references cited in the present disclosure, including patent applications, issued patents, published articles or other publications, are incorporated by reference in their entirety, which are for the purpose of providing methodologies that might be used in connection with the description provided herein. With respect to any term that is presented in one or more publications that is similar to, or identical with, a term that has been expressly defined in this disclosure, the definition of the term as expressly provided in this present disclosure will control in all respects.

[0067] All technical and scientific terms used, unless expressly defined otherwise, in this present disclosure, are generally deemed to have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs.

[0068] As used herein, i.e., throughout the whole disclosure, the articles "a," "an," and "the" are to be construed to mean "one or more" or "at least one" unless specified otherwise. By way of example, "a polypeptide complex" means one polypeptide complex or more than one polypeptide complex.

[0069] As used herein, the terms "about," "approximately," "around" or alike, refer to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In particular embodiments, the terms "about" or "approximately" when preceding a numerical value indicates the value plus or minus a range of 15%, 10%, 5%, or 1%.

[0070] In all occurrences in this application where there are a series of recited numerical values, it is to be understood that any of the recited numerical values may be the upper limit or lower limit of a numerical range. It is to be further understood that the invention encompasses all such numerical ranges, i.e., a range having a

combination of an upper numerical limit and a lower numerical limit, wherein the numerical value for each of the upper limit and the lower limit can be any numerical value recited herein. Ranges provided herein are understood to include all values within the range. For example, 1-10 is understood to include all of the values 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, and fractional values as appropriate.

[0071] As used herein, the terms "comprise," "comprises," "comprising," "include," "includes," "including," "contain," "contains," "containing", "have," "has," "having" and the like, are synonymous and are used inclusively, in an open-ended fashion, and do not exclude additional elements, features, steps, acts, operations, and so forth.

[0072] As used herein, the term "or" is used in its inclusive sense (and not in its exclusive sense) so that when used, for example, to connect a list of elements, the term "or" means one, some, or all of the elements in the list.

[0073] As used herein, the phrase "at least one" means one or more, i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. A phrase referring to "at least one of" a list of items is construed to refer to any combination of those items, including single members. As an example, "at least one of: A, B, or C" is intended to cover: A, B, C, A and B, A and C, B and C, and A, B, and C. Conjunctive language such as the phrase "at least one of X, Y and Z," unless specifically stated otherwise, is otherwise understood with the context as used in general to convey that an item, term, etc. may be at least one of X, Y or Z. Thus, such conjunctive language is not generally intended to imply that certain embodiment requires at least one of X, at least one of Y, and at least one of Z to each be present.

[0074] As used herein, the references "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," "some embodiments," "certain embodiments," or "a further embodiment" or combinations thereof, are to be understood to mean that a particular feature, structure or characteristic described in connection with this particular

embodiment is included in at least one embodiment of the present disclosure. Thus, the presences or appearances of the foregoing phrases in various places throughout this disclosure are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0075] Conditional language used herein, such as, among others, "can," "could," "might," "may," "*e.g.*," and the like, unless stated expressly otherwise, or otherwise understood within the context as used, is generally intended to convey that certain embodiments include, while other embodiments do not include, certain features, elements and/or steps. Thus, such conditional language is not generally intended to imply that features, elements and/or steps are in any way required for one or more embodiments.

[0076] In this section, definitions for some general terms are provided. Definition for other terms may be found in other sections of the disclosure that follow.

[0077] The terms "polypeptide", "peptide", and "protein" are used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds, which includes proteins, polypeptides, oligopeptides, peptides, and fragments thereof. The protein may be made up of naturally occurring amino acids and/or synthetic (*e.g.*, modified or non-naturally occurring) amino acids. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. The terms "polypeptide", "peptide", and "protein" includes fusion proteins, including, but not limited to, fusion proteins with a heterologous amino acid sequence, fusions with heterologous and homologous leader sequences, with or without N-terminal methionine residues; immunologically tagged proteins; fusion proteins with detectable fusion partners, *e.g.*, fusion proteins including as a fusion partner a fluorescent protein, β-galactosidase, luciferase, *etc.*; and the like.

[0078] As used herein, the term "amino acid" refers to a building block of a protein, a peptide, a polypeptide or an amino acid polymer, and the term "amino acid"

further refers to a naturally occurring or synthetic amino acid, as well as any amino acid analog and amino acid mimetics that functions in a manner similar to the naturally occurring amino acid. Naturally occurring amino acids are those encoded by the genetic codes, as well as those amino acids that are later modified, e.g., hydroxyproline, gamma-carboxyglutamate, and O-phosphoserine. As used within this application, naturally occurring amino acids include the group of naturally occurring carboxy alpha-amino acids comprising alanine (three letter code: Ala, one letter code: A), arginine (Arg, R), asparagine (Asn, N), aspartic acid (Asp, D), cysteine (Cys, C), glutamine (Gln, Q), glutamic acid (Glu, E), glycine (Gly, G), histidine (His, H), isoleucine (Ile, I), leucine (Leu, L), lysine (Lys, K), methionine (Met, M), phenylalanine (Phe, F), proline (Pro, P), serine (Ser, S), threonine (Thr, T), tryptophan (Trp, W), tyrosine (Tyr, Y), and valine (Val, V).

[0079] As used herein, the term "nucleic acid," "nucleic acid molecule," "nucleotide," "polynucleotide" or alike, is construed to refer to a nucleotide polymer of any length, can include both DNA and RNA, can be single-stranded or double-stranded, and includes analogs of naturally occurring polynucleotides in which one or more nucleotides are modified over naturally occurring nucleotides.

[0080] As used herein, the term "domain" refers to a structure formed by one or more regions of one or more polypeptide chains comprising secondary structures (such as peptide loops) that are stabilized, for example, by β-pleated sheet and/or intrachain disulfide bond(s). It is noted that in the present disclosure, the two terms "domain" and "region" may be used interchangeably.

[0081] The term "antibody" as used herein broadly encompasses any immunoglobulins (including native and modified) and proteins and protein complexes comprising at least one antigen-binding domain derived from an immunoglobulin. Examples of an antibody include monoclonal antibody, polyclonal antibody, chimeric antibody, humanized antibody, multi-specific antibody, bispecific antibody, bivalent antibody or multivalent antibody, among others. In the following, a description of an antibody, as well as terms relevant thereto, is provided in more details below.

[0082] In mammals such as human, depending on the different types of heavy chains present in the immunoglobulins, there are five different classes/isotypes (i.e. IgA, IgD, IgE, IgG, and IgM, corresponding to the five Ig heavy chain types α, δ, ε, γ, and μ, respectively) of antibodies, which typically have different molecular and biological properties, functional locations, physiological functionalities, and pathological implications in diseases. Certain antibody classes may further include subclasses. For example, in human, IgA may include IgA1 and IgA2 subclasses, and IgG may include four subclasses denoted as IgG1, IgG2, IgG3, and IgG4, respectively. With an immunoglobulin monomer as its basic functional unit, a mammalian antibody may exist as a monomer (*e.g.*, IgD, IgE, and IgG), a dimer (IgA), a tetramer (IgM), or a pentamer (IgM). In mammals, two types of light chain exist, including kappa (κ) chain and lambda (λ) chain.

[0083] Within the basic immunoglobulin unit, a native or naturally occurring antibody such as IgG generally includes two identical heavy (H) chains and two identical light (L) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond or linkage formed between a pair of cysteine residues present respectively in the light chain and the heavy chain, and the two heavy chains are further linked to one another through several disulfide bonds formed between cysteine residues in each heavy chain. The tetramer thus formed substantially takes a Y-like shape for the antibody, with the end of each fork arm containing an identical antigen-binding site (*i.e.*, paratope) that interacts specifically with a corresponding epitope of the antigen.

[0084] More specifically, in a native antibody, in a N-terminal-to-C-terminal direction, each heavy chain includes a variable region (VH, or HCVR), followed by three or four constant regions ("CHs", with IgA, IgD, IgG containing three CH regions CH1, CH2 and CH3; and IgE and IgM containing four CH regions CH1, CH2, CH3 and CH4), and each light chain includes a variable region (VL, or LCVR) and a constant region (CL). In the Y-shaped antibody, the variable region of each light chain (*i.e.*, the VL region) aligns or associates with the variable region of its

pairing heavy chain (*i.e.*, the VH region) to together form an antigen-binding site for the antibody. The term antibody as used in the present disclosure is also intended to encompass heavy chain antibody which has only heavy chain but no light chain.

[0085] The term "variable region" or "VR" as used herein means the region in heavy chain or light chain of an antibody that are responsible for antigen binding. In a native antibody, the heavy chain variable region (VH or HCVR) contains three highly variable loops called "complementarity determining regions" (CDRs), *i.e.*, heavy (H) chain CDRs including HCDR1, HCDR2, HCDR3, and the light chain variable region (VL or LCVR) contains three light (L) chain CDRs including LCDR1, LCDR2, and LCDR3. CDR boundaries for antibodies may be defined or identified by the conventions of Kabat, Chothia, or Al-Lazikani (Al-Lazikani, B., Chothia, C., Lesk, A. M., J. Mol. Biol., 273(4), 927 (1997); Chothia, C. *et al.*, J Mol. Biol. Dec 5;186(3):651-63 (1985); Chothia, C. and Lesk, A.M., J.Mol. Biol., 196,901 (1987); Chothia, C. *et al.*, Nature. Dec 21-28; 342(6252):877-83 (1989); Kabat E.A. *et al.*, National Institutes of Health, Bethesda, Md. (1991)). The three CDRs are interposed between flanking stretches known as "framework regions" (FRs), which are more highly conserved than the CDRs and form a scaffold to support the hypervariable loops. In a native antibody, each VH and VL comprises four FRs, and the CDRs and FRs are arranged from amino terminus to carboxyl terminus in the order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. However, it should be understood that the term "variable region" as used herein does not necessarily need to include all of the three CDRs or all of the four FRs, and should be construed to encompass any variant or derivative of a native variable region from a native antibody, as long as such variant or derivative retains antigen-binding activity.

[0086] The term "constant region" or "constant moiety" as used herein means the region in heavy chain or light chain of an antibody that are not directly involved in antigen binding. It should be understood that the term "constant region" or "constant moiety" as used herein does not necessarily need to include the full length of a native constant region of a native antibody, and should be construed to encompass any

variant or derivative of such a native constant region or constant moiety, as long as such variant or derivative retains the ability to, for example, support stability of the antigen-binding domain, or retains the intended biological function such as effector functions such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like.

[0087] The term "CL region" refers to the constant region of an immunoglobulin light chain that is adjacent to the VL region. In a native antibody, the constant region of each light chain (*i.e.*, the CL region) associates with the first constant region of a pairing heavy chain (*i.e.*, the CH1 region).

[0088] The term "CH1 region" as used herein encompasses the first (most amino terminal) constant region of an immunoglobulin heavy chain. The CH1 region is adjacent to the VH region, and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

[0089] The term "hinge region" in terms of an antibody includes the portion of a heavy chain molecule that joins the CH1 region to the CH2 region. The length of hinge region may vary depending on the defined boundaries of the CH1 region and of the CH2 region. The hinge region is normally flexible, thus allowing the two N-terminus antigen binding regions to move independently.

[0090] The term "CH2 region" or "CH2 domain" as used herein refers to the portion of a heavy chain immunoglobulin molecule that joins the hinge region to the CH3 region.

[0091] The term "CH3 region" or "CH3 domain" as used herein refers to the portion of a heavy chain immunoglobulin molecule that typically forms the C-terminal portion of the immunoglobulin like IgG, IgA, and IgD. In some immunoglobulins like IgE and IgM, however, additional domains may extend from CH3 domain to form the C-terminal portion of the molecule (*e.g.*, the CH4 domain in the μ chain of IgM and the ε chain of IgE).

[0092] "Fc" as used herein refers to a portion derived from an antibody, for example, IgG, mainly composed of the second (CH2) and third (CH3) constant regions of a first heavy chain bound to the CH2 and CH3 of a second heavy chain via one or more covalent bonds which are non-peptide bonds, for example, via disulfide bonding. The Fc portion of the antibody is responsible for various effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), etc., but does not function in antigen binding.

[0093] An "antigen" or "Ag" as used herein refers to a compound, composition, peptide, polypeptide, protein or substance (*e.g.*, polypeptide, carbohydrate, nucleic acid, lipid, or other naturally occurring or synthetic compound) that can be specifically recognized and bound by a component of the immune system, *e.g.*, an antibody. As used herein, the term "antigen" encompasses antigenic epitopes, *e.g.*, fragments of an antigen which are antigenic epitopes. The term "antigen" and "target" are used interchangeably in the present disclosure.

[0094] As used herein, the term "antigen-binding domain", such as PD-L1-binding domain, CD3-binding domain or the alike, means a fragment or a domain derived from a portion of an immunoglobulin, and this fragment or domain comprises one or more CDRs or otherwise binds to an antigen but does not comprise an intact native immunoglobulin structure. Examples of antigen-binding domains may include, without limitation, a variable domain, a variable region, a diabody, a Fab, a Fab', a $F(ab')_2$, an Fv fragment, a disulfide stabilized Fv fragment (dsFv), a $(dsFv)_2$, a bispecific dsFv (dsFv-dsFv'), a disulfide stabilized diabody (ds diabody), a multispecific antibody, a camelized single domain antibody, a nanobody, a domain antibody, and a bivalent domain antibody, etc. An antigen-binding domain is capable of binding to the same antigen to which the parent full-length antibody binds. An antigen-binding domain may comprise one or more CDRs from a particular animal or human antibody grafted to a framework region from one or more different animal or human antibodies. For more and detailed formats of antigen-binding moiety are

described in Spiess et al., 2015 (*Supra*), and Brinkman et al., *mAbs*, 9(2), pp.182–212 (2017), which are incorporated herein by entirety reference.

[0095] "Fv" with regard to an antibody refers to an antigen-binding domain that bear a complete antigen-binding site. An Fv domain comprises or consists of the variable domain of a single light chain bound to the variable domain of a single heavy chain. As used herein, the term Fv domain shall be construed to broadly encompass a domain formed by VH and VL, as well as a domain that further comprise scaffold domains fused to the VH and the VL, respectively. For example, Fv domain as used herein encompasses a Fab domain formed by VH and heavy chain constant region 1 (CH1), as well as a VL and a light chain constant region (CL). Fv domain can also contain other suitable pairing scaffold regions other than CH1 and CL, such as pairing TCR constant regions (such as TCR alpha/TCR beta, TCR gamma/TCR delta), or PRD (proline rich domain) and SH3 domain, obscurin and titin, IL2 and ligand binding domain of IL2 receptor, or IL15 and ligand binding domain of IL15 receptor. Fvs have also been multimerized to form diabodies and triabodies (Maynard *et al.*, *Annu Rev Biomed Eng* 2 339-376 (2000)). An Fv domain comprises or consists of the variable domain of a single light chain bound to the variable domain of a single heavy chain.

[0096] "Single-chain Fv antibody" or "scFv" refers to an engineered antibody consisting of a light chain variable region and a heavy chain variable region connected to one another directly or via a peptide linker sequence (Huston JS *et al. Proc Natl Acad Sci USA,* 85:5879(1988)).

[0097] A "dsFv" refers to a disulfide-stabilized Fv fragment that the linkage between the variable region of a single light chain and the variable region of a single heavy chain is a disulfide bond. In some embodiments, a "$(dsFv)_2$" comprises three peptide chains: two V_H moieties linked by a peptide linker (*e.g.,* a long flexible linker) and bound to two V_L moieties, respectively, via disulfide bridges.

[0098] "Fab" as used herein refers to a monovalent antigen-binding fragment of the immunoglobulin consisting of a single light chain (both variable and constant regions) bound to the variable region and first constant region of a single heavy chain by a disulfide bond. Fab can be obtained by papain digestion of an immunoglobulin at the residues proximal to the N-terminus of the disulfide bond between the heavy chains of the hinge region. In a native immunoglobulin, the Fab domain corresponds substantially to one arm of the immunoglobulin, typically retains the ability to recognize and bind to its corresponding antigen.

[0099] "Fab'" refers to a Fab fragment that includes a portion of the hinge region.

[00100] "$F(ab')_2$"refers to a dimer of Fab'.

[00101] "Camelized single domain antibody," "heavy chain antibody," "VHH", "nanobody", or "HCAb" refers to an antibody that contains two V_H domains and no light chains (Riechmann L. and Muyldermans S., *J Immunol Methods.* Dec 10; 231(1-2):25-38 (1999); Muyldermans S., *J Biotechnol.* Jun;74(4):277-302 (2001); WO94/04678; WO94/25591; U.S. Patent No. 6,005,079). Heavy chain antibodies were originally derived from *Camelidae* (camels, dromedaries, and llamas). Although devoid of light chains, camelized antibodies have an authentic antigen-binding repertoire (Hamers-Casterman C. *et al.*, *Nature*. Jun 3; 363(6428):446-8 (1993); Nguyen VK. *et al. Immunogenetics*. Apr;54(1):39-47 (2002); Nguyen VK. *et al. Immunology*. May; 109(1):93-101 (2003)). The variable domain of a heavy chain antibody (VHH domain) represents the smallest known antigen-binding unit generated by adaptive immune responses (Koch-Nolte F. *et al.*, *FASEB J.* Nov; 21(13):3490-8. Epub 2007 Jun 15 (2007)).

[00102] Antibody and antigen-binding domains thereof according to the present disclosure encompass multi-specific antibodies and domains thereof.

[00103] As used herein, the term "multi-specific polypeptide complex" refers to an artificial or engineered polypeptide complex that can simultaneously bind to at least two different epitopes. The multi-specific polypeptide complex can be bispecific,

trispecific, tetraspecific, and so on. In certain embodiments, the multi-specific polypeptide complex can be a multi-specific antibody.

[00104] As used herein, the term "multi-specific antibody" refers to an artificial or engineered antibody that can simultaneously bind to at least two different epitopes or at least two different antigens. A bispecific antibody is substantially a type of a multi-specific antibody. In addition, other multi-specific antibodies may include trispecific antibodies, which have three different antigen-binding specificities, tetraspecific antibodies, which have four different antigen-binding specificities, and so on. Multi-specific antibodies comprise two or more physically separable antigen-binding domains which differ from one another in their antigen specificity. For example, a multi-specific antibody is an artificial antibody which has fragments derived from two or more different monoclonal antibodies and is capable of binding to two or more different epitopes. The two or more epitopes may present on the same antigen, or they may present on two or more different antigens. It is in contrast to a naturally occurring antibody which has two physically separable antigen-binding moieties that are structurally identical and thus have the same antigen specificity. Multi-specific antibodies may resemble single antibodies (or antibody domains) but have two or more different antigen binding sites. Multi-specific antibodies and domains can also be in form of heteroantibodies. Heteroantibodies are two or more antibodies, or antibody binding domains (*e.g.*, Fab) linked together, each antibody or domain having a different specificity.

[00105] Throughout the disclosure, the numbers indicating the positions of amino acid residues in a variable region, a hinge region or a constant region of an antibody, can be based on the EU numbering, as described in Edelman, G.M. *et al.*, *Proc. Natl. Acad.* USA, 63, 78-85 (1969), IMGT numbering or Kabat index, as described in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991); Marie-Paule Lefranc *et al.*, *Developmental and Comparative Immunology*, 27: 55-77 (2003); Marie-Paule Lefranc *et al.*, *Immunome Research*, 1(3), (2005); Marie-Paule Lefranc, Molecular

Biology of B cells (second edition), chapter 26, 481-514, (2015). These numberings are also available from the IMGT scientific chart, accessible from the website of international ImMunoGeneTics information system.

[00106] The term "chimeric" as used herein, means an antibody or antigen-binding domain, having a portion of heavy and/or light chain derived from one species, and the rest of the heavy and/or light chain derived from a different species. In an illustrative example, a chimeric antibody may comprise a constant region derived from human and a variable region from a non-human animal, such as from mouse. In some embodiments, the non-human animal is a mammal, for example, a mouse, a rat, a rabbit, a goat, a sheep, a guinea pig, or a hamster.

[00107] The term "valent" as used herein refers to the presence of a specified number of antigen binding sites in a given molecule. The term "monovalent" refers to an antibody or an antigen-binding domain having only one single antigen-binding site; and the term "multivalent" refers to an antibody or an antigen-binding fragment having multiple antigen-binding sites. As such, the terms "bivalent", "tetravalent", and "hexavalent" denote the presence of two binding sites, four binding sites, and six binding sites, respectively, in an antigen-binding molecule. In some embodiments, the antibody or antigen-binding fragment thereof is bivalent.

[00108] The term "specifically binding" or "antigen-specific" in the context of a characteristic of an antibody or antigen-binding domain refers to the ability of an antibody or an antigen-binding domain to preferentially bind to a particular antigen that is present in a mixture of different antigens. In certain embodiments, a specific binding interaction will discriminate between desirable and undesirable antigens (or "target" and "non-target" antigens) in a sample, in some embodiments more than about 10 to 100-fold or more. Specific binding of an antibody or antigen-binding domain provided herein to an antigen can be characterized in affinity, as represented by K_D (dissociation constant).

[00109] K_D used herein refers to the ratio of the dissociation rate to the association rate (k_{off}/k_{on}) when the binding between the antigen and antigen-binding molecule (such as an antibody) reaches equilibrium. Low affinity (i.e., higher K_D value) generally means the antigen-binding molecule binds to the antigen slowly and/or tends to dissociate readily, whereas high affinity (i.e., lower K_D value) generally means the antigen-binding molecule binds to the antigen faster and tend to remain bound longer. K_D may be determined by using any conventional method known in the art, including but are not limited to surface plasmon resonance (SPR) method, Biolayer Interferometry method, microscale thermophoresis method, HPLC-MS method and flow cytometry (such as FACS) method.

[00110] SPR method can be carried out using instruments such as Biacore (GE Healthcare) (see, for example, Murphy, M. et al, Current protocols in protein science, Chapter 19, unit 19.14, 2006), using immobilized antigen CM5 chips at a proper response units (RU).

[00111] A specific illustrative and exemplary embodiment for measuring binding affinity is described in the following and in the Examples below.

[00112] In certain embodiments, KD is measured by surface plasmon resonance. In certain embodiments, KD is measured by Biacore. Systems (see, for example, Paul Leonard, et al, Measuring Protein-Protein Interactions Using Biacore, Methods Mol Biol,. 2017:1485:339-354.).

[00113] In on embodiment, to analyze the interaction between the antigen and antibody, His-tagged recombinant antigen is captured by an anti-Penta His antibody immobilized on CM5 chips and the multi-specific antibodies provided herein are used as analytes. Briefly, carboxymethylated dextran biosensor chips (CM5, GE Healthcare) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier' s instructions. Anti Penta-His antibody is diluted with sodium acetate, pH 4.5, to 0 μg/ml before injection at a flow rate of 10 μl/min to achieve approximately 6500

response units (RU) of coupled protein. Following the injection of the ligand, 1 M ethanolamine is injected to block unreacted groups. Subsequently the antigen is captured for 60 s at 1μg/ml. For kinetic measurements, two-fold serial dilutions of the antibody (range between 50 nM and 0.1 nM) are injected in HBS-EP (GE Healthcare, pH 7.4) at 25 °C at a flow rate of 30 μl/min for 180 s, and the dissociation is monitored for 400 s.

[00114] In certain embodiments, specific binding means an antibody or antigen-binding molecule binds to the antigen at a K_D value of no more than 10^{-6}M as measured by SPR method.

[00115] Biolayer Interferometry (BLI) is a label-free technology used to measure the binding kinetics and affinity of molecular interactions, including antibody-antigen interactions. BLI operates by monitoring changes in the interference pattern of white light reflected off a biosensor surface where the interaction occurs. Antibodies or antigens are immobilized on the biosensor tip, which is then dipped into solutions containing the interacting molecule. As binding occurs, the mass on the biosensor surface changes, leading to a shift in the interference pattern. This shift is measured in real time, providing kinetic data such as association and dissociation rates, and enabling the determination of the binding affinity. BLI is advantageous due to its simplicity, high sensitivity, and the ability to perform measurements in complex biological samples without the need for labels or extensive sample preparation. A nonlimiting exemplary device for biolayer interferometry is ForteBio Octet® RED96 system (Pall Corporation). See, e.g., Abdiche et al., 2008, Anal. Biochem.377: 209-277.

[00116] The term "operably link" or "operably linked" refers to a juxtaposition, with or without a spacer or linker, of two or more biological sequences of interest in such a way that they are in a relationship permitting them to function in an intended manner. When used with respect to proteins, it is intended to mean that the protein sequences are linked in such a way that permits the linked product to have the intended biological function. The term may also be used with respect to polynucleotides. For

instance, when a polynucleotide encoding a protein is operably linked to a regulatory sequence (e.g., promoter, enhancer, silencer sequence, etc.), it is intended to mean that the polynucleotide sequences are linked in such a way that permits regulated expression of the protein from the polynucleotide.

[00117] The term "vector" as used herein refers to a vehicle into which a genetic element may be operably inserted so as to bring about the expression of that genetic element, such as to produce the protein, RNA or DNA encoded by the genetic element, or to replicate the genetic element. A vector may be used to transform, transduce, or transfect a host cell so as to bring about expression of the genetic element it carries within the host cell. Examples of vectors include plasmids, phagemids, cosmids, and artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses. A vector may contain a variety of elements for controlling expression, including promoter sequences, transcription initiation sequences, enhancer sequences, selectable elements, and reporter genes. In addition, the vector may contain an origin of replication. A vector may also include materials to aid in its entry into the cell, including but not limited to a viral particle, a liposome, or a protein coating. A vector can be an expression vector or a cloning vector. The present disclosure provides vectors (*e.g.*, expression vectors) containing the nucleic acid sequence provided herein encoding the antibody or an antigen-binding fragment thereof, at least one promoter (*e.g.*, SV40, CMV, EF-1α) operably linked to the nucleic acid sequence, and at least one selection marker.

[00118] The phrase "host cell" as used herein refers to a cell into which an exogenous polynucleotide and/or a vector can be or has been introduced.

II. Multi-specific Polypeptide Complexes

[00119] In one aspect, the present disclosure provides a novel multi-specific polypeptide complex, comprising an immune stimulatory target binding domain, an immune checkpoint target binding domain, and a disease antigen binding domain.

[00120] In certain embodiments, the immune stimulatory target is CD3. In certain embodiments, the immune checkpoint target is PD-L1.

[00121] In some embodiments, the multi-specific polypeptide complex provided herein comprises a) a CD3-binding domain capable of specifically binding to CD3, b) a PD-L1-binding domain capable of specifically binding to PD-L1, and c) a first disease antigen binding domain, wherein the CD3-binding domain, PD-L1 binding domain and the disease antigen binding domain are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1, CD3 and the disease antigen.

[00122] In certain embodiments, the multi-specific polypeptide complex is a multi-specific antibody.

[00123] Multi-specific antibodies targeting CD3 and PD-L1, and optionally further targeting a tumor antigen have been reported to show good anti-tumor efficacy in mouse studies, although they were reported to be associated with side effects of cytokine release. Despite of the efficacy observed in mouse studies, no such multi-specific antibodies have reached clinical trial, and the reasons are largely unknown.

[00124] Through extensive and in-depth research, inventors of the present disclosure found that multi-specific antibodies targeting both CD3 and PD-L1 (optionally further targeting a disease antigen) are prone to rapid clearance in primates, making it unable to achieve a desired half-life in primates and hence impractical for any further clinical development in humans.

[00125] The present inventors unexpectedly found that, by reducing the binding affinity to PD-L1 (*e.g.*, human PD-L1) to an optimized range, the half-life of such a multi-specific antibody targeting both CD3 and PD-L1 can be significantly improved. Moreover, surprisingly, despite of reduced binding affinity to PD-L1, the therapeutic efficacy of such a multi-specific antibody can be substantially maintained.

[00126] Accordingly, in certain embodiments, the multi-specific polypeptide complex provided herein comprises a PD-L1-binding domain that is a low-affinity PD-L1-binding domain capable of specifically binding to PD-L1 at a K_D value of no less than 10^{-8} M as measured by Surface Plasmon Resonance (SPR) or by Bio-Layer Interferometry (BLI).

[00127] In certain embodiments, the multi-specific polypeptide complex further comprises a dimerization domain (*e.g.*, an Fc domain) that is operably linked to the CD3-binding domain, the PD-L1 binding domain and the disease antigen binding domain. In certain embodiments, the dimerization domain comprises a CH3 domain, which dimerizes to form a dimer. In certain embodiments, the dimerization domain further comprises a CH2 domain, which is operably linked to the CH3 domain. In certain embodiments, the dimerization domain comprises an Fc domain.

[00128] In some embodiments, the CH3 domain or the Fc domain is derived from human IgG1 or human IgG4. In some embodiments, the human IgG1 Fc comprises the amino acid sequence of SEQ ID NO: 57. In some embodiments, the human IgG4 Fc comprises the amino acid sequence of SEQ ID NO: 55.

[00129] It is yet another surprising finding by the present inventors that, by reducing or eliminating binding affinity of the multi-specific polypeptide complex to certain Fc receptors such as CD32a, the half-life of such a multi-specific polypeptide complex in primates can be further extended. In addition, unexpectedly, such reduction in Fc receptors binding can reduce the side effects of the multi-specific polypeptide complexes in cytokine release and/or reduction in CD45+ and/or CD3+ cells.

[00130] Accordingly, in certain embodiments, the multi-specific polypeptide complex provided herein comprises an Fc domain that is modified and has reduced binding or lacks substantial binding to human CD32a. In certain embodiments, the Fc domain further has reduced binding or lacks substantial binding to human CD16 and/or human CD64. In certain embodiments, the Fc domain has reduced binding or lacks substantial binding to human CD32a, human CD16 and human CD64. In some

embodiments, the Fc domain has reduced binding affinity to human CD32a and comprises the amino acid sequence of SEQ ID NO: 115 or SEQ ID NO: 114. In some embodiments, the Fc domain has reduced binding affinity to human CD64 and comprises the amino acid sequence of SEQ ID NO: 114.

[00131] It is still another surprising finding by the present inventors that, by reducing the binding affinity to CD3 (*e.g.*, human CD3) to an optimized range, the side effects of the multi-specific polypeptide complexes in cytokine release can be substantially reduced, yet the therapeutic efficacy of such a multi-specific antibody can be substantially maintained, thereby significantly increasing the therapeutic window.

[00132] Accordingly, in certain embodiments of the present disclosure, the CD3-binding domain is a low-affinity CD3-binding domain capable of specifically binding to CD3 at a K_D value of no less than 10^{-8} M as measured by Surface Plasmon Resonance (SPR).

[00133] Taken together, the present disclosure provides optimized multi-specific antibodies targeting both CD3 and PD-L1 (optionally further targeting a disease antigen) that have superior advantages in multiple aspects including efficacy, safety, and pharmacokinetics.

(i) Low-Affinity PD-L1 binding Domain

[00134] In some embodiments, the multi-specific poly peptide complex provided herein comprises a PD-L1 binding domain that is a low-affinity PD-L1-binding domain. The term "low-affinity" as used herein means that the binding domain has reduced binding affinity to its target. The binding domain can be derived from an antibody that has naturally or inherently low binding affinity to its target, or alternatively can be modified from a high-affinity parent antibody to reduce the original binding affinity to its target, or further alternatively can be modified from a very low-affinity parent antibody to slightly improve its binding affinity to its target, such that the modified antibody has the suitable binding affinity to its target as

described herein. The binding domain can also be replaced by a different domain having weaker binding to the target.

[00135] In some embodiments, the low-affinity PD-L1-binding domain is capable of specifically binding to PD-L1 (*e.g.* human PD-L1) at a K_D value of no less than 10^{-9} M or no less than 10^{-8} M as measured by Bio-Layer Interferometry (BLI). In the present disclosure, scientific notations such as 3E-8M and 3×10^{-8} M and $3*10^{-8}$ M, are equivalent expressions used interchangeably to represent the same concentrations. In certain embodiments, the K_D of the low-affinity PD-L1-binding domain is determined in accordance with the method as described in section 2.2 of Example 2 in the present disclosure.

[00136] In some embodiments, the PD-L1 binding domain is capable of specifically binding to human PD-L1 at a K_D value of no less than 1E-9 M, no less than 1E-8 M, no less than 2E-8 M, no less than 3E-8 M, no less than 4E-8 M, no less than 5E-8 M, no less than 6E-8 M, no less than 7E-8 M, no less than 8E-8 M, no less than 9E-8 M, no less than 1E-7 M as measured by Bio-Layer Interferometry(BLI).

[00137] In some embodiments, the PD-L1 binding domain is capable of specifically binding to human PD-L1 at a K_D value of no more than 1E-5 M, no more than 1E-6 M, or no more than 9E-7 M, or no more than 8E-7 M, no more than 7E-7 M, no more than 6E-7 M, or no more than 5E-7 M as measured by Bio-Layer Interferometry (BLI).

[00138] In some embodiments, the PD-L1 binding domain is capable of specifically binding to human PD-L1 at a K_D value ranging from 7E-6 M to 1E-9 M, from 4E-6 M to 1E-9 M, from 1E-6 M to 1E-9 M, 7E-6 M to 1E-8 M, from 4E-6 M to 1E-8 M, from 1E-6 M to 1E-8 M, 8E-7 M to 1E-8 M, from 8E-7 M to 2E-8 M, from 8E-7 M to 3E-8 M, from 8E-7 M to 4E-8 M, from 8E-7 M to 5E-8 M, from 8E-7 M to 6E-8 M, from 8E-7 M to 7E-8 M, from 8E-7 M to 8E-8 M, from 8E-7 M to 9E-8 M, from 8E-7 M to 1E-7 M, from 3E-6 M to 3E-8 M, from 7E-6 M to 3E-8 M, from 1E-6 M to 3E-8

M, 9E-7 M to 3E-8 M, 8E-7 M to 3E-8 M, 7E-7 M to 3E-8 M, or 3E-7 M to 3E-8 M as measured by BLI.

[00139] In some embodiments, the low-affinity PD-L1 binding domain is capable of specifically binding to human PD-L1 at a K_D value ranging from no less than 2.98E-8 M to no more than 2.11E-7 M as measured by BLI.

[00140] In some embodiments, the low-affinity PD-L1 binding domain of the multi-specific polypeptide complex is engineered to have reduced binding affinity to PD-L1, as compared to a non-engineered parent PD-L1 binding domain. In one such embodiment the low-affinity PD-L1 binding domain (or the multi-specific polypeptide complex comprising said low-affinity PD-L1 binding domain) exhibits lower binding affinity to PD-L1 (e.g. human PD-L1), as compared to a non-engineered parent PD-L1 binding domain (or the multi-specific polypeptide complex comprising said low-affinity PD-L1 binding domain).

[00141] In some embodiments, the low-affinity PD-L1 binding domain of the multi-specific polypeptide complex is engineered to have slightly improved binding affinity to PD-L1, as compared to a non-engineered parent PD-L1 binding domain which originally has very low binding affinity to PD-L1 (e.g. having a K_D value of more than 1E-6). In one such embodiment the low-affinity PD-L1 binding domain (or the multi-specific polypeptide complex comprising said low-affinity PD-L1 binding domain) exhibits higher binding affinity to PD-L1 (e.g. human PD-L1), as compared to a non-engineered parent PD-L1 binding domain (or the multi-specific polypeptide complex comprising said low-affinity PD-L1 binding domain).

[00142] Low-affinity binding domains can be obtained using methods known in the art. In certain embodiments, one may select an existing antibody for modification to reduce its binding affinity to the antigen. This can be done by mutating one or more amino acid residues in the antigen binding domain that are involved in or would otherwise impact on antigen binding, such that the binding affinity to the antigen can be reduced or optimized. Such amino acid residues for mutation can be in one or more

CDR regions and/or in one or more framework regions, and in particular, may be within, adjacent to, or near the paratope or binding interface of the antibody ("interface residues"), that makes direct contact with the antigen and form the antigen binding site.

[00143] For a given antibody, methods are known to a skilled person in the art to identify interface residues. For example, interface residues on the antibody may be identified through alanine scanning mutagenesis, by individually mutating residues in the CDRs of an antibody to alanine, followed by identification of residues whose mutation reduces binding affinity.

[00144] For another example, interface residues can also be identified based on co-crystal structure of the antibody and antigen complex. Specifically, an antigen and a Fab binding to the antigen can be allowed to form a complex, and the antigen-Fab complex can be co-crystallized and the co-crystal structure can be solved at certain resolution. The interface residues can be identified with the co-crystal structure through experimental methods like X-ray crystallography, Nuclear Magnetic Resonance, or cryo-electron microscopy, or through computational methods if the structure of the antibody is already known. Certain public databases such as Protein Data Bank (PDB) also provides published cocrystal structures of antigen-Fab complexes. Such high-resolution crystal structure can allow identification of the interface between the antigen and the antibodies, as well as mapping of the specific amino acid residues involved in the antigen binding and recognition site.

[00145] To determine the interface residues on the antigen, methods based on solvent exposure difference can be used, where the solvent accessible surface area (ASA) can be calculated for each residue in epitope domain in the antigen-antibody complex (such as epitope-Fab complex) and compared to the solvent ASA of the corresponding residue stripped from complex. All amino acids with different ASA are considered interface residues.

[00146] Alternatively, one can use a predefined distance to the partner protein of amino acid residues, wherein the interface residues can be selected that have at least one atom within such predefined distance to its partner protein.

[00147] In addition, interface residues can also be analyzed through computational methods, including PISA (Protein Interfaces, Surfaces and Assemblies offered by PDBe.

[00148] The spatial requirement and the nature of the interaction for every interface residue can be also elucidated. Important residues on the antigen that form hydrogen bond, electrostatic interactions or salt bridge interactions with Fab can be identified. Using similar methods, the paratope residues for the antibodies can be also identified.

[00149] Mutations can be introduced to the antibody at or near the interface residues or paratope residues, for example, to disrupt or reduce at least some of the identified hydrophobic interactions, electrostatic interactions, hydrogen bonds, and so on.

[00150] Computational tools can also be used to predict possible mutations and impacts on the binding affinity, considering the physiochemical properties of the amino acids, the confirmation of the binding site, and the energetics of the binding. Examples of such tools include FoldX, Rosetta, and Schrödinger's BioLuminate.

[00151] The predicated mutations can be further validated in experiments, for example by SPR, enzyme-linked immunosorbent assay (ELISA), or bio-layer interferometry (BLI). K_D values of the mutants can be determined, and those with suitable K_D values can be selected as the low-affinity PD-L1 binding domain in the multi-specific polypeptide complexes provided herein.

[00152] In some embodiments, the low-affinity PD-L1 binding domain is derived from a low-affinity variant of a parent anti-PD-L1 antibody, wherein the low-affinity variant has reduced binding affinity to human PD-L1 than the parent antibody. In some embodiments, the low-affinity variant specifically binds to human PD-L1 at a K_D value at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 10-fold, 15-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold higher or even higher than that of the

parent antibody. In some embodiments, the low-affinity PD-L1 binding domain of the multi-specific polypeptide complex specifically binds to human PD-L1 at a K_D value at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, or 6-fold higher than that of the parent PD-L1 binding domain of the multi-specific polypeptide complex.

[00153] In some embodiments, the low-affinity PD-L1 binding domain is derived from a low-affinity variant of a very low-affinity parent anti-PD-L1 antibody (e.g. having a K_D value of more than 1E-6), wherein the low-affinity variant has improved binding affinity to human PD-L1 than the very low-affinity parent antibody. In some embodiments, the low-affinity variant specifically binds to human PD-L1 at a K_D value at least 20% lower, preferably 50% lower, more preferably 1-fold lower, or even 10-fold lower than that of the parent antibody. In some embodiments, the low-affinity PD-L1 binding domain of the multi-specific polypeptide complex specifically binds to human PD-L1 at a K_D value at least 20% lower, preferably 50% lower, more preferably 1-fold lower, or even 10-fold lower than that of the parent PD-L1 binding domain of the multi-specific polypeptide complex.

[00154] In some embodiments, the low-affinity PD-L1 binding domain is derived from a low-affinity variant of a parent anti-PD-L1 antibody selected from the group consisting of MDX-1105, atezolizumab, avelumab, durvalumab, adebrelimab, sugemalimab, envafolimab, cosibelimab, garivulimab, manelimab, opucolimab, pacmilimab, sugemalimab, wherein the affinity variant has reduced binding affinity to human PD-L1 than the parent antibody. In some embodiments, the low-affinity variant has one or more mutations in one or more of the CDR regions, in the heavy chain variable region and/or the light chain region of the parent antibody. For example, if there is antibody-antigen complex crystal structure, then binding sites can be identified from the antibody sequence, which would provide positions for mutation consideration. Mutants can be made and tested, to obtain low-affinity variants of such parent antibodies.

[00155] In some embodiments, the low-affinity PD-L1 binding domain is derived from a low-affinity variant of a parent anti-PD-L1 antibody MDX-1105. In certain

embodiment, the parent anti-PD-L1 antibody MDX-1105 comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 [GDTFSSYA] or SEQ ID NO: 39 [GDTFSTYA], HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 [IIPIFGRA] or SEQ ID NO: 40 [IIPIFGKA], HCDR3 comprising the amino acid sequence of SEQ ID NO: 3 [ARKFHFVSGSPFGMDV], LCDR1 comprising the amino acid sequence of SEQ ID NO: 4 [QSVSSY], LCDR2 comprising the amino acid sequence of SEQ ID NO: 5 [DAS], and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6 [QQRSNWPT]. In certain embodiment, the parent anti-PD-L1 antibody MDX-1105 comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.

[00156] In a certain embodiment, the parent anti-PD-L1 antibody MDX-1105 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8. In a certain embodiment, the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 113, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8.

[00157] In some embodiments, the low-affinity PD-L1 binding domain is derived from a low-affinity variant of a parent anti-PD-L1 antibody atezolizumab. In certain embodiment, the parent anti-PD-L1 antibody atezolizumab comprises an HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131; LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134.

[00158] In a certain embodiment, the parent anti-PD-L1 antibody atezolizumab comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 135, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 136.

[00159] Table 1. Sequences of an exemplary parent PD-L1 binding domain (MDX-1105 and atezolizumab)

Target		CDR1	CDR2	CDR3
PD-L1 (MDX-1105)	HCDR	SEQ ID NO: 1 GDTFSSYA	SEQ ID NO: 2 IIPIFGRA	SEQ ID NO: 3 ARKFHFVSGSP FGMDV
		SEQ ID NO: 39 GDTFSTYA	SEQ ID NO: 40 IIPIFGKA	
	LCDR	SEQ ID NO: 4 QSVSSY	SEQ ID NO: 5 DAS	SEQ ID NO: 6 QQRSNWPT
	VH	SEQ ID NO: 7 QVQLVQSGAEVKKPGSSVKVSCKTS**GDTFSTYA**IS WVRQAPGQGLEWMGG**IIPIFGKA**HYAQKFQGRVTI TADESTSTAYMELSSLRSEDTAVYFC**ARKFHFVSGS PFGMDV**WGQGTTVTVSS		
		SEQ ID NO: 113 QVQLVQSGAEVKKPGSSVKVSCKTS**GDTFSSYA**ISW VRQAPGQGLEWMGG**IIPIFGRA**HYAQKFQGRVTIT ADESTSTAYMELSSLRSEDTAVYFC**ARKFHFVSGSP FGMDV**WGQGTTVTVSS		
	VL	SEQ ID NO: 8 EIVLTQSPATLSLSPGERATLSCRAS**QSVSSY**LAWYQ QKPGQAPRLLIY**DAS**NRATGIPARFSGSGSGTDFTLTI SSLEPEDFAVYYC**QQRSNWPT**FGQGTKVEIK		
atezolizumab	HCDR	SEQ ID NO: 129 GFTFSDSW	SEQ ID NO: 130 ISPYGGST	SEQ ID NO: 131 ARRHWPGGFD Y
	LCDR	SEQ ID NO: 132 QDVSTA	SEQ ID NO: 133 SA	SEQ ID NO: 134 QQYLYHPAT
	VH	SEQ ID NO: 135 EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSDSW**IH WVRQAPGKGLEWVAW**ISPYGGST**YYADSVKGRFTI SADTSKNTAYLQMNSLRAEDTAVYYC**ARRHWPGG FDY**WGQGTLVTVSS		
	VL	SEQ ID NO: 136 DIQMTQSPSSLSASVGDRVTITCRAS**QDVSTA**VAWY QQKPGKAPKLLIY**SA**SFLYSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYC**QQYLYHPAT**FGQGTKVEIK		

[00160] In some embodiments, the low-affinity variant has one or more mutations in one or more of the CDR regions, in the heavy chain variable region and/or the light chain region of MDX-1105. In some embodiments, the low-affinity variant of the parent anti-PD-L1 antibody has a mutation at position 56 or position 58 (based on Kabat numbering) in the heavy chain variable region of the parent anti-PD-L1 antibody, *e.g.* MDX-1105. In some embodiments, the low-affinity variant has a mutation at position 32 or position 94 (based on KABAT numbering) in the light chain variable region of the parent anti-PD-L1 antibody, *e.g.* MDX-1105. In some embodiments, the mutation at position 56 is selected from the group consisting of: 56D, 56A, and 56F. In some embodiments, the mutation at position 32 is 32E, or the mutation at position 94 is selected from the group consisting of 94D and 94A.

[00161] In some embodiments, the low-affinity variant of MDX-1105 comprises a mutation at position K56 or H58 of the heavy chain variable region (SEQ ID NO: 7 or SEQ ID NO: 113), or at the position Y32 or W94 of the light chain variable region (SEQ ID NO: 8), according to KABAT numbering system.

[00162] In some embodiments, the low-affinity variant of MDX-1105 has a mutation at K56 or H58 (based on KABAT numbering) in the heavy chain variable region. In some embodiments, the mutation at K56 is selected from the group consisting of: K56D, K56A, and K56F. In some embodiments, the heavy chain variable region of the low-affinity variant of MDX-1105 comprises a HCDR2 comprising the amino acid sequence of SEQ ID NO: 41 [IIPIFG**D**A], SEQ ID NO: 42 [IIPIFG**A**A], or SEQ ID NO: 43 [IIPIFG**F**A].

[00163] In some embodiments, the low-affinity variant MDX-1105 has a mutation at Y32 or W94 (based on KABAT numbering) in the light chain variable region. In some embodiments, the mutation at Y32 is selected from the group consisting of Y32E, or the mutation W94 is selected from the group consisting of W94D and W94A. In some embodiments, the light chain variable region of the low-affinity variant of MDX-1105 comprises a LCDR1 comprising the amino acid sequence of SEQ ID NO: 44 [QSVSS**E**]. In some embodiments, the light chain variable region of

the low-affinity variant of MDX-1105 comprises a LCDR3 comprising the amino acid sequence of SEQ ID NO: 45 [QQRSN**D**PT], SEQ ID NO: 46 [QQRSN**A**PT].

[00164] In some embodiments, the low-affinity variant MDX-1105 comprises:

a) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 42, or SEQ ID NO: 43, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6;

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 44, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6; or

c) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 45 or SEQ ID NO: 46.

[00165] In a certain embodiment, the low-affinity variant of the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25 (K56D in VH), SEQ ID NO: 26 (K56A in VH), SEQ ID NO: 27 (K56F in VH), or SEQ ID NO: 28 (H58D in VH) and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8. In a certain embodiment, the low-affinity variant of the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7

and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 29 (Y32E in VL), SEQ ID NO: 30 (W94D in VL), or SEQ ID NO: 31 (W94A in VL).

[00166] In some embodiments, the low-affinity variant has one or more mutations in one or more of the CDR regions, in the heavy chain variable region and/or the light chain region of atezolizumab. In some embodiments, the low-affinity variant of the parent anti-PD-L1 antibody has a mutation at position 56 or position 53 (based on Kabat numbering) in the heavy chain variable region of the parent anti-PD-L1 antibody, *e.g.* atezolizumab. In some embodiments, the low-affinity variant has a mutation at position 30 (based on KABAT numbering) in the light chain variable region of the parent anti-PD-L1 antibody, *e.g.* atezolizumab. In some embodiments, the mutation at position 56 is 56G. In some embodiments, the mutation at position 53 is selected from the group consisting of: 53C, 53G, 53H, and 53Q. In some embodiments, the mutation at position 30 is selected from the group consisting of: 30H, 30T, and 30E.

[00167] In some embodiments, the low-affinity variant of atezolizumab comprises a mutation at position Y53 or S56 of the heavy chain variable region (SEQ ID NO: 135), or at the position S30 of the light chain variable region (SEQ ID NO: 136), according to KABAT numbering system.

[00168] In some embodiments, the low-affinity variant derived from atezolizumab has a mutation at Y53 or S56 (based on KABAT numbering) in the heavy chain variable region. In some embodiments, the mutation at Y53 is selected from the group consisting of Y53C, Y53G, Y53H and Y53Q, or the mutation S56 is S56G. In some embodiments, the light chain variable region of the low-affinity variant of atezolizumab comprises an HCDR2 comprising the amino acid sequence of SEQ ID NO: 137 [ISP**C**GGST], SEQ ID NO: 138 [ISP**G**GGST], SEQ ID NO: 139 [ISP**H**GGST], SEQ ID NO: 140 [ISP**Q**GGST] or SEQ ID NO: 141 [ISPYGG**G**T]. In some embodiments, the light chain variable region of the low-affinity variant of atezolizumab comprises an LCDR1 comprising the amino acid sequence of SEQ ID

NO: 142 [QDV**H**TA], SEQ ID NO: 143 [QDV**T**TA] or SEQ ID NO: 144 [QDV**E**TA].

[00169] In some embodiments, the low-affinity variant atezolizumab comprises:

a) an HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 137, SEQ ID NO: 138, or SEQ ID NO: 139, SEQ ID NO: 140 or SEQ ID NO: 141, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134; or

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 142, SEQ ID NO: 143 or SEQ ID NO: 144, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134.

[00170] In a certain embodiment, the low-affinity variant of the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 147 (S56G in VH), SEQ ID NO: 148 (Y53C in VH), SEQ ID NO: 149 (Y53G in VH), SEQ ID NO: 150 (Y53H in VH) or SEQ ID NO: 152 (Y53Q in VH) and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 136. In a certain embodiment, the low-affinity variant of the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 135 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 145 (S30H in VL), SEQ ID NO: 146 (S30T in VL), or SEQ ID NO: 151 (S30E in VL).

[00171] In another aspect, the present disclosure also provides the low-affinity anti-PD-L1 antibodies comprising the CDR sequences as disclosed above. In another

aspect, the present disclosure also provides the low-affinity anti-PD-L1 antibodies comprising the VH and/or the VL sequences as disclosed above.

[00172] Without wishing to be bound by any theory, it is believed the low-affinity PD-L1 binding domain helps extend the serum half-life of the multi-specific polypeptide complex provided herein, in particular in primates and/or humans. In some embodiments, the multi-specific polypeptide complex provided herein exhibits a serum half-life in a primate (*e.g.* monkey or human) of no less than 8 hours (no less than 12 hours, 18 hours, 24 hours, or 32 hours). In certain embodiments, the serum half-life in a primate is obtained after a single-dose administration at a dose of at least 1mg/kg. In certain embodiments, the serum half-life in a primate is obtained using methods described in the Example 1.4.

[00173] In addition, despite of the low-affinity in PD-L1 binding, the multi-specific polypeptide complex provided herein substantially maintains its anti-tumor efficacy.

[00174] In another aspect, the present disclosure further provides methods of modifying a parent multi-specific polypeptide complex to produce a modified multi-specific polypeptide complex having improved half life in a primate, wherein the multi-specific polypeptide complex comprises a CD3-binding domain capable of specifically binding to CD3, a PD-L1 binding domain capable of specifically binding to PD-L1, wherein the method comprises: introducing one or more mutations to the PD-L1 binding domain to produce a modified PD-L1 binding domain having reduced binding affinity to PD-L1 characterized in a Kd value of no less than 10^{-8} M as measured by BLI.

[00175] In some embodiments, the modified PD-L1 binding domain binds to human PD-L1 at a Kd value ranging from 10^{-5} M to 10^{-8} M, 7E-6 M to 1E-8 M, from 4E-6 M to 1E-8 M, from 1E-6 M to 1E-8 M, 8E-7 M to 1E-8 M, from 8E-7 M to 2E-8 M, from 8E-7 M to 3E-8 M, from 8E-7 M to 4E-8 M, from 8E-7 M to 5E-8 M, from 8E-7 M to 6E-8 M, from 8E-7 M to 7E-8 M, from 8E-7 M to 8E-8 M, from 8E-7 M to 9E-8 M, from 8E-7 M to 1E-7 M, from 3E-6 M to 3E-8 M, from 7E-6 M to 3E-8 M, from

1E-6 M to 3E-8 M, 9E-7 M to 3E-8 M, 8E-7 M to 3E-8 M, 7E-7 M to 3E-8 M, or 3E-7 M to 3E-8 M as measured by BLI.

[00176] In some embodiments, the multi-specific polypeptide complex further comprises a first disease antigen binding domain.

[00177] In some embodiments, the modified multi-specific polypeptide complex has a half-life extended by at least 4 hours or at least 8 hours in a primate relative to the parent multi-specific polypeptide complex.

[00178] In some embodiments, the multi-specific polypeptide complex further comprises an Fc domain. In some embodiments, the Fc domain is modified and has reduced binding or lack binding to human CD32a. In some embodiments, the modified multi-specific polypeptide complex has a half-life extended by at least 8 hours or at least 12 hours in a primate relative to the parent multi-specific polypeptide complex.

(ii) CD3 Binding Domain

[00179] In some embodiments, the multi-specific polypeptide complex provided herein comprises a CD3-binding domain that specifically binds to CD3, in particular, human CD3. In some embodiments, the CD3-binding domain is capable of specifically binding to CD3 at a K_D value of no more than 10^{-6} as measured by Surface Plasmon Resonance (SPR).

[00180] In some embodiments, the CD3-binding domain can be derived from any anti-CD3 antibodies. Examples of anti-CD3 antibodies include, without limitation, SP34, L2K, UCHT1, TR66, and 38E4, or the humanized version thereof. SP34 is a mouse antibody (see, EMBO J.1985.4(2):337-344; J.Immunol.1986,137(4):1097-100), and the sequences for humanized VH and VL are set forth in SEQ ID NOs: 15 and 16).

[00181] The VH sequence and VL sequence of the antibodies L2K, UCHT1, TR66, and 38E4 are provided below:

[00182] Antibody UCHT1:

DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSR LHSGVPSKFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGGTKLEIK (SEQ ID NO: 121)

EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWMGLI NPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYY GDSDWYFDVWGQGTTLTVFS (SEQ ID NO: 122)

[00183] Antibody OKT3:

[00184] DIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRW IYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGS GTKLEINRADTAPT (SEQ ID NO: 123)

[00185] QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQG LEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYC ARYYDDHYSLDYWGQGTTLTVSS (SEQ ID NO: 124)

[00186] Antibody L2K:

[00187] DIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRW IYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGA GTKLELK(SEQ ID NO: 125)

[00188] DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGL EWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCA RYYDDHYCLDYWGQGTTLTVSS (SEQ ID NO: 126)

[00189] Antibody 38E4:

[00190] QVQLQQSGPELVKPGASVTISCTASGFTFTSYYIHWVRQRPGQGLE WIGWIYPENDNTKYNEKFKDKATLTADTSSSTAYMQLSSLTSEDSAVYYCAR DGYSRYYFDYWGQGTTLTVSS(SEQ ID NO: 127)

[00191] DIVMTQSPSFLAVSAGEKVTMSCKSSQSLLNSRTRKNYLAWYQQRP GQSPKLLIYWTSTRKSGVPDRFTGSGSGTDFTLTISSVQAEDLAIYYCKQSFIL RTFGGGTKLEIK(SEQ ID NO: 128)

[00192] In certain embodiment, the CD3-binding domain is derived from the antibody huSP34 and comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14.

(iii) Low Affinity CD3 Binding Domain

[00193] In some embodiments, in the multi-specific polypeptide complex provided herein, the CD3-binding domain is a low-affinity CD3-binding domain. In some embodiments, the low-affinity CD3-binding domain is capable of specifically binding to CD3 at a K_D value of no less than 5E-8 as measured by BLI method or Surface Plasmon Resonance (SPR). In certain embodiments, the K_D of the low-affinity CD3-binding domain is determined in accordance with the method as described in section 2.2 of Example 2 and section 3.2.2 of Example 3 in the present disclosure.

[00194] In some embodiments, the CD3 binding domain is capable of specifically binding to human CD3 at a K_D value of no less than 1E-8 M, no less than 5E-8 M, no less than 6E-8 M, no less than 7E-8 M, no less than 8E-8 M, or no less than 9E-8 M as measured by Surface Plasmon Resonance (SPR).

[00195] In some embodiments, the CD3 binding domain is capable of specifically binding to human CD3 at a K_D value of no more than 5E-7 M, or no more than 4E-7 M, no more than 3E-7 M, or no more than 2E-7 M as measured by SPR.

[00196] In some embodiments, the CD3 binding domain is capable of specifically binding to human CD3 at a K_D value ranging from 5E-7 M to 1E-8 M, 2E-7 M to 1E-8 M, from 2E-7 M to 2E-8 M, from 2E-7 M to 3E-8 M, from 2E-7 M to 4E-8 M, from

2E-7 M to 5E-8 M, from 2E-7 M to 6E-8 M, from 2E-7 M to 7E-8 M, from 2E-7 M to 8E-8 M, from 2E-7 M to 9E-8 M, from 2E-7 M to 1E-7 M, from 3E-7 M to 5E-8 M, or from 5E-7 M to 5E-8 M as measured by SPR.

[00197] In some embodiments, the low-affinity CD3 binding domain is capable of specifically binding to human CD3 at a K_D value ranging from 5.28E-8 M to 2E-7 M, 5.28E-8 M to 1.77E-7 M, 5.28E-8 M to 1.5E-7 M, 5.28E-8 M and 1.18E-7 M as measured by SPR.

[00198] Low-affinity CD3 binding domains can be obtained using different methods known in the art, including those methods described above under low-affinity PD-L1 binding domain, and is incorporated herein.

[00199] For example, the binding site of the antibody can be identified, and mutations introduced to disrupt at least some of the hydrophobic interactions, electrostatic interactions, and/or hydrogen bonds. Mutants can be validated to determine the K_D value, and those with suitable K_D values can be selected as the low-affinity PD-L1 binding domain in the multi-specific polypeptide complexes provided herein.

[00200] In some embodiments, the low-affinity CD3 binding domain is derived from a low-affinity variant of a parent anti- CD3 antibody, wherein the low-affinity variant has reduced binding affinity to human CD3 than the parent antibody. In some embodiments, the low-affinity variant specifically binds to human CD3 at a K_D value at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%. 90%, 100%, 150%, 200%, 3-fold, or 4-fold higher than that of the parent antibody. In some embodiments, the low-affinity CD3 binding domain of the multi-specific polypeptide complex specifically binds to human CD3 at a K_D value at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%. 90%, 100%, 150%, 200%, 3-fold, 4-fold, 10-fold, 15-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold higher or even higher than that of the parent CD3 binding domain of the multi-specific polypeptide complex.

[00201] In some embodiments, the low-affinity CD3 binding domain is derived from a low-affinity variant of a very low-affinity parent anti- CD3 antibody (e.g. having a K_D value of more than 1E-6), wherein the low-affinity variant has improved binding affinity to human CD3 than the very low-affinity parent antibody. In some embodiments, the low-affinity variant specifically binds to human CD3 at a K_D value at least 20% lower, preferably 50% lower, more preferably 1-fold lower, or even 10-fold lower than that of the parent antibody. In some embodiments, the low-affinity CD3 binding domain of the multi-specific polypeptide complex specifically binds to human CD3 at a K_D value at least 20% lower, preferably 50% lower, more preferably 1-fold lower, or even 10-fold lower than that of the parent CD3 binding domain of the multi-specific polypeptide complex.

[00202] In some embodiments, the low-affinity variant has one or more mutations in the amino acid residues on the heavy chain variable region and/or the light chain region.

[00203] In some embodiments, the low-affinity CD3 binding domain is derived from a low-affinity variant of a parent anti-CD3 antibody selected from the group consisting of SP34, L2K, UCHT1, TR66, 38E4, and their humanized variants thereof, wherein the low-affinity variant has reduced binding affinity to human CD3 than the parent antibody.

[00204] In some embodiments, the low-affinity CD3 binding domain is derived from a low-affinity variant of a parent anti-CD3 antibody SP34 or its humanized variant huSP34. In certain embodiment, the parent anti-CD3 antibody huSP34 comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14.

[00205] In a certain embodiment, the parent anti-CD3 antibody huSP34 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 15, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16.

[00206] Table 2. sequences of an exemplary CD3 binding domain

Target		CDR1	CDR2	CDR3
CD3 (huSP34)	HCDR	SEQ ID NO: 9 GFTFSTYA	SEQ ID NO: 10 IRSKYNNYAT	SEQ ID NO: 11 VRHGNFGTSY VSWFAY
	LCDR	SEQ ID NO: 12 TGAVTTSNY	SEQ ID NO: 13 GTN	SEQ ID NO: 14 ALWYSNLWV
	VH	SEQ ID NO: 15 EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSTYA**MN WVRQAPGKGLEWVGR**IRSKYNNYAT**YYADSVKGR FTISRDDSKNSLYLQMNSLRAEDTAVYYC**VRHGNF GTSYVSWFAY**WGQGTLVTVSS		
	VL	SEQ ID NO: 16 QAVVTQEPSLTVSPGGTVTLTCRSS**TGAVTTSNY**AN WVQQKPGQAPRGLIG**GTN**KRAPWTPARFSGSLLGG KAALTLSGAQAEDEADYYC**ALWYSNLWV**FGGGTK LTVL		

[00207] In some embodiments, the low-affinity variant has one or more mutations in one or more of the CDR regions, in the heavy chain variable region and/or the light chain region of huSP34.

[00208] In some embodiments, the low-affinity variant of the parent anti-CD3 antibody has a mutation at position 52B, 52C, 28, or 100C (based on Kabat numbering) in the heavy chain variable region of the parent anti-CD3 antibody, *e.g.* huSP34. In some embodiments, the mutation at position 52B is selected from the group consisting of: 52B Q and 52B H. In some embodiments, the mutation at position 52C is 52C F. In some embodiments, the mutation at position 28 is 28V. In some embodiments, the mutation at position 100C is 100C A.

[00209] In some embodiments, the low-affinity variant of huSP34 comprises a mutation at K52B, Y52C, T28, V100C or S100D in the heavy chain variable region of the parent anti-CD3 antibody, according to the KABAT numbering system. In some embodiments, the mutation at K52B is selected from the group consisting of: K52B Q

and K52B H. In some embodiments, the mutation at Y52C is selected from the group consisting of Y52C F. In some embodiments, the mutation at T28 is selected from the group consisting of T28V. In some embodiments, the mutation at V100C is selected from the group consisting of V100C A. In some embodiments, the mutation at S100D is selected from the group consisting of S100D T. In some embodiments, the heavy chain variable region of the low-affinity variant of huSP34 comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 68 [GF**V**FSTYA]. In some embodiments, the heavy chain variable region of the low-affinity variant of huSP34 comprises a HCDR2 comprising the amino acid sequence of SEQ ID NO: 69 [IRS**H**YNNYAT], SEQ ID NO: 71 [IRS**Q**YNNYAT], SEQ ID NO: 73 [IRSK**F**NNYAT], or SEQ ID NO: 103 [IRS**D**YNNYAT]. In some embodiments, the heavy chain variable region of the low-affinity variant of huSP34 comprises a HCDR3 comprising the amino acid sequence of SEQ ID NO: 75 [VRHGNFGTSY**A**SWFAY] or SEQ ID NO: 102 [VRHGNFGTSYV**T**WFAY].

[00210] In some embodiments, the low-affinity variant huSP34 comprises:

a) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 68, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14;

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, or SEQ ID NO: 103, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14; or

c) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the

amino acid sequence of SEQ ID NO: 75 or SEQ ID NO: 102, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14.

[00211] In a certain embodiment, the low-affinity variant of huSP34 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 32, 33, 34, 35, 36, 37 or 38; and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16. In some embodiments, the low-affinity variant of huSP34 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 34, and the VL1 comprises an amino acid sequence of SEQ ID NO: 16.

[00212] In a certain embodiment, the low-affinity variant of huSP34 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 34 (K52B Q in VH) and SEQ ID NO: 33 (K52B H in VH), and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16.

[00213] In another aspect, the present disclosure also provides the low-affinity anti-CD3 antibodies comprising the CDR sequences as disclosed above. In another aspect, the present disclosure also provides the low-affinity anti-CD3 antibodies comprising the VH and/or the VL sequences as disclosed above.

(iv) Disease Antigen Binding Domain

[00214] In some embodiments, the multi-specific polypeptide complex comprises a first disease antigen binding domain.

[00215] The "disease antigen" as used herein encompass all kinds of antigens or targets that are associated with a disease or with a condition desired to be treated, and can be recognized by an antibody or antigen-binding domain. Disease antigen can be proteins, polypeptide or fragment thereof, nucleic acids, small molecules, and so on.

[00216] Examples of a disease antigen can be a tumor-associated antigen, an immune-associated antigen, an inflammation-associated antigen, an antigen associated with an eye disorder, an antigen associated with a central nervous system disease, an antigen associated with an infectious disease, or an antigen associated with a coagulation disorder, among others.

[00217] In some embodiments, the disease antigen is a tumor-associated antigen (TAA). In some embodiments, the disease antigen is an immune-associated antigen. Examples of immune-associated antigen include, without limitation, antigens relating to autoimmune diseases, an immune checkpoint target, or a molecule expressed on an immune cell. In some embodiments, the disease antigen is an inflammation-associated antigen.

[00218] A tumor-associated antigen include antigens presented on a tumor cell surface, located on or within tumor cells, presented only by tumor cells and not by normal, *i.e.*, non-tumor cells, representing a protein harboring one or more tumor-specific mutations compared to non-tumor cells, overexpressed in tumor cells when compared to non-tumor cells; accessible for antibody binding in tumor cells due to the less compact structure of the tumor tissue compared to non-tumor tissue, and presented on the vasculature of a tumor, etc. is meant an antigenic substance produced in tumor cells, *i.e.*, it triggers an immune response in the host. In some embodiments, a tumor-associated antigen is an antigen that is present in a tumor that is not present in normal organs, tissues, and/or cells. In some embodiments, a tumor-associated antigen is an antigen that is more prevalent in a tumor than in normal organs, tissues, and /or cells. In some embodiments, a tumor-associated antigen is an antigen that is more prevalent in malignant cancer cells than in normal cells.

[00219] Examples of tumor-associated antigens include, but are not limited to: CD19, CD20, CD38, CD30, BCMA, GPRC5D, Her2/neu/ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high

molecular weight-melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, and the like. Tumor-associated antigens also include, e.g., 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNTO888, CTLA-4, DRS, EGFR, EpCAM, CD3, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HER2/neu, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, L1-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin α5β1, integrin αvβ3, MORAb-009, MS4A1, MUC1, mucin CanAg, N-glycolylneuraminic acid, NPC-1C, PDGF-R α, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF-β, TRAIL-R1, TRAIL-R2, tumor-associated antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, and vimentin, etc.

[00220] In some embodiments, the tumor-associated antigen is CEA, EGFR, CD20, CLDN6, GPRC5D, or the variants or analogs thereof.

[00221] In some embodiments, the tumor-associated antigen is CEA, and the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 17, HCDR2 comprising the amino acid sequence of SEQ ID NO: 18, HCDR3 comprising the amino acid sequence of SEQ ID NO: 19, LCDR1 comprising the amino acid sequence of SEQ ID NO: 20, LCDR2 comprising the amino acid sequence of SEQ ID NO: 21, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 22. In some embodiments, the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 23, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 24.

[00222] In some embodiments, the tumor-associated antigen is EGFR, and the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 78, HCDR2 comprising the amino acid sequence of SEQ ID NO: 79, HCDR3 comprising the amino acid sequence of SEQ ID NO: 80, LCDR1 comprising the amino acid sequence of SEQ ID NO: 81, LCDR2 comprising the amino acid sequence of SEQ ID NO: 82, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 83. In some embodiments, the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 84, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 85.

[00223] In some embodiments, the tumor-associated antigen is CD20, and the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 105, HCDR2 comprising the amino acid sequence of SEQ ID NO: 106, HCDR3 comprising the amino acid sequence of SEQ ID NO: 107, LCDR1 comprising the amino acid sequence of SEQ ID NO: 108, LCDR2 comprising the amino acid sequence of SEQ ID NO: 109, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 110. In some embodiments, the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 111, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 112.

[00224] In some embodiments, the tumor-associated antigen is CLND6, and the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 86, HCDR2 comprising the amino acid sequence of SEQ ID NO: 87, HCDR3 comprising the amino acid sequence of SEQ ID NO: 88, LCDR1 comprising the amino acid sequence of SEQ ID NO: 89, LCDR2 comprising the amino acid sequence of SEQ ID NO: 90, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 91. In some

embodiments, the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 92, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 93.

[00225] In some embodiments, the tumor-associated antigen is GPRC5D, and the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 94, HCDR2 comprising the amino acid sequence of SEQ ID NO: 95, HCDR3 comprising the amino acid sequence of SEQ ID NO: 96, LCDR1 comprising the amino acid sequence of SEQ ID NO: 97, LCDR2 comprising the amino acid sequence of SEQ ID NO: 98, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 99. In some embodiments, the first disease antigen comprised in the multi-specific polypeptide complex provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 100, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 101.

[00226] Table 3. Sequences of exemplary TAA binding domains

Target		CDR1	CDR2	CDR3
CEA hAb009	HCDR	SEQ ID NO: 17 GFTFTDYY	SEQ ID NO: 18 IRNKVNGDTT	SEQ ID NO: 19 ARDKGIAYYFDY
	LCDR	SEQ ID NO: 20 QSVSY	SEQ ID NO: 21 GTS	SEQ ID NO: 22 QQWSSNLST
	VH	SEQ ID NO: 23 EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMN WVRQAPGKALEWLGFIRNKVNGDTTEYAASVKGRF TISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAY YFDYWGQGTLVTVSS		
	VL	SEQ ID NO: 24 EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQ KPGQSPRPWIHGTSNLATGVPARFSGSGSGTDFTLTI SSLEPEDAAVYYCQQWSSNLSTFGGGTKVEIK		
EGFR nimotuzumab	HCDR	SEQ ID NO: 78 GYTFTNYY	SEQ ID NO: 79 INPTSGGS	SEQ ID NO: 80 ARQGLWFDSD GRGFDF
	LCDR	SEQ ID NO: 81 QNIVHSNGNTY	SEQ ID NO: 82 KV	SEQ ID NO: 83 FQYSHVPWT
	VH	SEQ ID NO: 84		

		QVQLQQSGAEVKKPGSSVKVSCKASGYTFTNYYIY WVRQAPGQGLEWIGGINPTSGGSNFNEKFKTRVTIT VDESTNTAYMELSSLRSEDTAFYFCARQGLWFDSDG RGFDFWGQGSTVTVSS		
	VL	SEQ ID NO: 85 DIQMTQSPSSLSASVGDRVTITCRSSQNIVHSNGNTY LDWYQQTPGKAPKLLIYKVSNRFSGVPSRFSGSGSG TDFTFTISSLQPEDIATYYCFQYSHVPWTFGQGTKLQ IT		
CLDN6 (GB7008-ch01)	HCDR	SEQ ID NO: 86 GYSITSGYY	SEQ ID NO: 87 ISYSGSN	SEQ ID NO: 88 ARGGYYFDY
	LCDR	SEQ ID NO: 89 QSLLNTNNQK NY	SEQ ID NO: 90 FA	SEQ ID NO: 91 QQHYNAPRT
	VH	SEQ ID NO: 92 QVQLQESGPGLVKPSETLSLTCTVSGYSITSGYYWN WIRQPPGKGLEWMGYISYSGSNNYNPSLKNRVTISR DTSKNQFSLKLSSVTAADTAVYYCARGGYYFDYWG QGTLVTVSS		
	VL	SEQ ID NO: 93 DIVMTQSPDSLAVSLGERATINCKSSQSLLNTNNQK NYLAWYQQKPGQPPKLLIYFASTRESGVPDRFSGSG SGTDFTLTISSLQAEDVAVYFCQQHYNAPRTFGGGT KVEIK		
GPRC5D 72c7	HCDR	SEQ ID NO: 94 GYPFTNYW	SEQ ID NO: 95 INPSNGRT	SEQ ID NO: 96 ARGFAY
	LCDR	SEQ ID NO: 97 SSVSF	SEQ ID NO: 98 DT	SEQ ID NO: 99 QQWNSHPLT
	VH	SEQ ID NO: 100 QVQLQESGPGLVKPSETLSLTCTASGYPFTNYWMH WVRQPPGKGLEWIGEINPSNGRTNYNEKFKSRATLS LDKSKNQASLNLDSVSAADTAIYYCARGFAYWGKG STVTVSS		
	VL	SEQ ID NO: 101 AIQLTQSPSSLSASVGDRVTITCSASSSVSFMHWYQQ KPGKAPKRWIYDTTKLASGVPSRFSGSGSGTDYTFTI SSLQPEDIATYYCQQWNSHPLTFGPGTKVDIK		
CD20 obinutuzumab	HCDR	SEQ ID NO: 105 GYAFSYSW	SEQ ID NO: 106 IFPGDGDT	SEQ ID NO: 107 ARNVFDGYWL VY
	LCDR	SEQ ID NO: 108 KSLLHSNGITY	SEQ ID NO: 109 QM	SEQ ID NO: 110 AQNLELPYT
	VH	SEQ ID NO: 111 QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWIN WVRQAPGQGLEWMGRIFPGDGDTDYNGKFKGRVTI TADKSTSTAYMELSSLRSEDTAVYYCARNVFDGYW LVYWGQGTLVTVSS		

	VL	SEQ ID NO: 112 DIVMTQTPLSLPVTPGEPASISCRSSKSLLHSNGITYL YWYLQKPGQSPQLLIYQMSNLVSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCAQNLELPYTFGGGTKVE IK

(v) Modified Fc Domain having low affinity or null binding to FcγR

[00227] In some embodiments, the multi-specific polypeptide complex provided herein further comprises a dimerization domain. In some embodiments, the dimerization domain comprises an Fc domain. In some embodiments, the Fc domain in the multi-specific polypeptide complex comprises a first Fc chain and a second Fc chain, wherein the first Fc chain and the second Fc chain are associated to form a dimer.

[00228] In certain embodiments, the multi-specific polypeptide complex comprises an Fc domain that is modified and has reduced binding or lacks substantial binding to certain human Fcγ receptor (FcγR). FcγR family includes several members, such as FcγRI (CD64), FcγRIIA (CD32a), FcγRIIB (CD32b), FcγRIIIA (CD16a), FcγRIIIB (CD16b), which differ in their antibody affinities due to their different molecular structure.

[00229] In certain embodiments, the multi-specific polypeptide complex comprises an Fc domain that is modified and has reduced binding or lacks substantial binding to human CD32a. The present inventors found that in addition to the modified low affinity to PD-L1, the modifications on the Fc domain of the multi-specific polypeptide complex can also result in unexpected effects, including lowering level of cytokine release, restoration of the reduced anti-tumor activity caused by the reduced affinity to PD-L1, and further extension of the half-life of the multi-specific polypeptide complex.

[00230] In some embodiments, the modified Fc domain has reduced binding to human CD32a relative to the counterpart wild-type Fc domain. In some embodiments, the modified Fc domain binds to human CD32a at a K_D value of no less than 10^{-7} M, no less than 10^{-6} M, no less than 10^{-5} M as measured by BLI. In some embodiments,

the Fc domain lacks substantial binding to human CD32a, such that K_D value cannot be calculated by BLI method. In some embodiments, the Fc domain does not show detectable binding to human CD32a. In certain embodiments, the K_D of the Fc domain binding to human CD32a is determined in accordance to the method as described in section 5.2 of Example 5 in the present disclosure.

[00231] In certain embodiments, the modified Fc domain further has reduced binding or lacks substantial binding to human CD16 and/or human CD64.

[00232] In some embodiments, the modified Fc domain further has reduced binding to human CD16 relative to the counterpart wild-type Fc domain. In some embodiments, the modified Fc domain binds to human CD16 at a K_D value of no less than 10^{-7} M, no less than 10^{-6} M, no less than 10^{-5} M, or even higher as measured by BLI. In some embodiments, the Fc domain lacks substantial binding to human CD16, such that K_D value cannot be calculated by BLI method. In some embodiments, the Fc domain does not show detectable binding to human CD16. In certain embodiments, the K_D of the Fc domain binding to human CD16 is determined in accordance to the method as described in section 5.2 of Example 5 in the present disclosure.

[00233] In some embodiments, the modified Fc domain further has reduced binding to human CD64 relative to the counterpart wild-type Fc domain. In some embodiments, the modified Fc domain binds to human CD64 at a K_D value of no less than 10^{-7} M, no less than 10^{-6} M, no less than 10^{-5} M, or even higher as measured by BLI. In some embodiments, the Fc domain lacks substantial binding to human CD64, such that K_D value cannot be calculated by BLI method. In some embodiments, the Fc domain does not show detectable binding to human CD64. In certain embodiments, the K_D of the Fc domain binding to human CD64 is determined in accordance with the method as described in section 5.2 of Example 5 in the present disclosure.

[00234] In certain embodiments, the Fc domain has reduced binding or lacks substantial binding to human CD32a, human CD16 and human CD64.

[00235] In some embodiments, the modified Fc domain of the multi-specific polypeptide complex is engineered to have reduced binding affinity to CD32a, human CD16 and/or human CD64, as compared to a non-engineered parent Fc domain. In one such embodiment the modified Fc domain (or the multi-specific polypeptide complex comprising said Fc domain) exhibits less than 50%, preferably less than 20%, more preferably less than 10% and most preferably less than 5% of the binding affinity to human CD32a, human CD16 and/or human CD64, as compared to a non-engineered Fc domain (or the multi-specific polypeptide complex comprising said Fc domain).

[00236] Modified Fc domains having reduced binding or lacks substantial binding to certain human FcγR can be obtained using different methods known in the art. In certain embodiments, one may select an existing Fc domain for modification to reduce its binding affinity to FcγR. This can be done by mutating one or more residues involved in binding to FcγR, such that the binding affinity to FcγR can be reduced or optimized. For a given Fc domain, a skilled person would understand how to identify the amino acid residues to mutation, and also to expect what amino acid residue to mutate into.

[00237] In some embodiments, the Fc domain comprised in the multi-specific polypeptide complex provided herein is derived from human IgG4.

[00238] In some embodiments, the Fc domain comprised in the multi-specific polypeptide complex provided herein is derived from human IgG1, and optionally has a mutation at position 247 and/or 248, according to Kabat numbering system. In some embodiments, the mutation at position 247 is selected from the group consisting of L247A and L248F; and/or the mutation at position 248 is selected from the group consisting of L248A and L248E. In some embodiments, the mutations at position 247 and position 248 are selected from the group consisting of: a) L247A/L248A, and b) L247F/L248E.

[00239] In some embodiments, the Fc domain comprised in the multi-specific polypeptide complex provided herein further comprises one or more additional mutations at the positions selected from the group consisting of position 278, 249, 314, 341, 348, and 350, according to Kabat numbering system. In some embodiments, the Fc domain further comprises one or more additional mutations at the positions selected from the group consisting of D278A, G249R, N314A, N314Q, K341A, P348G, and P350S.

[00240] In some embodiments, the Fc domain further comprises one or more additional mutations selected from the group consisting of a) L247F/L248E/P350S; b) L247A/L248A/P348G; c) L247F/L248E/P350S; and d) L247A/L248A/K341A.

[00241] In some embodiments, the Fc domain comprised in the multi-specific polypeptide complex provided herein is derived from human IgG4.

[00242] In some embodiments, the Fc domain comprises the amino acid sequence of SEQ ID NO: 114 or 115.

[00243] In another aspect, the present disclosure further provides methods of modifying a multi-specific polypeptide complex to reduce its ability to induce cytokine release in a subject, wherein the multi-specific polypeptide complex comprises a CD3-binding domain capable of specifically binding to CD3 and an Fc domain that are operably linked to allow the multi-specific polypeptide complex to bind to CD3, the method comprises: introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a. In some embodiment, the multi-specific polypeptide complex further comprises a disease antigen binding domain, wherein the disease antigen binding domain and the CD3-binding domain and the Fc domain are operably linked to allow the multi-specific polypeptide complex to bind to CD3 and the disease antigen.

[00244] In another aspect, the present disclosure further provides methods of modifying a multi-specific polypeptide complex to improve its potency or improve its

therapeutic window in a subject, wherein the multi-specific polypeptide complex comprises: a) a low-affinity CD3-binding domain capable of specifically binding to CD3 at a Kd value of no less than 10^{-7} M or no less than 10^{-8} M as measured by Surface Plasmon Resonance (SPR), and b) an Fc domain, that are operably linked to allow the multi-specific polypeptide complex to bind to CD3 and the disease antigen, the method comprises: introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a. In some embodiment, the multi-specific polypeptide complex further comprises a disease antigen binding domain. In some embodiment, the low-affinity CD3-binding domain binds to human CD3 at a Kd value ranging from 10^{-5} M to 10^{-8} M, 10^{-6} M to 10^{-8} M, 5E-7 M to 1E-8 M, 2E-7 M to 1E-8 M, from 2E-7 M to 2E-8 M, from 2E-7 M to 3E-8 M, from 2E-7 M to 4E-8 M, from 2E-7 M to 5E-8 M, from 2E-7 M to 6E-8 M, from 2E-7 M to 7E-8 M, from 2E-7 M to 8E-8 M, from 2E-7 M to 9E-8 M, from 2E-7 M to 1E-7 M, from 3E-7 M to 5E-8 M, or from 5E-7 M to 5E-8 M as measured by SPR.

[00245] In another aspect, the present disclosure further provides methods of modifying a multi-specific polypeptide complex to improve its potency or improve its therapeutic window in a subject, wherein the multi-specific polypeptide complex comprises: a) a low-affinity PD-L1-binding domain capable of specifically binding to PD-L1 at a Kd value of no less than 10^{-8} M as measured by BLI, b) a CD3-binding domain, and c) an Fc domain, that are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1 and CD3, the method comprises: introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a. In some embodiment, the multi-specific polypeptide complex further comprises a disease antigen binding domain, wherein the disease antigen binding domain and the CD3-binding domain, the PD-L1 binding domain and the Fc domain are operably linked to allow the multi-specific polypeptide complex to bind to CD3, PD-L1 and the disease antigen.

[00246] In some embodiment, the low-affinity PD-L1-binding domain binds to human PD-L1 at a Kd value ranging from 10^{-5} M to 10^{-8} M as measured by BLI (for example, from 7E-6 M to 1E-8 M, from 4E-6 M to 1E-8 M, from 1E-6 M to 1E-8 M, from 8E-7 M to 1E-8 M, from 8E-7 M to 2E-8 M, from 8E-7 M to 3E-8 M, from 8E-7 M to 4E-8 M, from 8E-7 M to 5E-8 M, from 8E-7 M to 6E-8 M, from 8E-7 M to 7E-8 M, from 8E-7 M to 8E-8 M, from 8E-7 M to 9E-8 M, from 8E-7 M to 1E-7 M, from 3E-6 M to 3E-8 M, from 7E-6 M to 3E-8 M, from 1E-6 M to 3E-8 M, 9E-7 M to 3E-8 M, 8E-7 M to 3E-8 M, 7E-7 M to 3E-8 M, or 3E-7 M to 3E-8 M as measured by BLI.

[00247] In some embodiment, the CD3-binding domain is a low-affinity CD3-binding domain capable of specifically binding to CD3 at a Kd value of no less than 10^{-7} M as measured by Surface Plasmon Resonance (SPR). In some embodiments, the CD3 binding domain is capable of specifically binding to human CD3 at a K_D value of no less than $1*10^{-8}$ M, no less than $5*10^{-8}$ M, no less than $6*10^{-8}$ M, no less than $7*10^{-8}$ M, no less than $8*10^{-8}$ M, or no less than $9*10^{-8}$ M as measured by SPR (for example, from 5E-7 M to 1E-8 M, 2E-7 M to 1E-8 M, from 2E-7 M to 2E-8 M, from 2E-7 M to 3E-8 M, from 2E-7 M to 4E-8 M, from 2E-7 M to 5E-8 M, from 2E-7 M to 6E-8 M, from 2E-7 M to 7E-8 M, from 2E-7 M to 8E-8 M, from 2E-7 M to 9E-8 M, from 2E-7 M to 1E-7 M, from 3E-7 M to 5E-8 M, from 5E-7 M to 5E-8 M, or from 1E-6 M to 5E-8 M as measured by SPR).

[00248] In some embodiment, the modified Fc domain has reduced binding or lacks substantial binding to both human CD32a and human CD64.

[00249] In some embodiment, the modified Fc domain has reduced binding or lacks substantial binding to each of human CD32a, human CD64, and human CD16a.

[00250] In some embodiment, the Fc domain is derived from human IgG1, and the one or more mutations is at position 247 and/or 248 (Kabat numbering).

[00251] In some embodiment, the mutation a position 234 is selected from the group consisting of L247A and L248F; and/or the mutation at position 235 is selected from the group consisting of L247A and L248E.

[00252] In some embodiment, the mutations at position 247 and position 248 are selected from the group consisting of: a) L247A/L248A, and b) L247F/L248E.

[00253] In some embodiment, the modified Fc domain further comprises one or more additional mutations at the positions selected from the group consisting of position 278, 249, 314, 341, 348, and 350.

[00254] In some embodiment, the one or more additional mutations comprises D278A, G249R, N314A, N314Q, K341A, P348G, P350S, or any combination thereof.

[00255] In some embodiment, the one or more additional mutations are selected from the group consisting of a) L247F/L248E/P350S; b) L247A/L248A/P348G; c) L247F/L248E/P350S; and d) L247A/L248A/K341A.

[00256] In some embodiment, the modified multi-specific polypeptide complex exhibits at least 40% increase (e.g. at least 50%, 60%, 70%, 80%, 90%, 1-fold, 2-fold, 3-fold, 4-fold, or 5-fold increase) in EC50 in inducing IL-6 release from the CD3-expressing immune cell (*e.g.*, T cell).

[00257] In some embodiment, the modified multi-specific polypeptide complex exhibits at least 5 fold increase (e.g. at least 6-fold, 8-fold, 10-fold, 15-fold, 20-fold, or 30 fold increase) in therapeutic window, wherein the therapeutic window is represented by a ratio of EC50 in inducing IL-6 release from the CD3-expressing immune cell (*e.g.*, T cell), to EC50 in killing a disease-antigen expressing cell in the presence of a CD3-expressin immune cell (*e.g.*, T cell).

(vi) Format of the Multi-specific Polypeptide Complex

[00258] In some embodiments, the CD3-binding domain, PD-L1 binding domain and the first disease antigen binding domain are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1, CD3 and the first disease antigen.

[00259] In some embodiments, the CD3-binding domain, PD-L1 binding domain, the first disease antigen binding domain and the dimerization domain (*e.g.*, the Fc

domain) are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1, CD3 and the first disease antigen.

[00260] In some embodiments, the dimerization domain comprises an Fc domain. In some embodiments, the Fc domain in the multi-specific polypeptide complex comprises a first Fc chain and a second Fc chain, wherein the first Fc chain and the second Fc chain are associated to form a dimer.

[00261] In some embodiments, the multi-specific polypeptide complex further comprises a second disease antigen binding domain. In some embodiments, the first disease antigen binding domain and the second disease antigen binding domain bind to the same target. In some embodiments, the first disease antigen binding domain and the second disease antigen binding domain are identical.

[00262] In some embodiments, the whole structure of the multi-specific polypeptide complex is symmetric or asymmetric.

[00263] In some embodiments, the CD3-binding domain, PD-L1 binding domain, the first disease antigen binding domain, the second disease antigen binding domain and the dimerization domain (*e.g.*, the Fc domain) are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1, CD3, the first disease antigen and the second disease antigen.

[00264] In some embodiments, the PD-L1 binding domain is operably linked to the first disease antigen binding domain, or to the CD3-binding domain, or to dimerization domain (*e.g.*, the Fc domain, or more specifically, to the first Fc chain or to the second Fc chain), or to the second disease antigen binding domain.

[00265] In some embodiments, the CD3 binding domain is operably linked to the first disease antigen binding domain, or to the PD-L1-binding domain, or to the dimerization domain (*e.g.*, the Fc domain, or more specifically, to the first Fc chain or to the second Fc chain), or to the second disease antigen binding domain.

[00266] In some embodiments, the first disease antigen binding domain is operably linked to the PD-L1 binding domain, or to the CD3-binding domain, or to the dimerization domain (*e.g.,* the Fc domain, or more specifically, to the first Fc chain or to the second Fc chain), or to the second disease antigen binding domain. In some embodiments, the second disease antigen binding domain is operably linked to the PD-L1 binding domain, or to the CD3-binding domain, or to the dimerization domain (*e.g.,* the Fc domain, or more specifically, to the first Fc chain or to the second Fc chain), or to the first disease antigen binding domain.

[00267] In some embodiments, the PD-L1 binding domain is operably linked to the first Fc chain and the CD3-binding domain is operably linked to the second Fc chain. In some embodiments, the first disease antigen binding domain is operably linked to the first Fc chain and the second disease antigen binding domain is operably linked to the second Fc chain, or vice versa.

[00268] The multi-specific polypeptide complex disclosed herein can be in any format known in the art. Examples of multi-specific antibody formats known in the art include, without limitation, (i) a multi-specific antibody with symmetric Fc, (ii) a multi-specific antibody with asymmetric Fc, (iii) a regular antibody appended with an additional antigen-binding moiety, (iv) a multi-specific antibody fragment, and (v) a regular antibody fragment appended with an additional antigen-binding moiety, to name just a few.

[00269] Bispecific IgG (BsIgG) binds to each antigen in a monovalent fashion and can be synthesized via the co-expression of dual light and heavy chains in a singular host cell. The BsIgG structure can be modified by attaching additional antigen-binding units to either the amino or carboxy termini of the light or heavy chains. These extra antigen-binding units can be unpaired single domain antibodies (like DVD-IgG), paired antibody variable domains (such as Fv or scFv), or constructed protein scaffolds.

[00270] In certain embodiments, the multi-specific polypeptide complex as provided herein is based on the format of a "complete" antibody, such as entire IgG or IgG-like molecules, and compact recombinant formats like tandem single chain variable fragment molecules (taFvs), diabodies (Dbs), single chain diabodies (scDbs), and their various derivatives (cf. bispecific antibody formats as described by Byrne H. et al. (2013) Trends Biotech, 31 (11): 621-632). Examples of the multi-specific polypeptide complex are based on formats such as quadroma, chemically coupled Fab, and BiTE (bispecific T cell engager).

[00271] In certain embodiments, the multi-specific polypeptide complex as provided herein comprises a multi-specific format selected from Triomabs; hybrid hybridoma (quadroma); Multispecific anticalin platform (Pieris); Diabodies; Single chain diabodies; Tandem single chain Fv fragments; TandAbs, Trispecific Abs (Affimed); Darts (dual affinity retargeting; Macrogenics); Bispecific Xmabs (Xencor); Bispecific T cell engagers (Bites; Amgen; 55 kDa); Triplebodies; Tribody (Fab-scFv) Fusion Protein (CreativeBiolabs) multifunctional recombinant antibody derivates; Duobody platform (Genmab); Dock and lock platform; Knob into hole (KIH) platform; Humanized bispecific IgG antibody (REGN1979) (Regeneron); Mab_2 bispecific antibodies (F-Star); DVD-Ig (dual variable domain immunoglobulin) (Abbvie); kappa-lambda bodies; TBTI (tetravalent bispecific tandem Ig); and CrossMab.

[00272] In certain embodiments, the multi-specific polypeptide complex as provided herein comprises a multi-specific format selected from multi-specific IgG-like antibodies (BsIgG) comprising CrossMab; DAF (two-in-one); DAF (four-in-one); DutaMab; DT-IgG; Knobs-in-holes common LC; Knobs-in-holes assembly; Charge pair; Fab-arm exchange; SEEDbody; Triomab; LUZ-Y; Fcab; kappa-lamda-body; and Orthogonal Fab. For detailed description of the bispecific antibody formats please see Spiess C., Zhai Q. and Carter P. J. (2015) Molecular Immunology 67: 95-106, which is incorporated herein by reference to its entirety.

[00273] In certain embodiments, the multi-specific polypeptide complex as provided herein comprises a multi-specific format selected from IgG-appended antibodies with

an additional antigen-binding moiety comprising DVD-IgG; IgG(H)-scFv; scFv-(H)IgG; IgG(L)-scFv; scFV-(L)IgG; IgG(L,H)-Fv; IgG(H)-V; V(H)-IgG; IgG(L)-V; V(L)-IgG; KIH IgG-scFab; 2scFv-IgG; IgG-2scFv; scFv4-Ig; scFv4-Ig; Zybody; and DVI-IgG (four-in-one) (see *Id.*).

[00274] In certain embodiments, the multi-specific polypeptide complex as provided herein comprises a multi-specific format selected from multi-specific antibody fragments comprising Nanobody; Nanobody-HAS; BiTE; Diabody; DART; TandAb; scDiabody; sc-Diabody-CH3; Diabody-CH3; Triple Body; Miniantibody; Minibody; TriBi minibody; scFv-CH3 KIH; Fab-scFv; scFv-CH-CL-scFv; F(ab')2; F(ab')2-scFv2; scFv-KIH; Fab-scFv-Fc; Tetravalent HCAb; scDiabody-Fc; Diabody-Fc; Tandem scFv-Fc; and Intrabody (see *Id.*).

[00275] In certain embodiments, the multi-specific polypeptide complex as provided herein comprises a multi-specific format such as Dock and Lock; ImmTAC; HSAbody; scDiabody-HAS; and Tandem scFv-Toxin (see *Id.*).

[00276] In certain embodiments, the multi-specific polypeptide complex as provided herein comprises a multi-specific selected from bispecific antibody conjugates comprising IgG-IgG; Cov-X-Body; and scFv1-PEG-scFv2 (see *Id.*).

[00277] In some embodiments, the PD-L1 binding domain provided herein comprises an antibody-derived domain that comprises one or more CDR sequences of an anti-PD-L1 antibody provided herein and is capable of specifically binding to PD-L1. In some embodiments, the anti-PD-L1 antibody is an IgG antibody, and the PD-L1 binding domain comprises the 6 CDR sequences of the anti-PD-L1 IgG antibody, or comprises both the VH and the VL sequences of the anti-PD-L1 IgG antibody. In some embodiments, the anti-PD-L1 antibody is a heavy chain antibody and the PD-L1 binding domain comprises the 3 CDR sequences of the anti-PD-L1 heavy chain antibody, or comprises the VHH sequence of the anti-PD-L1 heavy chain antibody.

[00278] In some embodiments, the PD-L1 binding domain is an antibody-derived domain selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv, disulfide

stabilized Fv fragment (dsFv), $(dsFv)_2$, single-chain Fv antibody (scFv), and heavy chain antibody (VHH).

[00279] In some embodiments, the CD3 binding domain provided herein comprises an antibody-derived domain that comprises one or more CDR sequences of an anti-CD3 antibody provided herein and is capable of specifically binding to CD3. In some embodiments, the anti-CD3 antibody is an IgG antibody and the CD3 binding domain comprises the 6 CDR sequences of the anti- CD3 IgG antibody, or comprises both the VH and the VL sequences of the anti- CD3 IgG antibody. In some embodiments, the anti- CD3 antibody is a heavy chain antibody and the CD3 binding domain comprises the 3 CDR sequences of the anti- CD3 heavy chain antibody, or comprises the VHH sequence of the anti- CD3 heavy chain antibody.

[00280] In some embodiments, the CD3 binding domain is an antibody-derived domain selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv, dsFv, $(dsFv)_2$, scFv, and VHH.

[00281] In some embodiments, the disease antigen binding domain provided herein comprises an antibody-derived domain that comprises one or more CDR sequences of an anti-disease antigen antibody provided herein and is capable of specifically binding to disease antigen. In some embodiments, the anti-disease antigen antibody is an IgG antibody and the disease antigen binding domain comprises the 6 CDR sequences of the anti- disease antigen IgG antibody, or comprises both the VH and the VL sequences of the anti- disease antigen IgG antibody. In some embodiments, the anti-disease antigen antibody is a heavy chain antibody and the disease antigen binding domain comprises the 3 CDR sequences of the anti-disease antigen heavy chain antibody, or comprises the VHH sequence of the anti- disease antigen heavy chain antibody.

[00282] In some embodiments, the first disease antigen binding domain or the second disease antigen binding domain is an antibody-derived domain selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv, dsFv, $(dsFv)_2$, scFv, and VHH.

[00283] In some embodiments, the PD-L1 binding domain provided herein and the CD3-binding domain provided herein are assembled to form a bi-specific-binding domain in the multi-specific polypeptide complex provided herein.

[00284] In some embodiments, the PD-L1 binding domain is operably linked to the CD3-binding domain to form a bispecific-binding domain. In some embodiment, the bispecific binding domain is a **DICAD domain**. In some embodiments, the bispecific-binding domain is operably linked to the one terminus (e.g. N terminus or C terminus) of the dimerization domain (e.g. the CH3 domain, the CH2-CH3 domain, or the Fc domain). In some embodiments, the bispecific-binding domain is operably linked to the N terminus of the first Fc chain.

[00285] In some embodiments, the bispecific-binding domain is further operably linked to the first disease antigen binding domain to form a tri-specific-binding domain in the multi-specific polypeptide complex provided herein. In some embodiments, the first disease antigen binding domain is operably linked to the PD-L1 binding domain or to the CD3-binding domain in the bispecific-binding domain (e.g. the DICAD domain). In some embodiments, the tri-specific-binding domain is operably linked to one terminus (e.g. N terminus or C terminus) of the dimerization domain (e.g. the CH3 domain, the CH2-CH3 domain, or the Fc domain). In some embodiments, the tri-specific-binding domain is operably linked to the N terminus of the first Fc chain. In some embodiments, one of the C termini of the tri-specific-binding domain is operably linked to the N-terminus of the first Fc chain.

[00286] In some embodiments, the first disease antigen binding domain comprises a first **Fv domain**. In some embodiments, the tri-specific-binding domain comprises the DICAD domain and the first Fv domain. An exemplary structure is shown in Figure 2, Type E structure.

[00287] In some embodiments, the multi-specific polypeptide complex further comprises a second disease antigen binding domain. In some embodiments, the first disease antigen binding domain and the second disease antigen binding domain bind

to the same target. In some embodiments, the first disease antigen binding domain and the second disease antigen binding domain are identical. In some embodiments, the second disease antigen binding domain is operably linked to another terminus (e.g. N terminus or C terminus) of the dimerization domain (e.g. the CH3 domain, the CH2-CH3 domain, or the Fc domain). In some embodiments, the second disease antigen binding domain is operably linked to N terminus of the second Fc chain. An exemplary structure is shown in Figure 2, Type E structure.

[00288] In some embodiments, the second disease antigen binding domain comprises a second **Fv domain**. In some embodiments, the first Fv domain is identical to the second Fv domain.

[00289] ***DICAD Domain***

[00290] As used herein, the term "DICAD" is short for "Disulfide and Charge Adjusted Diabody", which refers to a diabody structure introduced with covalent bonds and electrostatic charges at the VH-VL interface, and is disclosed in, for example PCT application WO2019/120245, incorporated herein to its entirety. This DICAD structure have the following advantages: (1) retain the avidity, affinity, potency and other properties of each individual targeting domain; (a) have high stability and less aggregation compared with other diabodies; (3) are easy for expression and purification.

[00291] In some embodiments, the DICAD domain comprises: i) a first polypeptide fragment comprising a first heavy chain variable domain (VH1) linked to a second light chain variable domain (VL2), and ii) a second polypeptide fragment comprising a second heavy chain variable domain (VH2) linked to a first light chain variable domain (VL1); wherein the VL1 and the VH1 associate to form a first domain capable of binding to a first target, and the VL2 and the VH2 associate to form a second domain capable of binding to a second target; wherein one of the first target and the second target is PD-L1, and the other of first target and the second target second target is CD3.

[00292] In some embodiments, the first target is human PD-L1, and the second target is human CD3. In some embodiments, the first domain can be any PD-L1 binding domain described under the section "***Low-Affinity PD-L1 binding Domain***" of the present disclosure. In some embodiments, the VH1 and the VL1 can be any VH and VL described under the section "***Low-Affinity PD-L1 binding Domain***" of the present disclosure. In some embodiments, the second domain can be any CD3 binding domain described under the section "***Low-Affinity CD3 binding Domain***" of the present disclosure. In some embodiments, the VH2 and the VL2 can be any VH and VL described under the section "***Low-Affinity CD3 binding Domain***" of the present disclosure.

[00293] In some embodiments, the first target is human CD3, and the second target is human PD-L1. In some embodiments, the first domain can be any CD3 binding domain described under the section "***Low-Affinity CD3 binding Domain***" of the present disclosure. In some embodiments, the VH1 and the VL1 can be any VH and VL described under the section "***Low-Affinity CD3 binding Domain***" of the present disclosure. In some embodiments, the second domain can be any PD-L1 binding domain described under the section "***Low-Affinity PD-L1 binding Domain***" of the present disclosure. In some embodiments, the VH2 and the VL2 can be any VH and VL described under the section "***Low-Affinity PD-L1 binding Domain***" of the present disclosure.

[00294] In some embodiments, the N-terminus of said VH1 is linked to C-terminus of said VL2, and N-terminus of said VH2 is linked to C-terminus of said VL1. In some embodiments, the N-terminus of said VL1 is linked to C-terminus of said VH2, and N-terminus of said VL2 is covalently linked to C-terminus of said VH1.

[00295] In some embodiments, the VH2 and the VL1 and/or the VH1 and the VL2 are covalently linked either directly or indirectly, for example, via a linker, for example a peptide linker. The term "peptide linker" as used herein can be any suitable polypeptide capable of bonding two entities to thereby form one molecule, or maintaining association of the two entities in sufficiently close proximity, yet without

substantially interference to the respective biological activities of the two entities. The linker can be made up of amino acid residues linked together by peptide bonds, yet may optionally further comprise one or more non-natural amino acids. Any suitable polypeptide can be used as a linker. In some embodiments, the polypeptide linker can be made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. In some embodiments, linkers are polyglycines, polyalanines, combinations of glycine and alanine (such as poly(Gly-Ala)), or combinations of glycine and serine (such as poly (Gly-Ser)). In some embodiments, the VL1 is linked to the VH2 via a first peptide linker, and wherein the VL2 is linked to the VH1 via a second peptide linker. In some embodiments, the first peptide linker and the second peptide linker each independently comprises 5 to 9 amino acids. In some embodiments, the first peptide linker and the second peptide linker each independently comprises the amino acid sequence of SEQ ID NO: 53 [RTVAA]. In some embodiments, the first peptide linker and the second peptide linker each independently comprises the amino acid sequence of SEQ ID NO: 104 [GGGGSGGGGS].

[00296] a) Disulfide bond introduced in the VH-VL regions

[00297] In some embodiments, non-native covalent bonds can be introduced, and/or electrostatic interactions can be introduced to the VH1-VL1 interface, or VH2-VL2 interface.

[00298] In some embodiments, one of the first domain (formed by association of VH1 and VL1) and the second domain (formed by association of VH2 and VL2) comprises a first non-native covalent bond formed between a first pair of two amino acid residues.

[00299] In some embodiments, the other one of the first domain and the second domain is an accompanying domain and does not comprise a non-native covalent bond or alternatively comprises a second non-native covalent bond formed between a

pair of two amino acid residues different from the pair of amino acid residues corresponding to the first pair of two amino acid residues.

[00300] In certain of these embodiments, the other one of the first domain and the second domain does not comprise a non-native covalent bond. Alternatively, the other one of the first domain and the second domain comprises a second non-native covalent bond that is distinct from the first non-native covalent bond. For example, the second non-native covalent bond is formed between a pair of two amino acid residues different from the pair of amino acid residues corresponding to the first pair of two amino acid residues.

[00301] In some embodiments, the first non-native covalent bond can be a non-native disulfide bond. In such embodiments, at least one of the first domain and the second domain is a disulfide-stabilized Fv. Analysis on crystal structure of antibodies revealed that cysteine mutations could be introduced in some of the relatively conserved sequences at the VL-VH interface to form disulfide bonds between VL and VH, so they are covalently connected. Covalent bonds between VLs and VHs significantly improved stability of the antibodies. The initial dsFv (disulfide Fv) was constructed by introducing disulfide bonds to VH-VL interface via covalent interaction between cysteine residues in CDRs of each fragment respectively (see Glockshuber, R., Malia, M., Pfitzinger, I. and Pluckthun, A. A comparison of strategies to stabilize immunoglobulin Fv-fragments. (1990) *Biochemistry*, 29, 1362-1367.). Though activity of antibodies was not affected using this method, detailed structure information on the CDRs of the original antibodies was required for "customized" design to avoid interference with antigen-recognizing/binding capability of the CDRs, which made it difficult for the method to become a universal solution for construction of various antibodies. To ensure broad application of the method, it is crucial that only amino acids at selected sites in conserved FR are engaged in the construction of dsFv.

[00302] Since 1993, several paired sites for VH-VL covalent bond formation have been discovered, including VH44-VL100, VH105-VL43, VH100b-VL49, VH100-

VL50, and VH101–VL46, etc., based on the Kabat index (see Reiter,Y., Brinkmann,R.J., Kreitman,R.J., etc. Stabilization of the Fv Fragments in Recombinant Immunotoxins by Disulfide Bonds Engineered Into Conserved Framework Regions. (1994) *Biochemistry*, 33, 5451–5459., Jung, S.H., Pastan, I. and Lee,B. Design of interchain disulfide bonds in the framework region of the Fv fragment of the monoclonal antibody B3. (1994) *Proteins, Struc. Func. Genet.*, 19, 35–47., Glockshuber, R., Malia, M., Pfitzinger, I. and Plu¨ckthun, A. A comparison of strategies to stabilize immunoglobulin Fv-fragments. (1990) *Biochemistry*, 29, 1362–1367., and Zhu, Z., Presta, L.G., Zapata, G. and Carter,P. Remodeling domain interfaces to enhance heterodimer formation. (1997) *Prot. Sci.*, 6, 781–788.). Among them VH44-VL100 and VH105-VL43 are, to different extent, superior to the others in many aspects such as protein expression level, mono rate, Tm and affinity etc., and are thus subjected to broader application.

[00303] In some embodiments, the first non-native disulfide bond is formed between two non-native cysteine residues. In some embodiments, the two non-native cysteine residues are at position 44 in the VH and position 100 in the VL, or at position 105 in the VH and position 43 in the VL, or at position 100 in the VH and position 49 in the VL, or at position 100 in the VH and position 150 in the VL, wherein numbering is according to the Kabat index.

[00304] In some embodiments, the VL1 and the VH1 of the DICAD domain are associated with the first non-native disulfide bond. In some embodiments, the first non-native disulfide bond is formed between two non-native cysteine residues in the VL1 and the VH1, respectively. In some embodiments, the two non-native cysteine residues are in the framework region (FR) of the VL1 and FR of the VH1, respectively. In some embodiments, the two non-native cysteine residues are in FR2 of the VL1 and an amino acid residue in FR4 of the VH1, respectively.

[00305] In some embodiments, the two non-native cysteine residues are at position 44 in the VH1 and position 100 in the VL1, or at position 105 in the VH1 and position 43 in the VL1, or at position 100 in the VH1 and position 49 in the VL1, or at position

100 in the VH1 and position 150 in the VL1. Unless otherwise specified, all position numberings in the present disclosure are according to Kabat index. In some embodiments, the non-native covalent bond is formed between introduced amino acid residues at position 44 in the VH1 and position 100 in the VL1, or cysteine residues at position 105 in the VH1 and position 43 in the VL1. In some embodiments, the non-native covalent bond is formed between cysteine residues at position 44 in the VH1 and position 100 in the VL1. In some embodiments, the two non-native cysteine residues are 100C in the VL1 and 44C in the VH1.

[00306] In some embodiments, the VL2 and the VH2 of the DICAD domain do not comprise any introduced non-native disulfide bond.

[00307] In some other embodiments, the VL2 and the VH2 of the DICAD domain are associated with a first non-native disulfide bond. In some embodiments, the first non-native disulfide bond is formed between two non-native cysteine residues in the VL2 and the VH2, respectively. In some embodiments, the two non-native cysteine residues are in the framework region (FR) of the VL2 and FR of the VH2, respectively. In some embodiments, the two non-native cysteine residues are in FR2 of the VL2 and an amino acid residue in FR4 of the VH2, respectively. In some embodiments, the two non-native cysteine residues are at position 44 in the VH2 and position 100 in the VL2, or at position 105 in the VH2 and position 43 in the VL2, or at position 100 in the VH2 and position 49 in the VL2, or at position 100 in the VH2 and position 150 in the VL2. Unless otherwise specified, all position numberings in the present disclosure are according to Kabat index. In some embodiments, the non-native covalent bond is formed between introduced amino acid residues at position 44 in the VH2 and position 100 in the VL2, or cysteine residues at position 105 in the VH2 and position 43 in the VL2. In some embodiments, the non-native covalent bond is formed between cysteine residues at position 44 in the VH2 and position 100 in the VL2. In some embodiments, the two non-native cysteine residues are 100C in the VL2 and 44C in the VH2. In some of these embodiments, the VL1 and the VH1 of the DICAD domain do not comprise any introduced non-native disulfide bond.

[00308]

[00309] b) Substitution with charged amino acids

[00310] In some embodiments, one of the first domain and the second domain comprises a first non-native pair of oppositely charged amino acid residues that introduces non-native electrostatic interactions, such that the pairing between the VH and the VL in such a domain is facilitated or favored. In some embodiments, the non-native charged residue in the VL is in FR (*e.g.*, FR2). In some embodiments, the non-native charged residue in the VH is in FR (*e.g.*, FR2).

[00311] In some embodiments, the other one of the first domain and the second domain does not comprise a non-native pair of oppositely charged amino acid residues that introduces non-native electrostatic interactions.

[00312] In some embodiments, the first domain comprising the VH1 and the VL1 comprises the first non-native pair of oppositely charged amino acid residues, wherein

(a) the two oppositely charged residues are at position 38 in the VL1 and position 39 in the VH1, respectively;
(b) the two oppositely charged residues are at position 40 in the VL1 and position 39 in the VH1, respectively; or
(c) the two oppositely charged residues are at position 37 in the VL1 and position 39 in the VH1, respectively; or
(d) the two oppositely charged residues are at position 44 in the VL and position 103 in the VH, respectively; wherein numbering is according to the Kabat index.

[00313] In some other embodiments, the second domain comprising the VH2 and the VL2 comprises the first non-native pair of oppositely charged amino acid residues, wherein

(a) the two oppositely charged residues are at position 38 in the VL2 and position 39 in the VH2, respectively;
(b) the two oppositely charged residues are at position 40 in the VL2 and position 39 in the VH2, respectively;

(c) the two oppositely charged residues are at position 37 in the VL2 and position 39 in the VH2, respectively; or

(d) the two oppositely charged residues are at position 44 in the VL2 and position 103 in the VH2, respectively;

wherein numbering is according to the Kabat index.

[00314] W103 of VH and P44 of VL are both at the side chain of the hydrophobic core and positioned in close proximity. Electrostatic interaction between W103-P44 was also examined during development of DICAD and found to be superior.

[00315] In some embodiments, the negatively charged amino acid residue selected from aspartic acid (D) or glutamic acid (E). In some embodiments, the positively charged amino acid residue selected from lysine (K), histidine (H) or arginine (R).

[00316] In some embodiments, the two oppositely charged residues comprises a negatively charged amino acid residue selected from aspartic acid (D) or glutamic acid (E), and a positively charged amino acid residue selected from lysine (K), histidine (H) or arginine (R).

[00317] In some embodiments, the two oppositely charged residues comprises a pair of aspartic acid (D) and lysine (K).

[00318]

[00319] In some embodiments, the other one of the first domain and the second domain comprises a second pair of two oppositely charged residues that pairing between the VH and the VL in such a domain, with the proviso that the cross pairing between the VH in one domain and the VL in the other domain is discouraged, for example, due to electrostatic repulsion.

[00320] In some embodiments, the DICAD domain provided herein comprises the first pair of two oppositely charged residues that promote electrostatic interactions between the VL1 and the VH1, as well as the second pair of two oppositely charged residues that promote electrostatic interactions between the VL2 and the VH2, with the proviso that the pairing between the VH1 and the VL2 and the pairing between the

VH2 and the VL1 are discouraged, for example, due to electrostatic repulsion. For example, the charged residues introduced to the VL2 and the VH1 are like-charged residues, and/or the charged residues introduced to the VH2 and the VL1 are like-charged residues, such that mispairing between VL2 and VH1 or between VL1 and VH2 are discouraged.

[00321] In some embodiments, the first pair of two oppositely charged residues are introduced to replace position 38 (*e.g.* Q38) in the VL1 and position (*e.g.* Q39) in the VH1, respectively, wherein numbering is according to the Kabat index. In some embodiments, the second pair of two oppositely charged residues are introduced to replace position 38 (*e.g.* Q38) in the VL2 and position 39 (*e.g.* Q39) in the VH2, respectively, wherein numbering is according to the Kabat index. In such embodiments, the charged residues introduced to the VL2 and the VH1 are like-charged residues, and/or the charged residues introduced to the VH2 and the VL1 are like-charged residues, such that mispairing between VL2 and VH1 or between VL1 and VH2 are discouraged.

[00322] In some embodiments, the two oppositely charged residues are composed of a positively charged residue and a negatively charged residue.

[00323] In certain embodiments, the first pair of two oppositely charged residues comprises a negatively charged residue in the VL1 and a positively charged residue in the VH1. In certain embodiments, the second pair of two oppositely charged residues comprises a positively charged residue in the VL2 and a negatively charged residue in the VH2.

[00324] In some embodiments, the first pair of two oppositely charged residues comprise 38D in the VL1 and 39K in the VH1, respectively, wherein numbering is according to the Kabat index. In some embodiments, the second pair of two oppositely charged residues comprise 39D in the VH2 and 40K in the VL2, respectively, wherein numbering is according to the Kabat index.

[00325] In certain embodiment, the VH1 and the VL1 associates to form the first domain that is a PD-L1 binding domain. In a certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 25 (K56D in VH), SEQ ID NO: 26 (K56A in VH), SEQ ID NO: 27 (K56F in VH), or SEQ ID NO: 28 (H58D in VH) and VL1 comprises the amino acid sequence of SEQ ID NO: 8, and the VH1 and the VL1 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00326] In a certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 7 and the VL1 comprises the amino acid sequence of SEQ ID NO: 29 (Y32E in VL), SEQ ID NO: 30 (W94D in VL), or SEQ ID NO: 31 (W94A in VL), and the VH1 and the VL1 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00327] In certain embodiment, the VH1 and the VL1 associates to form the first domain that is a PD-L1 binding domain. In certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 147 (S56G in VH), SEQ ID NO: 148 (Y53C in VH), SEQ ID NO: 149 (Y53G in VH), SEQ ID NO: 150 (Y53H in VH) or SEQ ID NO: 152 (Y53Q in VH) and VL1 comprises the amino acid sequence of SEQ ID NO: 136, and the VH1 and the VL1 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00328] In certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 135 and the VL1 comprises the amino acid sequence of SEQ ID NO: 136, SEQ ID NO: 145 (S30H in VL), SEQ ID NO: 146 (S30T in VL), or SEQ ID NO: 151 (S30E in VL), and the VH1 and the VL1 are modified to introduce the two non-native

cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00329] In certain embodiment, the VH1 and the VL1 associates to form the first domain that is a CD3 binding domain. In certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 32, 33, 34, 35, 36, 37 or 38 and VL1 comprises the amino acid sequence of SEQ ID NO: 16, and the VH1 and the VL1 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00330] In certain embodiment, the VH2 and the VL2 associates to form the second domain that is a PD-L1 binding domain. In a certain embodiment, the VH2 comprises the amino acid sequence of SEQ ID NO: 25 (K56D in VH), SEQ ID NO: 26 (K56A in VH), SEQ ID NO: 27 (K56F in VH), or SEQ ID NO: 28 (H58D in VH) and VL2 comprises the amino acid sequence of SEQ ID NO: 8, and the VH2 and the VL2 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00331] In a certain embodiment, the VH2 comprises the amino acid sequence of SEQ ID NO: 7 and the VL2 comprises the amino acid sequence of SEQ ID NO: 29 (Y32E in VL), SEQ ID NO: 30 (W94D in VL), or SEQ ID NO: 31 (W94A in VL), and the VH2 and the VL2 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00332] In certain embodiment, the VH2 and the VL2 associates to form the second domain that is a PD-L1 binding domain. In a certain embodiment, the VH2 comprises the amino acid sequence of SEQ ID NO: 147 (S56G in VH), SEQ ID NO: 148 (Y53C in VH), SEQ ID NO: 149 (Y53G in VH), SEQ ID NO: 150 (Y53H in VH) or SEQ ID NO: 152 (Y53Q in VH) and VL2 comprises the amino acid sequence of SEQ ID NO: 136, and the VH2 and the VL2 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00333] In certain embodiment, the VH2 comprises the amino acid sequence of SEQ ID NO: 135 and the VL2 comprises the amino acid sequence of SEQ ID NO: 145 (S30H in VL), SEQ ID NO: 146 (S30T in VL), or SEQ ID NO: 151 (S30E in VL), and the VH2 and the VL2 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00334] In certain embodiment, the VH2 and the VL2 associates to form the second domain that is a CD3 binding domain. In certain embodiment, the VH2 comprises the amino acid sequence of SEQ ID NO: 32, 33, 34, 35, 36, 37 or 38 and VL2 comprises the amino acid sequence of SEQ ID NO: 16, and the VH2 and the VL2 are modified to introduce the two non-native cysteine residues as disclosed herein to allow formation of the first non-native disulfide bond, and/or are modified to introduce the pair of two oppositely charged residues as disclosed herein to introduce non-native electrostatic interactions.

[00335] Strategies to enhancing the stably binding of corresponding VH and VL in the bispecific antibodies by introducing charged amino acids are well known in the art. Tan *et al.* managed to influence stability of the scFv (single chain F variant) by adjusting amino acids at VH-VL interface based on their electrostatic properties (see Philip H. Tan, Brenda M. Sandmaier, Patrick S. Stayton. Contributions of a Highly

Conserved VH VL Hydrogen Bonding Interaction to scFv Folding Stability and Refolding Efficiency. *Biophys J.* 1998 Sep; 75(3): 1473–1482.). Later, Igawa *et al.* adapted the method to modify scDb. Two pairs of Q39-Q38 in 4 V fragments respectively were replaced with amino acids with proper electrostatic charge to either promote or inhibit certain isoforms, in order to improve homogeneity of the product (see Igawa T, Tsunoda H, Kikuchi Y, etc. VH/VL interface engineering to promote selective expression and inhibit conformational isomerization of thrombopoietin receptor agonist single-chain diabody. *Protein Eng Des Sel.* 2010 Aug;23(8):667-77., and WO2006106905A1). Gunasekaran *et al.* at Amgen did further research on the method and engaged it in modification of the Fab arms of antibodies. Adjusting electrostatic steering at the CH1-CL interface, together with modifications at 38-39 of VH-VL, facilitated specific interaction between CH1-VH and CL-VL (see Gunasekaran K, Pentony M, Shen M, *etc.* Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem. 2010 Jun 18;285(25):19637-46.; and Liu Z, Leng EC2, Gunasekaran K3, *etc.* A novel antibody engineering strategy for making monovalent bispecific heterodimeric IgG antibodies by electrostatic steering mechanism. *J Biol Chem.* 2015 Mar 20;290(12):7535-62.). By these methods, each HC of the bispecific antibodies is able to interact with the corresponding LC, which results in bispecific antibodies that could bind to two antigens at the same time.

[00336] Methods of introduction of positively or negatively charged amino acid into an antibody are also known in the art.

[00337] In some embodiments, the DICAD domain provided herein has modified electrostatic steering of selected regions in addition to introduction of non-native disulfide bonds, and by doing so managed to minimize unwanted non-specific interactions.

[00338] In some embodiments, the one of the first domain and the second domain that comprises the first non-native covalent bond further comprises a first non-native pair of oppositely charged amino acid residues that introduces non-native electrostatic

interactions, such that the pairing between the VH and the VL in such a domain is facilitated or favored.

[00339] In some embodiments, the first domain comprising the VH1 and the VL1 comprises the first non-native disulfide bond which is formed between the two non-native cysteine residues, and further comprises the first non-native pair of oppositely charged amino acid residues. In some embodiments, the second domain comprising the VH2 and the VL2 comprises the second pair of two oppositely charged residues, with the proviso that the pairing between the VH1 and the VL2 and the pairing between the VH2 and the VL1 are discouraged, for example, due to electrostatic repulsion. For example, the charged residues introduced to the VL2 and the VH1 are like-charged residues, and/or the charged residues introduced to the VH2 and the VL1 are like-charged residues, such that mispairing between VL2 and VH1 or between VL1 and VH2 are discouraged.

[00340] Alternatively, in some other embodiments, the second domain comprising the VH2 and the VL2 comprises the first non-native disulfide bond which is formed between the two non-native cysteine residues, and further comprises the first non-native pair of oppositely charged amino acid residues. In some embodiments, the first domain comprising the VH1 and the VL1 comprises the second pair of two oppositely charged residues, with the proviso that the pairing between the VH2 and the VL1 and the pairing between the VH1 and the VL2 are discouraged, for example, due to electrostatic repulsion. For example, the charged residues introduced to the VL2 and the VH1 are like-charged residues, and/or the charged residues introduced to the VH2 and the VL1 are like-charged residues, such that mispairing between VL2 and VH1 or between VL1 and VH2 are discouraged.

[00341] In some embodiments, the VL1 comprises a non-native cysteine residue at position 100 and the VH1 comprises a non-native cysteine residue at position 44 to form the non-native disulfide bond, meanwhile the VL1 and the VH1 further comprises two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00342] In some embodiments, the VL2 comprises a non-native cysteine residue at position 100 and the VH2 comprises a non-native cysteine residue at position 44 to form the non-native disulfide bond, meanwhile the VL2 and the VH2 further comprises two oppositely charged residues at position 38 in the VL2 and 39 in the VH2, respectively, wherein numbering is according to the Kabat index.

[00343] The modification to introduce electrostatic interactions can improve stability and uniformity of the polypeptide complexes, help to remove obstacles in downstream development process, and increase probability of success in development of multi-specific polypeptide complexes.

[00344] In some embodiments, the first target is PD-L1, and the VL1 comprises a non-native cysteine residue at position 100 and the VH1 comprises a non-native cysteine residue at position 44 to form the non-native disulfide bond, meanwhile the VL1 and the VH1 further comprises two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00345] In certain embodiment, the VH1 and the VL1 associates to form the first domain that is a PD-L1 binding domain. In a certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 25 (K56D in VH), SEQ ID NO: 26 (K56A in VH), SEQ ID NO: 27 (K56F in VH), or SEQ ID NO: 28 (H58D in VH) and VL1 comprises the amino acid sequence of SEQ ID NO: 8, and the VL1 is modified to introduce a non-native cysteine residue at position 100 and the VH1 is modified to introduce a non-native cysteine residue at position 44 to form the non-native disulfide bond, wherein numbering is according to the Kabat index. In a certain embodiment, the VL1 and the VH1 is further modified to introduce two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00346] In a certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 7 and the VL1 comprises the amino acid sequence of SEQ ID NO: 29

(Y32E in VL), SEQ ID NO: 30 (W94D in VL), or SEQ ID NO: 31 (W94A in VL) , and the VL1 is modified to introduce a non-native cysteine residue at position 100 and the VH1 is modified to introduce a non-native cysteine residue at position 44 to form the non-native disulfide bond, wherein numbering is according to the Kabat index. In a certain embodiment, the VL1 and the VH1 is further modified to introduce two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00347] In a certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 147 (S56G in VH), SEQ ID NO: 148 (Y53C in VH), SEQ ID NO: 149 (Y53G in VH), SEQ ID NO: 150 (Y53H in VH) or SEQ ID NO: 152 (Y53Q in VH) and VL1 comprises the amino acid sequence of SEQ ID NO: 136, and the VL1 is modified to introduce a non-native cysteine residue at position 100 and the VH1 is modified to introduce a non-native cysteine residue at position 44 to form the non-native disulfide bond, wherein numbering is according to the Kabat index. In a certain embodiment, the VL1 and the VH1 is further modified to introduce two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00348] In a certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 135 and the VL1 comprises the amino acid sequence of SEQ ID NO: 145 (S30H in VL), SEQ ID NO: 146 (S30T in VL), or SEQ ID NO: 151 (S30E in VL), and the VL1 is modified to introduce a non-native cysteine residue at position 100 and the VH1 is modified to introduce a non-native cysteine residue at position 44 to form the non-native disulfide bond, wherein numbering is according to the Kabat index. In a certain embodiment, the VL1 and the VH1 is further modified to introduce two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00349] In certain embodiment, the VH1 and the VL1 associates to form the first domain that is a CD3 binding domain. In certain embodiment, the VH1 comprises the amino acid sequence of SEQ ID NO: 32, 33, 34, 35, 36, 37 or 38 and VL1 comprises

the amino acid sequence of SEQ ID NO: 16, and the VL1 is modified to introduce a non-native cysteine residue at position 100 and the VH1 is modified to introduce a non-native cysteine residue at position 44 to form the non-native disulfide bond, wherein numbering is according to the Kabat index. In a certain embodiment, the VL1 and the VH1 is further modified to introduce two oppositely charged residues at position 38 in the VL1 and 39 in the VH1, respectively, wherein numbering is according to the Kabat index.

[00350] In some embodiments, the VH1 comprises an amino acid sequence of SEQ ID NO: 47, and the VL1 comprises an amino acid sequence of SEQ ID NO: 48. In some embodiments, the VH1 or the VL1 further comprises one or more mutations that reduces binding affinity to PD-L1. In some embodiments, the VH1 comprises an amino acid sequence of SEQ ID NO: 116, and the VL1 comprises an amino acid sequence of SEQ ID NO: 48. In some embodiments, the VH1 comprises an amino acid sequence of SEQ ID NO: 153, and the VL1 comprises an amino acid sequence of SEQ ID NO: 154. In some embodiments, the VH1 comprises an amino acid sequence of SEQ ID NO: 160, and the VL1 comprises an amino acid sequence of SEQ ID NO: 154.

[00351] In some embodiments, the second target is CD3. In some embodiments, the VH2 comprises the amino acid sequence of SEQ ID NO: 15, and the VL2 comprises the amino acid sequence of SEQ ID NO: 16. In some embodiments, the VH2 or the VL2 further comprises one or more mutations that reduces binding affinity to CD3. In some embodiments, the VH2 comprises an amino acid sequence of SEQ ID NO: 34, and the VL1 comprises an amino acid sequence of SEQ ID NO: 16.

Table 4. Sequences of exemplary PD-L1 binding domain in the DICAD domain

Target		Sequence
PD-L1	VH	SEQ ID NO: 47 QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSSYAISWV RDAPGQCLEWMGGIIPIFGRAHYAQKFQGRVTITADES TSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDV WGQGTTVTVSS
	VH	SEQ ID NO: 116

		(further comprising the low affinity mutation of K56F based o n SEQ ID NO: 47) QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSTYAISWV RDAPGQCLEWMGGIIPIFGFAHYAQKFQGRVTITADES TSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDV WGQGTTVTVSS
	VH	SEQ ID NO: 160 EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWV R**D**APGK**C**LEWVAWISPYGGSTYYADSVKGRFTISADT SKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWG QGTLVTVSS
	VH	SEQ ID NO: 153 *(further comprising the low affinity mutation of Y53H based o n SEQ ID NO: 160)* EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWV RDAPGKCLEWVAWISP**H**GGSTYYADSVKGRFTISADT SKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWG QGTLVTVSS
	VL	SEQ ID NO: 48 EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQK KPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISS LEPEDFAVYYCQQRSNWPTFGCGTKVEIK
	VL	SEQ ID NO: 154 DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQ KKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQYLYHPATFGCGTKVEIK
CD3	VH	SEQ ID NO: 15 EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWV RQAPGKGLEWVGRIRSKYNNYATYYADSVKGRFTISR DDSKNSLYLQMNSLRAEDTAVYYCVRHGNFGTSYVS WFAYWGQGTLVTVSS
	VH	SEQ ID NO: 34 *(further comprising the low affinity mutation of K52B Q)* EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWV RQAPGKGLEWVGRIRSQYNNYATYYADSVKGRFTISR DDSKNSLYLQMNSLRAEDTAVYYCVRHGNFGTSYVS WFAYWGQGTLVTVSS
	VL	SEQ ID NO: 16 QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANW VQQKPGQAPRGLIGGTNKRAPWTPARFSGSLLGGKAA LTLSGAQAEDEADYYCALWYSNLWVFGGGTKLTVL

[00352] In some embodiments, the first polypeptide fragment of DICAD comprises a VH1 comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 47, 116, and a VL2 comprising the amino acid sequence of SEQ ID NO: 16. In some embodiments, the second polypeptide fragment of DICAD comprises a VH2 comprising the amino acid sequence selected from the group

consisting of SEQ ID NOs: 15 and 34, and a VL1 comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 48.

[00353] In some embodiments, the first polypeptide fragment of DICAD comprises a VH1 comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 160 and 153, and a VL2 comprising the amino acid sequence of SEQ ID NO: 16. In some embodiments, the second polypeptide fragment of DICAD comprises a VH2 comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 15 and 34, and a VL1 comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 154.

[00354] In some embodiments, the first polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 49, and the second polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 50.

[00355] Chain 1 of the DICAD domain (SEQ ID NO: 49)

[00356] QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAP
RGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAEDEADYYCALWYSNL
WVFGGGTKLTVLRTVAAQVQLVQSGAEVKKPGSSVKVSCKTSGDTFSSYAIS
WVRDAPGQCLEWMGGIIPIFGRAHYAQKFQGRVTITADESTSTAYMELSSLR
SEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVTVSSASEPKSSD

[00357] Chain 2 of the DICAD domain (SEQ ID NO: 50)

[00358] EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQKKPGQAPRLLI
YDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGCGT
KVEIKRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAP
GKGLEWVGRIRSKYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAED
TAVYYCVRHGNFGTSYVSWFAYWGQGTLVTVSS

[00359] In some embodiments, the first polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 118, and the second polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 119.

[00360] Chain 1 of the DICAD domain (SEQ ID NO: 118)

[00361] QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAP RGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAEDEADYYCALWYSNL WVFGGGTKLTVLRTVAAQVQLVQSGAEVKKPGSSVKVSCKTSGDTFSTYAIS WVRDAPGQCLEWMGGIIPIFGFAHYAQKFQGRVTITADESTSTAYMELSSLRS EDTAVYFCARKFHFVSGSPFGMDVWGQGTTVTVSSASEPKSSD

[00362] Chain 2 of the DICAD domain (SEQ ID NO: 119)

[00363] EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQKKPGQAPRLLI YDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGCGT KVEIKRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAP GKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAED TAVYYCVRHGNFGTSYVSWFAYWGQGTLVTVSS

[00364] In some embodiments, the first polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 161, and the second polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 156.

[00365] Chain 1 of the DICAD domain (SEQ ID NO: 161)

[00366] QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAP RGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAEDEADYYCALWYSNL WVFGGGTKLTVLRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIH WVRDAPGKCLEWVAWISPYGGSTYYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS

[00367] Chain 2 of the DICAD domain (SEQ ID NO: 156)

[00368] DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQKKPGKAPKL LIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGC GTKVEIKRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQ APGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSYVSWFAYWGQGTLVTVSS

[00369] In some embodiments, the first polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 155, and the second polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 156.

[00370] Chain 1 of the DICAD domain (SEQ ID NO: 155)

[00371] QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAP RGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAEDEADYYCALWYSNL WVFGGGTKLTVLRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIH WVRDAPGKCLEWVAWISPHGGSTYYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS

[00372] Chain 2 of the DICAD domain (SEQ ID NO: 156)

[00373] DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQKKPGKAPKL LIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGC GTKVEIKRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQ APGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSYVSWFAYWGQGTLVTVSS

[00374] ***Fv Domain***

[00375] In some embodiments, the first disease antigen binding domain of the multi-specific polypeptide complex comprises a first Fv domain comprising: i) a third polypeptide fragment comprising a third heavy chain variable domain (VH3), and ii) a fourth poly peptide fragment comprising a third light chain variable domain (VL3); wherein the VH3 and the VL3 associate to form a third domain capable of binding to the disease antigen.

[00376] In some embodiments, the Fv domains further comprise scaffold domains fused to the VH3 and VL3, respectively. For example, a scaffold region can be fused to VH3, and a pairing scaffold region can be fused to VL3. Binding of the scaffold region to the pairing scaffold region can allow association of the VH3 region and the VL3 region, thereby forming the Fv domain that is capable of binding to the target

antigen. Any suitable binding partners can be used as the scaffold regions. In certain embodiments, the scaffold region can be selected from antibody CH1 domain and CL domain, pairing TCR constant regions (such as TCR alpha/TCR beta, TCR gamma/TCR delta), or PRD (proline rich domain) and SH3 domain, or obscurin and titin, IL2 and ligand binding domain of IL2 receptor, or IL15 and ligand binding domain of IL15 receptor.

[00377] ***In*** some embodiments, the Fv domain comprises a CH1 domain covalently linked to one of VH3 and VL3, and a CL domain covalently linked to the other one of VH3 and VL3.

[00378] In some embodiments, the third polypeptide fragment further comprises an antibody heavy chain constant region (CH1) operably linked to the C terminus of the VH3 domain, and/or the fourth polypeptide fragment further comprise an antibody light chain constant region (CL) operably linked to the C terminus of the VL3 domain. In some embodiments, the Fv domain comprises a Fab domain.

[00379] In some embodiments, the first disease antigen is CEA. In some embodiments, the third polypeptide fragment comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3, each comprising respectively the amino acid sequences of: SEQ ID NOs: 17-22, respectively. In some embodiments, the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 23, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 24.

[00380] ***In*** some embodiments, the first disease antigen is EGFR. In some embodiments, the third polypeptide fragment comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3, each comprising respectively the amino acid sequences of: SEQ ID NOs: 78-83, respectively. In some embodiments, the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 84, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 85.

[00381] In some embodiments, the first disease antigen is CD20. In some embodiments, the third polypeptide fragment comprises a HCDR1, a HCDR2, a

HCDR3, a LCDR1, a LCDR2, and a LCDR3, each comprising respectively the amino acid sequences of: SEQ ID NOs: 105-110, respectively. In some embodiments, the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 111, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 112.

[00382] In some embodiments, the first disease antigen is CLDN6. In some embodiments, the third polypeptide fragment comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3, each comprising respectively the amino acid sequences of: SEQ ID NOs: 86-91, respectively. In some embodiments, the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 92, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 93.

[00383] In some embodiments, the first disease antigen is GPRC5D. In some embodiments, the third polypeptide fragment comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3, each comprising respectively the amino acid sequences of: SEQ ID NOs: 94-99, respectively. In some embodiments, the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 100, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 101.

[00384] The DICAD domains and the Fv domains can be linked in any suitable manners in the multi-specific polypeptide complex, as long as these domains can substantially retain their antigen-binding activity.

[00385] In some embodiments, one of the termini of the DICAD domain is operably linked to one of the termini of the Fv domain.

[00386] In some embodiments, one of the N termini of the DICAD domain is operably linked to one of the C termini of the first Fv domain.

[00387] In some embodiments, one of the N termini of the DICAD domain is operably linked to the C termini of the third polypeptide fragment the first Fv domain. In some embodiments, the N termini of the second polypeptide fragment of the DICAD domain is operably linked to the C termini of the third polypeptide fragment

of the first Fv domain. In some embodiments, the N termini of the first polypeptide fragment of the DICAD domain is operably linked to the C termini of the third polypeptide fragment of the first Fv domain.

[00388] In some embodiments, one of the N termini of the DICAD domain is operably linked to the C termini of the fourth polypeptide fragment of the first Fv domain. In some embodiments, the N termini of the first polypeptide fragment of the DICAD domain is operably linked to the C termini of the fourth polypeptide fragment of the first Fv domain. In some embodiments, the N termini of first polypeptide fragment of the DICAD domain is operably linked to the C termini of the fourth polypeptide fragment of the first Fv domain.

[00389] ***Linkages between the DICAD domain or Fv domain to the dimerization domain***

[00390] In some embodiments, the multi-specific polypeptide complex provided herein comprises: (a) **the first Fc chain**; and (b) the tri-specific-binding domain comprising the **DICAD domain** and **the first Fv domain**; and one of the termini of the DICAD domain is operably linked to one of termini of the first Fv domain.

[00391] ***In*** some embodiments, one of the C termini of the DICAD domain is operably linked to N termini of the first Fc chain.

[00392] ***In*** certain embodiments, the C termini of the third polypeptide fragment of the first Fv domain is covalently linked to the N termini of the second polypeptide fragment of the DICAD domain, and the C termini of the first polypeptide fragment of the DICAD domain is covalently linked to the N termini of the first Fc chain.

[00393] In some embodiments, the multi-specific polypeptide complex further comprises a second antigen-binding domain and a second Fc chain, wherein the second Fc chain associates with the first Fc chain to form a dimer, wherein one of the C termini of the second antigen-binding domain is operably linked to the N-terminus of the second Fc chain.

[00394] In some embodiments, the first antigen-binding domain and the second antigen-binding domain are identical. In some embodiments, the second antigen-binding domain is different from the first antigen-binding domain.

[00395] In some embodiments, the first Fc chain and the second Fc chain are identical. In some embodiments, the second Fc chain is different from the first Fc chain and associates with the first Fc chain to form a heterodimer.

[00396] In some embodiments, the C termini of the first polypeptide fragment of the DICAD domain is operably linked to an N-terminus of the dimerization domain (*e.g.* the CH3 domain, the CH2-CH3 domain, or the Fc domain).

[00397] ***In*** some embodiments, the C termini of the first polypeptide fragment of the DICAD domain is operably linked to the N-terminus of the first Fc chain. In some embodiments, the second disease antigen-binding domain is operably linked to the N-terminus of the second Fc chain.

[00398] In some embodiments, the second disease antigen-binding domain is identical to the first disease antigen-binding domain. In some embodiments, the second disease antigen-binding domain comprises a second Fv domain. In some embodiments, the second Fv domain is identical to the first Fv domain, and the second Fv domain comprises a fifth polypeptide fragment identical to the third polypeptide fragment comprising the third heavy chain variable domain (VH3), and a sixth polypeptide fragment identical to the fourth polypeptide fragment comprising the third light chain variable domain (VL3). The VH3 and the VL3 associate to form the third domain capable of binding to the first disease antigen.

[00399] ***In*** some embodiments, the third polypeptide fragment further comprises an antibody heavy chain constant region (CH1) operably linked to the C terminus of the VH3 domain, and/or the fourth polypeptide fragment further comprise an antibody light chain constant region (CL) operably linked to the C terminus of the VL3 domain. In some embodiments, the Fv domain comprises a Fab domain.

[00400] In some embodiments, the C termini of heavy chain of the second Fv domain is operably linked to the N-terminus of the second dimerization domain. In some embodiments, the C termini of the fifth polypeptide fragment of the second Fv domain is operably linked to the N-terminus of the second Fc chain of the Fc domain.

[00401] ***Modifications in Fc domain for heterodimerization***

[00402] ***The*** multi-specific polypeptide complex disclosed herein further comprises modifications in the Fc domains, facilitating the heterodimerization.

[00403] ***In*** some embodiments, the Fc chains comprise CH3 domain of IgG. In some embodiments, the Fc chains further comprise a CH2 domain and/or a hinge region. In some embodiments, the Fc chains comprise an Fc region derived from IgG1, IgG2, IgG3 or IgG4. In some embodiments, the Fc chains comprise CH3 domain of IgG.

[00404] ***In*** certain embodiments, the polypeptide complex disclosed herein comprise one or more amino acid substitution(s) in the interface of the CH3 domain to facilitate and/or promote heterodimerization. These modifications comprise introduction of a protuberance into a first CH3 domain and a cavity into a second CH3 domain, wherein the protuberance can be positioned in the cavity so as to promote interaction of the first and second dimerization domains to form a heterodimer or a complex (also referred to as knob-into-hole structure). Methods of generating antibodies with these modifications are known in the art, e.g. as described in U.S. Pat. No. 5,731,168.

[00405] ***In*** some embodiments, a "knob" is generated by replacing one or more small amino acid side chains from the interface of the first antibody molecule with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory "holes" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine).

[00406] ***In*** some embodiments, the Fc domain comprises a first mutation in one Fc chain and a second mutation in the other Fc chain.

[00407] ***In*** some embodiments, a) the first mutation comprises T389W or S375C, and the second mutation comprises Y370C, T389S, L391A, or Y438V; b) the first mutation comprises D427K or E377K, and the second mutation comprises K420D, or K440D; c) the first mutation comprises E377K, E378K, or D427K, and the second mutation comprises K393E, K440D, or K470E; d) the first mutation comprises S387H, or F436A, and the second mutation comprises Y370T, or T422F; e) the first mutation comprises S387H, or T422F, and the second mutation comprises Y422T, or F436A; f) the first mutation comprises K393D, or K440D, and the second mutation comprises E378K, or D427K; or g) the first mutation comprises L372D, or L391E, and the second mutation comprises L372K, or T389K, wherein numbering is according to the Kabat index.

[00408] ***In*** some embodiments, the polypeptide complex disclosed herein comprises a first CH3 region and a second CH3 region, wherein the first CH3 region or the second CH3 region comprises an amino acid sequence differing from wild-type IgG amino acid sequence such that one or more positive-charged amino acids (*e.g.*, lysine, histidine and arginine) in the wild-type human IgG amino acid sequence are replaced with one or more negative-charged amino acids (*e.g.*, aspartic acid and glutamic acid) at the corresponding position(s) in the CH3 region. Alternatively, the first CH3 region or the second CH3 region comprises van amino acid sequence differing from wild-type IgG amino acid sequence such that one or more negative-charged amino acids in the wild-type human IgG amino acid sequence are replaced with one or more positive-charged amino acids at the corresponding position(s) in the CH3 region.

[00409] In some embodiments, a) the first mutation comprises K393E/D427K/K470D, and the second mutation comprises D377K/E378K/K440D; b) the first mutation comprises K440D, and the second mutation comprises D427K; c) the first mutation comprises K440E, and the second mutation comprises D427K; d) the first mutation comprises K440E, and the second mutation comprises D427R; e) the first mutation comprises K440D, and the second mutation comprises D427R; f) the first mutation comprises D427K, and the second mutation comprises E377K; g)

the first mutation comprises E377K/D427K, and the second mutation comprises K420D/K440D; h) the first mutation comprises E377K/D427K, and the second mutation comprises K440D/K470D; i) the first mutation comprises E378K/D427K, and the second mutation comprises K393D/K440D; j) the first mutation comprises E377K/E378K/D427K, and the second mutation comprises K393D/K420D/K440D; k) the first mutation comprises E378K/D427K, and the second mutation comprises K420D/K440D; l) the first mutation comprises K420D/K440D, and the second mutation comprises D427K; m) the first mutation comprises K383D/K440D, and the second mutation comprises D427K, wherein numbering is according to the Kabat index.

[00410] In some embodiments, the polypeptide complex disclosed herein comprise one or more amino acid substitution(s) in the CH3 domain to facilitate purification of heterodimers. In some embodiments, the one or more amino acid substitution(s) exist in one of the Fc chains. In some embodiments, the one or more amino acid substitution(s) comprise H466R and Y467F. Methods of generating antibodies with these modifications are known in the art, e.g. as described in PCT publication WO2010151792A1.

(vii) Multi-specific Polypeptide Complexes with Exemplary Sequences

[00411] In certain embodiments, the multi-specific polypeptide complexes provided herein comprise: a) a CD3-binding domain, b) a PD-L1 domain, and c) a first disease antigen binding domain, wherein the CD3-binding domain and the PD-L1 domain are assembled to form a bispecific binding domain which is a DICAD domain, wherein: **the DICAD domain** comprises: i) a first polypeptide fragment comprising a first heavy chain variable domain (VH1) linked to a second light chain variable domain (VL2), and ii) a second polypeptide fragment comprising a second heavy chain variable domain (VH2) linked to a first light chain variable domain (VL1), wherein the VL1 and the VH1 associate to form a first domain capable of binding to a first target, and the VL2 and the VH2 associate to form a second domain capable of binding to a second target; wherein the first domain is a PD-L1 binding domain

provided herein (in particular, a low-affinity PD-L1 binding domain provided herein), and the second domain is a CD3 binding domain provided herein (in particular, a low-affinity CD3 binding domain provided herein).

[00412] ***In*** certain embodiments, the first disease antigen binding domain comprises a **first Fv** domain comprising: i) a third polypeptide fragment comprising a third heavy chain variable domain (VH3), and ii) a fourth polypeptide fragment comprising a third light chain variable domain (VL3); wherein the VH3 and the VL3 associate to form a third domain capable of binding to the disease antigen. In certain embodiments, the VH3 and the VL3 comprise the heavy chain variable domain sequence and the light chain variable domain sequence of a disease antigen binding domain provided herein.

[00413] ***In*** any of the above embodiments, the multi-specific polypeptide complexes further comprise a first Fc chain and a second Fc chain.

[00414] ***In*** certain embodiments, one of the termini of the DICAD domain is operably linked to one of the termini of the first Fv domain. In some embodiments, one of the C termini of the DICAD domain is operably linked to N termini of the first Fc chain. In certain embodiments, the C termini of the third polypeptide fragment of the first Fv domain is covalently linked to the N termini of the second polypeptide fragment of the DICAD domain, and the C termini of the first polypeptide fragment of the DICAD domain is covalently linked to the N termini of the first Fc chain.

[00415] ***In*** some embodiments, the multi-specific polypeptide complex further comprises a second disease antigen-binding domain and a second Fc chain, wherein the second Fc chain associates with the first Fc chain to form a dimer, wherein one of the C termini of the second antigen-binding domain is operably linked to the N-terminus of the second Fc chain. In some embodiments, the first disease antigen-binding domain and the second disease antigen-binding domain are identical.

[00416] ***In*** certain embodiments, the VH1, the VL1, the VH2 and the VL2 comprise the set of amino acid sequences of:

a) SEQ ID NOs: 25, 8, 36, and 16, respectively;

b) SEQ ID NOs: 26, 8, 36, and 16, respectively;

c) SEQ ID NOs: 27, 8, 36, and 16, respectively;

d) SEQ ID NOs: 28, 8, 36, and 16, respectively;

e) SEQ ID NOs: 7, 29, 36, and 16, respectively;

f) SEQ ID NOs: 7, 30, 36, and 16, respectively;

g) SEQ ID NOs: 7, 31, 36, and 16, respectively;

h) SEQ ID NOs: 27, 8, 34 and 16, respectively;

i) SEQ ID NOs: 135, 136, 34 and 16, respectively;

j) SEQ ID NOs: 148, 136, 34 and 16, respectively;

k) SEQ ID NOs: 149, 136, 34 and 16, respectively;

l) SEQ ID NOs: 150, 136, 34 and 16, respectively;

m) SEQ ID NOs: 135, 151, 34 and 16, respectively; or

n) SEQ ID NOs: 152, 136, 34 and 16, respectively;

[00417] ***In*** certain embodiments, the VH3 and the VL3 comprise a set of 6 CDR sequences comprising: a) SEQ ID NOs: 17-22; b) SEQ ID NOs:78-83; c) SEQ ID NOs: 105-110; d) SEQ ID NOs: 86-91, or e) SEQ ID NOs: 94-99.

[00418] In certain embodiments, the VH3 and the VL3 comprise a pair of amino acid sequences of: a) SEQ ID NOs: 23 and 24; b) SEQ ID NOs: 84 and 85; c) SEQ ID NOs: 111 and 112; d) SEQ ID NOs: 92 and 93; or e) SEQ ID NOs: 100 and 101.

[00419] An exemplary multi-specific polypeptide complex is 12A56, 12A57, 12A58, 12A59, 12A60, 12A61, 12A62, 12A70, 12A94, 12A98, 12A99, 12A106, 12A107, or 12A108. An exemplary multi-specific polypeptide complex is 5A52, replacing 12A73 in the CEA-binding domain with the EGFR-binding domain

provided herein; 2A42, replacing 12A73 in the CEA-binding domain with the CD20-binding domain provided herein; 24A3, replacing 12A73 in the CEA-binding domain with the Claudin6-binding domain provided herein; or 22A18, replacing 12A73 in the CEA-binding domain with the GPRC5D -binding domain provided herein.

[00420] In some embodiments, the first Fc chain and the second Fc chain comprise knob-in hole mutations to facilitate the interaction thereof. In some embodiments, the knob-in-hole mutations comprise T389W in one of the first Fc chain and the second Fc chain, and T389S, L391A, Y438V, H466R and Y467F in the other one, wherein numbering is according to the Kabat index..

[00421] ***In*** some embodiments, the first Fc chain and the second Fc chain comprise mutations to reduced binding to human CD32a, human CD16 and human CD64. In some embodiments, the first Fc chain and the second Fc chain comprise mutations of L247A and L248A, wherein numbering is according to the Kabat index.. In some embodiments, the first Fc chain and the second Fc chain comprise mutations of L247A, L248A and D278A, wherein numbering is according to the Kabat index.

[00422] ***In*** some embodiments, the first Fc chain and the second Fc chain comprises amino acid sequences of SEQ ID NO: 56 and SEQ ID NO: 77. In some embodiments, the first Fc chain and the second Fc chain comprise the amino acid sequence of SEQ ID NO: 58 and SEQ ID NO: 162. In certain embodiments, the first Fc chain and and the second Fc chain comprise the amino acid sequence of SEQ ID NO: 60 and SEQ ID NO: 61.

[00423] ***In*** certain embodiments, the multi-specific polypeptide complex 12A39 comprises four polypeptide chains having the following amino acid sequences respectively: SEQ ID NO: 70, SEQ ID NO: 63, SEQ ID NO: 72 and SEQ ID NO: 65.

[00424] ***In*** certain embodiments, the multi-specific polypeptide complex 12A73 comprises four polypeptide chains having the following amino acid sequences respectively: SEQ ID NO: 74, SEQ ID NO: 67, SEQ ID NO: 76 and SEQ ID NO: 65.

[00425] In certain embodiments, the multi-specific polypeptide complex 12A94 comprises four polypeptide chains having the following amino acid sequences respectively: SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 76 and SEQ ID NO: 65.

[00426] ***the*** multi-specific polypeptide complex 12A106 comprises four polypeptide chains having the following amino acid sequences respectively: SEQ ID NO: 159, SEQ ID NO: 158, SEQ ID NO: 76 and SEQ ID NO: 65.

[00427] The sequences and mutation information for 12A39, 12A73, 12A94 and 12A106 are provided below in Table 5.

Table 5. Exemplary multi-specific polypeptide complex

Structure	**Peptide (SEQ ID NO)**	**Sequence**	**Mutations** (Kabat numbering)
12A39	Chain 1 (SEQ ID NO: 70)	VL2(CD3)-**Linker 1**-VH1(PD-L1)-*Hinge 2*-Fc(knob) QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTPAR FSGSLLGGKAALTLSGAQAEDEADYYCALWYSNLWVFGGGTKLTVL**RTVAA**QVQLVQSGAE VKKPGSSVKVSCKTSGDTFSSYAISWVR**D**APGQ**C**LEWMGGIIPIFGKAHYAQKFQGRVTITADE STSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVTVSS*ASEPKSSDKTHTCPPC P*APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVKFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSRDEL TKNQVSL**W**CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSP F1: the dashed underline shows the sequence of VL2(CD3)-**linker 1**-VH1(PD-L1), which is set forth as SEQ ID NO: 62 VL2(CD3): SEQ ID NO: 16 Linker 1: SEQ ID NO: 53 VH1(PD-L1): SEQ ID NO: 47 Hinge2: SEQ ID NO: 120 Fc(knob): SEQ ID NO: 56	VL2: N/A VH1: Q39D and G44C Fc-(knob): T389W

Chain 2 (SEQ ID NO: 63)	VH3(CEA)-CH1-***Linker 2***-VL1(PD-L1)-**Linker 1**-VH2(CD3) EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD***GGGGSGGGGS***EIVLTQSPATLSLSPGERAT LSCRASQSVSSYLAWYQ**K**KPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVY YCQQRSNWPTFG**C**GTKVEIK**RTVAA**EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWV RQAPGKGLEWVGRIRSKYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAEDTAVYYCV RHGNFGTSYVSWFAYWGQGTLVTVSS F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 F2: the double underline shows the sequence of VL1(PD-L1)-Linker 1-VH2(CD3), which is set forth as SEQ ID NO: 50 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Linker2: SEQ ID NO: 104 VL1(PD-L1): SEQ ID NO: 48 Linker 1: SEQ ID NO: 53VH2(CD3): SEQ ID NO: 15	VH3: N/A CH1: N/A VL1: Q38K and G100C VH2: N/A
Chain 3 (SEQ ID NO: 72)	VH3(CEA)-CH1-**Hinge 1**-*Fc(hole)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD**KTHTCPPCP***APPVAGPSVFLFPPKPKDTLM* *ISRTPEVTCVVVDVSQEDPEVKFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY* *KCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPE*	VH3: N/A CH1: N/A Fc (hole): T389S, L391A, Y438V, H466R

		*NNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGNVFSCSVMHEALHN**R**F**T**QKSLSLSP* F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Hinge 1: SEQ ID NO: 54 Fc(Hole): SEQ ID NO: 77	and Y467F
	Chain 4 (SEQ ID NO: 65)	VL3(CEA)-CL EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPRPWIHGTSNLATGVPARFSGSG SGTDFTLTISSLEPEDAAVYYCQQWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC VL3(CEA): SEQ ID NO: 24 CL: SEQ ID NO: 52	VL3: N/A CL: N/A
12A73	Chain 1 (SEQ ID NO: 74)	VL2(CD3)-**Linker 1**-VH1(PD-L1)-*Hinge 2*-Fc(knob) QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTPAR FSGSLLGGKAALTLSGAQAEDEADYYCALWYSNLWVFGGGTKLTVL**RTVAAQVQLVQSGAE** VKKPGSSVKVSCKTSGDTFSTYAISWVR**D**APGQ**C**LEWMGGIIPIFG**F**AHYAQKFQGRVTITADE STSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVTVSS*ASEPKSSDKTHTCPPC* *PAPEAA*GGPSVFLFPPKPKDTLMISRTPEVTCVVV**A**VSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD ELTKNQVSL**W**CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPG	VL2: N/A VH1: Q39D, G44C and K56F Fc-(knob): L247A, L248A, D278A, T389W

		F1: the dashed underline shows the sequence of VL2(CD3)-**linker 1**-VH1(PD-L1), which is set forth as SEQ ID NO: 118 VL2(CD3): SEQ ID NO: 16 Linker 1: SEQ ID NO: 53 VH1(PD-L1): SEQ ID NO: 116 Hinge2: SEQ ID NO: 120 Fc(knob): SEQ ID NO: 59	
	Chain 2 (SEQ ID NO: 67)	VH3(CEA)-CH1-***Linker 2***-VL1(PD-L1)-**Linker 1**-VH2(CD3) EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD***GGGGSGGGGS***EIVLTQSPATLSLSPGERAT LSCRASQSVSSYLAWYQ**K**KPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVY YCQQRSNWPTFG**C**GTKVEIK**RTVAA**EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWV RQAPGKGLEWVGRIRS**Q**YNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAEDTAVYYCV RHGNFGTSYVSWFAYWGQGTLVTVSS F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 F2: the double underline shows the sequence of VL1(PD-L1)-Linker 1-VH2(CD3), which is set forth as SEQ ID NO: 119 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Linker2: SEQ ID NO: 104 VL1(PD-L1): SEQ ID NO: 48	VH3: N/A CH1: N/A VL1: Q38K and G100C VH2: K52B Q

		Linker 1: SEQ ID NO: 53 VH2(CD3): SEQ ID NO: 34	
	Chain 3 (SEQ ID NO: 76)	VH3(CEA)-CH1-**Hinge 1**-*Fc(hole)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD**KTHTCPPCP***APEAAGGPSVFLFPPKPKDTL* *MISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE* *YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQP* *ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRFTQKSLSLSPG* F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Hinge 1: SEQ ID NO: 54 Fc(Hole): SEQ ID NO: 61	VH3: N/A CH1: N/A Fc (hole): L247A, L248A, D278A, T389S, L391A, Y438V, H466R and Y467F
	Chain 4 (SEQ ID NO: 65)	VL3(CEA)-CL EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPRPWIHGTSNLATGVPARFSGSG SGTDFTLTISSLEPEDAAVYYCQQWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC VL3(CEA): SEQ ID NO: 24	VL3: N/A CL: N/A

12A94	Chain 1 (SEQ ID NO: 157)	CL: SEQ ID NO: 52 VL2(CD3)-**Linker 1**-VH1(PD-L1)-*Hinge 2*-Fc(knob) QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTPAR FSGSLLGGKAALTLSGAQAEDEADYYCALWYSNLWVFGGGTKLTVL**RTVAA**EVQLVESGGGL VQPGGSLRLSCAASGFTFSDSWIHWVR**D**APGK**C**LEWVAWISPYGGSTYYADSVKGRFTISADTS KNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS*ASEPKSSDKTHTCPPCP*APE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK NQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPG F1: the dashed underline shows the sequence of VL2(CD3)-**linker 1**-VH1(PD-L1), which is set forth as SEQ ID NO: 161 VL2(CD3): SEQ ID NO: 16 Linker 1: SEQ ID NO: 53 VH1(PD-L1): SEQ ID NO: 160 Hinge2: SEQ ID NO: 120 Fc(knob): SEQ ID NO: 56	VL2: N/A VH1: Q39D, G44C Fc-(knob): L247A, L248A, D278A, T389W
	Chain 2 (SEQ ID NO: 158)	VH3(CEA)-CH1-***Linker 2***-VL1(PD-L1)-**Linker 1**-VH2(CD3) EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD***GGGGSGGGGS***DIQMTQSPSSLSASVGDRV TITCRASQDVSTAVAWYQ**K**KPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFAT YYCQQYLYHPATFGC**G**TKVEIK**RTVAA**EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMN	VH3: N/A CH1: N/A VL1: Q38K and G100C

		WVRQAPGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAEDTAVYY CVRHGNFGTSYVSWFAYWGQGTLVTVSS F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 F2: the double underline shows the sequence of VL1(PD-L1)-Linker 1-VH2(CD3), which is set forth as SEQ ID NO: 156 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Linker2: SEQ ID NO: 104 VL1(PD-L1): SEQ ID NO: 154 Linker 1: SEQ ID NO: 53 VH2(CD3): SEQ ID NO: 34	VH2: K52B Q
	Chain 3 (SEQ ID NO: 76)	VH3(CEA)-CH1-**Hinge 1**-*Fc(hole)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD**KTHTCPPCP***APEA4GGPSVFLFPPKPKDTL MISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLS**C**AVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLV**S**KLTVDKSRWQQGNVFSCSVMHEALHNR**R**FTQKSLSLSPG* F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Hinge 1: SEQ ID NO: 54	VH3: N/A CH1: N/A Fc (hole): L247A, L248A, D278A, T389S, L391A, Y438V, H466R and Y467F

		Fc(Hole): SEQ ID NO: 61	
	Chain 4 (SEQ ID NO: 65)	VL3(CEA)-CL EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPRPWIHGTSNLATGVPARFSGSG SGTDFTLTISSLEPEDAAVYYCQQWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC VL3(CEA): SEQ ID NO: 24 CL: SEQ ID NO: 52	VL3: N/A CL: N/A
12A106	Chain 1 (SEQ ID NO: 159)	VL2(CD3)-**Linker 1**-VH1(PD-L1)-*Hinge 2*-Fc(knob) QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIGGTNKRAPWTPAR FSGSLLGGKAALTLSGAQAEDEADYYCALWYSNLWVFGGGTKLTVL**RTVAA**EVQLVESGGGL VQPGGSLRLSCAASGFTFSDSWIHWVR**D**APGK**C**LEWVAWISP**H**GGSTYYADSVKGRFTISADT SKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSS*ASEPKSSDKTHTCPPCP*AP EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPG F1: the dashed underline shows the sequence of VL2(CD3)-**linker 1**-VH1(PD-L1), which is set forth as SEQ ID NO: 155 VL2(CD3): SEQ ID NO: 16 Linker 1: SEQ ID NO: 53 VH1(PD-L1): SEQ ID NO: 153	VL2: N/A VH1: Q39D, G44C and Y53H Fc-(knob): L247A, L248A, D278A, T389W

		Hinge2: SEQ ID NO: 120 Fc(knob): SEQ ID NO: 56	
	Chain 2 (SEQ ID NO: 158)	VH3(CEA)-CH1-***Linker 2***-VL1(PD-L1)-**Linker 1**-VH2(CD3) EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD***GGGGSGGGGS***DIQMTQSPSSLSASVGDRV TITCRASQDVSTAVAWYQ**K**KPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFAT YYCQQYLYHPATFG**C**GTKVEIK**RTVAA**EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMN WVRQAPGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAEDTAVYY CVRHGNFGTSYVSWFAYWGQGTLVTVSS F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 F2: the double underline shows the sequence of VL1(PD-L1)-Linker 1-VH2(CD3), which is set forth as SEQ ID NO: 156 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Linker2: SEQ ID NO: 104 VL1(PD-L1): SEQ ID NO: 154 Linker 1: SEQ ID NO: 53 VH2(CD3): SEQ ID NO: 34	VH3: N/A CH1: N/A VL1: Q38K and G100C VH2: K52B Q

Chain 3 (SEQ ID NO: 76)	VH3(CEA)-CH1-**Hinge 1**-*Fc(hole)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPGKALEWLGFIRNKVNGDTTEY. AASVKGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD**KTHTCPPCP***APEAAGGPSVFLFPPKPKDTL* *MISRTPEVTCVVVAVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE* *YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQP* *ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRFTQKSLSLSPG* F3: the dashed underline shows the sequence of VH3(CEA)-CH1, which is set forth as SEQ ID NO: SEQ ID NO: 64 VH3(CEA): SEQ ID NO: 23 CH1: SEQ ID NO: 51 Hinge 1: SEQ ID NO: 54 Fc(Hole): SEQ ID NO: 61	VH3: N/A CH1: N/A Fc (hole): L247A, L248A, D278A, T389S, L391A, Y438V, H466R and Y467F
Chain 4 (SEQ ID NO: 65)	VL3(CEA)-CL EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPRPWIHGTSNLATGVPARFSGSG SGTDFTLTISSLEPEDAAVYYCQQWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGEC VL3(CEA): SEQ ID NO: 24 CL: SEQ ID NO: 52	VL3: N/A CL: N/A

The present disclosure further provides additional multi-specific complexes that are modified based on 12A39, in particular, modified in the PD-L1 binding domain to reduce PD-L1 binding affinity, modified in the Fc domain to reduce some or all FcγR Binding, or modified in the CD3-binding domain to reduce CD3 binding affinity. The modifications are summarized below in Table 6, and the full-length sequences can be thus derived from the sequences of 12A39.

Table 6

	Low Affinity Mutations relative to Sequences in 12A39					PD-L1 Kd (M)	CD3 Kd (M)	FcγR Binding*
	VL2(CD3)	VH1(PD-L1)	VL1(PD-L1)	VH2(CD3)	Fc			
12A39	/	/	/	/	IgG4 isotype with E246P,F247V, L248A,G249-, Q287K,R440K, Q450E mutations + knob/hole (SEQ ID NOs: 56 and 77)	2.98E-08	5.28E-08 8.64E-08	CD32a:+
12A63	/	K56D	/	/	/	7.56E-07	5.34E-08	Same as 12A39
12A64	/	K56A	/	/	/	6.54E-07	5.47E-08	Same as 12A39
12A65	/	K56F	/	/	/	2.11E-07	4.84E-08	Same as 12A39
12A66	/	H58D	/	/	/	2.12E-05	5.57E-08	Same as 12A39

12A67	/	/	Y32E	/	/	1.09E-06	5.86E-08	Same as 12A39
12A68	/	/	W94D	/	/	1.68E-06	6.59E-08	Same as 12A39
12A69	/	/	W94A	/	/	7.28E-08	6.65E-08	Same as 12A39
12A42	/	/	/	T28V	/			Same as 12A39
12A43	/	/	/	K52B H	/			Same as 12A39
12A44	/	/	/	K52B Q	/		1.18E-07	Same as 12A39
12A45	/	/	/	Y52C F	/			Same as 12A39
12A46	/	/	/	V100C A	/		1.76E-07	Same as 12A39
12A47	/	/	/	S100DT	/			Same as 12A39
12A51	/	/	/	K52B D	/			Same as 12A39
12A56	/	/	K56D	/	V100C A	/	7.67E-07	1.08E-07	Same as 12A39
12A57	/	/	K56A	/	V100C A	/	9.69E-07	1.04E-07	Same as 12A39
12A58	/	/	K56F	/	V100C A	/	2.82E-07	1.07E-07	Same as 12A39
12A59	/	/	H58D	/	V100C A	/	7.00E-06	9.01E-08	Same as 12A39

12A60	/	/	Y32E	V100C A	/	2.64E-05	1.77E-07	Same as 12A39
12A61	/	/	W94D	V100C A	/	3.67E-06	9.59E-08	Same as 12A39
12A62	/	/	W94A	V100C A	/	7.88E-08	8.81E-08	Same as 12A39
12A70	/	K56F		K52B Q	/	2.82E-07	1.030E-07	Same as 12A39
12A52	/	/	/	/	IgG1 isotype with L247A/L248A mutation+ knob/hole (SEQ ID Nos: 58 and 121)			CD16a: - CD32a: - CD64: +
12A74	/	K56F	/	/	IgG1 isotype with L247A/L248A mutation and D278A+ knob/hole +RF mutations			Same as 12A73
12A73	/	K56F		K52B Q	IgG1 isotype with L247A/L248A mutation and D278A+ knob/hole+RF mutations			CD32a: - CD16a: - CD64: -

					(SEQ ID Nos: 60 and 61)			

*: + means positive binding; - means negative binding;

The present disclosure further provides additional multi-specific complexes that are modified based on 12A94, in particular, modified in the PD-L1 binding domain to reduce PD-L1 binding affinity, modified in the Fc domain to reduce some or all FcγR Binding, or modified in the CD3-binding domain to reduce CD3 binding affinity. The modifications are summarized below in Table 7, and the full-length sequences can be thus derived from the sequences of 12A94.

Table 7

	Low Affinity Mutations relative to Sequences in 12A39					PD-L1 Kd (M)	CD3 Kd (M)	FcγR Binding*
	VL2(CD3)	VH1(PD-L1)	VL1(PD-L1)	VH2(CD3)	Fc			
12A94	/	/	/	K52B Q	IgG1 isotype with L247A/L248A mutation and D278A+ knob/hole+RF mutations (SEQ ID Nos: 60 and 61)	4.127E-09	Same as 12A73	Same as 12A73
12A95	/	/	S30H	K52B Q	/	3.266E-09	Same as 12A73	Same as 12A73

12A96	/	/	S30T	K52B Q	/	4.094E-09	Same as 12A73	Same as 12A73
12A97	/	S56G	/	K52B Q	/	1.434E-09	Same as 12A73	Same as 12A73
12A98	/	Y53C	/	K52B Q	/		Same as 12A73	Same as 12A73
12A99	/	Y53G	/	K52B Q	/		Same as 12A73	Same as 12A73
12A106	/	Y53H	/	K52B Q	/	3.490E-08	Same as 12A73	Same as 12A73
12A107	/	/	S30E	K52B Q	/	3.809E-09	Same as 12A73	Same as 12A73
12A108	/	Y53Q	/	K52B Q	/	1.092E-07	Same as 12A73	Same as 12A73

III. Polynucleotides and Recombinant Methods

[00428] ***The*** present disclosure provides a nucleic acid comprising a nucleotide sequence that encodes the polypeptide complex provided herein. The term "nucleic acid" or "nucleotide sequence" as used herein refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless otherwise indicated, a particular polynucleotide sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (see Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

[00429] ***The*** polynucleotides encoding the polypeptide complex disclosed herein may be generated using methods known in the art. In certain embodiments, the sequence of the polynucleotides may be obtained based on the amino acid sequences of the polypeptide complex, and nucleic acids can be generated using synthetic methods. Alternatively, the polynucleotides provided herein can also be obtained from another available nucleic acid that encodes a polypeptide with a sequence homologous to the polypeptides in the polypeptide complex disclosed herein. Then a DNA manipulation process can be applied to manipulate the sequence of the parent antibody-encoding nucleic acid, such as introducing mutations, insertion, deletion, etc., so as to obtain the nucleic acid encoding the polypeptide complex disclosed herein.

[00430] ***The*** nucleotide sequence that encodes the polypeptide complex can be inserted into one or more vector(s) for further cloning (amplification of the DNA) or for expression, using recombinant techniques known in the art. Many vectors are

available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter (*e.g.*, SV40, CMV, EF-1α), a transcription termination sequence, and one or more other regulatory elements.

[00431] ***The*** present disclosure provides vectors comprising the nucleic acid provided herein. In certain embodiments, the nucleic acid provided herein encodes the antibodies, with at least one promoter (*e.g.*, SV40, CMV, EF-1α) operably linked to the nucleic acid sequence, and at least one selection marker. Examples of vectors include, but are not limited to, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.*, herpes simplex virus), poxvirus, baculovirus, papillomavirus, papovavirus (*e.g.*, SV40), lambda phage, and M13 phage, plasmid pcDNA3.3, pMD18-T, pOptivec, pCMV, pEGFP, pIRES, pQD-Hyg-GSeu, pALTER, pBAD, pcDNA, pCal, pL, pET, pGEMEX, pGEX, pCI, pEGFT, pSV2, pFUSE, pVITRO, pVIVO, pMAL, pMONO, pSELECT, pUNO, pDUO, Psg5L, pBABE, pWPXL, pBI, p15TV-L, pPro18, pTD, pRS10, pLexA, pACT2.2, pCMV-SCRIPT.RTM., pCDM8, pCDNA1.1/amp, pcDNA3.1, pRc/RSV, PCR 2.1, pEF-1, pFB, pSG5, pXT1, pCDEF3, pSVSPORT, pEF-Bos etc.

[00432] Vectors comprising the nucleotide sequence encoding the fusion polypeptide or the polypeptide complex can be introduced to a host cell for cloning or gene expression. Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis*, *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*.

[00433] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for the polypeptide complex-encoding

vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, e.g. *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g. *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

[00434] ***Suitable*** host cells for the expression of glycosylated polypeptide complex provided herein are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruiffly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx* mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

[00435] ***However***, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney

cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2). In some embodiments, the host cell is a mammalian cultured cell line, such as CHO, BHK, NS0, 293 and their derivatives.

[00436] Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In another embodiment, the antibodies may be produced by homologous recombination known in the art. In certain embodiments, the host cell is capable of producing the fusion polypeptide or the polypeptide complex provided herein.

[00437] The present disclosure also provides a method of expressing the polypeptide complex provided herein, comprising culturing the host cell provided herein under the condition at which the vector of the present disclosure is expressed. The host cells used to produce the antibodies provided herein may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem. 102:255 (1980), U.S. Pat. No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine),

antibiotics (such as GENTAMYCINTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to a person skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to a person skilled in the art.

[00438] ***When*** using recombinant techniques, the polypeptide complex can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the fusion polypeptide or the polypeptide complex is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter et al., Bio/Technology 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of E. coli. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the fusion polypeptide or the polypeptide complex is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[00439] The polypeptide complex prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, DEAE-cellulose ion exchange chromatography, ammonium sulfate precipitation, salting out, and affinity chromatography, with affinity chromatography being the preferred purification technique.

[00440] In certain embodiments, Protein A immobilized on a solid phase is used for immunoaffinity purification of the fusion polypeptide or the polypeptide complex.

The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the polypeptide complex. Protein A can be used to purify antibodies that are based on human gamma1, gamma2, or gamma4 heavy chains (Lindmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human gamma3 (Guss et al., EMBO J. 5:1567 1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the polypeptide complex comprises a CH3 domain, the Bakerbond ABXTM resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSETM chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[00441] ***Following*** any preliminary purification step(s), the mixture comprising the fusion polypeptide or the polypeptide complex of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

IV. Pharmaceutical Composition

[00442] The present invention further provides a pharmaceutical composition comprising the polypeptide complex described herein and a pharmaceutically acceptable carrier.

[00443] ***As*** used herein, the term "pharmaceutically acceptable" indicates that the designated carrier, vehicle, diluent, excipient(s), salt and/or medium is generally chemically and/or physiologically compatible with other ingredients, such as the

active ingredient (*i.e.*, the polypeptide complex or the heterodimeric antibody or antigen-binding fragment thereof) comprising the formulation, and is physiologically compatible with a subject receiving the pharmaceutical composition.

[00444] A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is bioactivity acceptable and nontoxic to a subject. In the context of the present disclosure, a pharmaceutical acceptable carrier for use in the pharmaceutical composition disclosed herein may include, for example, pharmaceutically acceptable liquid, gel or solid carriers, aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispending agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, other components known in the art, or various combinations thereof.

[00445] Herein, suitable "components" may include, for example, antioxidants, fillers, binders, disintegrants, buffers, preservatives, lubricants, flavorings, thickeners, coloring agents, emulsifiers or stabilizers such as sugars and cyclodextrins. Suitable "antioxidants" may include, for example, methionine, ascorbic acid, EDTA, sodium thiosulfate, platinum, catalase, citric acid, cysteine, thioglycerol, thioglycolic acid, thiosorbitol, butylated hydroxanisol, butylated hydroxytoluene, and/or propyl gallate. As disclosed herein, inclusion of one or more antioxidants such as methionine in a pharmaceutical composition provided herein decreases oxidation of the polypeptide complex or heterodimeric antibody or antigen-binding fragment thereof. This reduction in oxidation prevents or reduces loss of binding affinity, thereby improving protein stability and maximizing shelf-life. Therefore, in certain embodiments, a pharmaceutical composition is provided that comprise, in addition to the active ingredient (*i.e.*, the polypeptide complex or the heterodimeric antibody or antigen-binding fragment thereof disclosed herein), one or more antioxidants such as methionine.

[00446] ***The*** pharmaceutical acceptable carriers may include, for example, aqueous vehicles such as sodium chloride injection, Ringer's injection, isotonic dextrose

injection, sterile water injection, or dextrose and lactated Ringer's injection, nonaqueous vehicles such as fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil, or peanut oil, antimicrobial agents at bacteriostatic or fungistatic concentrations, isotonic agents such as sodium chloride or dextrose, buffers such as phosphate or citrate buffers, antioxidants such as sodium bisulfate, local anesthetics such as procaine hydrochloride, suspending and dispersing agents such as sodium carboxymethylcelluose, hydroxypropyl methylcellulose, or polyvinylpyrrolidone, emulsifying agents such as Polysorbate 80 (TWEEN-80), sequestering or chelating agents such as EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol tetraacetic acid), ethyl alcohol, polyethylene glycol, propylene glycol, sodium hydroxide, hydrochloric acid, citric acid, or lactic acid. Antimicrobial agents utilized as carriers may be added to pharmaceutical compositions in multiple-dose containers that include phenols or cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and benzethonium chloride. Suitable excipients may include, for example, water, saline, dextrose, glycerol, or ethanol. Suitable non-toxic auxiliary substances may include, for example, wetting or emulsifying agents, pH buffering agents, stabilizers, solubility enhancers, or agents such as sodium acetate, sorbitan monolaurate, triethanolamine oleate, or cyclodextrin.

[00447] ***Pharmaceutically*** acceptable "diluents" may include saline and aqueous buffer solutions.

[00448] Pharmaceutically acceptable "adjuvants" may include preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought

about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[00449] The pharmaceutical compositions can be a liquid solution, suspension, emulsion, pill, capsule, tablet, sustained release formulation, or powder. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, etc.

[00450] In embodiments, the pharmaceutical compositions are formulated into an injectable composition. The injectable pharmaceutical compositions may be prepared in any conventional form, such as for example liquid solution, suspension, emulsion, or solid forms suitable for generating liquid solution, suspension, or emulsion. Preparations for injection may include sterile and/or non-pyretic solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use, including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready to be combined with a vehicle just prior to use, and sterile and/or non-pyretic emulsions. The solutions may be either aqueous or nonaqueous.

[00451] In certain embodiments, unit-dose parenteral preparations are packaged in an ampoule, a vial or a syringe with a needle. All preparations for parenteral administration should be sterile and not pyretic, as is known and practiced in the art.

[00452] In certain embodiments, a sterile, lyophilized powder is prepared by dissolving the polypeptide complex as disclosed herein in a suitable solvent. The solvent may contain an excipient which improves the stability or other pharmacological components of the powder or reconstituted solution, prepared from the powder. Excipients that may be used include, but are not limited to, water, dextrose, sorbital, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agents. The solvent may contain a buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art at, in one

embodiment, about neutral pH. Subsequent sterile filtration of the solution followed by lyophilization under standard conditions known to those of skill in the art provides a desirable formulation. In one embodiment, the resulting solution will be apportioned into vials for lyophilization. Each vial can contain a single dosage or multiple dosages of the polypeptide complex, the polypeptide complex. Overfilling vials with a small amount above that needed for a dose or set of doses (e.g., about 10%) is acceptable so as to facilitate accurate sample withdrawal and accurate dosing. The lyophilized powder can be stored under appropriate conditions, such as at about 4 °C to room temperature.

[00453] ***Reconstitution*** of a lyophilized powder with water for injection provides a formulation for use in parenteral administration. In one embodiment, for reconstitution the sterile and/or non-pyretic water or other liquid suitable carrier is added to lyophilized powder. The precise amount depends upon the selected therapy being given, and can be empirically determined.

[00454] In certain embodiments, a composition is further provided, comprising a pharmaceutically acceptable carrier, diluent or adjuvant, and an active ingredient. The active ingredient can be the polypeptide complex disclosed herein, or a conjugate of the polypeptide complex disclosed herein.

V. Conjugates

[00455] The polypeptide complex as provided herein can be used in a non-conjugated form or in a conjugated form.

[00456] The terms "conjugated" and "joining" generally refer to a chemical linkage, either covalent or non-covalent that proximally associates one molecule with second molecule. The conjugates include any antibody of the present disclosure and an agent. The agent may be selected from a therapeutic agent, an imaging agent, a labeling agent, or an agent useful for therapeutic and/or labeling purposes.

[00457] In a conjugated form, the polypeptide complexes are conjugated to one or more desired conjugate moieties, *i.e.*, heterologous moieties, to realize certain functionalities, *e.g.*, to facilitate target detection or for imaging or therapy.

[00458] ***Herein***, the present disclosure provides a conjugate, which comprises the polypeptide complex provided herein, and a conjugate moiety (*e.g.*, payload) that is conjugated thereto. The payload can be any one of the group consisting of a radioactive label, a fluorescent label, an enzyme-substrate label, an affinity purification tag, a tracer molecule, an anticancer drug, and a cytotoxic molecule.

[00459] A variety of conjugates can be linked to the multi-specific polypeptide complex provided herein by covalent binding, affinity binding, intercalation, coordinate binding, complexation, association, blending, or addition, among others.(see, *e.g.*, "Conjugate Vaccines", Contributions to Microbiology and Immunology, J. M. Cruse and R. E. Lewis, Jr. (eds.), Carger Press, New York, (1989)).

[00460] ***In*** certain embodiments, the polypeptide complex provided herein may be engineered to contain specific sites outside the epitope binding portion that may be specifically utilized for binding to one or more conjugates. For example, such a site may include one or more reactive amino acid residues, such as for example cysteine or histidine residues, to facilitate covalent linkage to a conjugate.

[00461] In certain embodiments, the N-terminus and/or C-terminus of the polypeptide complex provided herein can also serve to provide reactive groups for conjugation. For example, the N-terminus can be conjugated to one moiety (*e.g.*, polyethylene glycol (PEG), *etc.*) and the C-terminus is conjugated to another moiety (*e.g.*, biotin, etc.).

[00462] ***In*** certain embodiments, the polypeptide complex provided herein may be linked to a conjugate directly, or indirectly for example through another conjugate or through a linker.

[00463] ***For*** example, the polypeptide complex provided herein having a reactive residue such as cysteine may be linked to a thiol-reactive agent in which the reactive group is, for example, a maleimide, an iodoacetamide, a pyridyl disulfide, or other thiol-reactive conjugation partner (Haugland, 2003, Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Inc.; Brinkley, 1992, Bioconjugate Chem. 3:2; Garman, 1997, Non-Radioactive Labelling: A Practical Approach, Academic Press, London; Means (1990) Bioconjugate Chem. 1:2; Hermanson, G. in Bioconjugate Techniques (1996) Academic Press, San Diego, pp. 40-55, 643-671).

[00464] ***For*** another example, the polypeptide complex provided herein may be conjugated to biotin, then indirectly conjugated to a second conjugate that is conjugated to avidin. For still another example, the polypeptide complex may be linked to a linker which further links to the conjugate. Examples of linkers include bifunctional coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suherate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and his-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., Biochem. J. 173:723-737 (1978)) and N-succinimidyl-4-(2-pyridylthio) pentanoate (SPP) to provide for a disulfide linkage.

[00465] In certain embodiments, the conjugate moiety comprises an agent for detection or isolation, such as a clearance-modifying agent, a chemotherapeutic agent, a toxin, a radioactive isotope, a lanthanide, a luminescent label, a fluorescent label, an enzyme-substrate label, a DNA-alkylator, a topoisomerase inhibitor, a tubulin-binder, or other anticancer drugs.

[00466] The conjugate moiety can be a detectable label, a pharmacokinetic modifying moiety, a purification moiety, a cytotoxic moiety or a therapeutic agent. Examples of detectable label may include a fluorescent labels (*e.g.*, fluorescein, rhodamine, dansyl, phycoerythrin, or Texas Red), enzyme-substrate labels (*e.g.*, horseradish peroxidase, alkaline phosphatase, luceriferases, glucoamylase, lysozyme, saccharide oxidases or β-D-galactosidase), radioisotopes (*e.g.*, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{35}S, ^{3}H, ^{111}In, ^{112}In, ^{14}C, ^{64}Cu, ^{67}Cu, ^{86}Y, ^{88}Y, ^{90}Y, ^{177}Lu, ^{211}At, ^{186}Re, ^{188}Re, ^{153}Sm, ^{212}Bi, and ^{32}P, other lanthanides, luminescent labels), chromophoric moiety, digoxigenin, biotin/avidin, a DNA molecule or gold for detection.

[00467] ***In*** certain embodiments, the conjugate moiety can be a pharmacokinetic modifying moiety such as PEG which helps increase half-life of the antibody. Other suitable polymers include, such as, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, copolymers of ethylene glycol/propylene glycol, and the like. The polymer may be of any molecular weight and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In certain embodiments, the conjugate can be a purification moiety such as a magnetic bead.

[00468] In certain embodiments, the conjugate moiety can be a cytotoxic moiety. A "cytotoxic moiety" can be any agent that is detrimental to cells or that can damage or kill cells. Examples of cytotoxic moiety include, without limitation, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin and analogs thereof, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin

(formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). In some embodiments, the conjugate moiety comprises an enzymatically active toxin or a fragment thereof, including but not limited to diphtheria A chain, non-binding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins, Momordica charantia inhibitor, curcin, crotin, Sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[00469] ***Methods*** for the conjugation of conjugate moieties to proteins such as antibodies, immunoglobulins or fragments thereof are found, for example, in U.S. Pat. No. 5,208,020; U.S. Pat. No. 6,4411,163; WO2005037992; WO2005081711; and WO2006/034488, which are incorporated herein by reference to the entirety.

[00470] In ***certain*** embodiments, the polypeptide complex provided herein are used as a base for a conjugate.

VI. Composition

[00471] In another aspect, the present invention provides a composition comprising the polypeptide complex or the conjugate described herein and a pharmaceutically acceptable carrier.

VII. Medical Use

[00472] In another aspect, the present invention provides a method for treating a disease condition in a subject that is in need of such treatment, comprising: administering to the subject a therapeutically effective amount of the polypeptide complex of the present invention, the pharmaceutical composition described herein, the conjugate described herein, or the composition described herein.

[00473] As used herein, the term "subject" or "individual" or "animal" or "patient" refers to human or non-human animal, including a mammal or a primate, in need of diagnosis, prognosis, amelioration, prevention and/or treatment of a disease or disorder. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sports, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, swine, cows, bears, and so on. In certain embodiments, the subject is human.

[00474] As used herein, "treatment" of a condition may include, alleviating a condition, slowing the onset or rate of development of a condition, delaying the development of symptoms associated with a condition, reducing or ending symptoms associated with a condition, generating a complete or partial regression of a condition, or some combinations thereof.

[00475] As used herein, the term "disorder," "disease," "condition" or alike, refers to a condition that affects a subject who would nonetheless benefits from treatment with the polypeptide complex.

[00476] As used herein, the term "therapeutically effective amount" of a therapeutic agent refers to an amount of the therapeutic agent that, when taken by a subject in an appropriate manner, can generate sufficient therapeutic effects to the subject. It is to be understood that just like other therapeutic drugs, the therapeutically effective amount of the polypeptide complex as provided above will be influenced by various factors known in the art, such as for example body weight, age, past medical history, present medications, state of health of the subject and potential for cross-reaction, allergies, sensitivities and adverse side-effects, as well as the administration route and extent of disease development. Dosages may be proportionally reduced or increased by one of ordinary skill in the art (*e.g.*, physician or veterinarian) as indicated by these and other circumstances or requirements.

[00477] In certain embodiments, the polypeptide complex as provided herein may be administered at a therapeutically effective amount of about 0.01 mg/kg to about 100 mg/kg. Dosage regimens may be adjusted to provide the optimum desired response

(*e.g.*, a therapeutic response). For example, a single dose may be administered, or several divided doses may be administered over time.

[00478] The polypeptide complex described above and the method disclosed herein can be applied to treat a wide variety of diseases. In humans, and other primates as well, the diseases that are contemplated to be treatable by the polypeptide complex described above and the method disclosed herein can include the following:

[00479] (1) cancers and other hyperproliferative disorders, including both benign or malignant tumors, leukemia and lymphoid malignancies. Depending on the cell type having cancers or hyperproliferative disorders, examples include neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, endothelial, and stromal malignancies. Depending on the organ/location afflicted with cancers or hyperproliferative disorders, examples include cancers of the head, neck, eye, mouth, throat, esophagus, chest, skin, bone, lung, colon, rectum, colorectal, stomach, spleen, kidney, skeletal muscle, subcutaneous tissue, metastatic melanoma, endometrial, prostate, breast, ovaries, testicles, thyroid, blood, lymph nodes, kidney, liver, pancreas, brain, or central nervous system;

[00480] (2) autoimmune and/or inflammatory disorders, including alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, Sjogren's syndrome, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasia (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation, sepsis, rheumatoid arthritis, peritonitis, Crohn's disease, reperfusion injury, septicemia, endotoxic shock, cystic fibrosis, endocarditis, psoriasis, arthritis (e.g., psoriatic arthritis), anaphylactic shock, organ ischemia, reperfusion injury, spinal cord injury and allograft rejection. autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic

inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, CREST syndrome, cold agglutinin disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erythematosus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychrondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynauld's phenomenon, Reiter's syndrome, Rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, takayasu arteritis, temporal arteristis/giant cell arteritis, ulcerative colitis, uveitis, vasculitides such as dermatitisherpetiformis vasculitis, vitiligo, and Wegener's granulomatosis. Inflammatory disorders, can further include, but are not limited to, asthma, encephalitis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), allergic disorders, septic shock, pulmonary fibrosis, undifferentiated spondyloarthropathy, undifferentiated arthropathy, arthritis, inflammatory osteolysis, and chronic inflammation resulting from chronic viral or bacterial infections; bispecific antibodies targeting CD3 and a disease antigen (e.g. T cell engagers) have been reported to be effective in treating autoimmune diseases, see, for example, Perico, L. et al, Front. Immunol., Volume 15 - 2024; Bucci L. et al, Nature Medicine, 30, pages1593–1601 (2024); Subklewe, M. et al, European Journal of Cancer 204 (2024) 114071).

[00481] (3) infectious and parasitic diseases, such as those caused by viruses (e.g. HBV, HCV, HIV, RSV, hMPV, PIV, coronaviruses, or influenza viruses, etc.), fungi (e.g. *Naegleria, Aspergillus, Blastomyces, Histoplasma, Candida* or *Tinea* genera, etc.), eukaryotic microbes (e.g. *Giardia, Toxoplasma, Plasmodium, Trypanosoma,*

and *Entamoeba* genera, etc.), and bacteria (*Staphylococcus, Streptococcus, Pseudomonas, Clostridium, Borrelia, Vibro and Neiserria genera*, etc.);

[00482] (4) other disease or disorders, including those not covered by an of the above in (1)-(3), such as cardiovascular diseases, neuropathies, neuropsychiatric conditions, injuries, or coagulation disorder, etc.

[00483] The polypeptide complex disclosed herein may be administered by any route known in the art, such as for example parenteral (e.g., subcutaneous, intraperitoneal, intravenous, including intravenous infusion, intramuscular, or intradermal injection) or non-parenteral (e.g., oral, intranasal, intraocular, sublingual, rectal, or topical) routes.

[00484] In some embodiments, the polypeptide complex disclosed herein may be administered alone or in combination with one or more additional therapeutic means or agents. For example, the polypeptide complex disclosed herein may be administered in combination with another therapeutic agent, for example, a chemotherapeutic agent or an anti-cancer drug.

[00485] In certain of these embodiments, the polypeptide complex as disclosed herein that is administered in combination with one or more additional therapeutic agents may be administered simultaneously with the one or more additional therapeutic agents, and in certain of these embodiments the polypeptide complex and the additional therapeutic agent(s) may be administered as part of the same pharmaceutical composition. However, the polypeptide complex administered "in combination" with another therapeutic agent does not have to be administered simultaneously with or in the same composition as the agent. The polypeptide complex administered prior to or after another agent is considered to be administered "in combination" with that agent as the phrase is used herein, even if the polypeptide complex and second agent are administered via different routes. Where possible, additional therapeutic agents administered in combination with the polypeptide complex disclosed herein are administered according to the schedule listed in the

product information sheet of the additional therapeutic agent, or according to the Physicians' Desk Reference 2003 (Physicians' Desk Reference, 57th Ed; Medical Economics Company; ISBN: 1563634457; 57th edition (November 2002)) or protocols well known in the art.

[00486] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. All specific compositions, materials, and methods described below, in whole or in part, fall within the scope of the present invention. These specific compositions, materials, and methods are not intended to limit the invention, but merely to illustrate specific embodiments falling within the scope of the invention. A person skilled in the art may develop equivalent compositions, materials, and methods without the exercise of inventive capacity and without departing from the scope of the invention. It will be understood that many variations can be made in the procedures herein described while still remaining within the bounds of the present invention. It is the intention of the inventors that such variations are included within the scope of the invention.

EXAMPLES:

Example 1: Construction and testing of 12A39

[00487] Construction of 2:1:1 Asymmetric Tri-specific Antibody

[00488] An asymmetric structure can be applied to construct a 2:1:1 tri-specific antibody by introducing a knobs-into-hole mutation in CH3 domain. Various 2:1:1 asymmetric tri-specific antibody constructs could be formed depending on different construction methods (Table 3).

[00489] A series of 2:1:1 symmetric tri-specific antibodies were constructed, namely 12A22 (see FIG 1, in TRIAD structure), 12A39 (see FIG 2, in TYPE E structure), in two different formats or structures. Each of these tri-specific antibody is composed of two asymmetric halves, bundled together via heterodimerized Fc domains. 12A22 is composed of four different polypeptide chains. Each polypeptide chain comprises one or more polypeptide fragments, namely, polypeptide fragment AL (AL), second

polypeptide fragment AH (AH), third polypeptide fragment BH (BH), and fourth polypeptide fragment BL (BL), which are shown in FIG 1 as AL, AH, BH and BL.

[00490] 12A39 is composed of five polypeptide chains, in which two are identical, hence making a total of four different polypeptide chains. Each polypeptide chain comprises one or more polypeptide fragments, namely, first polypeptide fragment (F1), second polypeptide fragment (F2), third polypeptide fragment (F3), and fourth polypeptide fragment (F4), which are shown in FIG 2 as ①, ②, ③ and ④. Each polypeptide fragment is composed of one or more domains, as outlined below in the Table 8, which provides a more detailed description of the domains in the tri-specific antibodies.

Table 8. Structures of different TRIAD-constructed tri-specific antibodies and 2:1:1 asymmetric tri-specific antibodies

	Structure	Peptide	Domain 1	Domain 2	Domain 3	Domain 4	Domain 5	Domain 6	Domain 7
12A22 (FIG 1)	**TRIAD**	12A22 AL (SEQ ID NO: 63)	VL1(CEA) (Q38D, Q100C)	VH2(CD3)	/	/	/	/	/
		12A22 AH (SEQ ID NO: 64)	VL2(CD3)	VH1(CEA) (Q39K, G44C)	hinge	CH2-CH3(knob)		/	/
		12A22 BH (SEQ ID NO: 65)	VH3(CEA)	CH1	hinge	CH2-CH3(hole)		/	/
		12A22 BL (SEQ ID NO: 66)	VL3(CEA)	CL	/	/	/	/	/
12A39 (FIG 2)	**Type E**	12A39 F3-F2 (SEQ ID NO: 71)	VH3(CEA)	CH1	linker	VL1(**PDL1**) (SEQ ID NO: 8)	VH2(CD3)	/	/
		12A39 F1 (SEQ ID NO: 72)	VL2(CD3)	VH1(**PDL1**) (SEQ ID NO: 7)	hinge	CH2-CH3(knob)		/	/
		12A39 F3 (SEQ ID NO: 73)	VH3(CEA)	CH1	hinge	CH2-CH3(hole)		/	/
		12A39 F4 (SEQ ID NO: 74)	VL3(CEA)	CL	/	/	/	/	/

F1: first polypeptide fragment; F2: second polypeptide fragment; F3: third polypeptide fragment; F4: fourth polypeptide fragment;

1.1 In vitro Cell killing assay

[00491] Experimental Methods:

[00492] LS174T cells were cultivated to a good condition and the culture medium was removed. The cells were then digested with trypsin, counted and an appropriate amount of the cells was taken, centrifuged at 1000rpm for 5 minutes, resuspended with RPMI-1640 medium supplemented with 2% FBS to 300,000/ml, seeded in a 96-well cell culture plate (with clear bottom).

[00493] Peripheral blood mononuclear cells (PBMCs) were taken and centrifuged at 1000rpm for 5 minutes. Then PBMCs were resuspended with 3 ml of RPMI-1640 medium supplemented with 2% FBS, diluted 4 times, counted, the density of PBMCs was adjusted with RPMI-1640 medium supplemented with 2% FBS to 3,000,000/ml, and 50 μl per well of each adjusted PBMCs solution was added into each well of the LS174T cell plate. The test trispecific antibodies (*i.e.*, 12A22 and 12A39) were diluted to 200nM with RPMI-1640 medium supplemented with 2% FBS, and then a 5-fold gradient dilution was performed (40 μl + 160 μl), resulting in a total of 9 points. Each well of the cell culture plate was subsequently added with 50 μl of the diluted antibody drug. The cell culture plates were incubated at 37°C with 5% CO2 for 24 hours.

[00494] Each well was added with 100 μl of PBS, followed by the addition of 100 μl of CTG reagent per well. The cells were thoroughly lysed by repeated pipetting and agitation using a pipette, and approximately 10 minutes later, a white film was attached to the bottom of the 96-well plate, and the luminescence value was read using an ELISA reader.

[00495] The upper culture medium (150 μl/well) of the aforementioned experiment was taken and centrifuged at 1000rpm for 5 minutes. The cytokine IL-6 in the samples was then detected using the Luminex Discovery Assay Human Premixed Multi-Analyte Kit (LXSAHM).

[00496] Experimental Results:

[00497] The analysis of the cell cytotoxicity and cytokine (*i.e.*, IL-6) release data reveals that the trend of cytokine release for 12A22 is generally consistent with the cytotoxicity (See FIG 3). On the other hand, 12A39 exhibits stronger cell cytotoxicity but lower cytokine release than 12A22, and thus achieved a certain safety window between cell cytotoxicity and cytokine release (See FIG 4). However, overall, the level of IL-6 release was still relatively high.

1.2 In vivo animal pharmacodynamics (PD) study

[00498] Experimental Methods:

[00499] Female huPBMC-NCG mice were inoculated subcutaneously with 0.1 ml LS174T cells (5×10^6/each) in the right back of each mouse. Three days after inoculation, each mouse was injected intraperitoneally with 0.1ml PBMCs (1×10^7/each). After 10 days of inoculation, the mice developing a tumor volume of 113.7-282.3mm^3 were selected, and randomly divided into 4 groups of 6 mice according to the tumor volume and the weight of the selected mice. A negative control (PBS, i.e., Vehicle), 12A22(5 mg per kg body weight (mpk)), 12A22 (1 mpk), 12A22 (0.2mpk) and 12A39 (5 mpk) were administered at a dose of 10ml/kg/mouse through the tail vein of mice from different groups two times a week for 3 weeks. The tumor diameter was measured twice a week with vernier calipers, and the tumor volume (V) was calculated by the following formula:

[00500] Criteria for Measurements:

[00501] $V=1/2\times a\times b^2$

[00502] in which a and b denote length and width, respectively.

[00503] Based on the experiment above, a dose-dependent study of the effect of 12A39 on LS174T tumor growth was further conducted using dosages of 0.2mpk, 1mpk, and 5mpk. The administration was carried out twice a week to observe the

inhibitory effect of 12A39 on tumor growth.12A40 was used as control. 12A40 is a trispecific antibody in TRIAD format targeting to CD3, PD-L1 and CEA, see Fig 1, lower panel, with amino acid sequences for VHs and VLs same as those in 12A39.

[00504] Experimental Results:

[00505] The tumor-bearing mice were given negative control (i.e., vehicle), 12A22 (5mpk), 12A22 (1mpk), 12A22 (0.2mpk) and 12A39 (0.2mpk) by tail vein injection, two times a week for 2 weeks and then discontinued for 1 week of observation. After discontinuation of the drug (from Day 15), the tumor volume increased more significantly for groups treated with 12A22 compared to groups treated with 12A39.

[00506] In conclusion, the results showed that 12A39 demonstrates incomparable tumor growth inhibitory effect, with 12A39 showing higher and more sustained inhibitory effect than 12A22 (See FIG 5A & 5B).

1.3 In vivo cytokine release study

[00507] Experimental methods:

[00508] 12A22(0.3 mpk) and 12A39 (0.3 mpk) were administered in administration volume of 10 ml/kg/monkey via intravenous injection routes from different groups. The level of IL-6 was detected by cytometric bead array (CBA) 4 hours after administration of 12A22 and 12A39.

[00509] Experimental results:

[00510] As shown in Table 9, the IL-6 level induced in 4 hours after the administration of 12A22 was 32,985.94 pg/ml. The IL-6 level induced in 4 hours after the administration of 12A39 was 1,780.28 pg/ml

[00511] The results show that both 12A22 and 12A39 have been found to increase IL-6 release in monkey models. Although 12A39 increases the level of IL-6 significantly less than 12A22 does, it has also been observed that 12A39 still exhibits high cytokine release in in vitro experiments.

Table 9. IL-6 level induced in monkey model after the administration of 12A22 and 12A39

	12A22	12A39
Dosage	0.3mg/kg	0.3mg/kg
IL6 level(Post 4h of administration) (pg/ml)	32985.94	1780.28

1.4 In vivo animal pharmacokinetics (PK) study

[00512] Experimental Methods:

[00513] 12A22 (0.1 mpk) and 12A39 (0.1 mpk) were administered to cynomolgus monkeys from different groups through intravenous injection. The concentrations of the tested antibodies were determined on 2min, 2h, 4h, 8h, 24h, 36h, 48h and 72h after administration of 12A22 and 12A39.

[00514] Experimental Results:

[00515] The results show that when administered at a dosage of 0.1 mg/kg, 12A22 exhibited a longer half-life. In contrast, however, 12A39 showed rapid clearance (see FIG 6), although 12A39 only differs from 12A22 in that 12A39 was engineered based on 12A22 to further introduce a PD-L1-binding domain. After intravenous administration of 12A39 to cynomolgus monkeys, 12A39 could no longer be detected in the peripheral blood after 8 hours.

Example 2: Construction and testing of mutants of 12A39 in its PD-L1-binding domain

2.1 Construction of PD-L1-binding domain mutations

[00516] Based on the results of the aforementioned examples, 12A39 showed incomparable tumor inhibitory effect in the animal study, indicating the advantages of Type E antibody structure by introducing a PDL1 binding domain. However, the level

of IL6 release induced by 12A39 remained relatively high and the half-life of 12A39 in vivo was still too short.

[00517] In order to investigate the cytotoxic potency at different levels of PD-L1 affinity, mutations were introduced into the VH or VL sequences of the PD-L1-binding domain of 12A39. These mutations were designed to reduce the affinity of the PD-L1-binding domain towards PD-L1. The efficacy of the mutated 12A39 variants and the cytokine release activity were then observed.

[00518] The VH and VL sequences of the PD-L1-binding domains of 12A39 antibody are as follows:

[00519] VH sequence (SEQ ID NO: 7):

QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSTYAISWVRQAPGQGLEW
MGGIIPIFGKAHYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCA
RKFHFVSGSPFGMDVWGQGTTVTVSS

[00520] VL sequence (SEQ ID NO: 8):

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYD
ASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQG
TKVEIKR

[00521] The mutations introduced towards the PD-L1-binding domain of 12A39 were listed in Table 10.

Table 10. Mutations of PDL1-binding domain based on 12A39 antibody

KABAT	PDL1 mutation (KABAT)	
	VH	VL
12A39	-	-
12A63	K56D (SEQ ID NOs: 41, 25)	-
12A64	K56A (SEQ ID NOs: 42, 26)	-
12A65	K56F (SEQ ID NO: 43, 27)	-
12A66	H58D (SEQ ID NO: 28)	-

12A67	-	Y32E (SEQ ID NOs: 44, 29)
12A68	-	W94D (SEQ ID NOs: 45, 30)
12A69	-	W94A (SEQ ID NOs: 46, 31)

2.2 Affinity of the mutations towards the PD-L1-binding domain

[00522] Experimental Methods:

[00523] The affinity of the antibody variants with the aforementioned mutations introduced towards the PD-L1-binding domain were investigated through Bio-Layer Interferometry (BLI) method using the OctetR8 system, the procedures of BLI method were as follows:

[00524] Human PD-L1 was diluted to a concentration of 2 μg/ml in KB buffer;

[00525] 12A63-12A69 were diluted to a concentration of 100 nM in KB buffer;

[00526] Each well was filled with 200 μl of the prepared mixture;

[00527] Experimental Results:

[00528] The affinity (K_D value) of the aforementioned antibody variants towards PD-L1 were listed in Table 11.

[00529] The results show that the mutations introduced into the PD-L1 binding domains of 12A39 reduced the binding affinity to PD-L1. Specifically, the mutations of the VH sequence of the PD-L1-binding domain of 12A39 to W94A (12A69) or K56F (12A65) significantly reduced the affinity of the antibody towards PD-L1, with the affinity (Kd) was of 7.28E-08M and 2.11E-07M, respectively.

Table 11. Affinity towards PD-L1 of the antibody variants with the mutation introduced into the PD-L1-binding domains of 12A39

	Affinity (M)
	PDL1
12A39	2.98E-08
12A63	7.56E-07
12A64	6.54E-07

12A65	2.11E-07
12A66	2.12E-05
12A67	1.09E-06
12A68	1.68E-06
12A69	7.28E-08

2.3 In vitro cell killing assay

[00530] Experimental Methods:

[00531] The experimental procedures of Example 2.3 are similar to those of Example 1.2, except that the antibody variants investigated herein are 12A39, 12A64, 12A65, 12A66, 12A67, 12A68, and 12A69.

[00532] Experimental Results:

[00533] The results show that despite of the reduced affinity, the variants 12A69 (which has mutation of W94A in the VH sequence of the PD-L1-binding domain of 12A39), and 12A65 (which has mutation of K56F also in the VH sequence) still maintained their original cytotoxic activity. On the other hand, when the affinity towards PD-L1 was lower than 2.11E-07M, it noticeably affects the cytotoxic activity of these antibody variants mentioned above (See FIG 7A & 7B).

2.4 In vivo animal pharmacodynamic study

[00534] Experimental Methods:

[00535] The experimental procedures of Example 2.4 are similar to those of Example 1.3, except for the following: 1) the antibody variants investigated herein are 12A39 and 12A65, and 2) the dosage of all the antibody variants tested herein is 1mg/kg, respectively.

[00536] Experimental Results:

[00537] The results show that at a dosage of 1 mg/kg, the K56F (12A65) mutation in PD-L1-binding domain leads to a slight decrease in anti-tumor activity compared to 12A39 (See FIG 8).

2.5 In vivo animal pharmacokinetic study

[00538] Experimental Methods:

[00539] The experimental procedures of Example 2.5 are the same as those of Example 1.5 (i.e., the same study), except that data for 12A65 is further included here.

[00540] Experimental Results:

[00541] The results show that when administered at a dosage of 0.1 mg/kg, 12A39 demonstrated a significantly rapid clearance compared to 12A22 as indicated in Example 1.5. Based on 12A39, the variant 12A65 which has mutation of K56F in the VH sequence of the anti-PD-L1 domain was developed and showed reduced the affinity towards PD-L1. Surprisingly, 12A65 showed an unexpectedly extended half-life exceeding 36 hours in vivo.

[00542] In conclusion, 12A65 shows a significant improvement in its half-life compared to 12A39, suggesting that the binding to PD-L1 plays a crucial role in maintaining the level of 12A39 in the body, and hence extending the otherwise short half-life in vivo.

[00543] Based on the aforementioned results, it is speculated that upon introducing the PD-L1-binding domain to construct 12A39, the T-cell engager may undergo rapid clearance of 12A39 due to its binding to target cells expressing PD-L1. This pathway may be associated with the activation of monocytes and is primarily concentrated in peripheral blood. Based on the above findings, it is possible to avoid rapid clearance of the antibody through the peripheral blood pathway by reducing the binding affinity to PD-L1. However, it is important to ensure that the reduction in PD-L1 binding affinity does not compromise the functional activity of PD-L1 in the tumor

microenvironment. This balance allows for the retention of therapeutic efficacy while increasing the half-life and systemic level of the antibody variant.

Example 3: Construction and testing of mutants of 12A39 in its CD3-binding domain

3.1 Construction of CD3-binding domain mutations

[00544] In order to investigate the cytokine-mediated cytotoxicity potency, mutations were introduced into the CD3-binding domain based on the structure of 12A39. These mutations were designed to reduce the affinity of the antibody variants towards CD3 to improve the safety window of 12A39. The efficacy of the mutated antibody and its cytokine release activity were then observed.

[00545] The VH sequences of the CD3-binding domains of 12A39 antibody are as follows:

[00546] VH sequence (SEQ ID NO: 15):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEW
VGRIRSKYNNYATYYADSVKGRFTISRDDSKNSLYLQMNSLRAEDTAV
YYCVRHGNFGTSYVSWFAYWGQGTLVTVSS

[00547] VL sequence (SEQ ID NO: 16):

QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRG
LIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAEDEADYYCALWYSN
LWVFGGGTKLTVL

[00548] The mutations introduced towards the VH sequence of CD3-binding domain based on the structure of 12A39 were listed in Table 12.

Table 12. Mutations of the VH sequence of CD3-binding domain based on the structure of 12A39

Code	KABAT
12A42	T28V (SEQ ID NOs: 68, 32)

12A43	K52B H (SEQ ID NOs: 69, 33)
12A44	K52B Q (SEQ ID NOs: 71, 34)
12A45	Y52C F (SEQ ID NOs: 73, 35)
12A46	V100C A (SEQ ID NOs: 75, 36)
12A47	S100DT (SEQ ID NOs: 102, 37)
12A51	K52B D (SEQ ID NOs: 103, 38)

3.2 Affinity of the mutations towards the CD3-binding domain

3.2.1 Determined by BLI method

[00549] Experimental Methods:

[00550] The experimental procedures of Example 3.2.1 are similar to those of Example 2.2, except for the following: 1) the antigen diluted in step 1 was human CD3 epsilon, 2) the antibody variants diluted in step 2 were 12A42-12A47 and 12A51. Experimental Results:

The results show that the affinity of the antibodies 12A39, 12A42-12A47 and 12A51 for CD3, from highest to lowest, is as follows: 12A39, 12A43 (12A45), 12A42 (12A44), 12A46, 12A47, 12A51.

3.2.2 Determined by SPR (Surface Plasmon Resonance) assay

[00551] Experimental Methods:

[00552] Based on the experimental result of BLI method, the affinity towards CD3 of 12A39, 12A44, 12A46 and 12A47 was further investigated using SPR method. The procedures of SPR assay were as followed:

1) Biacore Measurement:
2) Capture: Flow cell 2 path, Injection Antigen, Flow rate (10μl/min), Contact time (60s).
3) Analyte: Both flow cells1&2 path, Injection analyte Antibody, Flow rate (30μl/min), Contact time (180s), Dissociation(400s).
4) Regeneration: Both flow cells1&2 path, Injection 10 mM glycine -HCl, pH1.5, Flow rate (30μl/min), Contact time (30s).

5) Method: Multiple cycle kinetics/affinity using capture

[00553] Experimental Results:

[00554] The affinity towards CD3 of 12A39, 12A44 and 12A46 are listed in Table 13. The results show that mutations introduced into the CD3-binding domains of 12A39 reduced the binding affinity of the antibody variants towards CD3. Specifically, the mutations of the VH sequence of the CD3-binding domain of 12A39 to K52B Q (12A44) or V100C A (12A46) (based on KABAT numbering) significantly reduced the affinity of the antibody variants towards CD3, with the affinity (Kd) values of 1.18E-07M and 1.76E-07M, respectively.

Table 13. Affinity towards CD3 using SPR assay

Kinetics model	Capture 1 Solution	Analyte 1 Solution	ka (1/Ms)	kd (1/s)	KD (M)
1:1 binding	human CD3	12A39	3.19E+05	2.75E-02	8.64E-08
1:1 binding	human CD3	12A46	2.51E+05	4.43E-02	1.76E-07
1:1 binding	human CD3	12A44	3.65E+05	4.32E-02	1.18E-07

3.3 In vitro cell killing assay

[00555] Experimental Methods:

[00556] The experimental procedures of Example 2.3 are similar to those of Example 1.2, except that the antibody variants investigated herein are 12A22, 12A39, 12A42-12A47, 12A51.

[00557] Experimental Results:

[00558] The results show that when variants demonstrated higher affinity towards CD3 than 12A46 (having mutation V100C A) (based on KABAT numbering), the cytotoxicity potency of the variants are comparable to that of 12A39 (See FIG. 10A-C). Such variants include: 12A42 (which has mutation of T28V in the VH sequence of the CD3-binding domain of 12A39), 12A43 (having mutation of K52B H in the

VH sequence), 12A44 (having mutation of K52B Q in the VH sequence), and 12A45 (having mutation of Y52C F in the VH sequence). However, when the affinity towards CD3 is lower than that of 12A46, the cytotoxicity significantly decreased or even disappeared (See FIG. 10B). Such variants include: 12A47 (e.g., S100DT), and 12A51 (e.g., K52B D).

[00559] Based on the results above, the relationship between cell cytotoxicity and IL-6 release for 12A46 was further analyzed (See FIG 11). The results indicate that the V100C A (12A46) mutation maintains the original safety window of 12A39. However, once the affinity of an antibody variant is lower than S100D T (12A47), the antibody variant loses potency. Considering the above results, the CD3 affinity range is determined to be 86.4nM to 176nM by SPR. Therefore, the K52B Q (12A44) and V100C A (12A46) mutations are selected as the main directions for further optimization.

3.4 In vivo animal pharmacokinetic study in primates

[00560] Experimental Methods:

[00561] The experimental procedures of Example 3.5 are same as those of Example 1.5 (i.e., the same study), except that data for 12A44 is further included here.

[00562] Experimental Results:

[00563] The results show that when administered at a dosage of 0.1 mg/kg, 12A39 demonstrated a significantly rapid clearance compared to 12A22 as indicated in Example 1.5. Based on 12A39, the variant 12A44 which has mutation of K52B Q in the VH sequence was developed and showed reducing the affinity towards CD3. Similar to 12A39, 12A44 also became undetectable in peripheral blood after 8 hours of administration.

Example 4: Construction and testing of mutants of 12A39 in both its PD-L1-binding domain and CD3 binding domain

Construction of mutants of 12A39 in both PD-L1-binding domain and CD3 binding domain

[00564] In order to investigate the cytotoxic potency at different levels of PD-L1 binding affinity and CD3 binding affinity, mutations were introduced into the VH or VL sequences of the PD-L1-binding domain and CD3-binding domain based on the structure of 12A39. These mutations were designed to reduce the affinity of the antibody towards both PD-L1 and CD3. The efficacy of the mutated antibody and its cytokine release activity were then observed.

[00565] The mutations introduced towards the PD-L1-binding domain and/or CD3-binding domain based on the structure of 12A39 were listed in Table 14A.

Table 14A. Mutations of PDL1-binding domain and/or CD3-binding domain based on the structure of 12A39

KABAT	CD3 mutation (KABAT)	PDL1 mutation (KABAT)	
	HC	HC	LC
12A56	V100C A (SEQ ID NO: 75)	K56D (SEQ ID NO: 41)	-
12A57	V100C A (SEQ ID NO: 75)	K56A (SEQ ID NO: 42)	-
12A58	V100C A (SEQ ID NO: 75)	K56F (SEQ ID NO: 43)	-
12A59	V100C A (SEQ ID NO: 75)	H58D (SEQ ID NO: 28)	-
12A60	V100C A (SEQ ID NO: 75)	-	Y32E (SEQ ID NO: 44)
12A61	V100C A (SEQ ID NO: 75)	-	W94D (SEQ ID NO: 45)
12A62	V100C A (SEQ ID NO: 75)	-	W94A (SEQ ID NO: 46)
12A70	K52B Q (SEQ ID NO: 34)	K56F (SEQ ID NO: 43)	

4.2 Affinity of the mutations towards the PD-L1-bidning domain and the CD3-binding domain

4.2.1 Determined by BLI method

[00566] The experimental procedures here were similar to those of Example 2.2 and Example 3.2, except that the tested mutations and variants are as listed in Table 14B.

Table 14B. Affinity towards PD-L1 and CD3 of the antibody variants with the mutation introduced into the PD-L1- and CD3-binding domains of 12A39

	Affinity(M)	
	PDL1	CD3
12A39	2.98E-08	5.28E-08
12A56	7.67E-07	1.08E-07
12A57	9.69E-07	1.04E-07
12A58	2.82E-07	1.07E-07
12A59	7.00E-06	9.01E-08
12A60	2.64E-05	1.77E-07
12A61	3.67E-06	9.59E-08
12A62	7.88E-08	8.81E-08
12A70	2.82E-07	1.030E-07

4.3 In vitro cell killing assay

[00567] Experimental Methods:

[00568] The experimental procedures of Example 4.1 were similar to those of Example 1.2, except that the antibody variants investigated herein were 12A39, 12A56-12A62 and 12A70.

[00569] Experimental Results:

[00570] The results show that when the mutation V100C A of the CD3-binding domain was introduced, the cytotoxicity potency of the antibody variants was significantly reduced (See FIGs 12A-C). Based on the above results, a further expansion of the safety window was achieved by simultaneously introducing the K52B Q mutation in CD3 and the K56F mutation in PD-L1, resulting in the construction of 12A70 from 12A39 (See FIG 13).

4.4 In vivo animal PD study

[00571] Experimental Methods:

[00572] The experimental procedures of Example 2.4 are same as those of Example 1.3, except that data for 12A44 is further included here. for the following: 1) the antibody variants investigated herein are 12A39, 12A44, 12A46, 12A58, 12A65, and 2) the dosage of all the antibody variants tested herein is 1mg/kg, respectively.

[00573] Experimental Results:

[00574] The results show that under a dosage of 1 mg/kg, 12A46 and 12A58 did not show good anti-tumor activity, whereas 12A44 and 12A65 retained most of the anti-tumor activity, with 12A44 showed anti-tumor activity almost comparable to that 12A39 (See FIG 14).

[00575] The results indicated that the V100C A mutation in CD3-binding domain (12A46 and 12A58) appeared to significantly impair the anti-tumor activity, whereas the K52B Q mutation in CD3-binding domain (12A44) and the K56F mutation in PD-L1-binding domain (12A65) appeared to have less impact on the anti-tumor activity (See FIG 14).

[00576] 12A70 also shows anti-tumor activity in vivo.

Example 5: Construction and testing of mutants of 12A39 in Fc

5.1 Construction of mutants of 12A39 in Fc

[00577] In addition to the aforementioned studies, the impact of antibody binding to FcγR on therapeutic efficacy was also investigated. While 12A39 utilized an Fc domain with low affinity for FcγR (E246P, F247V, L248A, G249-, Q287K, R440K, and Q450E, Kabat numbering), it still exhibited some degree of affinity for certain FcγR subtypes, which may interfere with the efficacy of T-cell engagers. To reduce the affinity for FcγR, the Fc domain of 12A39 was replaced by the Fc domain of IgG1 isotype with L247A/L248A mutation (based on the Kabat numbering system), and thus providing 12A52. This mutation can further reduce the affinity to FcγRII and may potentially decrease cytokine release caused by monocyte binding.

[00578] In order to investigate the reduction of affinity for FcγR, 12A52 was constructed. The efficacy of the mutated antibody and its cytokine release activity were then observed in vitro and in vivo.

5.2 Affinity of the mutations towards FcγR

[00579] Experimental Methods:

[00580] The experimental procedures of Example 5.1 are similar to those of Example 2.2, except for the following: 1) the antigen diluted in step 1 was human CD16 alpha, human CD32 alpha or human CD64 2) the antibody variants diluted in step 2 were 12A39 and 12A52.

[00581] Experimental Results:

[00582] The results indicate that 12A39 exhibits weak affinity for CD32a, with an affinity of approximately 5.266E-07M, whereas no significant affinity of 12A52 for CD32a was observed. However, the affinity of 12A52 for CD64 is slightly higher compared to 12A39 (See FIG 15A-F).

5.3 In vitro cell killing assay

[00583] Experimental Methods:

[00584] The experimental procedures of Example 5.2 were similar to those of Example 1.2, except that the antibody variants investigated herein were 12A22, 12A39, and 12A52.

[00585] Experimental Results:

[00586] The results show that the cytotoxicity potency of 12A52 was not significantly affected by replacing the Fc domain of 12A39 with IgG1 with the L247A/L248A mutations (See FIG 16). Based on the cytotoxicity potency results, a study on cytokine release to observe the impact of FcγR binding on cytokine release was further conducted. The results show that by solely adjusting the Fc domain to

reduce CD32a binding, 12A52 achieved reduction in IL-6 release compared to 12A39 (See FIG 17). Such reduction indicates that the binding of FcγRII may affect the cytokine release of T-engagers due to its interaction with monocytes.

5.4 In vivo animal PD study

[00587] Experimental Methods:

[00588] The experimental procedures of Example 5.3 are similar to those of Example 1.3, except for the following: 1) the antibody variants investigated herein are 12A39 and 12A52, 2) the dosage of all the antibody variants tested herein is 1mg/kg, respectively, and 3) 3 weeks after inoculation, the peripheral blood of each group was collected and tested by flow cytometry for hCD45 and hCD3.

[00589] Experimental Results:

[00590] The results show that 12A52 demonstrated a comparable efficacy to 12A39 (See FIG 18), but it shows a significant increase in the levels of human CD45 and CD3 in the mouse model with human transplants (See FIGs 19 & 20). Such increase suggests that the binding of FcγRII may have a positive impact on the expansion and maintenance of CD45 by T-engagers. In comparison to 12A39, 12A52 induced lesser reduction in CD45+ cells and T cells.

Example 6: Construction and testing of mutants of 12A39 in its PD-L1-binding domain and Fc variant

Construction of mutants of 12A39 in its PD-L1-binding domain and Fc domain

[00591] To investigate the impact of FcγR binding and PD-L1 binding affinity on the therapeutic efficacy and safety of an antibody variant, 12A74 was constructed based on 12A65 (CD3wt, PDL1 HK56F) to introduce a modified Fc domain of IgG1 isotype having L247A/L248A mutations and D278A mutations.

[00592] Experimental Methods:

[00593] The experimental procedures of Example 6.1 are similar to those of Example 2.2, except for the following: 1) the antigen diluted in step 1 was human CD16 alpha, human CD32 alpha or human CD64 2) the antibody variants diluted in step 2 were 12A74.

[00594] Experimental Results:

[00595] The results show that the Fc domain of 12A74 do not exhibit significant affinity to CD16a, CD32a, and CD64. In comparison to the mutation in 12A52, which solely utilized L247A/L248A mutations, the additional D278A mutation in 12A74 further eliminated the binding to CD64, thereby avoiding non-specific killing and cytokine release caused by CD64 binding.

[00596] Experimental Methods:

[00597] The experimental procedures of Example 6.3 are similar to those of Example 1.3, except for the following: 1) the antibody variants investigated herein are 12A39 and 12A74, 2) the dosage of all the antibody variants tested herein is 1mg/kg, respectively, and 3) 3 weeks after inoculation, the peripheral blood of each group was collected and tested by flow cytometry for hCD45 and hCD3.

[00598] Experimental Results:

[00599] The results show that the main difference between 12A74 and 12A65 was the variation of FcγR binding. 12A65 was shown to have significantly decreased in vivo anti-tumor activity compared to 12A39 (see FIG. 8), however, by simply modifying the Fc domain to have reduced CD32a binding, 12A74 demonstrated significant increase in in vivo antitumor activity relative to 12A65, and the anti-tumor activity was even comparable to that of 12A39 (See FIG 21). Furthermore, as the affinity for PDL1 was also reduced, 12A74 exhibited a lower reduction on CD45+ and CD3+ cells compared to 12A39 (See FIGs 22 & 23). Such results highlight the

unexpected effects of combination of reduced FcγR binding and reduced PDL1 binding affinity in increasing therapeutic efficacy and in the meantime reducing side effects in cytokine release and in reduction of CD45+ and/or CD3+ cell counts.

Example 7: Construction and testing **of mutants of 12A39 in its PD-L1-binding domain, Fc variant and CD3-binding domain**

[00600] To investigate the impact of FcγR binding, PD-L1 binding affinity and CD3-binding affinity on the therapeutic efficacy and safety of an antibody variant, 12A73 was constructed based on 12A70 (CD3 K52B Q, PDL1 K56F) to introduce a modified Fc domain of IgG1 isotype having L247A/L248A mutations and D278A mutations. The efficacy of the mutated antibody and its cytokine release activity were then observed.

[00601] Experimental Methods:

[00602] The experimental procedures of Example 7.1 are similar to those of Example 2.2, except for the following: 1) the antigen diluted in step 1 was human CD16 alpha, human CD32 alpha or human CD64 2) the antibody variants diluted in step 2 were 12A73.

[00603] Experimental Results:

[00604] The results showed that the Fc domain of 12A73 did not exhibit significant affinity to CD16a, CD32a, and CD64 (See FIGs 24A-C). In comparison to the mutation in 12A52, which solely utilized L247A/L248A mutations, the additional D278A mutation in 12A73 further eliminated the binding to CD64, thereby avoiding non-specific killing and cytokine release caused by CD64 binding.

[00605] Experimental Methods:

[00606] The experimental procedures of Example 7.3 were similar to those of Example 1.2, except that the antibody variants investigated here are 12A39, 12A70, 12A73.

[00607] Experimental Results:

[00608] The results show that 12A73 demonstrated comparable anti-tumor efficacy to 12A39 (See FIG 25), while significantly reducing the levels of cytokine release. Compared to 12A39, 12A73 has a larger cytotoxicity/cytokine release window (See FIG 26), indicating improved efficacy and safety performance.

[00609] Experimental Methods:

[00610] The experimental procedures of Example 7.3 are similar to those of Example 1.3, except for the following: 1) the antibody variants investigated here are 12A39 and 12A73, 2) the dosage of 12A39 tested is 1mg/kg, and the dosages of 12A73 tested are 1mg/kg and 3 mg/kg, respectively, and 3) 3 weeks after inoculation, the peripheral blood of each group was collected and tested by flow cytometry for hCD45 and hCD3.

[00611] Experimental Results:

[00612] The results show that at a dosage of 1 mg/kg, similar to 12A39, 12A73 was able to achieve near-complete tumor clearance (See FIG 27). Moreover, 12A73 showed significantly less reduction on human CD45+ cells (representing the entire white blood cells) and CD3+ cells (representing T lymphocytes) relative to 12A39 The level of human CD45+ cells and CD3+ cells after treatment with 12A73 at 1mg/kg was almost comparable to that of negative control, and only slightly reduced after treatment with 12A73 at 3mg/kg (See FIGs 28A & 28B).

7.5 In vivo cytokine release study

[00613] Experimental Methods:

[00614] The experimental procedures of Example 7.5 are the same as those of Example 1.4 (i.e., the same study), except that data for 12A73 is further included here.

[00615] Experimental Results:

[00616] The results demonstrate that the step-up dosing from 0.3mpk, 1mpk, to 3mpk in cynomolgus monkeys was well tolerated. Peripheral blood IL6 levels were measured after each administration, and it was observed that the IL6 level at 4 hours post-administration with 0.3 mpk was only 910.88 pg/ml, which subsequently decreased to 30-50 pg/ml. The animals did not show any significant abnormalities. Compared to the IL6 elevation of more than 30,000 pg/ml observed after administration of 12A22, the safety of 12A73 is significantly improved.

Table 15. IL-6 level induced in monkey model after the administration of 12A22, 12A39 and 12A73

	12A22	12A39	12A73
Dosage	0.3mg/kg	0.3mg/kg	0.3mg/kg
IL6 level (Post 4h of administration) (pg/ml)	32985.94	1780.28	910.88

7.6 In vivo animal PK study

[00617] Experimental Methods:

[00618] The experimental procedures of Example 7.6 are the same as those of Example 1.5 (i.e., the same study), except that data for 12A44, 12A65, and 12A73 is further included here.

[00619] Experimental Results:

[00620] The results show that when administered at a dosage of 0.1 mg/kg, 12A39 demonstrated a significantly rapid clearance compared to 12A22 as indicated in Example 1.5. Based on 12A39, the variant 12A44 having K52B Q in VH of anti-CD3 domain also became undetectable in peripheral blood after 8 hours of administration, as indicated in Example 3.4. Based on 12A39, the variant 12A65 having K56F in VH

of anti-PD-L1 domain was developed and showed reduced affinity towards PD-L1, whereas 12A65 showed an extended half-life exceeding 36 hours in vivo, as indicated in Example 2.5. The variant 12A73, however, demonstrated an even more impressive extended half-life of 33 hours, which is almost comparable to the half-life of 12A22 in vivo (See FIG 29). The increased half-life of 12A73 demonstrated the unexpected effects of combination of reduced/eliminated FcγR binding and reduced PDL1 binding affinity in extending half-life.

Example 8: Construction and testing of mutants of 12A58 in Fc domain

Construction of 12A78

[00621] To investigate whether reduced/eliminated FcγR binding can be sufficient to improve therapeutic efficacy of an antibody variant having very low CD3 binding and reduced PD-L1 binding affinity, 12A78 was constructed based on 12A58 (CD3 V100C A, PDL1 K56F) to introduce a modified Fc domain of IgG1 isotype having L247A/L248A mutations and D278A mutations. The efficacy of the mutated antibody and its cytokine release activity were then observed.

8.2 In vitro cell killing assay

[00622] Experimental Methods:

[00623] The experimental procedures of Example 8.2 were similar to those of Example 1.2, except that the antibody variants investigated herein were 12A39, 12A58, and 12A78.

[00624] Experimental Results:

[00625] The results show that after introducing the modified the Fc domain, the activity of 12A78 did not show significant improvement over 12A58, and the in vitro activity of 12A78 was almost comparable to that of 12A58 (see FIG 30).

8.3 In vivo animal PD study

[00626] Experimental Methods:

[00627] The experimental procedures of Example 8.3 are similar to those of Example 1.3, except for the following: 1) the antibody variants investigated herein 12A78, and 2) the dosage of 12A78 tested herein was 10mpk, and 3) 3 weeks after inoculation, the peripheral blood of each group was collected and tested by flow cytometry for hCD45 and hCD3.

[00628] Experimental Results:

[00629] The results show that when administered at a high dose of 10 mg/kg, the anti-tumor efficacy of 12A78 only showed weak anti-tumor efficacy (see FIG 31A). This is consistent with the observations with 12A58 in similar animal PD studies (see FIG. 14, copied as FIG 31B for ease of comparison), where 12A58 at 9 mg/kg merely showed weak anti-tumor activity. This is consistent with the observations in vitro for cytotoxicity.

[00630] In contrast, after introducing the same modified Fc domain having reduced FcγR binding, 12A74 demonstrated significant increase in in vivo antitumor activity relative to 12A65 (See FIG 21, Example 6.3). 12A65 (having wild type anti-CD3 and K56F in anti-PD-L1 VH) differed from 12A58 (having V100C A in anti-CD3 VH and K56F in anti-PD-L1 VH) only in the anti-CD3 domain, in particular, 12A65 has higher binding affinity to CD3 than 12A58.

[00631] These findings indicate that when the CD3 affinity is lower than a certain threshold, complete loss of effector function in the Fc region, as compared to the Fc used in 12A58 and 12A39, does not further improve efficacy. In other words, at this affinity level, an antibody that binds to CD3 does not direct T cells to attack cells expressing CD32. Therefore, adjusting the Fc region does not enhance its efficacy.

[00632] This may also suggest that there could be certain minimal requirement for CD3 binding affinity (e.g. above 1.76E-07M as measured by SPR, as with the V100C A mutation in 12A58) in order to have increased anti-tumor activity by modifying the Fc domain to reduce or eliminate certain FcγR binding. Indeed, 12A73 has been

shown to have low-affinity anti-CD3 domain yet still demonstrate impressive anti-tumor activity in vivo. The CD3 binding affinity of 12A73 is 1.18E-7M as measured by SPR and contains K52B Q mutation in VH of anti-CD3 domain.

[00633] When peripheral blood samples from experimental animals were analyzed using FACS, it was observed that 12A58 induced minimal reduction in the counts of human CD45+ and CD3+ cells and was almost comparable with the negative control animals (See FIGs 32A&B), indicating the 12A58 has good safety. This is in direct contrast to 12A39, which induced significant reduction in the counts of human CD45+ and CD3+ cells. Additionally, when counting human lymphocytes transplanted in mice, the impact of 12A78 on lymphocyte counts was similar to that of 12A58 (See FIGs 33A&B).

Example 9: Construction and testing of CD3/PDL1/EGFR antibodies

Construction of CD3/PDL1/EGFR antibody

[00634] To investigate the impact of specific binding to EGFR instead of specific binding to CEA on therapeutic efficacy, 5A52 was constructed by replacing the CEA-binding domain of 12A73 (CD3 K52B Q, PDL1 K56F, Fc L247A/L248A and D278A) with an EGFR-binding domain (VH: SEQ ID NO: 84; VL: SEQ ID NO: 85). The efficacy of the newly constructed antibody 5A52 were then observed in vitro and in vivo.

9.2 In vitro cell killing assay

[00635] Experimental Methods:

[00636] The experimental procedures of Example 9.2 were similar to those of Example 1.2, except for the following: 1) the antibody variants investigated herein was 5A52, and 2) the target cells used herein were A431, HT29, Kato3 and Ls174t cell lines.

[00637] Experimental Results:

[00638] The results show that 5A52 exhibits a significant anti-tumor efficacy across different tumor cell lines including A431, Kato3, HT29, and LS174T, with an EC50 for cytotoxicity at nM level (See FIGs 34A-D).

9.3 In vivo animal PD study

[00639] Experimental Methods:

[00640] The experimental procedures of Example 9.3 were similar to those of Example 1.3, except for the following: 1) the antibody variants investigated herein was 5A52, and 2) the dosage of all the antibody variants tested herein is 1mg/kg, respectively.

[00641] Experimental Results:

[00642] The results show that under a dosage of 1 mg/kg, the 5A52 demonstrated significant tumor-inhibiting activity compared to negative control (See FIG 35). While mice administered with PBS only had uncontrolled tumor growth 25 days after treatment, mice administered with 5A52 had only minimal tumor growth and the tumor volume was significantly lower than that of PBS control 25 days after treatment.

9.4 In vivo animal PK study

[00643] Experimental Methods:

[00644] The experimental procedures of Example 9.4 are similar to those of Example 1.5, except for the following: 1) the antibody variants tested herein are 5A52, and 2) the dosage of all the antibody variants tested herein is 0.1mg/kg, respectively.

[00645] Experimental Results:

[00646] The results show that the half-life of 5A52 in vivo reaches the expected half-life after administration.

Example 10: Construction and testing of CD3/PDL1/ CD20 antibodies

10.1 Construction of CD3/PDL1/EGFR antibody

[00647] To investigate the impact of specific binding to CD20 instead of specific binding to CEA on therapeutic efficacy, 2A42 was constructed by replacing the CEA-binding domain of 12A73 (CD3 K52B Q, PDL1 K56F, Fc L247A/L248A and D278A) with an CD20-binding domain (VH: SEQ ID NO: 111, VL: SEQ ID NO: 112). The efficacy of the newly constructed antibody 5A52 were then observed in vitro and in vivo.

10.2 In vitro cell killing assay

[00648] Experimental Methods:

[00649] The experimental procedures of Example 10.2 were similar to those of Example 1.2, except for the following: 1) the antibody variants investigated herein was 2A42, and 2) the target cells used herein was Raji cell line.

[00650] Experimental Results:

[00651] The results show that 2A42 exhibits a significant anti-tumor efficacy in Raji cell line at an EC50 of nM level for cytotoxicity (See FIG 36).

10.3 In vivo animal PD study

[00652] Experimental Methods:

[00653] The experimental procedures of Example 10.3 were similar to those of Example 1.3, except for the following: 1) the antibody variants investigated herein was 2A42, and 2) the dosage of all the antibody variants tested herein is 0.5mg/kg, respectively.

[00654] Experimental Results:

[00655] The results show that under a dosage of 0.5 mg/kg, the 2A42 demonstrated significant tumor-inhibiting activity compared to negative control (See FIG 37). While mice administered with PBS only had uncontrolled tumor growth 25 days after treatment, mice administered with 2A42 had only minimal tumor growth and the tumor volume was significantly lower than that of PBS control 25 days after treatment.

Example 11: Construction and testing of CD3/PDL1/CLDN6 antibodies

11.1 Construction of CD3/PDL1/CLDN6 antibody

[00656] To investigate the impact of specific binding to Claudin6 instead of specific binding to CEA on therapeutic efficacy, 24A3 was constructed by replacing the CEA-binding domain of 12A73 (CD3 K52B Q, PDL1 K56F, Fc L247A/L248A and D278A) with an Claudin6-binding domain (VH: SEQ ID NO: 92; VL: SEQ ID NO: 93). The efficacy of the newly constructed antibody 5A52 were then observed in vitro and in vivo.

11.2 In vitro cell killing assay

[00657] Experimental Methods:

[00658] The experimental procedures of Example 11.2 were similar to those of Example 1.2, except for the following: 1) the antibody variants investigated herein was 24A3, and 2) the target cells used herein was OV90 cell line.

[00659] Experimental Results:

[00660] The results show that 24A3 exhibits a significant anti-tumor efficacy in OV90 cell line at nM level for cytotoxicity (See FIG 38).

11.3 In vivo animal PK study

[00661] Experimental Methods:

[00662] The experimental procedures of Example 11.3 were similar to those of Example 1.5, except for the following: 1) the antibody variants tested herein was 24A3, and 2) the dosage of all the antibody variants tested herein was 0.1mg/kg, respectively.

[00663] Experimental Results:

[00664] The results show that the half-life of 24A3 in vivo reaches the expected half-life after administration.

Example 12: Construction and testing of CD3/PDL1/GPRC5D antibodies

12.1 Construction of CD3/PDL1/ GPRC5D antibody

[00665] To investigate the impact of specific binding to GPRC5D instead of specific binding to CEA on therapeutic efficacy, 22A18 was constructed by replacing the CEA-binding domain of 12A73 (CD3 K52B Q, PDL1 K56F, Fc L247A/L248A and D278A) with an GPRC5D-binding domain (VH: SEQ ID NO: 100; VL: SEQ ID NO: 101). The efficacy of the newly constructed antibody 5A52 were then observed in vitro and in vivo.

12.2 In vitro cell killing assay

[00666] Experimental Methods:

[00667] The experimental procedures of Example 12.2 were similar to those of Example 1.2, except for the following: 1) the antibody variants investigated herein was 22A18, and 2) the target cells used herein was H929 cell line.

[00668] Experimental Results:

[00669] H929 cells were used as target cells, and PBMCs were used as effector cells to evaluate the in vitro cytotoxicity of 22A18. The results show that 22A18 exhibits anti-tumor efficacy (See FIG 39).

12.3 in vivo animal PD study

[00670] Experimental Methods:

[00671] The experimental procedures of Example 11.2 were similar to those of Example 1.2, except for the following: 1) the antibody variants investigated herein was 22A18, and 2) the target cells used herein was OV90 cell line.

12.4 in vivo animal PK study

[00672] Experimental Methods:

[00673] The experimental procedures of Example 12.4 were similar to those of Example 1.5, except for the following: 1) the antibody variants tested herein was 22A18, and 2) the dosage of all the antibody variants tested herein was 0.1mg/kg, respectively.

[00674] Experimental Results:

[00675] The results show that the half-life of 22A18 in vivo reaches the expected half-life after administration.

Example 13: Construction and testing of 12A94, as well as PD-L1 binding domain mutants in 12A94

[00676] To further investigate the effects of mutations to reducing the PD-L1 binding affinity based on other PD-L1 parent antibodies, antigen-binding domain (i.e., CDRs from the heavy chain variable region and light chain variable region) of another known PD-L1 antibody, namely atezolizumab, was introduced in the Type E format.

[00677] Specifically, 12A94 has the same format with 12A73, while compared with 12A73, CDRs in the PD-L1 binding domain of 12A94 were replaced with those derived from atezolizumab.

13.1 Construction of PD-L1-binding domain mutations

[00678] In order to investigate the cytotoxic potency at different levels of PD-L1 affinity, mutations were introduced into the VH or VL sequences of the PD-L1-binding domain of 12A94. These mutations were designed to reduce the affinity of the PD-L1-binding domain towards PD-L1. The efficacy of the mutated 12A94 variants and the cytokine release activity were then observed.

[00679] The VH and VL sequences of the PD-L1-binding domains of 12A94 antibody are as follows:

[00680] VH sequence (SEQ ID NO: 135):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWV
AWISPYGGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC
ARRHWPGGFDYWGQGTLVTVSS

[00681] VL sequence (SEQ ID NO: 136):

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIY
SASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFG
QGTKVEIK

[00682] The mutations introduced towards the PD-L1-binding domain of 12A94 were listed in Table 16.

Table 16. Mutations of PDL1-binding domain based on 12A94 antibody

KABAT	PDL1 mutation (KABAT)	
	VH	VL
12A95	-	S30H (SEQ ID NOs: 142, 145)
12A96	-	S30T (SEQ ID NOs: 143, 146)
12A97	S56G (SEQ ID NOs: 141, 147)	-
12A98	Y53C (SEQ ID NOs: 137, 148)	-
12A99	Y53G (SEQ ID NOs: 138, 149)	-
12A106	Y53H (SEQ ID NOs: 139, 150)	-
12A107	-	S30E (SEQ ID NOs: 144, 151)
12A108	Y53Q (SEQ ID NOs: 140, 152)	-

13.2 Affinity of the mutations towards the PD-L1-binding domain

[00683] Experimental Methods:

[00684] The experimental procedures of Example 13.2 were the similar to those of Example 2.2, except that the antibody and antibody variants investigated herein were 12A39, 12A73, 12A94, 12A95, 12A96, 12A97, 12A98, 12A99, 12A106, 12A107 and 12A108.

[00685] Experimental Results:

[00686] The affinity (K_D value) of the aforementioned antibody variants towards PD-L1 were shown in FIG. 44 listed in Table 17.

[00687] The results show that the mutations introduced into the PD-L1 binding domains of 12A94 reduced the binding affinity to PD-L1. Specifically, the mutations of the VH sequence of the PD-L1-binding domain of 12A94 to Y63G (12A99) or Y53H (12A106) reduced the affinity of the antibody towards PD-L1 to the Kd of 9.782E-08 M and 3.490E-08 M, respectively, which is within the range of no less than 10^{-8} M and no more than 10^{-7} M.

Table 17. Affinity towards PD-L1 of the antibody variants with the mutation introduced into the PD-L1-binding domains of 12A94

Sample ID	KD (M)	ka (1/Ms)	kdis (1/s)
12A39	1.548E-08	5.549E05	8.592E-03
12A73	3.472E-08	5.263E05	1.828E-02
12A94	4.127E-09	2.817E05	1.162E-03
12A95	3.266E-09	3.187E05	1.041E-03
12A96	4.094E-09	2.888E05	1.182E-03
12A97	1.434E-09	3.576E05	5.128E-04
12A98	1.011E-07	3.314E05	3.351E-02
12A99	9.782E-08	4.400E05	4.305E-02
12A94	2.528E-09	4.216E05	1.066E-03
12A106	3.490E-08	4.116E05	1.436E-02
12A107	3.809E-09	3.216E05	1.225E-03
12A108	1.092E-07	3.082E05	3.366E-02

13.3 In vitro cell killing assay

[00688] Experimental Methods:

[00689] The experimental procedures of Example 13.3 were similar to those of Example 1.2, except that the antibody and antibody variants investigated herein were 12A39, 12A73, 12A94, 12A95, 12A96, 12A97, 12A98, 12A99, 12A106, 12A107 and 12A108.

[00690] Experimental Results:

[00691] The results show that when variants demonstrated lower affinity towards PD-L1 than 12A94 (i.e., 12A98, 12A99, 12A106, 12A108, having mutation of Y53C, Y53G, Y53H and Y53Q, based on KABAT numbering), the cytotoxicity potency of the variants are lower to that of 12A94 (See FIGs 44A and 44B). Such variants include: 12A98 (which has mutation of Y53C in the VH sequence of the PD-L1-binding domain of 12A94), 12A99 (having mutation of Y53G Q in the VH sequence), 12A106 (having mutation of Y53H in the VH sequence), and 12A108 (having mutation of Y53Q in the VH sequence).

[00692] Based on the above results, the relationship between cell cytotoxicity and IL-6 release for 12A94, 12A98, 12A99, 12A106, 12A107 and 12A108 were further analyzed (See FIGs. 45A-45F). The results indicate that the V100C A (12A46) mutation in the anti-CD3 binding domain maintains the original safety window of 12A39. However, with the decrease in the PD-L1 binding affinity, 12A99 and 12A106 exhibited a killing efficacy window of approximately 10 times of 12A94, with the PD-L1 affinity (Kd) value between 9.78E-8 M and 3.49E-8 M. When the PD-L1 affinity was weaker than the above range, such as 12A98 and 12A108, there was a decrease in the maximum cytotoxicity, and no further decrease in cytokine release was observed.

WHAT IS CLAIMED IS:

1. A multi-specific polypeptide complex comprising:
   a) a CD3-binding domain capable of specifically binding to CD3,
   b) a PD-L1-binding domain that is a low-affinity PD-L1-binding domain capable of specifically binding to PD-L1 at a Kd value of no less than 10^{-8} M as measured by Bio-Layer Interferometry (BLI), and
   c) a first disease antigen binding domain,
   wherein the CD3-binding domain, PD-L1 binding domain and the disease antigen binding domain are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1, CD3 and the disease antigen.
2. The multi-specific polypeptide complex of claim 1, further comprising a dimerization domain that is operably linked to the CD3-binding domain, PD-L1 binding domain and the disease antigen binding domain.
3. The multi-specific polypeptide complex of claim 2, wherein the dimerization domain comprises a CH3 domain, and optionally further comprises a CH2 domain, and further optionally comprises an Fc domain.
4. The multi-specific polypeptide complex of claim 3, wherein the Fc domain is modified and has reduced binding or lacks substantial binding to human CD32a.
5. The multi-specific polypeptide complex of claim 3, wherein the Fc domain further has reduced binding or lacks substantial binding to human CD16 and/or human CD64.
6. The multi-specific polypeptide complex of claim 3, wherein the Fc domain has reduced binding or lacks substantial binding to human CD32a, human CD16 and human CD64.
7. The multi-specific polypeptide complex of any one of the preceding claims, wherein the CD3-binding domain is a low-affinity CD3-binding domain capable of specifically binding to CD3 at a Kd value of no less than 10^{-8} M as measured by Bio-Layer Interferometry(BLI) or Surface Plasmon Resonance (SPR),
8. The multi-specific polypeptide complex of any one of the preceding claims, wherein the PD-L1 binding domain is capable of specifically binding to human PD-L1 at a Kd value no more than 7E-6 M, 4E-6 M, 1E-6 M, 8E-7 M or 5E-7 M.

9. The multi-specific polypeptide complex of any one of the preceding claims, wherein the low-affinity PD-L1 binding domain is capable of specifically binding to human PD-L1 at a Kd value ranging from no less than 2.98E-8 M to no more than 2.11E-7 M as measured by BLI.
10. The multi-specific polypeptide complex of any one of the preceding claims, wherein the low-affinity PD-L1 binding domain is derived from a low-affinity variant of a parent anti-PD-L1 antibody selected from the group consisting of MDX-1105, atezolizumab, avelumab, durvalumab, adebrelimab, sugemalimab, envafolimab, cosibelimab, garivulimab, manelimab, opucolimab, pacmilimab, sugemalimab, wherein the low-affinity variant has reduced binding affinity to human PD-L1 than the parent antibody.
11. The multi-specific polypeptide complex of claim 10, wherein the parent anti-PD-L1 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6.
12. The multi-specific polypeptide complex of claim 11, wherein the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7 or 113, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8.
13. The multi-specific polypeptide complex of claim 11, wherein the low-affinity variant of the parent anti-PD-L1 antibody has a mutation at position 56 (based on Kabat numbering) in the heavy chain variable region of the parent anti-PD-L1 antibody.
14. The multi-specific polypeptide complex of claim 13, wherein the mutation at position 56 is selected from the group consisting of: 56D, 56A, and 56F, optionally wherein when the parent anti-PD-L1 antibody comprises the HCDR2 sequence of SEQ ID NO: 40, the mutation at position 56 is selected from the group consisting of: K56D, K56A, and K56F, optionally wherein the low-affinity variant comprises a HCDR2 comprising the amino acid sequence of SEQ ID NO: 41, 42, or 43.

15. The multi-specific polypeptide complex of claim 11, wherein the low-affinity variant has a mutation at position 32 or position 94 (based on KABAT numbering) in the light chain variable region of the parent anti-PD-L1 antibody.
16. The multi-specific polypeptide complex of claim 15, wherein the mutation at position 32 is 32E, or the mutation at position 94 is selected from the group consisting of 94D and 94A, optionally wherein when the parent anti-PD-L1 antibody comprises the LCDR1 sequence of SEQ ID NO: 4, the mutation at position 32 is Y32E, when the parent anti-PD-L1 antibody comprises the LCDR3 sequence of SEQ ID NO: 6, the mutation at position 94 is selected from the group consisting of: W94D and W94A, optionally wherein the low-affinity variant comprises a LCDR1 comprising the amino acid sequence of SEQ ID NO: 44, and/or a LCDR3 comprising the amino acid sequence of SEQ ID NO: 45 or 46.
17. The multi-specific polypeptide complex of claim 10, wherein the low-affinity variant of the parent anti-PD-L1 antibody comprises

a) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 41, 42 or 43, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25, 26 or 27, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8;

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 44, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 29;

c) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 45 or 46; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 30 or 31.

18. The multi-specific polypeptide complex of claim 10, wherein the parent anti-PD-L1 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134.
19. The multi-specific polypeptide complex of claim 11, wherein the parent anti-PD-L1 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 135, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 136.
20. The multi-specific polypeptide complex of claim 11, wherein the low-affinity variant of the parent anti-PD-L1 antibody has a mutation at position 56 or position 53 (based on Kabat numbering) in the heavy chain variable region of the parent anti-PD-L1 antibody.
21. The multi-specific polypeptide complex of claim 20, wherein the mutation at position 56 is 56G, or the mutation at position 53 is 53C, 53G, 53H, or 53Q, optionally wherein when the parent anti-PD-L1 antibody comprises the HCDR2 sequence of SEQ ID NO: 130, the mutation at position 56 is S56G, optionally wherein the low-affinity variant comprises a HCDR2 comprising the amino acid sequence of SEQ ID NO: 141 [ISPYGG**G**T]; or the mutation at position 53 is Y53C, Y53G, Y53H, or Y53Q, optionally wherein the low-affinity variant comprises a HCDR2 comprising the amino acid sequence of SEQ ID NO: 137

[ISP**C**GGST], SEQ ID NO: 138 [ISP**G**GGST], SEQ ID NO: 139 [ISP**H**GGST] or SEQ ID NO: 140 [ISP**Q**GGST]

22. The multi-specific polypeptide complex of claim 11, wherein the low-affinity variant has a mutation at position 30 (based on KABAT numbering) in the light chain variable region of the parent anti-PD-L1 antibody.
23. The multi-specific polypeptide complex of claim 22, wherein the mutation at position 30 is 30H, 30T, or 30E, optionally wherein when the parent anti-PD-L1 antibody comprises the LCDR1 sequence of SEQ ID NO: 132, the mutation at position 30 is S30H, S30T, or S30E, optionally wherein the low-affinity variant comprises a LCDR1 comprising the amino acid sequence of SEQ ID NO: 142 [QDV**H**TA], SEQ ID NO: 143 [QDV**T**TA] or SEQ ID NO: 144 [QDV**E**TA].

24. The multi-specific polypeptide complex of claim 11, wherein the low-affinity variant of the parent anti-PD-L1 antibody comprises

a) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 137, 138, 139, 140 or 141, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 147, 148, 149, 150 or 152, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 136;

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 142, 143 or 144, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 135, and a light chain variable region comprising the amino acid sequence of SEQ ID NO:145, 146 or 151.

25. The multi-specific polypeptide complex of any one of the preceding claims, wherein the CD3 binding domain is capable of specifically binding to human CD3 at a Kd value no more than 5E-7 M.
26. The multi-specific polypeptide complex of claim 7, wherein the low-affinity CD3 binding domain is capable of specifically binding to human CD3 at a Kd value ranging from no less than 5.28E-8 M to no more than 2E-7 M, 5.28E-8 M to no more than 1.77E-7 M, no less than 5.28E-8 M to no more than 1.5E-7 M, or no less than 5.28E-8 M to no more than 1.18E-7 M as measured by SPR.
27. The multi-specific polypeptide complex of any one of the preceding claims, wherein the CD3 binding domain is derived from an anti-CD3 antibody selected from the group consisting of SP34, L2K, UCHT1, TR66, and 38E4.
28. The multi-specific polypeptide complex of any one of claims 7-27, wherein the low-affinity CD3 binding domain is derived from a low-affinity variant of a parent anti-CD3 antibody selected from the group consisting of SP34, L2K, UCHT1, TR66, 38E4 and its humanized variant thereof, wherein the low-affinity variant has reduced binding affinity to human CD3 than the parent antibody.
29. The multi-specific polypeptide complex of claim 28, wherein the parent anti-CD3 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14.
30. The multi-specific polypeptide complex of claim 28, wherein the parent anti-CD3 antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 15, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16.
31. The multi-specific polypeptide complex of claim 28, wherein the low-affinity variant of a parent anti-CD3 antibody comprises a mutation at position 52B, 52C,

28, or 100C (based on Kabat numbering) in the heavy chain variable region of the parent anti-CD3 antibody.

32. The multi-specific polypeptide complex of claim 31, wherein the mutation at position 52B is selected from the group consisting of: 52B Q and 52B H; the mutation at position 52C is 52C F; the mutation at position 28 is 28V; and/or the mutation at position 100C is selected from the group consisting of 100C A, optionally wherein when the parent anti-CD3 antibody comprises the HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, the mutation at position 52B is selected from the group consisting of: K52B Q and K52B H, and the mutation at position 52C is Y52C F; optionally wherein the low-affinity variant comprises a HCDR2 comprising the amino acid sequence of SEQ ID NO: 69, 71, or 73;
optionally wherein when the parent anti-CD3 antibody comprises the HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, the mutation at position 28 is 28V, optionally wherein the low-affinity variant comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 68; or
optionally wherein when the parent anti-CD3 antibody comprises the HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, the mutation at position 100C is V100C A, optionally wherein the low-affinity variant comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 75.
33. The multi-specific polypeptide complex of claim 3, wherein the Fc domain is derived from human IgG1, and optionally has a mutation at position 247 and/or 248 (Kabat numbering).
34. The multi-specific polypeptide complex of claim 33, wherein the mutation at position 247 is selected from the group consisting of L247A and L247F; and/or the mutation at position 248 is selected from the group consisting of L248A and L248E.
35. The multi-specific polypeptide complex of claim 34, wherein the mutations at position 247 and position 248 are selected from the group consisting of: a) L247A/L248A, and b) L247F/L248E.
36. The multi-specific polypeptide complex of claim 34, wherein the Fc domain further comprises one or more additional mutations at the positions selected from the group consisting of position 278, 249, 314, 341, 348, and 350.

37. The multi-specific polypeptide complex of claim 34, where the Fc domain further comprises one or more additional mutations at the positions selected from the group consisting of D278A, G249R, N314A, N314Q, K341A, P348G, and P350S.
38. The multi-specific polypeptide complex of claim 34, where the Fc domain further comprises one or more additional mutations selected from the group consisting of a) L247F/L248E/P350S; b) L247A/L248A/P348G; c) L247F/L248E/P350S; and d) L247A/L248A/K341A.
39. The multi-specific polypeptide complex of any one of claims 3-38, wherein the Fc domain is derived from human IgG4, and optionally has a mutation at position 247 and/or 248 (Kabat numbering).
40. The multi-specific polypeptide complex of any one of the preceding claims, wherein the multi-specific polypeptide complex exhibits a serum half-life in a primate (*e.g.* monkey or human) of no less than 8 hours (no less than 12 hours, 18 hours, 24 hours, or 32 hours).
41. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen is selected from the group consisting of tumor associated antigen (TAA), immune-associated antigen, and an inflammation-associated antigen.
42. The multi-specific polypeptide complex of claim 41, wherein the TAA is CEA, CD20, CD22, BCMA, GPRC5D, EGFR, Her2, Her3, GPC3, PSMA, CLDN6, CLDN18.2, MUC16, or MUC17.
43. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen binding domain is a CEA binding domain that comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 17, HCDR2 comprising the amino acid sequence of SEQ ID NO: 18, HCDR3 comprising the amino acid sequence of SEQ ID NO: 19, LCDR1 comprising the amino acid sequence of SEQ ID NO: 20, LCDR2 comprising the amino acid sequence of SEQ ID NO: 21, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 22.
44. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen binding domain is a GPRC5D binding domain that comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 94, HCDR2 comprising the amino acid sequence of SEQ ID NO: 95, HCDR3

comprising the amino acid sequence of SEQ ID NO: 96, LCDR1 comprising the amino acid sequence of SEQ ID NO: 97, LCDR2 comprising the amino acid sequence of SEQ ID NO: 98, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 99.

45. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen binding domain is a EGFR binding domain that comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 78, HCDR2 comprising the amino acid sequence of SEQ ID NO: 79, HCDR3 comprising the amino acid sequence of SEQ ID NO: 80, LCDR1 comprising the amino acid sequence of SEQ ID NO: 81, LCDR2 comprising the amino acid sequence of SEQ ID NO: 82, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 83.
46. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen binding domain is a CD20 binding domain that comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 105, HCDR2 comprising the amino acid sequence of SEQ ID NO: 106, HCDR3 comprising the amino acid sequence of SEQ ID NO: 107, LCDR1 comprising the amino acid sequence of SEQ ID NO: 108, LCDR2 comprising the amino acid sequence of SEQ ID NO: 109, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 110.
47. The multi-specific polypeptide complex of any one of the preceding claims, wherein the PD-L1 binding domain and the CD3-binding domain are assembled to form a bispecific-binding domain.
48. The multi-specific polypeptide complex of claim 40, wherein the bispecific-binding domain is operably linked to the first disease antigen binding domain to form a tri-specific-binding domain.
49. The multi-specific polypeptide complex of claim 48, wherein the tri-specific-binding domain is operably linked to one terminus of the dimerization domain (*e.g.* the CH3 domain, the CH2-CH3 domain, or the Fc domain).
50. The multi-specific polypeptide complex of claim 48, wherein the tri-specific-binding domain is operably linked to the N terminus of the first Fc chain.
51. The multi-specific polypeptide complex of any one of the preceding claims, wherein the multi-specific polypeptide complex further comprises a second

disease antigen binding domain, optionally operably linked to another terminus the dimerization domain.

52. The multi-specific polypeptide complex of claim 51, wherein the second disease antigen binding domain is operably linked to N terminus of the second Fc chain.
53. The multi-specific polypeptide complex of any one of the preceding claims, wherein the PD-L1 binding domain is an antibody-derived domain selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv, disulfide stabilized Fv fragment (dsFv), $(dsFv)_2$, single-chain Fv antibody (scFv), and heavy chain antibody (VHH).
54. The multi-specific polypeptide complex of any one of the preceding claims, wherein the CD3 binding domain is an antibody-derived domain selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv, disulfide stabilized Fv fragment (dsFv), $(dsFv)_2$, single-chain Fv antibody (scFv), and heavy chain antibody (VHH).
55. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen binding domain or the second disease antigen binding domain is an antibody-derived domain selected from the group consisting of Fab, Fab', $F(ab')_2$, Fv, disulfide stabilized Fv fragment (dsFv), $(dsFv)_2$, single-chain Fv antibody (scFv), and heavy chain antibody (VHH).
56. The multi-specific polypeptide complex of any one of the preceding claims, wherein the first disease antigen binding domain and the second disease antigen binding domain bind to the same target, optionally the first disease antigen binding domain and the second disease antigen binding domain are identical.
57. The multi-specific polypeptide complex of any one of the preceding claims, wherein the CD3-binding domain and the PD-L1 domain are assembled to form a bispecific binding domain which is a DICAD domain,

    wherein:

    the DICAD domain comprises:

    i) a first polypeptide fragment comprising a first heavy chain variable domain (VH1) linked to a second light chain variable domain (VL2), and

    ii) a second polypeptide fragment comprising a second heavy chain variable domain (VH2) linked to a first light chain variable domain (VL1),

wherein the VL1 and the VH1 associate to form a first domain capable of binding to a first target, and the VL2 and the VH2 associate to form a second domain capable of binding to a second target;

wherein one of the first target and the second target is PD-L1, and the other one of the first target and the second target is CD3.

58. The multi-specific polypeptide complex of claim 57, wherein the first target is human PD-L1, and the second target is human CD3; or wherein the first target is human CD3, and the second target is human PD-L1.
59. The multi-specific polypeptide complex of claim 57, wherein the N-terminus of said VH1 is linked to C-terminus of said VL2, and N-terminus of said VH2 is linked to C-terminus of said VL1.
60. The multi-specific polypeptide complex of claim 57, wherein the N-terminus of said VL1 is linked to C-terminus of said VH2, and N-terminus of said VL2 is covalently linked to C-terminus of said VH1.
61. The multi-specific polypeptide complex of claim 57, wherein one of the first domain and the second domain is a bonding domain and comprises a first non-native covalent bond formed between a first pair of two amino acid residues.
62. The multi-specific polypeptide complex of claim 61, wherein the other one of the first domain and the second domain is an accompanying domain and does not comprise a non-native covalent bond or alternatively comprises a second non-native covalent bond formed between a pair of two amino acid residues different from the pair of amino acid residues corresponding to the first pair of two amino acid residues.
63. The multi-specific polypeptide complex of claim 61, wherein the first non-native covalent bond is a non-native disulfide bond.
64. The multi-specific polypeptide complex of claim 63, wherein the non-native disulfide bond is formed between two non-native cysteine residues.
65. The multi-specific polypeptide complex of claim 64, wherein the two non-native cysteine residues are present in FR2 of VL of the bonding domain and FR4 of VH of the bonding domain, respectively.
66. The multi-specific polypeptide complex of claim 64, wherein the two non-native cysteine residues are at position 44 in the VH and position 100 in the VL, or at position 105 in the VH and position 43 in the VL, or at position 100 in the VH

and position 49 in the VL, or at position 100 in the VH and position 150 in the VL, wherein numbering is according to the Kabat index.

67. The multi-specific polypeptide complex of claim 61, wherein the bonding domain further comprises a non-native pair of two oppositely charged amino acid residues in the VH and in the VL, respectively.

68. The multi-specific polypeptide complex of claim 67, wherein

(a) the two oppositely charged residues are at position 38 in the VL and position 39 in the VH, respectively;

(b) the two oppositely charged residues are at position 40 in the VL and position 39 in the VH, respectively;

(c) the two oppositely charged residues are at position 37 in the VL and position 39 in the VH, respectively; or

(d) the two oppositely charged residues are at position 44 in the VL and position 103 in the VH, respectively;

wherein numbering is according to the Kabat index.

69. The multi-specific polypeptide complex of claim 67, wherein the two oppositely charged residues comprises a negatively charged amino acid residue selected from aspartic acid (D) or glutamic acid (E), and a positively charged amino acid residue selected from lysine (K), histidine (H) or arginine (R).

70. The multi-specific polypeptide complex of claim 67, wherein the two oppositely charged residues comprise Q38D in the VL and Q39K in the VH, respectively; or Q38D in the VL and Q39K in the VH, respectively, wherein numbering is according to the Kabat index.

71. The multi-specific polypeptide complex of claim 57, wherein the VH1 comprises the amino acid sequence of SEQ ID NO: 47, and the VL1 comprises the amino acid sequence of SEQ ID NO:48.

72. The multi-specific polypeptide complex of claim 57, wherein the VH2 comprises the amino acid sequence of SEQ ID NO: 15, and the VL2 comprises the amino acid sequence of SEQ ID NO: 16.

73. The multi-specific polypeptide complex of claim 57, wherein the VL1 is linked to the VH2 via a first peptide linker, and wherein the VL2 is linked to the VH1 via a second peptide linker.

74. The multi-specific polypeptide complex of claim 57, wherein the first peptide linker and the second peptide linker each independently comprises 5 to 9 amino acids.
75. The multi-specific polypeptide complex of claim 57, wherein the first polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 49, and the second polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 50.
76. The multi-specific polypeptide complex of claim 57, wherein the first disease antigen binding domain comprises a first **Fv domain** comprising:
    i) a third polypeptide fragment comprising a third heavy chain variable domain (VH3), and
    ii) a fourth poly peptide fragment comprising a third light chain variable domain (VL3),

    wherein the VH3 and the VL3 associate to form a third domain capable of binding to the disease antigen.
77. The multi-specific polypeptide complex of claim 76, wherein the VH3 comprises the amino acid sequence of SEQ ID NO: 23, and the VL3 comprises the amino acid sequence of SEQ ID NO: 24; or the VH3 comprises the amino acid sequence of SEQ ID NO: 84 and the VL3 comprises the amino acid sequence of SEQ ID NO: 85; or the VH3 comprises the amino acid sequence of SEQ ID NO: 92 and the VL3 comprises the amino acid sequence of SEQ ID NO: 93; or the VH3 comprises the amino acid sequence of SEQ ID NO: 100 and the VL3 comprises the amino acid sequence of SEQ ID NO: 101; or the VH3 comprises the amino acid sequence of SEQ ID NO: 111 and the VL3 comprises the amino acid sequence of SEQ ID NO: 112.
78. The multi-specific polypeptide complex of claim 76, wherein the third polypeptide fragment further comprises an antibody heavy chain constant region (CH1) operably linked to the C terminus of the VH3 domain, and/or the fourth polypeptide fragment further comprises an antibody light chain constant region (CL) operably linked to the C terminus of the VL3 domain.
79. The multi-specific polypeptide complex of claim 76, wherein
    a) the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 23 or 64, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 24 or 65.

b) the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 84, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 85; or

c) the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 111, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 112;

d) the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 92, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 93; or

e) the third polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 100, and the fourth polypeptide fragment comprises the amino acid sequence of SEQ ID NO: 101.

80. The multi-specific polypeptide complex of claim 76, wherein one of the termini of the DICAD domain is operably linked to one of the termini of the Fv domain.

81. The multi-specific polypeptide complex of claim 76, wherein one of the **N** termini of the **DICAD** domain is operably linked to one of the **C** termini of the first **Fv** domain.

82. The multi-specific polypeptide complex of claim 76, wherein one of the N termini of the DICAD domain is operably linked to the C termini of the **third** polypeptide fragment of the first Fv domain.

83. The multi-specific polypeptide complex of claim 76, wherein the N termini of the **second** polypeptide fragment of the DICAD domain is operably linked to the C termini of the **third** polypeptide fragment of the first Fv domain.

84. The multi-specific polypeptide complex of claim 76, wherein the C termini of the first polypeptide fragment of the bispecific-binding domain or of the tri-specific-binding domain is operably linked to the N-terminus of the first dimerization domain (*e.g.* the CH3 domain, the CH2-CH3 domain, or the Fc domain).

85. The multi-specific polypeptide complex of claim 76, wherein the C termini of the **first** polypeptide fragment is operably linked to the N-terminus of the first Fc chain.

86. The multi-specific polypeptide complex of claim 76, wherein the second disease antigen-binding domain is operably linked to the N-terminus of the second Fc chain.

87. The multi-specific polypeptide complex of claim 51, wherein the second disease antigen-binding domain is identical to the first disease antigen-binding domain, optionally the second disease antigen-binding domain comprises a second Fv domain.
88. The multi-specific polypeptide complex of claim 87, wherein the second Fv domain is identical to the first Fv domain, and the second Fv domain comprises a fifth polypeptide fragment identical to the third polypeptide fragment comprising the third heavy chain variable domain (VH3), and a sixth polypeptide fragment identical to the fourth polypeptide fragment comprising the third light chain variable domain (VL3).
89. The multi-specific polypeptide complex of claim 88, wherein the C termini of the fifth polypeptide fragment of the second Fv domain is operably linked to the N-terminus of the second Fc chain of the Fc domain.
90. The multi-specific polypeptide complex of claim 3, wherein the Fc domain comprises a first mutation in one Fc chain and a second mutation in the other Fc chain,

wherein

a) the first mutation comprises T389W and/or S375C, and the second mutation comprises Y438V, T389S, L391A, and/or Y370C;
b) the first mutation comprises D427K and/or D377K, and the second mutation comprises K420D, and/or K440D;
c) the first mutation comprises D377K, E378K, and/or D427K, and the second mutation comprises K393E, K440D, and/or K470E;
d) the first mutation comprises S387H, and/or F436A, and the second mutation comprises Y370T, and/or T422F;
e) the first mutation comprises S387H, and/or T422F, and the second mutation comprises Y422T, and/or F436A;
f) the first mutation comprises K393D, and/or K440D, and the second mutation comprises E378K, and/or D427K; or
g) the first mutation comprises L372D, and/or L391E, and the second mutation comprises L372K, or T389K,

wherein numbering is according to the Kabat index.

91. The multi-specific polypeptide complex of claim 1, wherein the multi-specific polypeptide complex comprises polypeptide chains having the following amino acid sequences respectively:
    a) SEQ ID NO: 70, SEQ ID NO: 63, SEQ ID NO: 72, and SEQ ID NO: 65;
    b) SEQ ID NO: 74, SEQ ID NO: 67, SEQ ID NO: 76, and SEQ ID NO: 65.
    c) SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 76, and SEQ ID NO: 65; or
    d) SEQ ID NO: 159, SEQ ID NO: 158, SEQ ID NO: 76, and SEQ ID NO: 65.
92. The multi-specific polypeptide complex of any one of the preceding claims, which is linked to one or more conjugate moieties.
93. The multi-specific polypeptide complex of claim 93, wherein the conjugate moiety comprises an agent for detection or isolation, such as a clearance-modifying agent, a chemotherapeutic agent, a toxin, a radioactive isotope, a lanthanide, a luminescent label, a fluorescent label, an enzyme-substrate label, a DNA-alkylator, a topoisomerase inhibitor, a tubulin-binder, other anticancer drugs, immunomodulators (Toll-like receptor) or hormones.

94. An isolated polynucleotide encoding the multi-specific polypeptide complex of any one of the preceding claims.

95. A vector comprising the isolated polynucleotide of claim 94.

96. A host cell comprising the vector of claim 95.

97. A pharmaceutical composition, comprising:

(i) the multi-specific polypeptide complex of any one of claims 1-93, or the polynucleotide encoding the multi-specific polypeptide complex of any one of claims1-87; and

(ii) one or more pharmaceutically acceptable carriers, diluent, buffer or excipient.

98. The pharmaceutical composition of claim 97, further comprising an additional therapeutic agent.

99. A method of expressing the multi-specific polypeptide complex of any one of claims 1-93, comprising culturing the host cell of claim 96 under the condition at which the vector of claim 95 is expressed.

100. A method of treating, preventing or alleviating a disease or disorder in a subject, comprising administering to the subject a therapeutically effective amount of the multi-specific polypeptide complex of any one of claim 1-93.

101. A method of treating, preventing or alleviating a disease or disorder in a subject, comprising administering to the subject a therapeutically effective amount of the multi-specific polypeptide complex of any one of claims 1-93, or the polynucleotide encoding the multi-specific polypeptide complex of any one of claims 1-93, and/or the pharmaceutical composition of claim 97 or 98.

102. The method of any one of claims 99-101, wherein the subject is human.

103. The method of any one of claims 99-101, wherein the administration is via oral, nasal, intravenous, subcutaneous, sublingual, or intramuscular administration.

104. Use of the multi-specific polypeptide complex of any one of claims 1-93, the pharmaceutical composition of claim 97 or 98, and/or the polynucleotide encoding the multi-specific polypeptide complex of any one of claims 1-93 in the manufacture of a medicament for treating, preventing or alleviating a disease or disorder.

105. A method of modifying a parent multi-specific polypeptide complex to produce a modified multi-specific polypeptide complex having improved half life in a primate, wherein the multi-specific polypeptide complex comprises a CD3-binding domain capable of specifically binding to CD3, a PD-L1 binding domain capable of specifically binding to PD-L1, wherein the method comprises: introducing one or more mutations to the PD-L1 binding domain to produce a modified PD-L1 binding domain having reduced binding affinity to PD-L1 characterized in a Kd value of no less than 10^{-8} M as measured by BLI.

106. The method of claim 105, wherein the modified PD-L1 binding domain binds to human PD-L1 at a Kd value ranging from 10^{-5} M to 10^{-8} M as measured by BLI.

107. The method of claim 105, wherein the multi-specific polypeptide complex further comprises a first disease antigen binding domain.

108. The method of claim 105, wherein the modified multi-specific polypeptide complex has a half-life extended by at least 4 hours in a primate relative to the parent multi-specific polypeptide complex.

109. The method of claim 105, wherein the modified multi-specific polypeptide complex has a half-life of at least 8 hours in a primate.

110. The method of claim 105, wherein the multi-specific polypeptide complex further comprises an Fc domain.

111. The method of claim 110, wherein the Fc domain is modified and has reduced binding or lack binding to human CD32a.

112. The method of claim 105, wherein the modified multi-specific polypeptide complex has a half-life extended by at least 8 hours in a primate relative to the parent multi-specific polypeptide complex.

113. The method of claim 105, wherein the modified multi-specific polypeptide complex has a half-life of at least 12 hours in a primate.

114. A method of modifying a multi-specific polypeptide complex to reduce its ability to induce cytokine release in a subject,
wherein the multi-specific polypeptide complex comprises a CD3-binding domain capable of specifically binding to CD3 and an Fc domain that are operably linked to allow the multi-specific polypeptide complex to bind to CD3, the method comprises:
introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a.

115. The method of claim 114, wherein the multi-specific polypeptide complex further comprises a disease antigen binding domain, wherein the disease antigen binding domain and the CD3-binding domain and the Fc domain are operably linked to

allow the multi-specific polypeptide complex to bind to CD3 and the disease antigen.

116. A method of modifying a multi-specific polypeptide complex to improve its potency or improve its therapeutic window in a subject,
wherein the multi-specific polypeptide complex comprises:
a) a low-affinity CD3-binding domain capable of specifically binding to CD3 at a Kd value of no less than 10^{-7} M or no less than 10^{-8} M as measured by Surface Plasmon Resonance (SPR), and
b) an Fc domain,
that are operably linked to allow the multi-specific polypeptide complex to bind to CD3 and the disease antigen,
the method comprises:
introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a.

117. The method of claim 116, wherein the multi-specific polypeptide complex further comprises a disease antigen binding domain.

118. The method of claim 116, wherein the low-affinity CD3-binding domain binds to human CD3 at a Kd value ranging from 10^{-5} M to 10^{-8} M as measured by SPR.

119. A method of modifying a multi-specific polypeptide complex to improve its potency or improve its therapeutic window in a subject,
wherein the multi-specific polypeptide complex comprises:
a) a low-affinity PD-L1-binding domain capable of specifically binding to PD-L1 at a Kd value of no less than 10^{-8} M as measured by BLI,
b) a CD3-binding domain, and
c) an Fc domain,
that are operably linked to allow the multi-specific polypeptide complex to bind to PD-L1 and CD3,
the method comprises:
introducing one or more mutations to the Fc domain to produce a modified Fc domain having reduced binding or lacks substantial binding to human CD32a.

120. The method of claim 119, wherein the multi-specific polypeptide complex further comprises a disease antigen binding domain, wherein the disease antigen binding domain and the CD3-binding domain, the PDL-1 binding domain and the Fc

domain are operably linked to allow the multi-specific polypeptide complex to bind to CD3, PD-L1 and the disease antigen.

121. The method of claim 119, wherein the low-affinity PD-L1-binding domain binds to human PD-L1 at a Kd value ranging from 7E-6 M to 1E-8 M as measured by BLI.

122. The method of claim 119, wherein the CD3-binding domain is a low-affinity CD3-binding domain capable of specifically binding to CD3 at a Kd value of no less than 10^{-7} M as measured by Surface Plasmon Resonance (SPR).

123. The method of any one of claims 114, 116 and 119, wherein the modified Fc domain has reduced binding or lacks substantial binding to both human CD32a and human CD64.

124. The method of any one of claims 114, 116 and 119, wherein the modified Fc domain has reduced binding or lacks substantial binding to each of human CD32a, human CD64, and human CD16a.

125. The method of any one of claims 114, 116 and 119, wherein the Fc domain is derived from human IgG1, and the one or more mutations is at position 247 and/or 248 (Kabat numbering).

126. The method of claim 125, wherein the mutation at position 247 is selected from the group consisting of L247A and L248F; and/or the mutation at position 235 is selected from the group consisting of L247A and L248E.

127. The method of claim 126, wherein the mutations at position 247 and position 248 are selected from the group consisting of: a) L247A/L248A, and b) L247F/L248E.

128. The method of claim 125, wherein the modified Fc domain further comprises one or more additional mutations at the positions selected from the group consisting of position 278, 249, 314, 341, 348, and 350.

129. The method of claim 128, where the one or more additional mutations comprises D278A, G249R, N314A, N314Q, K341A, P348G, P350S, or any combination thereof.

130. The method of claim 129, where the one or more additional mutations are selected from the group consisting of a) L247F/L248E/P350S; b) L247A/L248A/P348G; c) L247F/L248E/P350S; and d) L247A/L248A/K341A.

131. The method of any one of claims 114, 116 and 119, wherein the modified multi-specific polypeptide complex exhibits at least 40% increase in EC50 in inducing IL-6 release from the CD3-expressing immune cell (*e.g.,* T cell).

132. The method of any one of claims 114, 116 and 119, wherein the modified multi-specific polypeptide complex exhibits at least 5 fold increase in therapeutic window, wherein the therapeutic window is represented by a ratio of EC50 in inducing IL-6 release from the CD3-expressing immune cell (*e.g.,* T cell), to EC50 in killing a disease-antigen expressing cell in the presence of a CD3-expressin immune cell (*e.g.,* T cell).

133. A PD-L1 binding polypeptide complex comprising an affinity variant of a parent anti-PD-L1 antibody, wherein

the parent anti-PD-L1 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6, or

the parent anti-PD-L1 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134; and

wherein the affinity variant is capable of specifically binding to human PD-L1 at a Kd value ranging from 10^{-5} M to 10^{-8} M as measured by BLI.

134. The PD-L1 binding polypeptide complex of claim 133, wherein the affinity variant has a mutation at K56 or H58D (based on KABAT numbering) in the heavy chain variable region of the parent anti-PD-L1 antibody; or a mutation at Y53 or S56 (based on KABAT numbering) in the heavy chain variable region of the parent anti-PD-L1 antibody.

135.The PD-L1 binding polypeptide complex of claim 134, wherein the mutation at K56 is selected from the group consisting of: K56D, K56A, and K56F.

136.The PD-L1 binding polypeptide complex of claim 134, wherein the mutation at Y53 is selected from the group consisting of: Y53C, Y53G, Y53H, and Y53Q; or the mutation at S56 is S56G.

137.The PD-L1 binding polypeptide complex of claim 133, wherein the affinity variant has a mutation at Y32 or W94 (based on KABAT numbering) in the light chain variable region of the parent anti-PD-L1 antibody; or a mutation at S30 (based on KABAT numbering) in the light chain variable region of the parent anti-PD-L1 antibody.

138.The PD-L1 binding polypeptide complex of claim 137, wherein the mutation at Y32 is selected from the group consisting of Y32E, or the mutation W94 is selected from the group consisting of W94D and W94A.

139.The PD-L1 binding polypeptide complex of claim 137, wherein the mutation at S30 is selected from the group consisting of S30H, S30T or S30E,

140.The PD-L1 binding polypeptide complex of claim 133, wherein the affinity variant comprises a HCDR1 comprising the amino acid sequence of

a) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 41, 42 or 43, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 25, 26 or 27, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 8;

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 44, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 6; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 29;

c) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1 or 39, HCDR2 comprising the amino acid sequence of SEQ ID NO: 2 or 40, HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 45 or 46;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 30 or 31;

d) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 137, 138, 139, 140 or 141, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 132, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 147, 148, 149, 150 or 152, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 136; or

e) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 129, HCDR2 comprising the amino acid sequence of SEQ ID NO: 130, HCDR3 comprising the amino acid sequence of SEQ ID NO: 131, LCDR1 comprising the amino acid sequence of SEQ ID NO: 142, 143 or 144, LCDR2 comprising the amino acid sequence of SEQ ID NO: 133, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 134;

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 135, and a light chain variable region comprising the amino acid sequence of SEQ ID NO:145, 146 or 151.

141. The PD-L1 binding polypeptide complex of claim 133, wherein the PD-L1 binding polypeptide complex comprises a bispecific antibody capable of specifically binding to PD-L1 and CD3.

142. The PD-L1 binding polypeptide complex of claim 133, wherein the PD-L1 binding polypeptide complex comprises a tri-specific antibody capable of specifically binding to PD-L1, CD3 and a disease antigen.

143. A CD3 binding polypeptide complex comprising an affinity variant of a parent anti-CD3 antibody, wherein the parent anti-CD3 antibody comprises a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14, and wherein the affinity variant is capable of specifically binding to human CD3 at a Kd value ranging from 10^{-6} M to 10^{-8} M (optionally from $5*10^{-7}$ M to $5*10^{-8}$ M) as measured by SPR.

144. The CD3 binding polypeptide complex of claim 143, wherein the affinity variant of a parent anti-CD3 antibody comprises a mutation at K52B , Y52C , T28 or V100C (based on KABAT numbering) in the heavy chain variable region of the parent anti-CD3 antibody.

145. The CD3 binding polypeptide complex of claim 144, Wherein the mutation at K52B is selected from the group consisting of: K52B Q and K52B H; the mutation at Y52C is selected from the group consisting of Y52C F; the mutation at T28 is selected from the group consisting of T28V; and/or the mutation at V100C is selected from the group consisting of V100C A.

146.The CD3 binding polypeptide complex of claim 143, wherein the affinity variant of the parent anti-CD3 antibody comprises

a) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 68, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14; or

a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 32, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16;

b) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 69, 71, 71 or 103 , HCDR3 comprising the amino acid sequence of SEQ ID NO: 11, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14; or

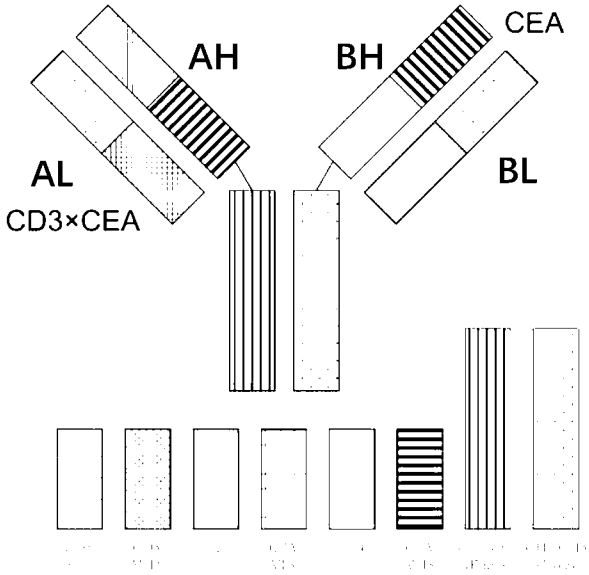
a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 33, 34, 35 or 36, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16;

c) a HCDR1 comprising the amino acid sequence of SEQ ID NO: 9, HCDR2 comprising the amino acid sequence of SEQ ID NO: 10, HCDR3 comprising the amino acid sequence of SEQ ID NO: 75 or 102, LCDR1 comprising the amino acid sequence of SEQ ID NO: 12, LCDR2 comprising the amino acid sequence of SEQ ID NO: 13, and LCDR3 comprising the amino acid sequence of SEQ ID NO: 14; or

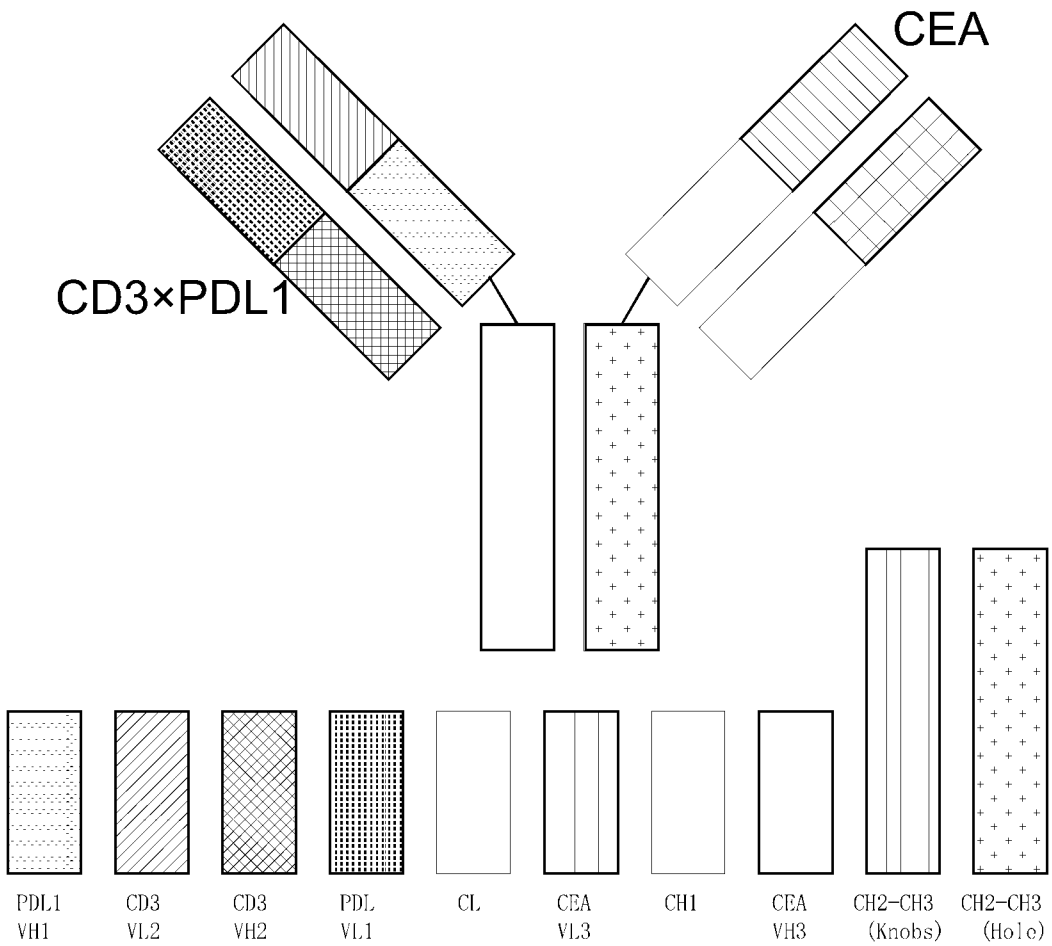
a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 36 or 37, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 16.

147. The CD3 binding polypeptide complex of claim 143, wherein the CD3 binding polypeptide comprises a bispecific antibody capable of specifically binding to PD-L1 and CD3.

148. The CD3 binding polypeptide complex of claim 143, wherein the CD3 binding polypeptide complex comprises a tri-specific antibody capable of specifically binding to PD-L1, CD3 and a disease antigen.

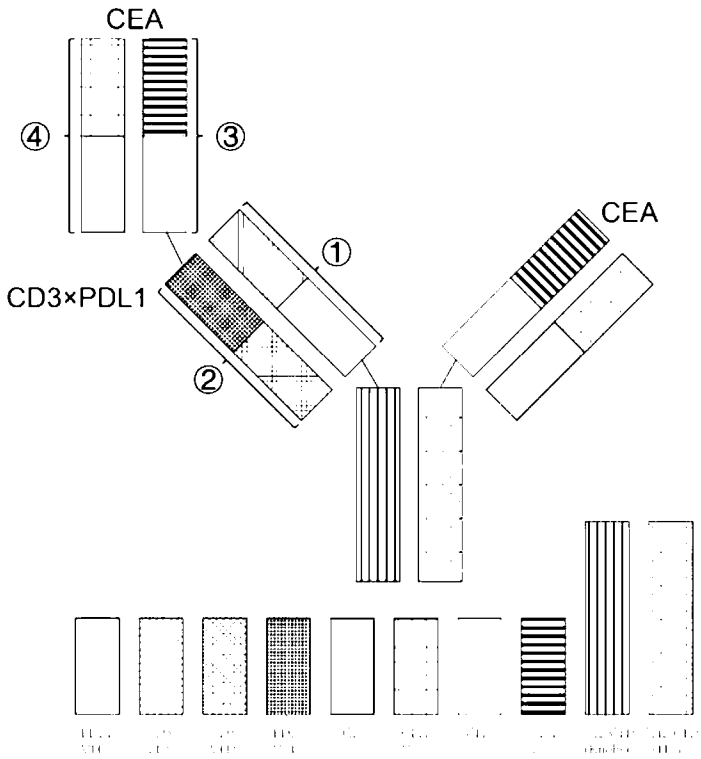


12A22 (TRIAD Structure)



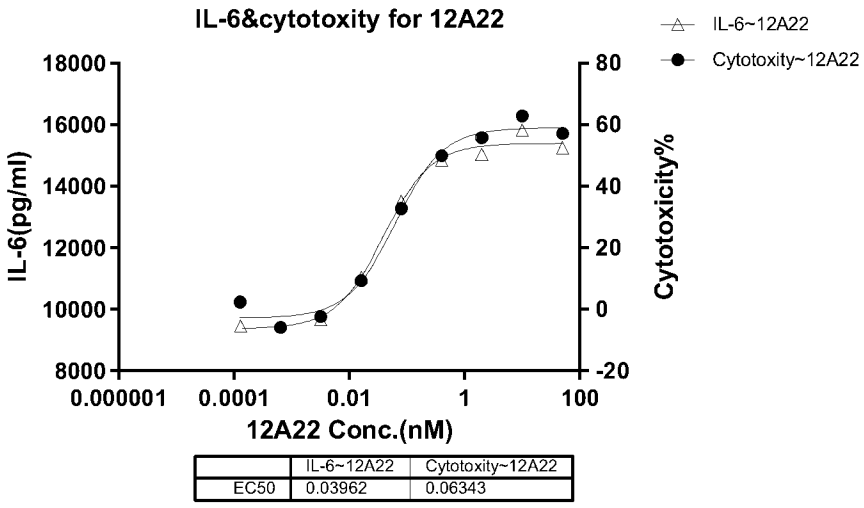
12A40(TRIAD Structure)

FIG 1



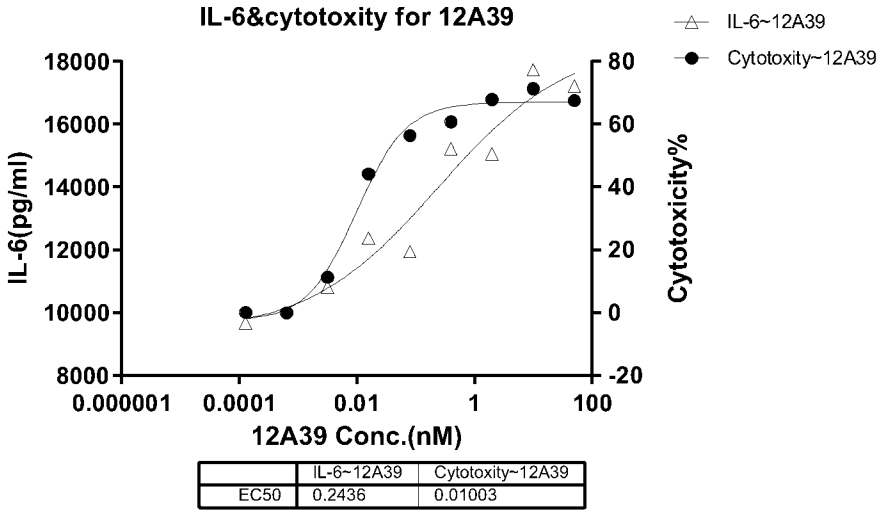
12A39 (TYPE E Structure)

FIG 2



	IL-6~12A22	Cytotoxity~12A22
EC50	0.03962	0.06343

FIG 3



	IL-6~12A39	Cytotoxity~12A39
EC50	0.2436	0.01003

FIG 4

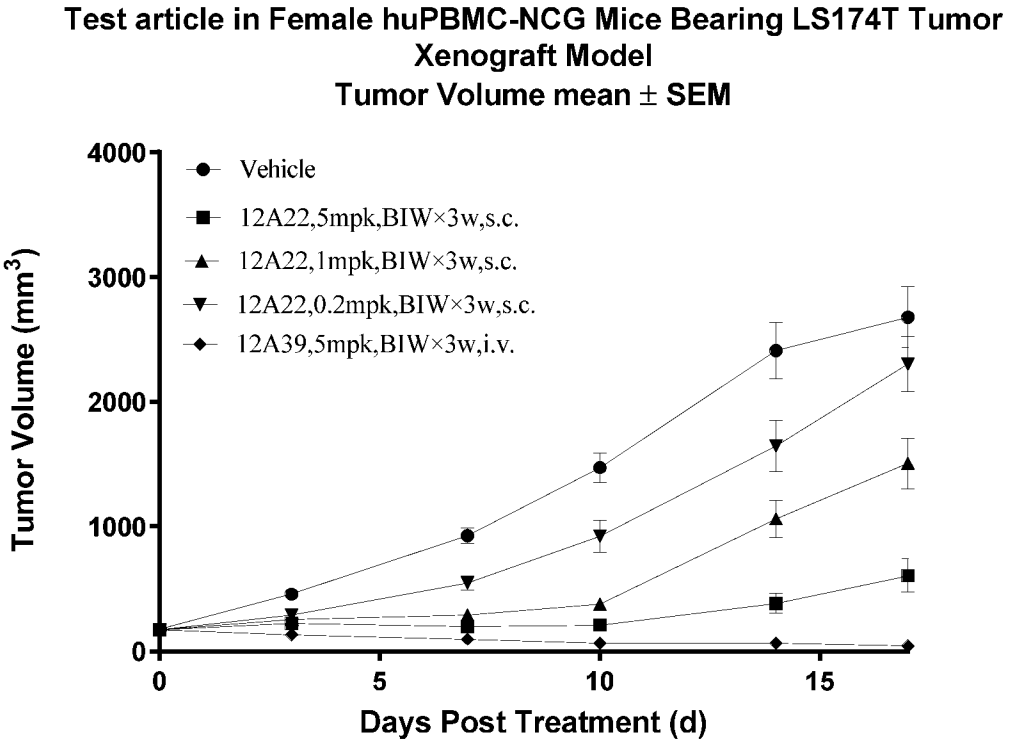


FIG 5A

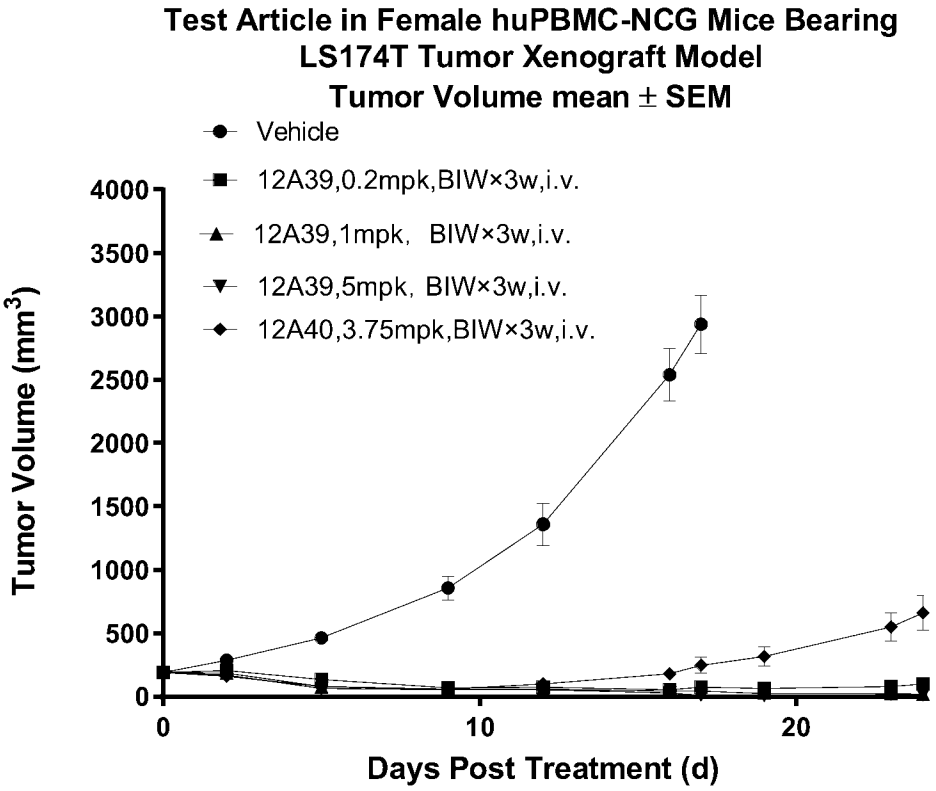

Test Article in Female huPBMC-NCG Mice Bearing
LS174T Tumor Xenograft Model
Tumor Volume mean ± SEM
Vehicle
12A39,0.2mpk,BIW×3w,i.v.
12A39,1mpk, BIW×3w,i.v.
12A39,5mpk, BIW×3w,i.v.
12A40,3.75mpk,BIW×3w,i.v.
Tumor Volume (mm3)
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
Days Post Treatment (d)

FIG 5B

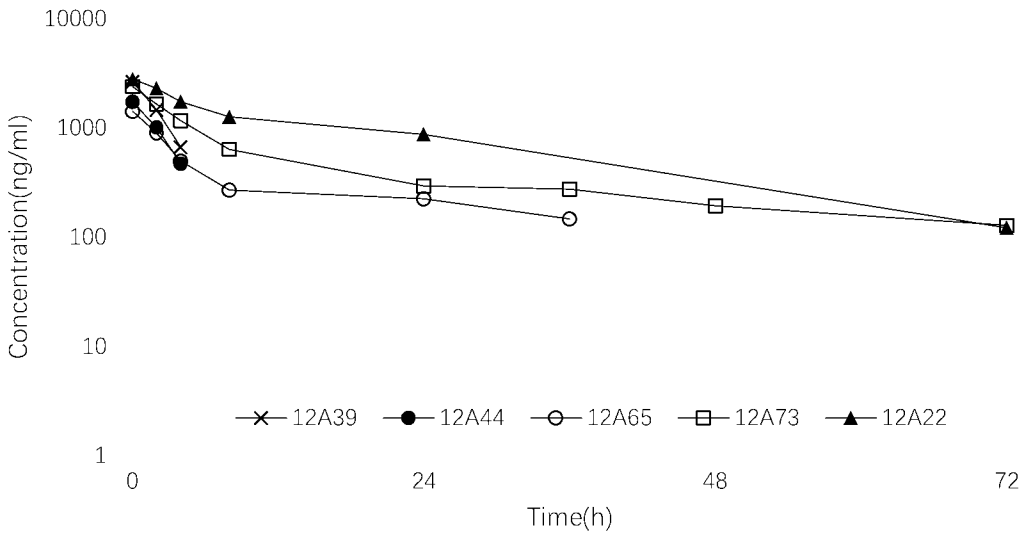
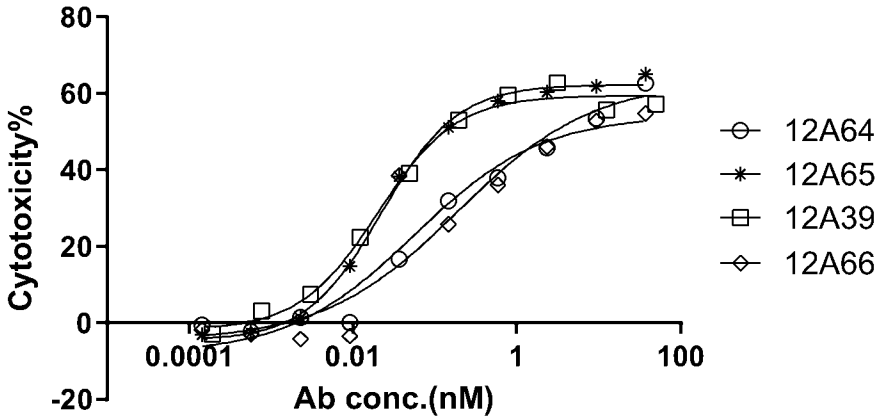
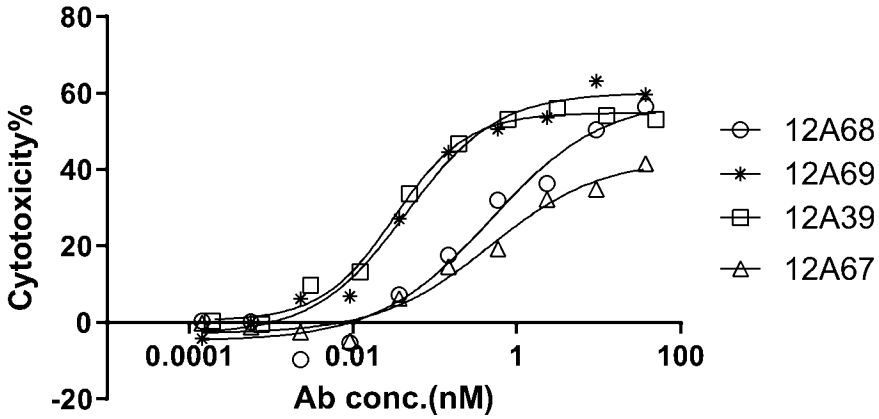

10000
1000
100
10
1
Concentration(ng/ml)
12A39
12A44
12A65
12A73
12A22
0
24
48
72
Time(h)

FIG 6



	12A39	12A64	12A65	12A66
IC50	0.02012	0.1940	0.02243	0.05656

FIG 7A



	12A39	12A67	12A68	12A69
IC50	0.03220	0.4671	0.4764	0.04460

FIG 7B

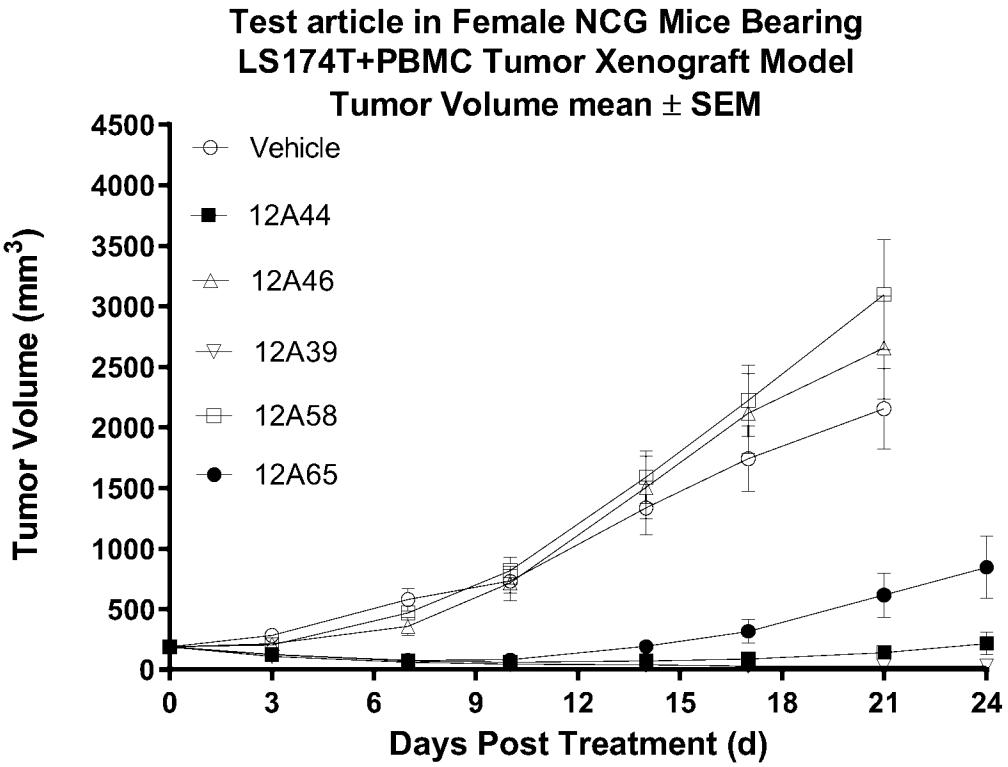

Test article in Female NCG Mice Bearing
LS174T+PBMC Tumor Xenograft Model
Tumor Volume mean ± SEM
Tumor Volume (mm3)
Days Post Treatment (d)
Vehicle
12A44
12A46
12A39
12A58
12A65
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
3
6
9
12
15
18
21
24

FIG 8

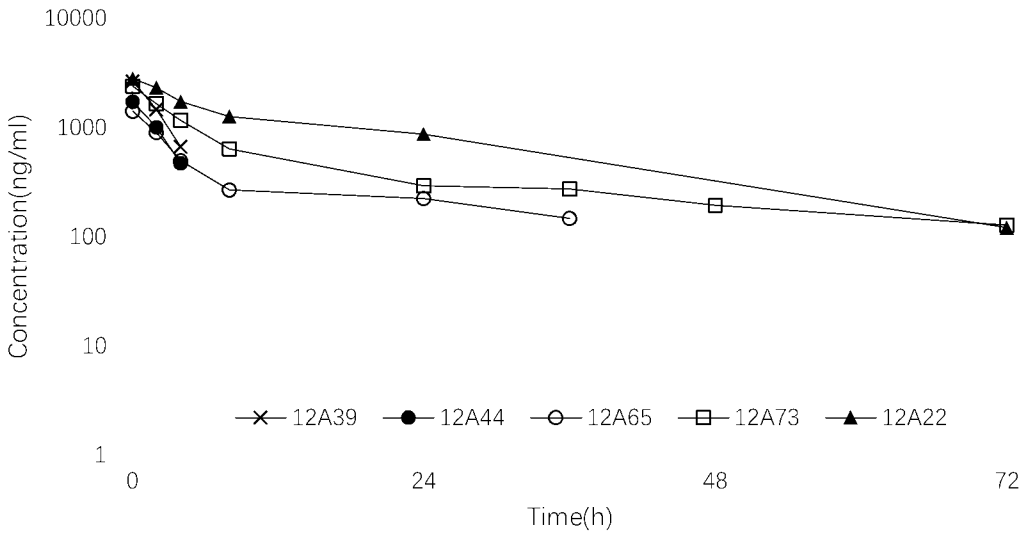
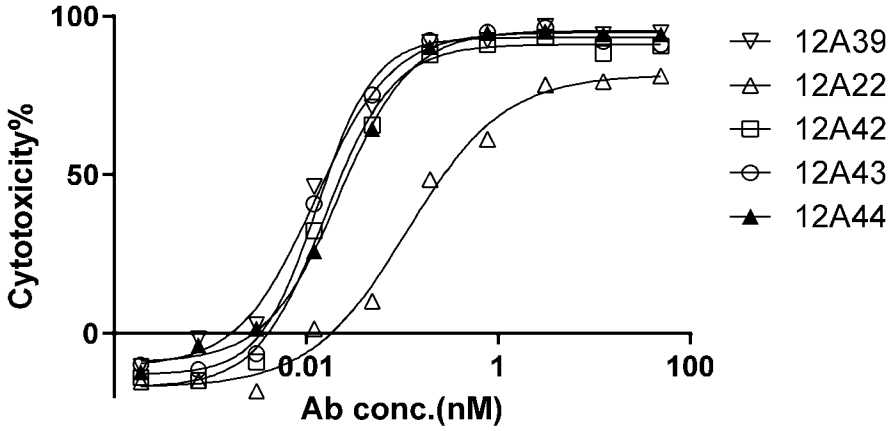
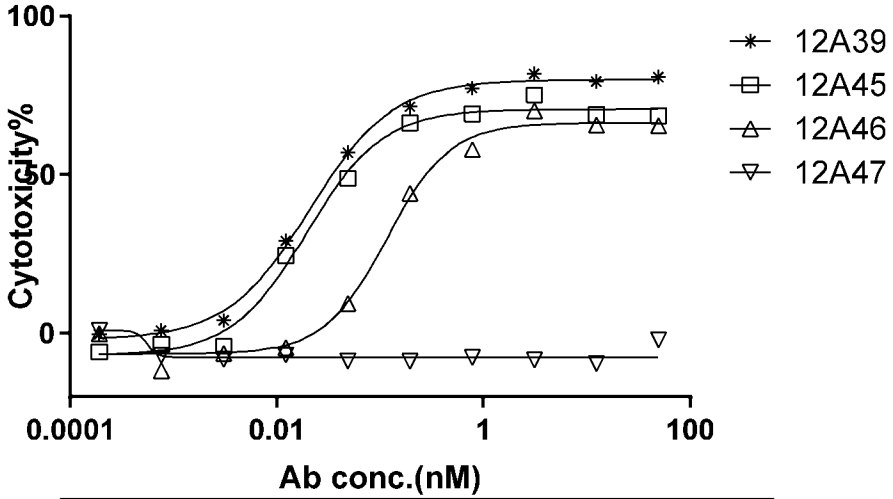

Concentration(ng/ml)
Time(h)
10000
1000
100
10
1
0
24
48
72
12A39
12A44
12A65
12A73
12A22

FIG 9



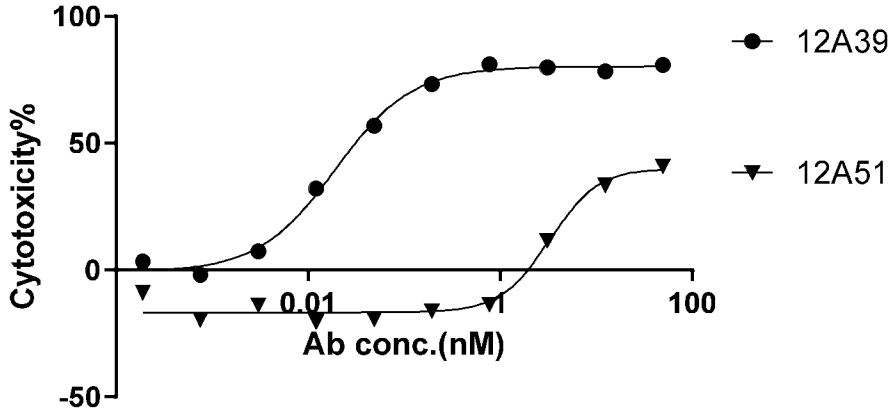
	12A39	12A22	12A42	12A43	12A44
IC50	0.01254	0.1131	0.01585	0.01319	0.02210

FIG 10A



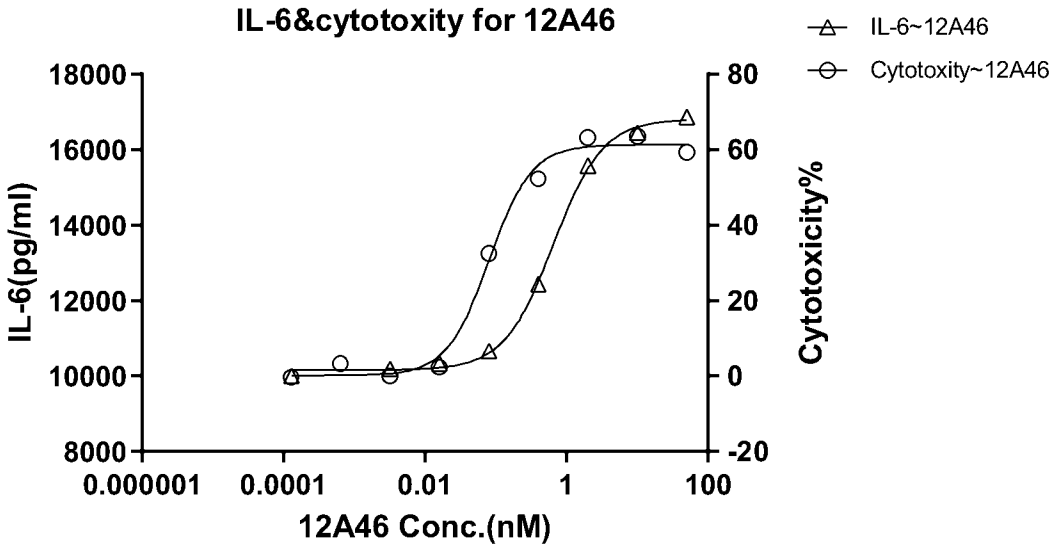
	12A39	12A45	12A46	12A47
IC50	0.02084	0.01959	0.1163	~ 0.0005558

FIG 10B



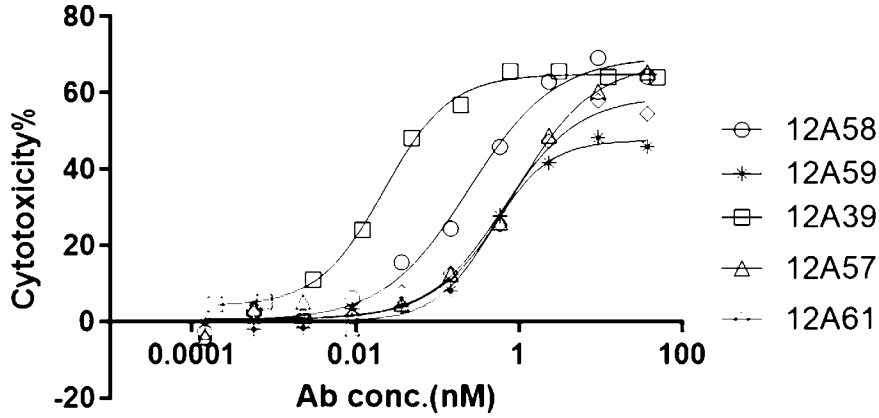
	12A39	12A51
IC50	0.01938	3.279

FIG 10C



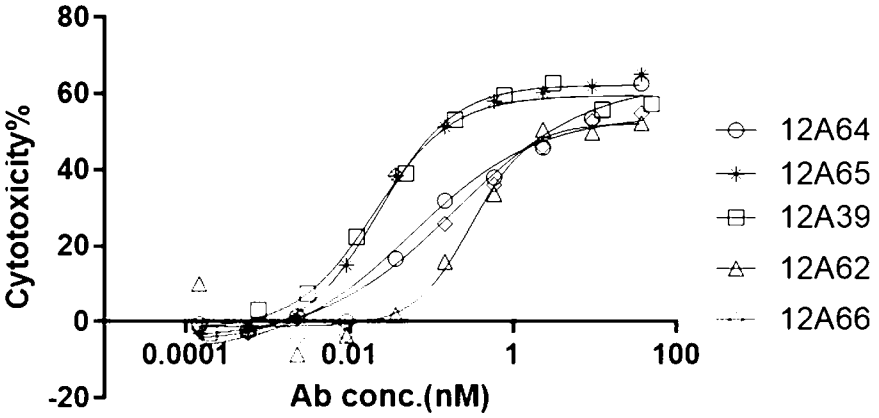
	IL-6~12A46	Cytotoxity~12A46
EC50	0.6538	0.08104

FIG 11



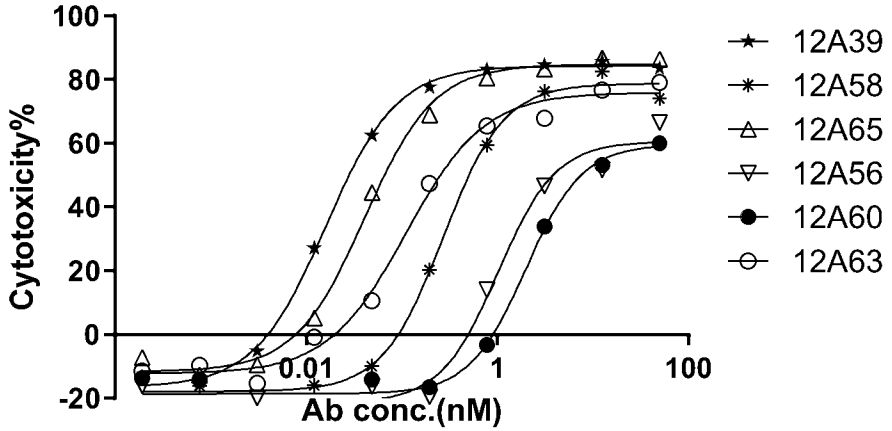
	12A39	12A57	12A58	12A59	12A61
IC50	0.02224	0.9087	0.2454	0.4601	0.6472

FIG 12A



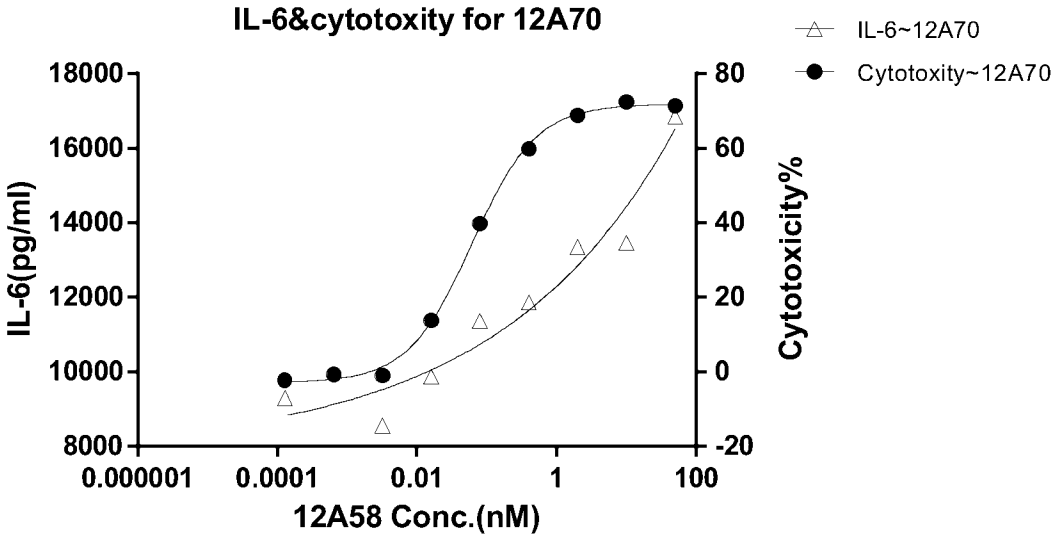
	12A39	12A62	12A64	12A65	12A66
IC50	0.02012	0.3076	0.1940	0.02243	0.05656

FIG 12B



	12A39	12A58	12A65	12A56	12A60	12A63
IC50	0.01601	0.2708	0.04094	1.023	1.988	0.1109

FIG 12C



	IL-6~12A46	Cytotoxity~12A46
EC50	~ 3.428e+015	0.06158

FIG 13

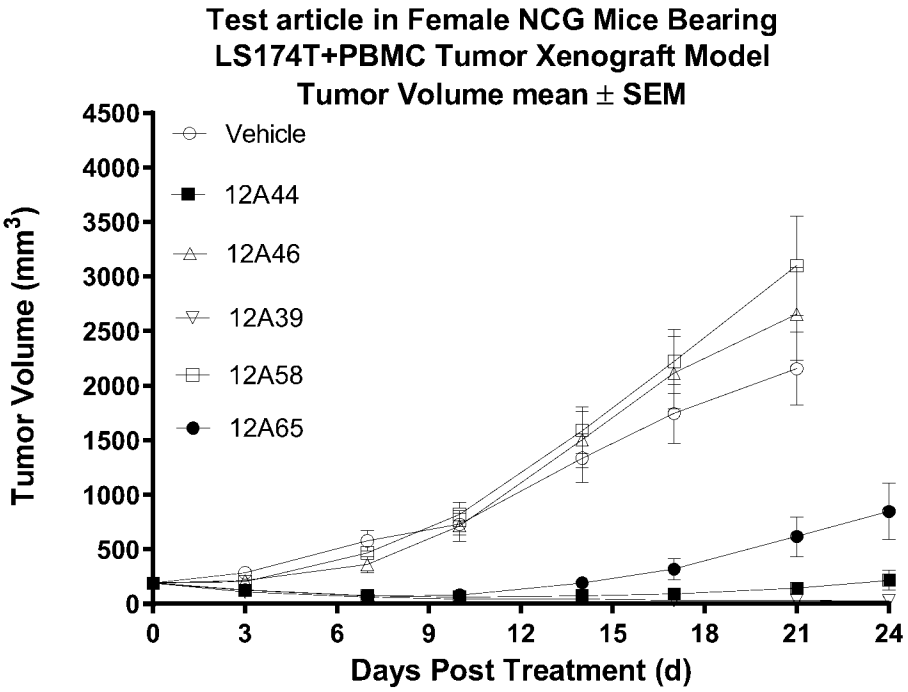

Test article in Female NCG Mice Bearing
LS174T+PBMC Tumor Xenograft Model
Tumor Volume mean ± SEM
Vehicle
12A44
12A46
12A39
12A58
12A65
Tumor Volume (mm3)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
3
6
9
12
15
18
21
24
Days Post Treatment (d)

FIG 14

12A39

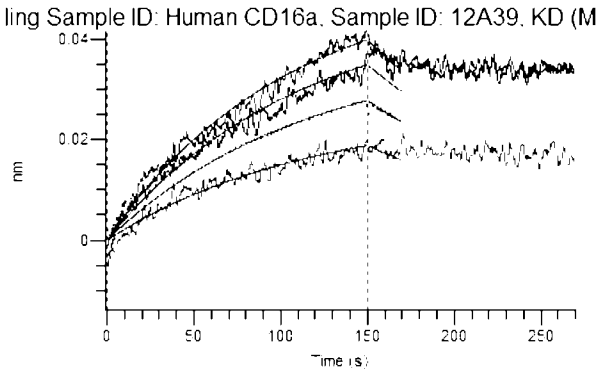


FIG 15A

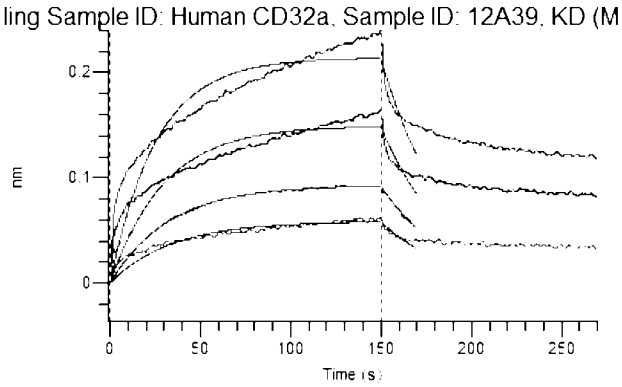


FIG 15B

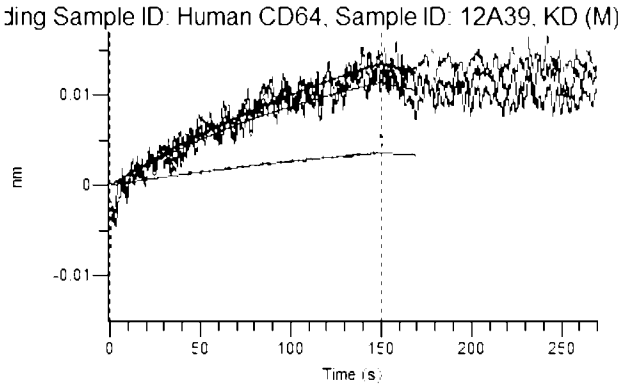


FIG 15C

12A52

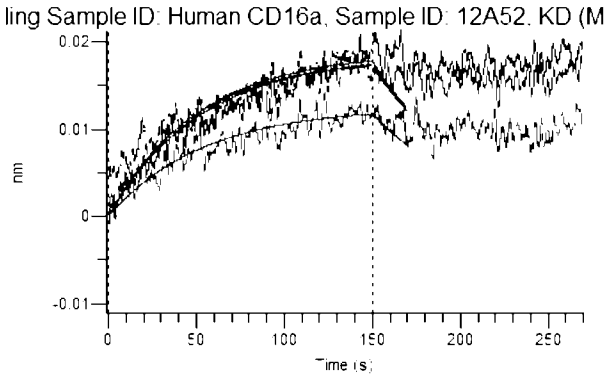


FIG 15D

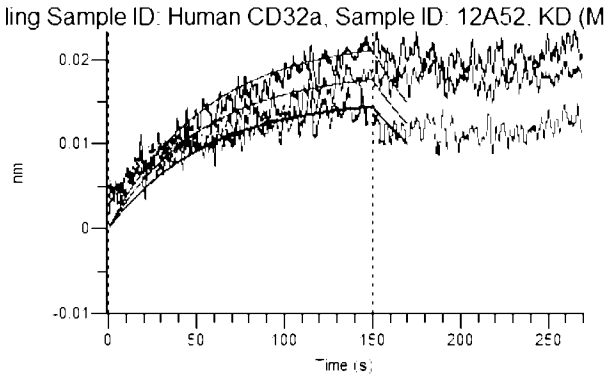


FIG 15E

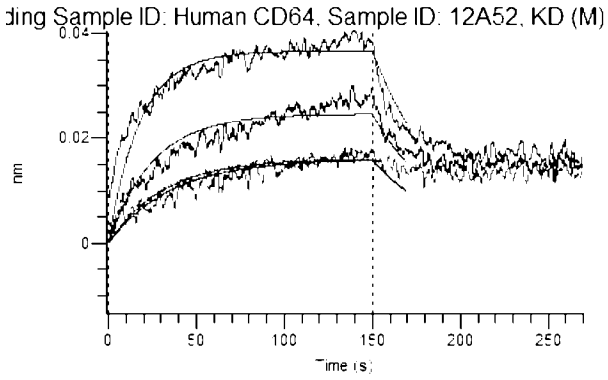
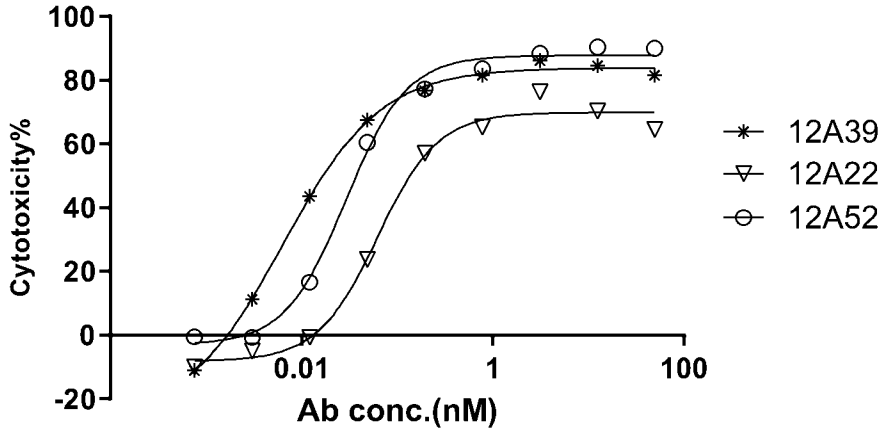


FIG 15F



	12A39	12A22	12A52
IC50	0.006432	0.06261	0.02920

FIG 16

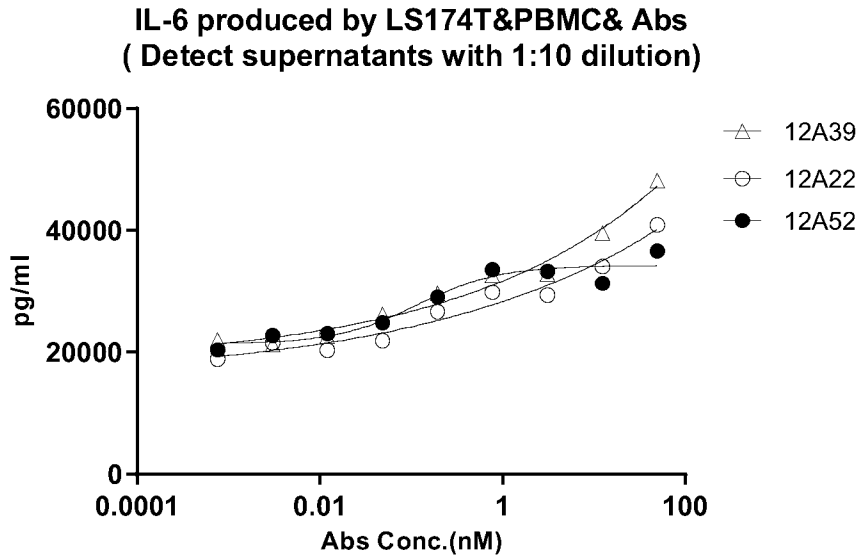


FIG 17

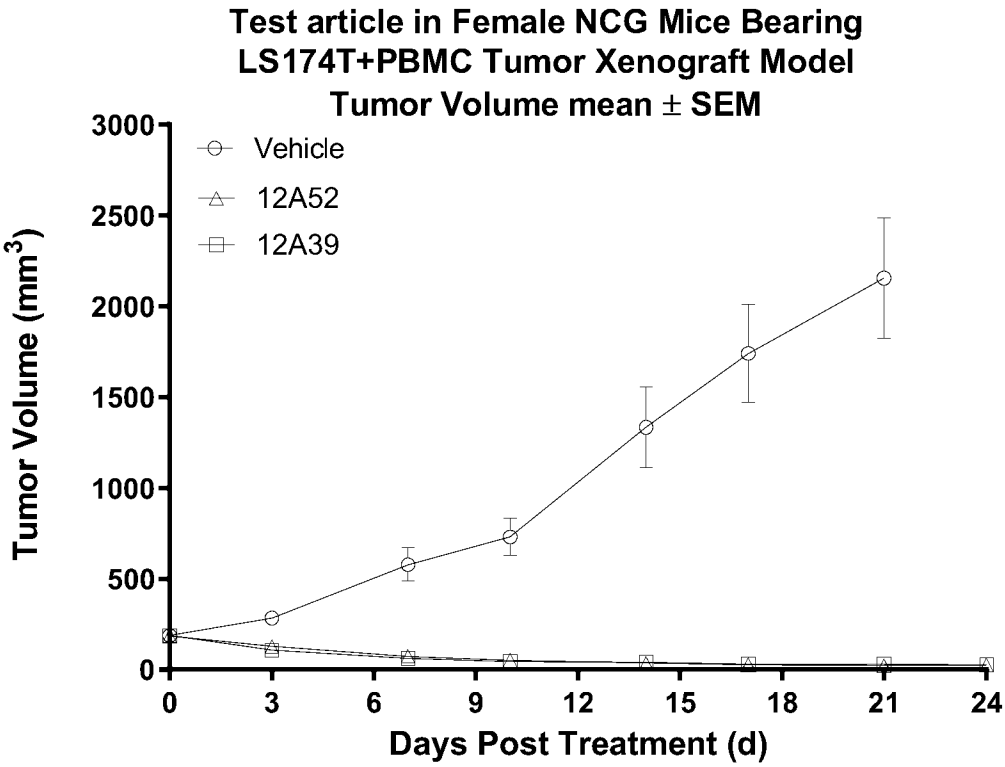

Test article in Female NCG Mice Bearing
LS174T+PBMC Tumor Xenograft Model
Tumor Volume mean ± SEM
Vehicle
12A52
12A39
Tumor Volume (mm3)
3000
2500
2000
1500
1000
500
0
0
3
6
9
12
15
18
21
24
Days Post Treatment (d)

FIG 18

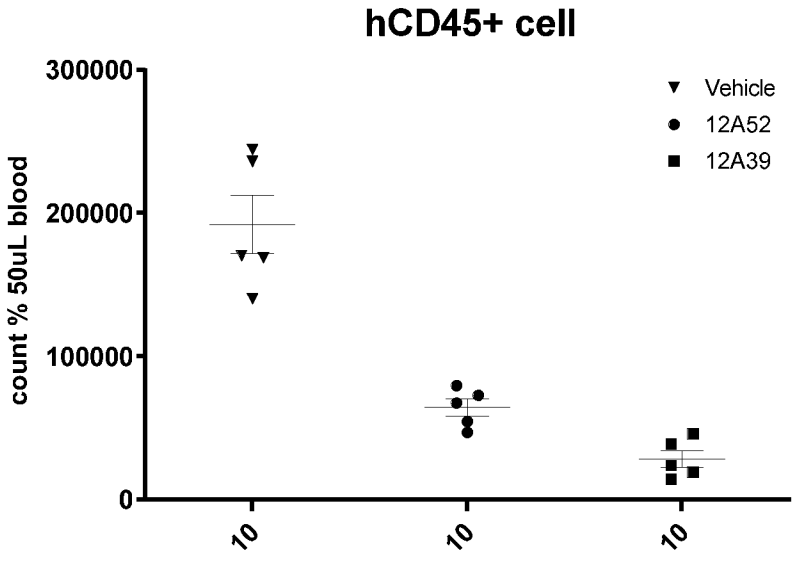

hCD45+ cell
Vehicle
12A52
12A39
count % 50uL blood
300000
200000
100000
0
10
10
10

FIG 19

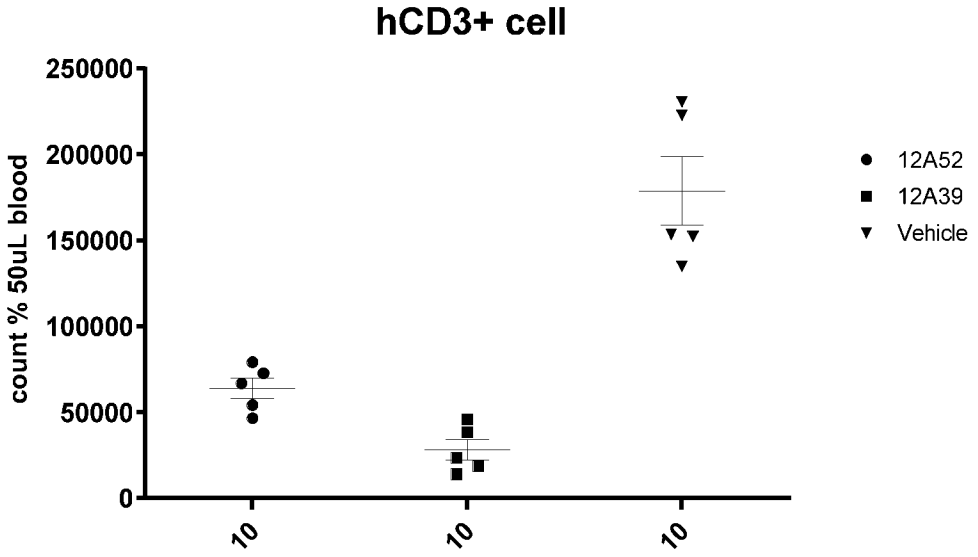

hCD3+ cell
count % 50uL blood
250000
200000
150000
100000
50000
0
10
10
10
12A52
12A39
Vehicle

FIG 20

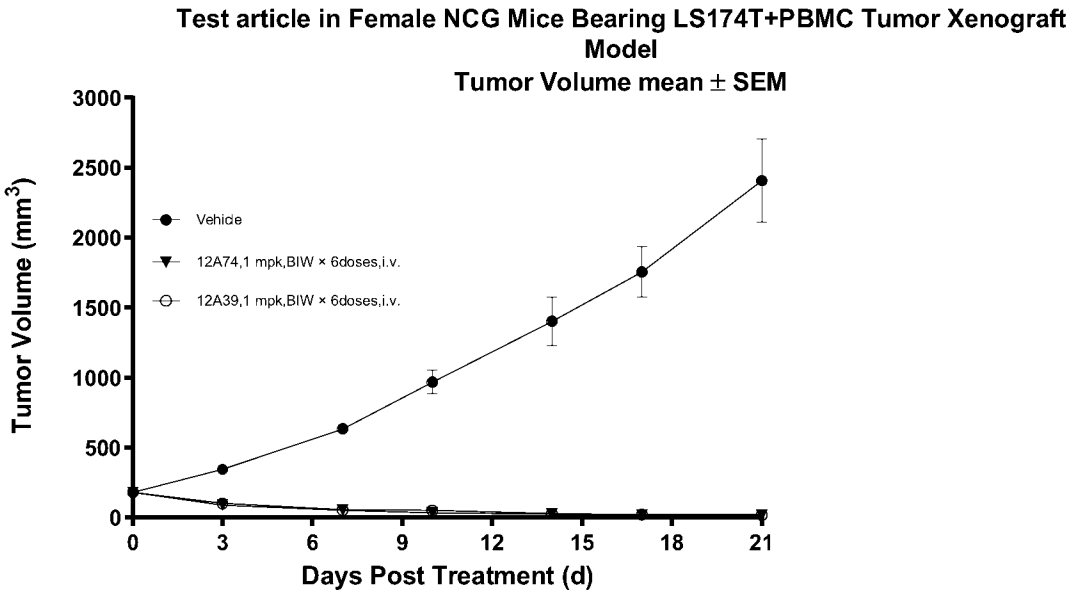

Test article in Female NCG Mice Bearing LS174T+PBMC Tumor Xenograft Model
Tumor Volume mean ± SEM
Tumor Volume (mm3)
3000
2500
2000
1500
1000
500
0
Vehicle
12A74,1 mpk,BIW × 6doses,i.v.
12A39,1 mpk,BIW × 6doses,i.v.
0
3
6
9
12
15
18
21
Days Post Treatment (d)

FIG 21

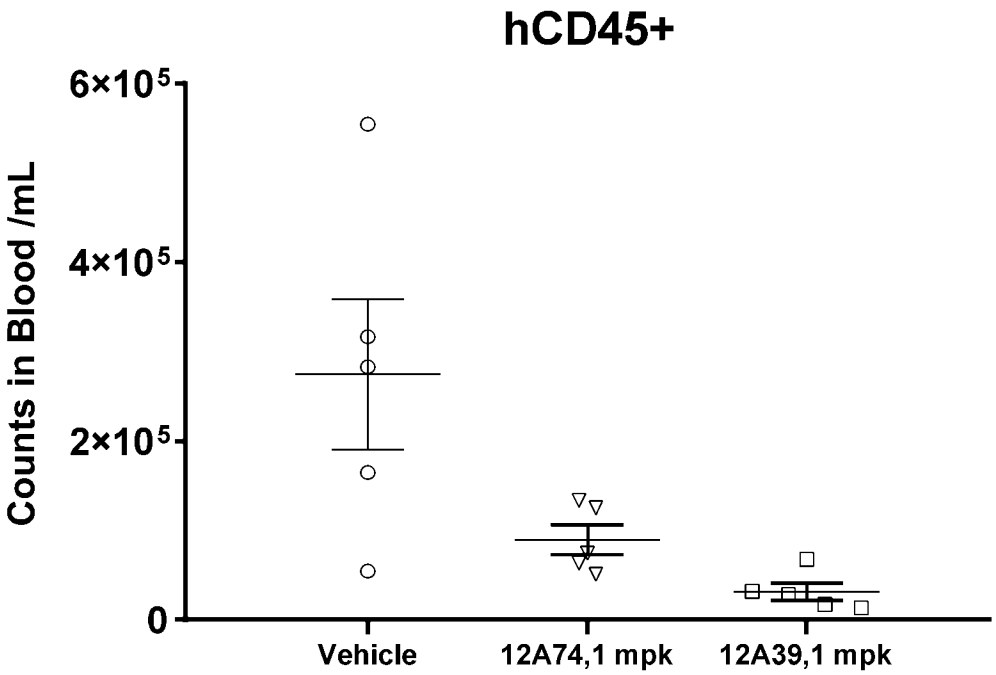

hCD45+
6×10^5
4×10^5
2×10^5
0
Counts in Blood /mL
Vehicle
12A74,1 mpk
12A39,1 mpk

FIG 22

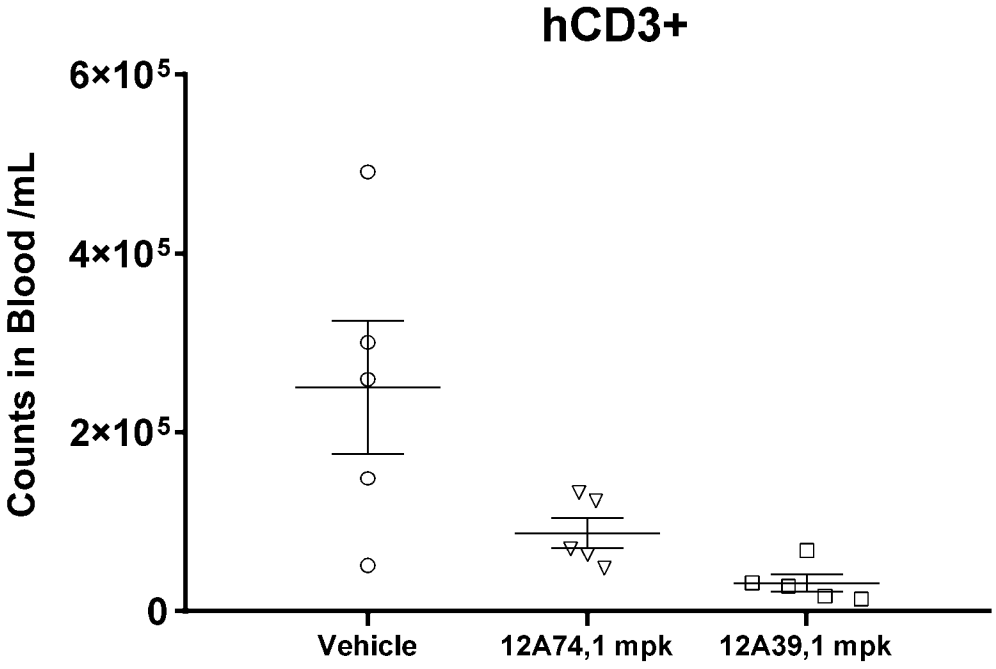

hCD3+
6×10^5
4×10^5
2×10^5
0
Counts in Blood /mL
Vehicle
12A74,1 mpk
12A39,1 mpk

FIG 23

Sample ID: 12A73, Loading Sample ID: Human CD16a

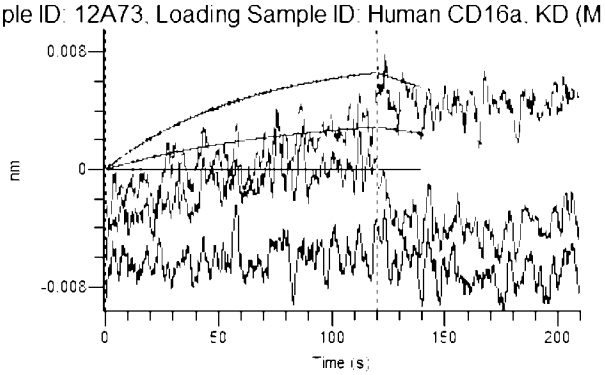


FIG 24A

Sample ID: 12A73, Loading Sample ID: Human CD32a

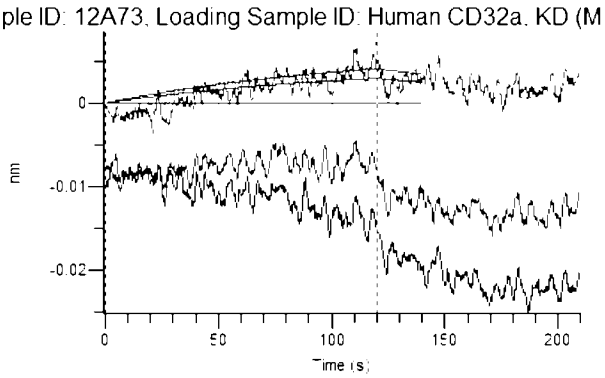


FIG 24B

Sample ID: 12A73, Loading Sample ID: Human CD64

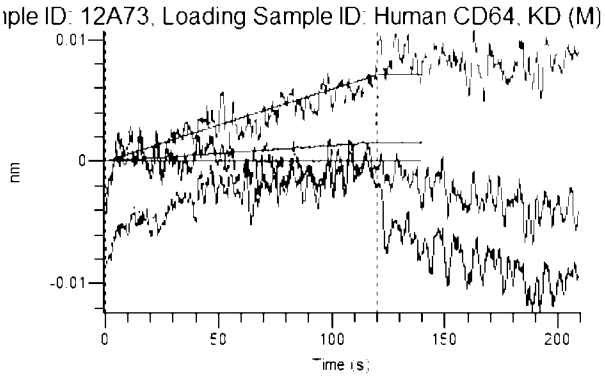
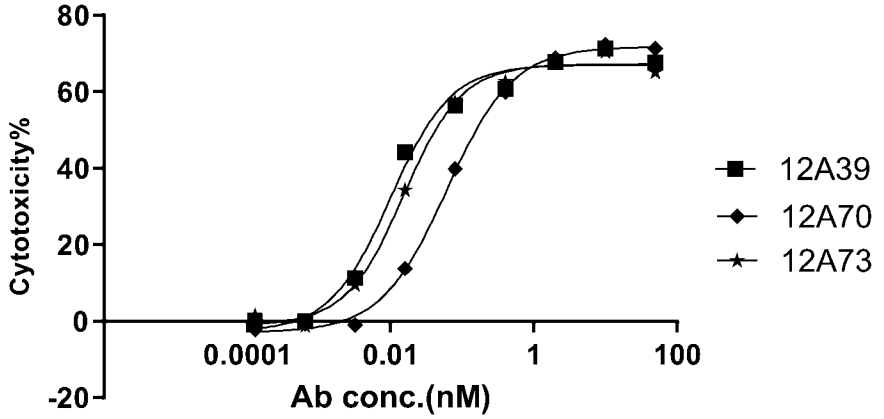
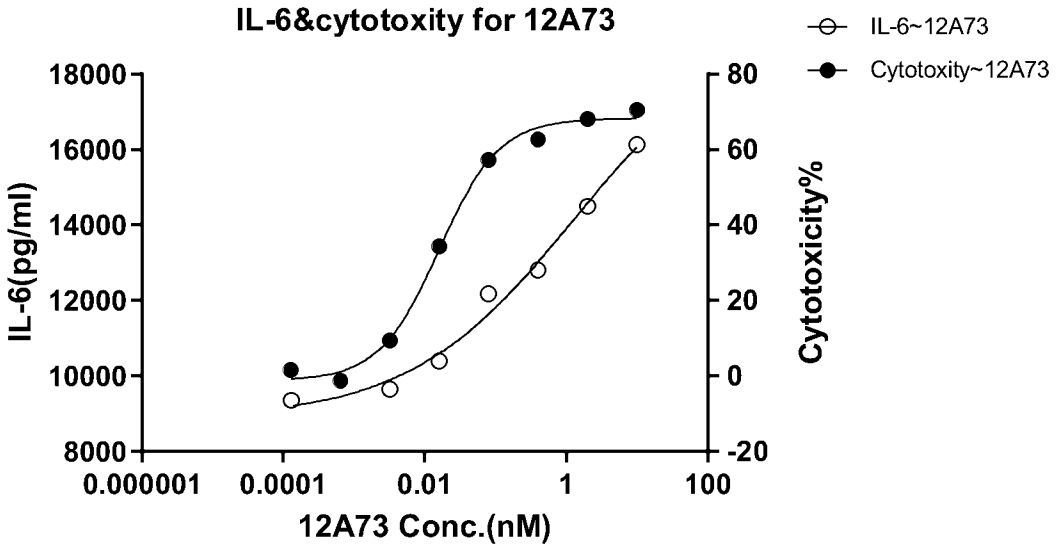


FIG 24C



	12A39	12A70	12A73
IC50	0.01003	0.06158	0.01548

FIG 25



	IL-6~12A73	Cytotoxity~12A73
EC50	1.289	0.01600

FIG 26

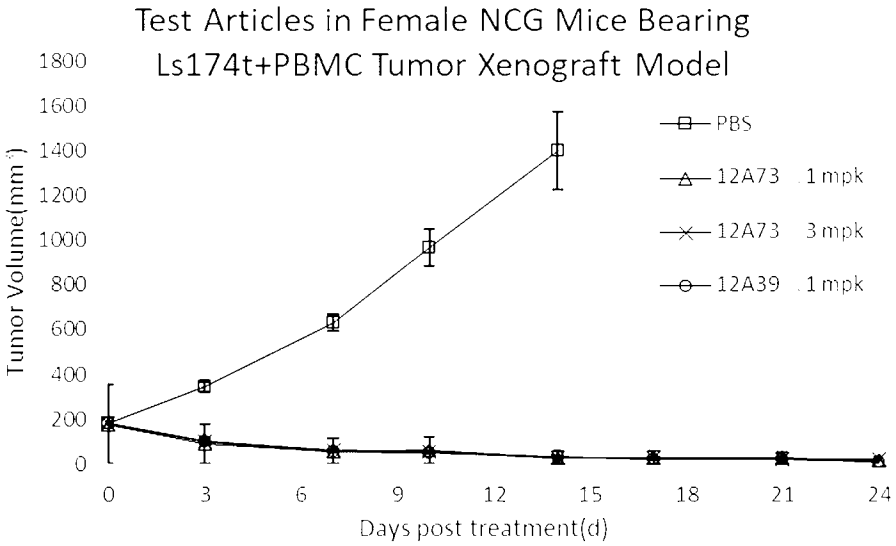


FIG 27

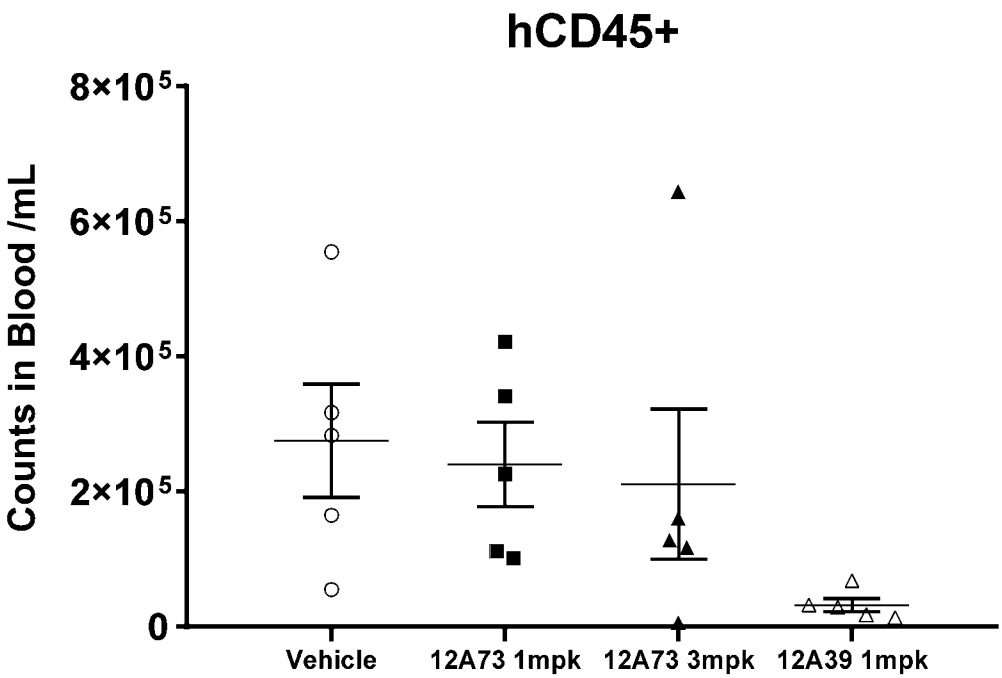


FIG 28A

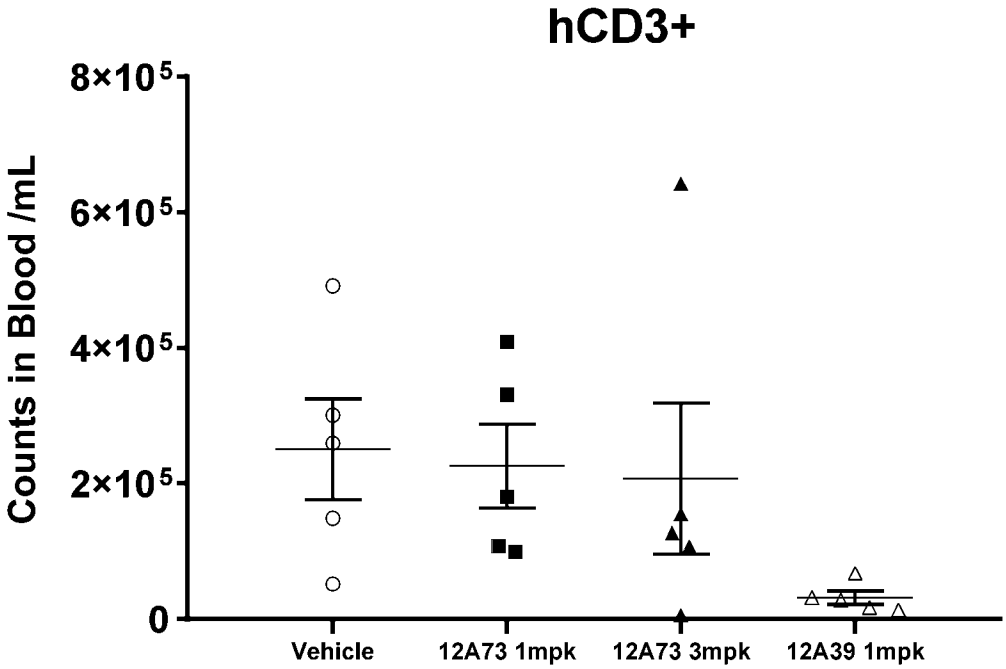


FIG 28B

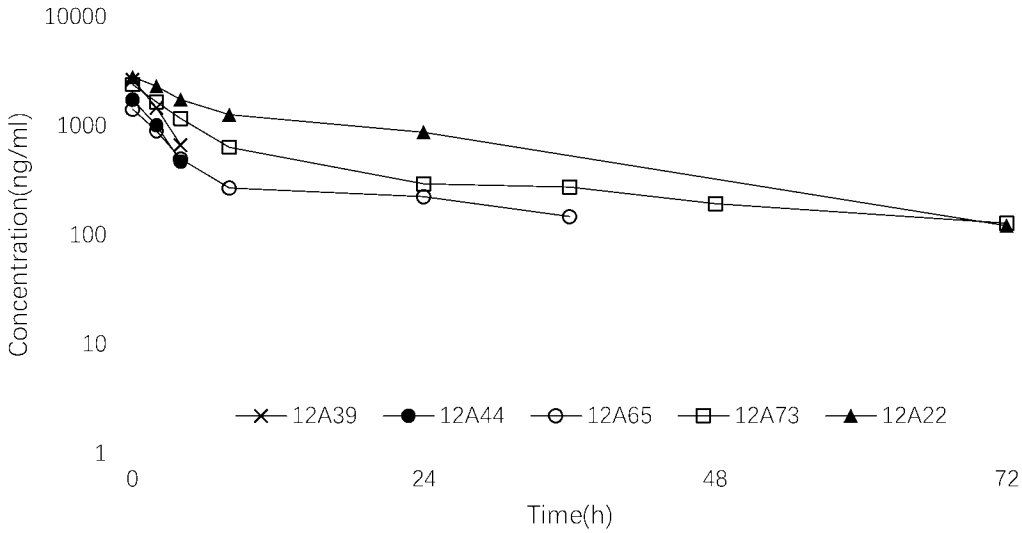
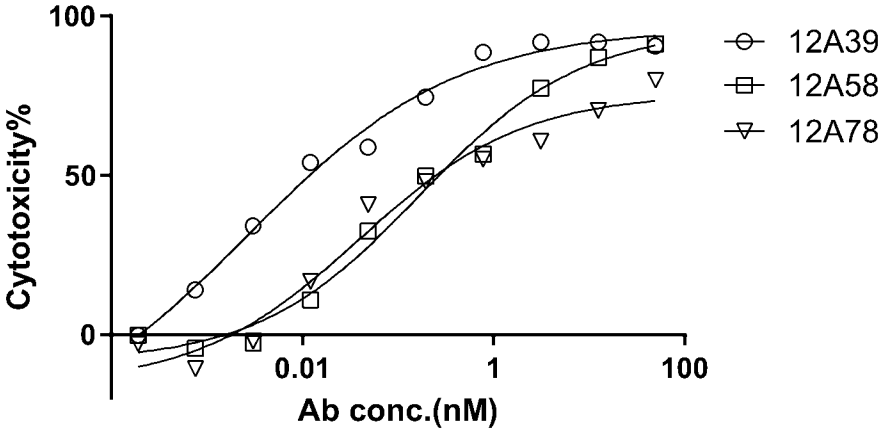


FIG 29



	12A39	12A58	12A78
IC50	0.002680	0.1686	0.03952

FIG 30

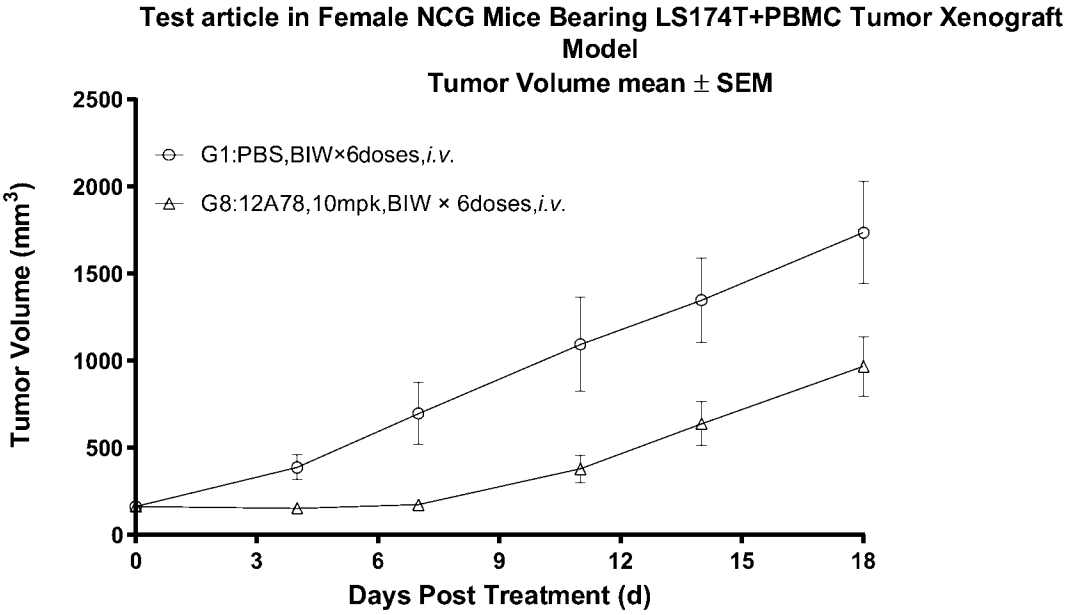


FIG 31A

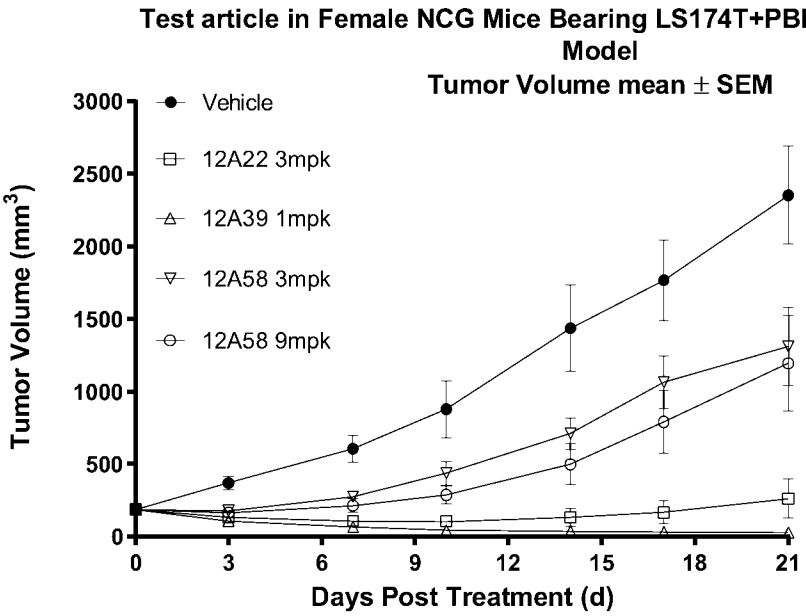


FIG 31B

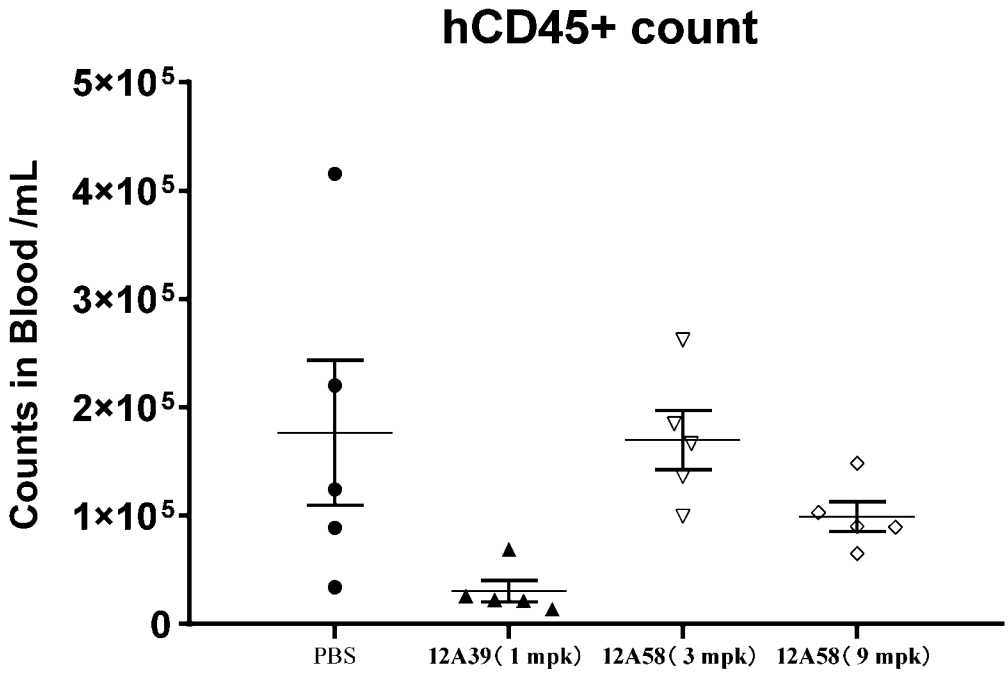


FIG 32A

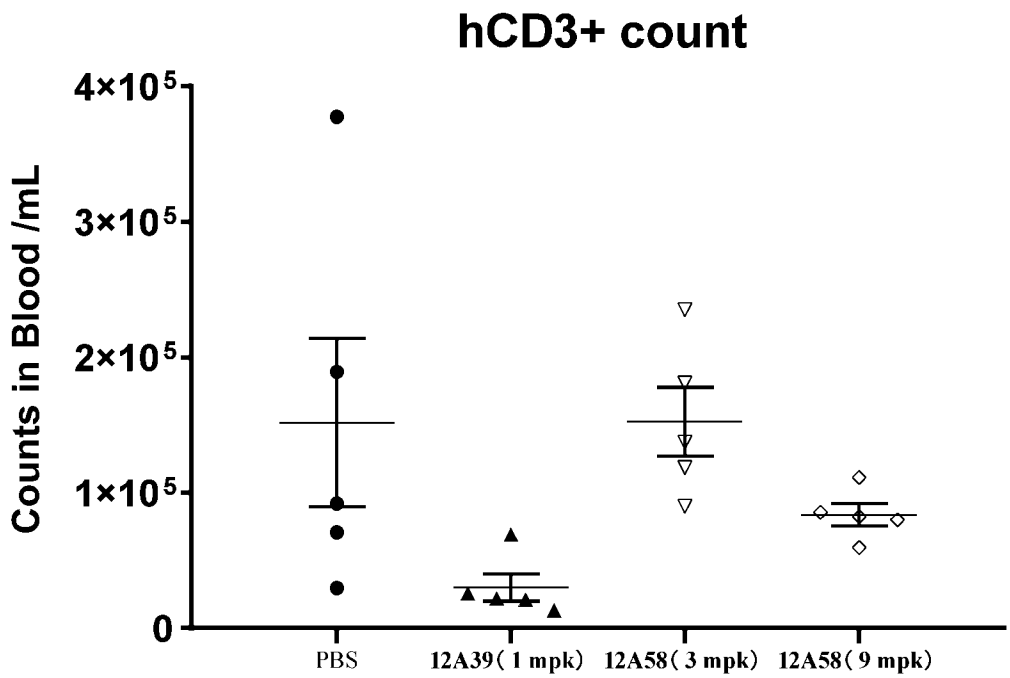


FIG 32B

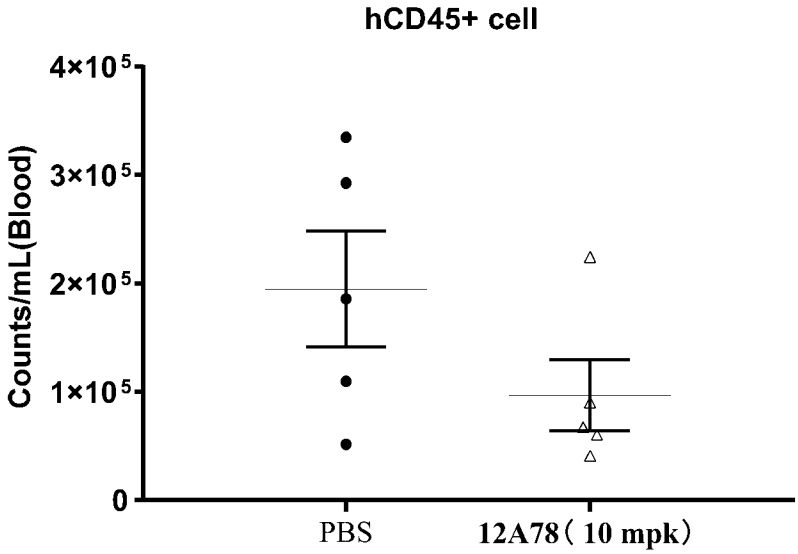


FIG 33A

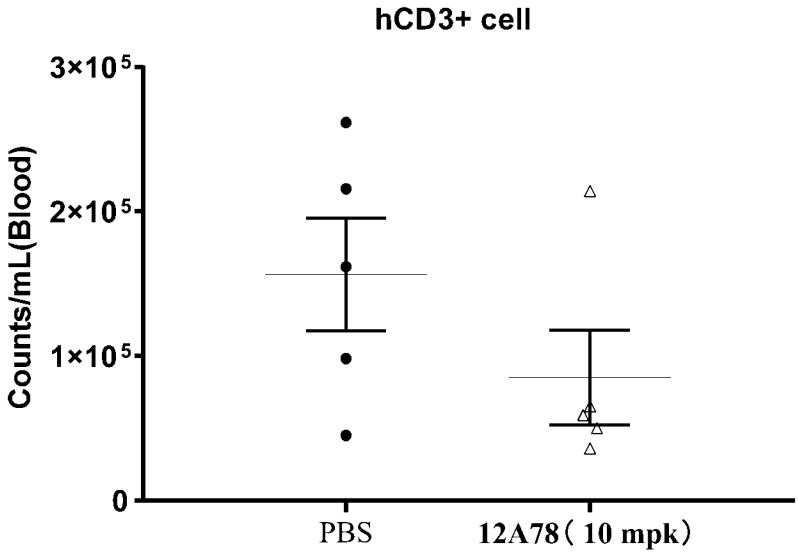


FIG 33B

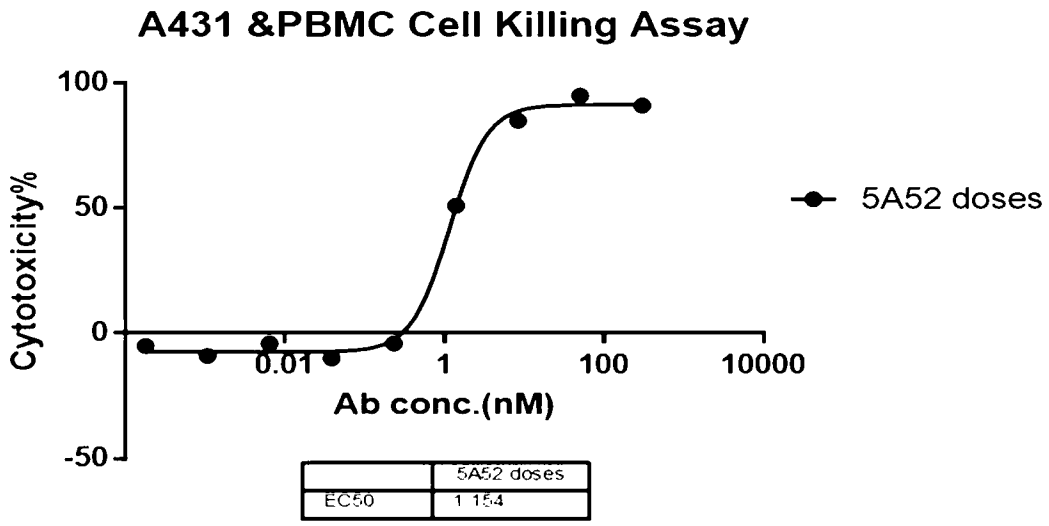
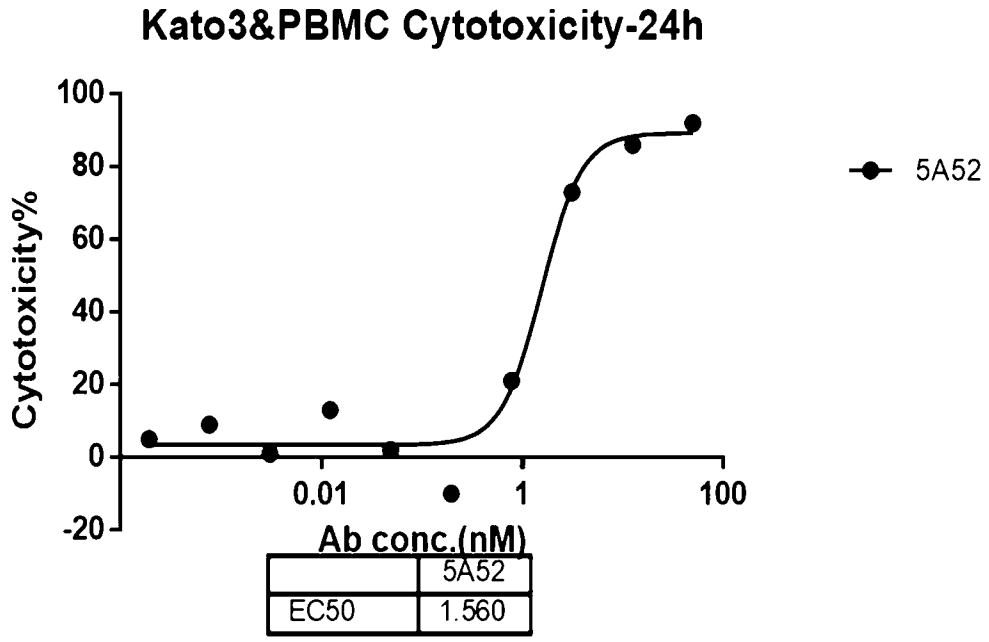
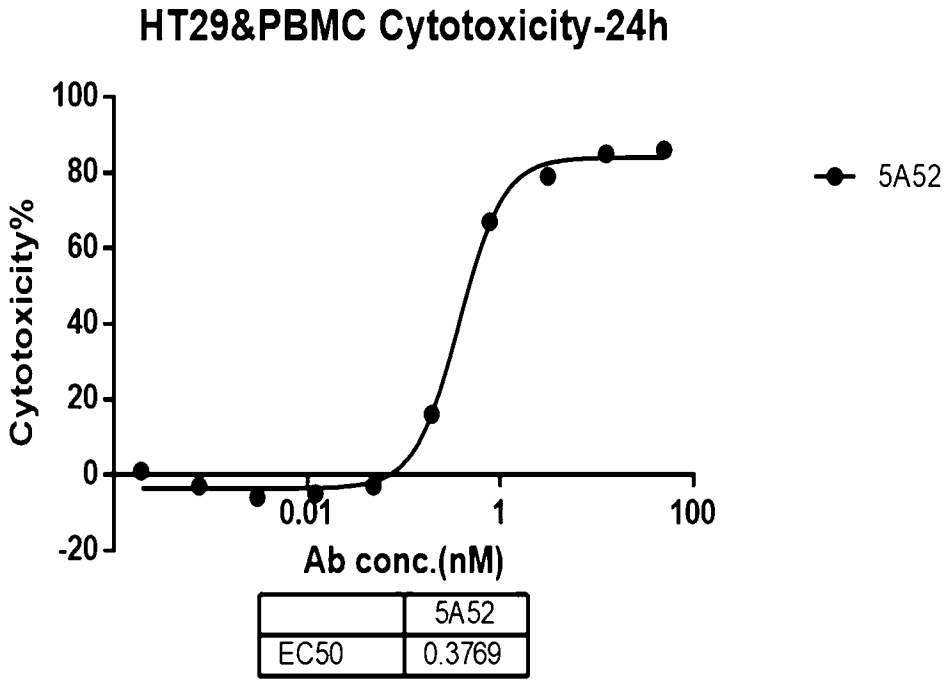


FIG 34A



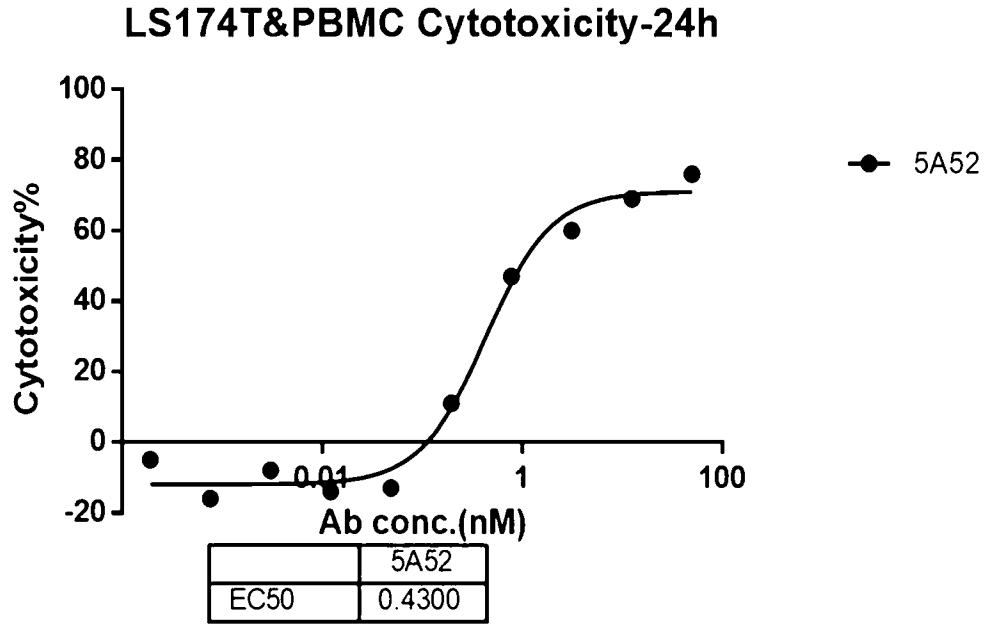
	5A52
EC50	1.560

FIG 34B



	5A52
EC50	0.3769

FIG 34C



	5A52
EC50	0.4300

FIG 34D

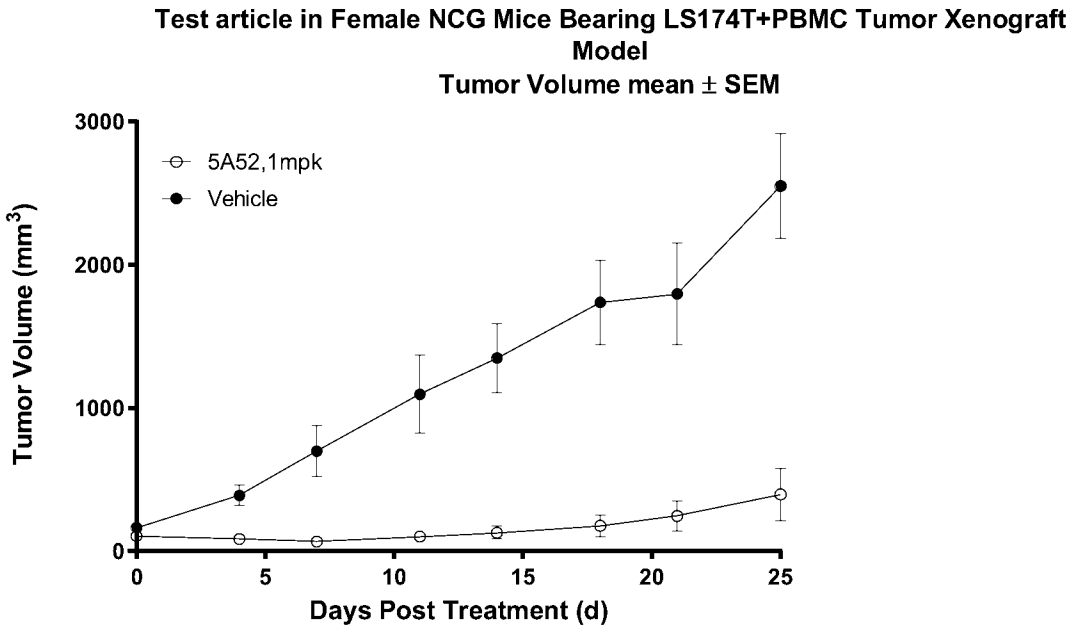
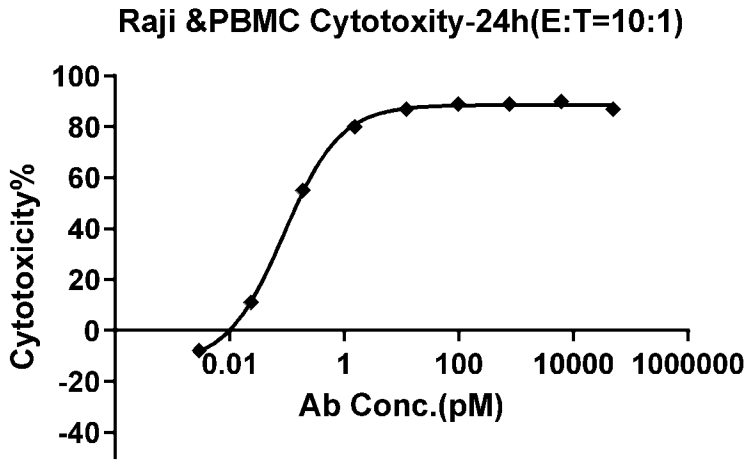


FIG 35



	2A42
EC50	0.08759

FIG 36

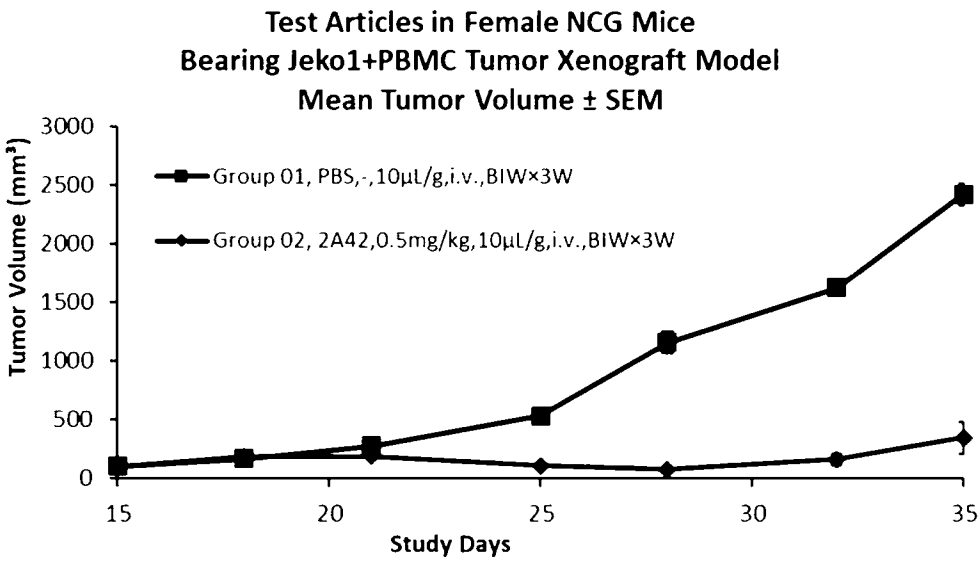
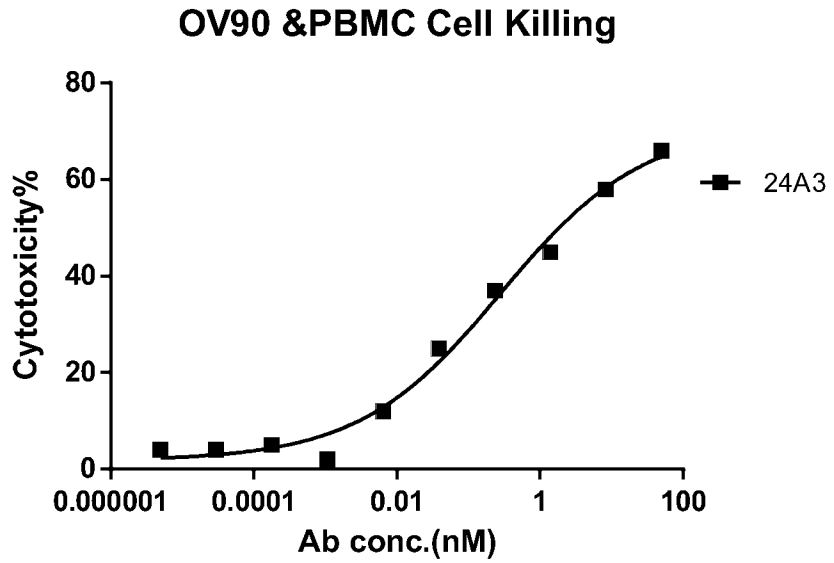
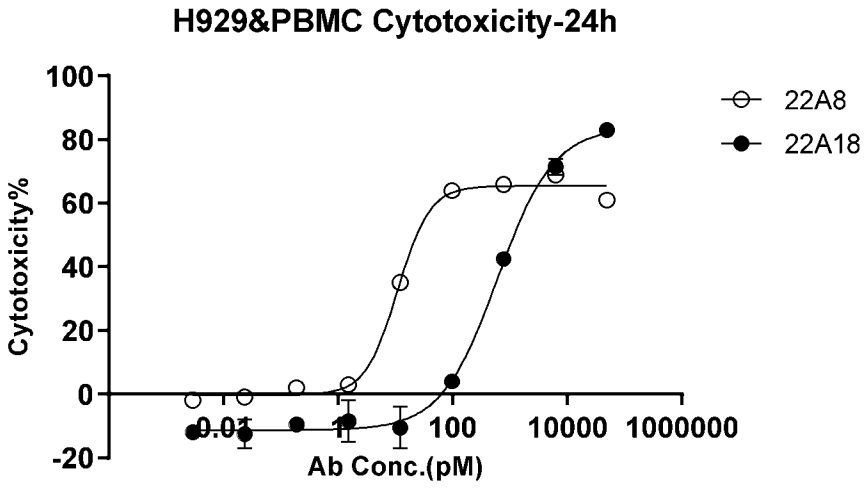


FIG 37



	24A3
EC50	0.2818

FIG 38



	22A8	22A18
EC50	11.05	607.5

FIG 39

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A63	SEQ ID NO: 41 (in CDR2) IIPIFG**D**A SEQ ID NO: 25 (K56D) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FG**D**AHYAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		
12A64	SEQ ID NO: 42 (in CDR2) IIPIFG**A**A SEQ ID NO: 26 (K56A) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FG**A**AHYAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		
12A65	SEQ ID NO: 43 (in CDR2) IIPIFG**F**A SEQ ID NO: 27 (K56F) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FG**F**AHYAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		
12A66	SEQ ID NO: 28 (H58D) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FGKA**D**YAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		

FIG 40

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A67		SEQ ID NO: 44 (in CDR1) QSVSS**E** SEQ ID NO: 29 (Y32E) EIVLTQSPATLSLSPGERA TLSCRASQSVSS**E**LAWY QQKPGQAPRLLIYDASN RATGIPARFSGSGSGTDF TLTISSLEPEDFAVYYCQ QRSNWPTFGQGTKVEIK R	
12A68		SEQ ID NO: 45 (in CDR3) QQRSN**D**PT SEQ ID NO: 30 (W94D) EIVLTQSPATLSLSPGERA TLSCRASQSVSSYLAWY QQKPGQAPRLLIYDASN RATGIPARFSGSGSGTDF TLTISSLEPEDFAVYYCQ QRSN**D**PTFGQGTKVEIK R	
12A69		SEQ ID NO: 46 (in CDR3) QQRSN**A**PT SEQ ID NO: 31 (W94A) EIVLTQSPATLSLSPGERA TLSCRASQSVSSYLAWY QQKPGQAPRLLIYDASN RATGIPARFSGSGSGTDF TLTISSLEPEDFAVYYCQ QRSN**A**PTFGQGTKVEIK R	
12A42			SEQ ID NO: 68 (in CDR1) GF**V**FSTYA SEQ ID NO: 32 (T28V) EVQLVESGGGLVQPGGS LRLSCAASGF**V**FSTYAM NWVRQAPGKGLEWVG RIRSKYNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS

FIG 40 (cont.)

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A43			SEQ ID NO: 69 (in CDR2) IRS**H**YNNYAT SEQ ID NO: 33 (K52B H) EVQLVESGGGLVQPGGSLRL SCAASGFTFSTYAMNWVRQ APGKGLEWVGRIRS**H**YNNY ATYYADSVKGRFTISRDDSK NSLYLQMNSLRAEDTAVYYC VRHGNFGTSYVSWFAYWGQ GTLVTVSS
12A44			SEQ ID NO: 71 (in CDR2) IRS**Q**YNNYAT SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGSLRL SCAASGFTFSTYAMNWVRQ APGKGLEWVGRIRS**Q**YNNY ATYYADSVKGRFTISRDDSK NSLYLQMNSLRAEDTAVYYC VRHGNFGTSYVSWFAYWGQ GTLVTVSS
12A45			SEQ ID NO: 73 (in CDR2) IRSK**F**NNYAT SEQ ID NO: 35 (Y52C F) EVQLVESGGGLVQPGGSLRL SCAASGFTFSTYAMNWVRQ APGKGLEWVGRIRSK**F**NNYA TYYADSVKGRFTISRDDSKN SLYLQMNSLRAEDTAVYYCV RHGNFGTSYVSWFAYWGQG TLVTVSS
12A46			SEQ ID NO: 75 (in CDR3) VRHGNFGTSY**A**SWFAY SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSLRL SCAASGFTFSTYAMNWVRQ APGKGLEWVGRIRSKYNNY ATYYADSVKGRFTISRDDSK NSLYLQMNSLRAEDTAVYYC VRHGNFGTSY**A**SWFAYWGQ GTLVTVSS

FIG 40 (cont.)

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A47			SEQ ID NO: 102 (in CDR3) VRHGNFGTSYV**T**WFAY SEQ ID NO: 37 (S100D T) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY V**T**WFAYWGQGTLVTVSS
12A51			SEQ ID NO: 103 (in CDR2) IRS**D**YNNYAT SEQ ID NO: 38 (K52B D) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRS**D** YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY VSWFAYWGQGTLVTVSS
12A56	SEQ ID NO: 25 (K56D) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FG**D**AHYAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS
12A57	SEQ ID NO: 26 (K56A) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FG**A**AHYAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS

FIG 40 (cont.)

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A58	SEQ ID NO: 27 (K56F) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FG**F**AHYAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS
12A59	SEQ ID NO: 28 (H58D) QVQLVQSGAEVKKPGSSV KVSCKTSGDTFSTYAISW VRQAPGQGLEWMGGIIPI FGKA**D**YAQKFQGRVTITA DESTSTAYMELSSLRSEDT AVYFCARKFHFVSGSPFG MDVWGQGTTVTVSS		SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS
12A60		SEQ ID NO: 29 (Y32E) EIVLTQSPATLSLSPGER ATLSCRASQSVSS**E**LA WYQQKPGQAPRLLIY DASNRATGIPARFSGSG SGTDFTLTISSLEPEDFA VYYCQQRSNWPTFGQ GTKVEIKR	SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS
12A61		SEQ ID NO: 30 (W94D) EIVLTQSPATLSLSPGER ATLSCRASQSVSSYLA WYQQKPGQAPRLLIY DASNRATGIPARFSGSG SGTDFTLTISSLEPEDFA VYYCQQRSN**D**PTFGQ GTKVEIKR	SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS
12A62		SEQ ID NO: 31 (W94A) EIVLTQSPATLSLSPGER ATLSCRASQSVSSYLA WYQQKPGQAPRLLIY DASNRATGIPARFSGSG SGTDFTLTISSLEPEDFA VYYCQQRSN**A**PTFGQ GTKVEIKR	SEQ ID NO: 36 (V100C A) EVQLVESGGGLVQPGGSL RLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSK YNNYATYYADSVKGRFTI SRDDSKNSLYLQMNSLRA EDTAVYYCVRHGNFGTSY **A**SWFAYWGQGTLVTVSS

FIG 40 (cont.)

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A70	SEQ ID NO: 27 (K56F) QVQLVQSGAEVKKPGSS VKVSCKTSGDTFSTYAIS WVRQAPGQGLEWMGGII PIFG**F**AHYAQKFQGRVTIT ADESTSTAYMELSSLRSE DTAVYFCARKFHFVSGSP FGMDVWGQGTTVTVSS		SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYL QMNSLRAEDTAVYYCV RHGNFGTSYVSWFAYW GQGTLVTVSS
12A94			SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAMN WVRQAPGKGLEWVGRI RS**Q**YNNYATYYADSVKG RFTISRDDSKNSLYLQMN SLRAEDTAVYYCVRHGN FGTSYVSWFAYWGQGTL VTVSS
12A95		SEQ ID NO: 145 (S30H) DIQMTQSPSSLSASVGD RVTITCRASQDV**H**TAVA WYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSGSG TDFTLTISSLQPEDFATY YCQQYLYHPATFGQGTK VEIK	SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAMN WVRQAPGKGLEWVGRI RS**Q**YNNYATYYADSVKG RFTISRDDSKNSLYLQMN SLRAEDTAVYYCVRHGN FGTSYVSWFAYWGQGTL VTVSS
12A96		SEQ ID NO: 146 (S30T) DIQMTQSPSSLSASVGD RVTITCRASQDV**T**TAVA WYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSGSG TDFTLTISSLQPEDFATY YCQQYLYHPATFGQGTK VEIK	SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAMN WVRQAPGKGLEWVGRI RS**Q**YNNYATYYADSVKG RFTISRDDSKNSLYLQMN SLRAEDTAVYYCVRHGN FGTSYVSWFAYWGQGTL VTVSS

FIG 40 (cont.)

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A97	SEQ ID NO: 147 (S56G) EVQLVESGGGLVQPGGSL RLSCAASGFTFSDSWIHW VRQAPGKGLEWVAWISPY GG**G**TYYADSVKGRFTISA DTSKNTAYLQMNSLRAED TAVYYCARRHWPGGFDY WGQGTLVTVSS		SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS
12A98	SEQ ID NO: 148 (Y53C) EVQLVESGGGLVQPGGSL RLSCAASGFTFSDSWIHW VRQAPGKGLEWVAWISP**C** GGSTYYADSVKGRFTISA DTSKNTAYLQMNSLRAED TAVYYCARRHWPGGFDY WGQGTLVTVSS		SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS
12A99	SEQ ID NO: 149 (Y53G) EVQLVESGGGLVQPGGSL RLSCAASGFTFSDSWIHW VRQAPGKGLEWVAWISP **G**GGSTYYADSVKGRFTIS ADTSKNTAYLQMNSLRAE DTAVYYCARRHWPGGFD YWGQGTLVTVSS		SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS
12A106	SEQ ID NO: 150 (Y53H) EVQLVESGGGLVQPGGSL RLSCAASGFTFSDSWIHW VRQAPGKGLEWVAWISP **H**GGSTYYADSVKGRFTIS ADTSKNTAYLQMNSLRAE DTAVYYCARRHWPGGFD YWGQGTLVTVSS		SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS

FIG 40 (cont.)

Name	PD-L1 (low affinity)		CD3 (low affinity)
	VH	VL	VH
12A107		SEQ ID NO: 151 (S30E) DIQMTQSPSSLSASVGD RVTITCRASQDV**E**TAVA WYQQKPGKAPKLLIYSA SFLYSGVPSRFSGSGSGT DFTLTISSLQPEDFATYY CQQYLYHPATFGQGTKV EIK	SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS
12A108	SEQ ID NO: 152 (Y53Q) EVQLVESGGGLVQPGGSL RLSCAASGFTFSDSWIHW VRQAPGKGLEWVAWISP **Q**GGSTYYADSVKGRFTIS ADTSKNTAYLQMNSLRAE DTAVYYCARRHWPGGFD YWGQGTLVTVSS		SEQ ID NO: 34 (K52B Q) EVQLVESGGGLVQPGGS LRLSCAASGFTFSTYAM NWVRQAPGKGLEWVG RIRS**Q**YNNYATYYADSV KGRFTISRDDSKNSLYLQ MNSLRAEDTAVYYCVR HGNFGTSYVSWFAYWG QGTLVTVSS

FIG 40 (cont.)

CH1	SEQ ID NO: 51 ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSCD
CL	SEQ ID NO: 52 RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC
Linker 1 (within DICAD)	SEQ ID NO: 53 RTVAA
Linker 2 (between Fab and DICAD)	SEQ ID NO: 104 GGGGSGGGGS
Hinge 1	SEQ ID NO: 54 KTHTCPPCP
Hinge 2	SEQ ID NO: 120 ASEPKSSDKTHTCPPCP

FIG 41

	Fc Sequences
Wild-type Fc (IgG4)	SEQ ID NO: 55 APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFN WYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEY KCKVSNKGLPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSP
12A39 Fc without Knob or Hole mutations	SEQ ID NO: 114 APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVKFNW YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
12A39 AH(Knobs)	SEQ ID NO: 56 APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVKFNW YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLWC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
12A39 BH(Hole)	SEQ ID NO: 77 APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVKFNW YVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCA VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDK SRWQQGNVFSCSVMHEALHNRFTQKSLSLSP
Wild type Fc (IgG1)	SEQ ID NO: 57 APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
12A52 Fc without Knob or Hole mutations	SEQ ID NO: 115 APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

FIG 42

12A52 (Fc with LALA mutations) Knobs	SEQ ID NO: 58 APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL WCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
12A52 (Fc with LALA mutations) Hole	SEQ ID NO: 162 APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLS CAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTV DKSRWQQGNVFSCSVMHEALHNRFTQKSLSLSPG
12A73 (Fc with LALA mutations +D278 mutations)	SEQ ID NO: 59 APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
12A73 (Fc with LALA mutations +D278 mutations + Knob)	SEQ ID NO: 60 APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL WCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
12A73 (Fc with LALA mutations +D278 mutations + Hole)	SEQ ID NO: 61 APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVAVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLS CAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTV DKSRWQQGNVFSCSVMHEALHNRFTQKSLSLSPG

FIG 42 (cont.)

Antibody		Sequence
12A39	Chain 1	SEQ ID NO: 62 *VL2(CD3)-linker1-VH1(PD-L1)* QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKP GQAPRGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAED EADYYCALWYSNLWVFGGGTKLTVLRTVAAQVQLVQSGAEV KKPGSSVKVSCKTSGDTFSSYAISWVRDAPGQCLEWMGGIIPIF GKAHYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCAR KFHFVSGSPFGMDVWGQGTTVTVSSASEPKSSD
	Chain 2	SEQ ID NO: 63 *VH3(CEA) -CH1-linker2-VL1(PD-L1)-linker1-VH2(CD3)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAP GKALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDGGGGSGGGGSEIVLTQSPATLSLSPGERAT LSCRASQSVSSYLAWYQKKPGQAPRLLIYDASNRATGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGCGTKVEIKR TVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVR QAPGKGLEWVGRIRSKYNNYATYYADSVKGRFTISRDDSKNS LYLQMNSLRAEDTAVYYCVRHGNFGTSYVSWFAYWGQGTLV TVSS
	Chain 3	SEQ ID NO: 64 *VH3(CEA) -CH1* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAP GKALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCD
	Chain 4	SEQ ID NO: 65 *VL3(CEA)-CL* EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPR PWIHGTSNLATGVPARFSGSGSGTDFTLTISSLEPEDAAVYYCQ QWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG 43

12A73	Chain 1	SEQ ID NO: 66 *VL2(CD3)-linker1-VH1(PD-L1)* QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKP GQAPRGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAED EADYYCALWYSNLWVFGGGTKLTVLRTVAAQVQLVQSGAEV KKPGSSVKVSCKTSGDTFSTYAISWVRDAPGQCLEWMGGIIPIF GFAHYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCAR KFHFVSGSPFGMDVWGQGTTVTVSSASEPKSSD
	Chain 2	SEQ ID NO: 67 *VH3(CEA)-CH1-linker2-VL1(PD-L1)-linker1-VH2(CD3)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAP GKALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDGGGGSGGGGSEIVLTQSPATLSLSPGERAT LSCRASQSVSSYLAWYQKKPGQAPRLLIYDASNRATGIPARFS GSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGCGTKVEIKR TVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVR QAPGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSKNS LYLQMNSLRAEDTAVYYCVRHGNFGTSYVSWFAYWGQGTLV TVSS
	Chain 3	SEQ ID NO: 64 *VH3(CEA)-CH1-Hinge* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAP GKALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCD
	Chain 4	SEQ ID NO: 65 *VL3(CEA)-CL* EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPR PWIHGTSNLATGVPARFSGSGSGTDFTLTISSLEPEDAAVYYCQ QWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

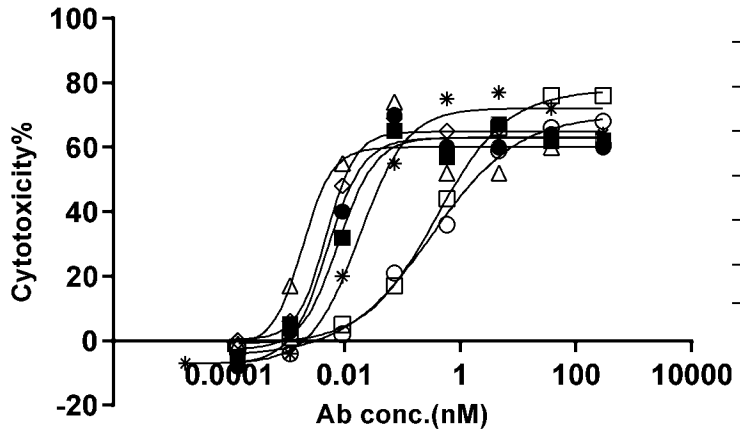
FIG 43 (cont.)

12A94	Chain 1	SEQ ID NO: 161 *VL2(CD3)-linker1-VH1(PD-L1)* QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKP GQAPRGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAED EADYYCALWYSNLWVFGGGTKLTVLRTVAAEVQLVESGGGL VQPGGSLRLSCAASGFTFSDSWIHWVRDAPGKCLEWVAWISP YGGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY CARRHWPGGFDYWGQGTLVTVSS
	Chain 2	SEQ ID NO: 158 *VH3(CEA)-CH1-linker2-VL1(PD-L1)-linker1-VH2(CD3)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPG KALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDGGGGSGGGGSDIQMTQSPSSLSASVGDRV TITCRASQDVSTAVAWYQKKPGKAPKLLIYSASFLYSGVPSRF SGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGCGTKVEI KRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSK NSLYLQMNSLRAEDTAVYYCVRHGNFGTSYVSWFAYWGQGT LVTVSS
	Chain 3	SEQ ID NO: 64 *VH3(CEA)-CH1-Hinge* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAP GKALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCD
	Chain 4	SEQ ID NO: 65 *VL3(CEA)-CL* EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPR PWIHGTSNLATGVPARFSGSGSGTDFTLTISSLEPEDAAVYYCQ QWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG 43 (cont.)

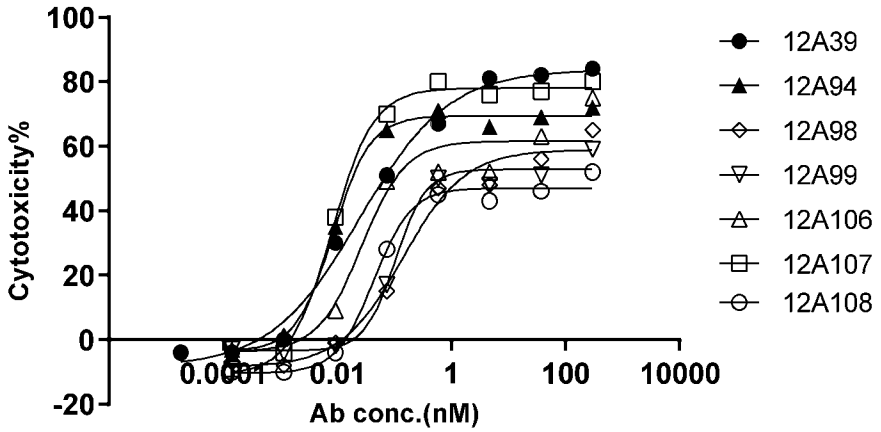
12A106	Chain 1	SEQ ID NO: 155 *VL2(CD3)-linker1-VH1(PD-L1)* QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKP GQAPRGLIGGTNKRAPWTPARFSGSLLGGKAALTLSGAQAED EADYYCALWYSNLWVFGGGTKLTVLRTVAAEVQLVESGGGL VQPGGSLRLSCAASGFTFSDSWIHWVRDAPGKCLEWVAWISP **H**GGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY CARRHWPGGFDYWGQGTLVTVSS
	Chain 2	SEQ ID NO: 158 *VH3(CEA)-CH1-linker2-VL1(PD-L1)-linker1-VH2(CD3)* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAPG KALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCDGGGGSGGGGSDIQMTQSPSSLSASVGDRV TITCRASQDVSTAVAWYQKKPGKAPKLLIYSASFLYSGVPSRF SGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGCGTKVEI KRTVAAEVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNW VRQAPGKGLEWVGRIRSQYNNYATYYADSVKGRFTISRDDSK NSLYLQMNSLRAEDTAVYYCVRHGNFGTSYVSWFAYWGQGT LVTVSS
	Chain 3	SEQ ID NO: 64 *VH3(CEA)-CH1-Hinge* EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYYMNWVRQAP GKALEWLGFIRNKVNGDTTEYAASVKGRFTISRDDSKNSLYLQ MNSLKTEDTAVYYCARDKGIAYYFDYWGQGTLVTVSSASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKKVEPKSCD
	Chain 4	SEQ ID NO: 65 *VL3(CEA)-CL* EIVLTQSPATLSASPGERATLSCRASQSVSYIHWYQQKPGQSPR PWIHGTSNLATGVPARFSGSGSGTDFTLTISSLEPEDAAVYYCQ QWSSNLSTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG 43 (cont.)



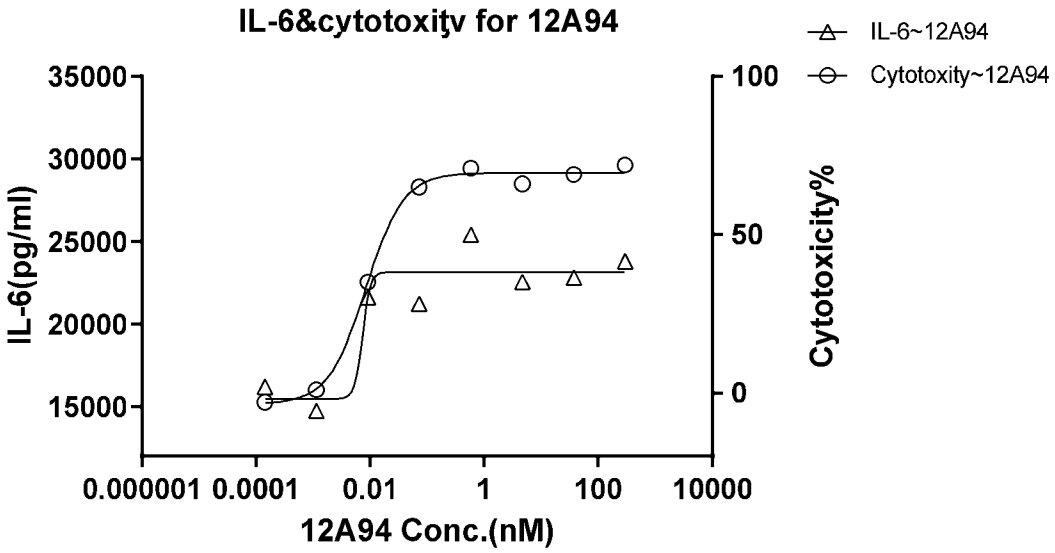
	12A39	12A94	12A95	12A96	12A97	12A98	12A99
EC50	0.01824	0.007845	0.005006	0.004886	0.001891	0.3901	0.3191

FIG 44A



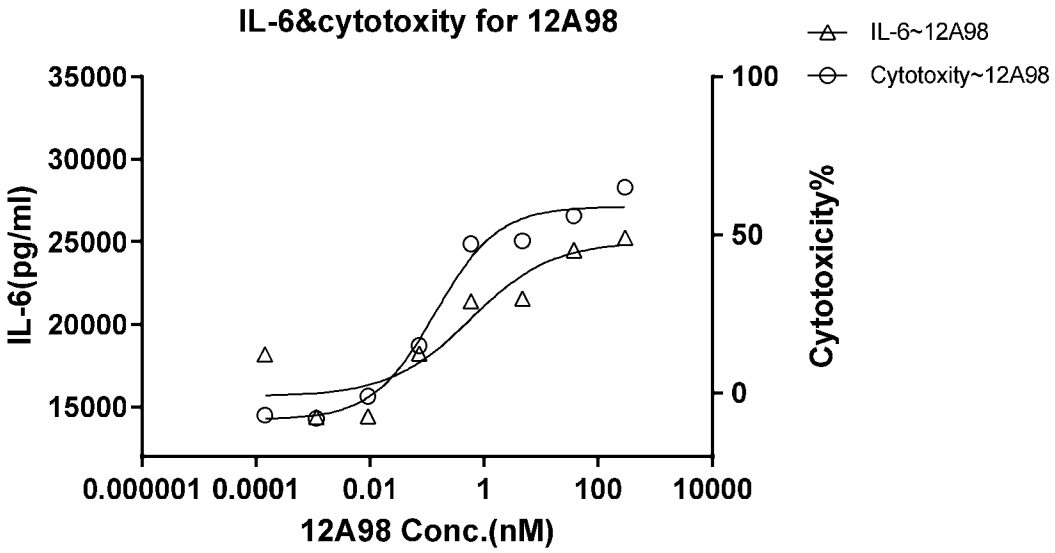
	12A39	12A94	12A98	12A99	12A106	12A107	12A108
EC50	0.02471	0.008466	0.1396	0.1037	0.02667	0.008014	0.04344

FIG 44B



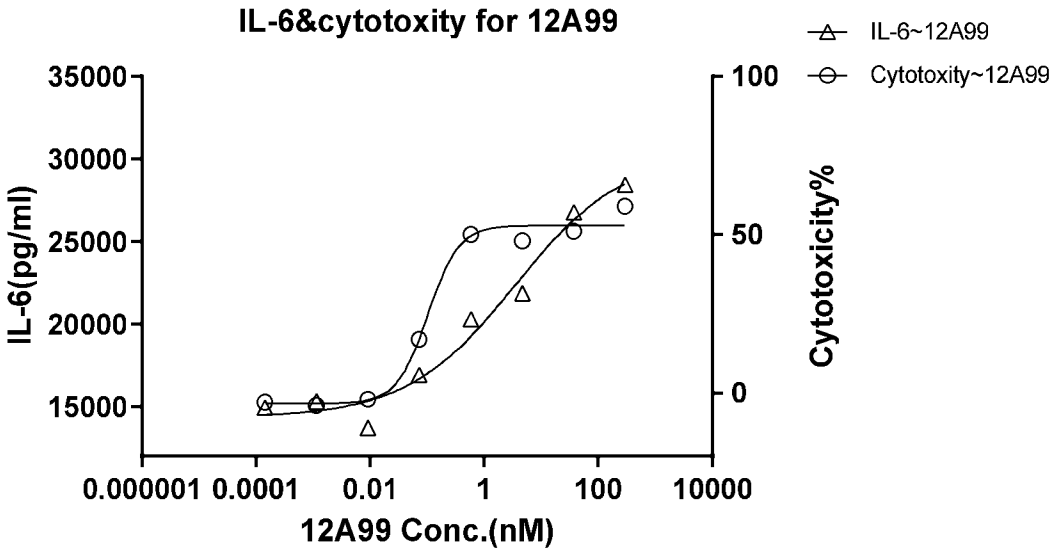
	IL-6~12A94	Cytotoxity~12A94
EC50	~ 0.007415	0.008465

FIG 45A



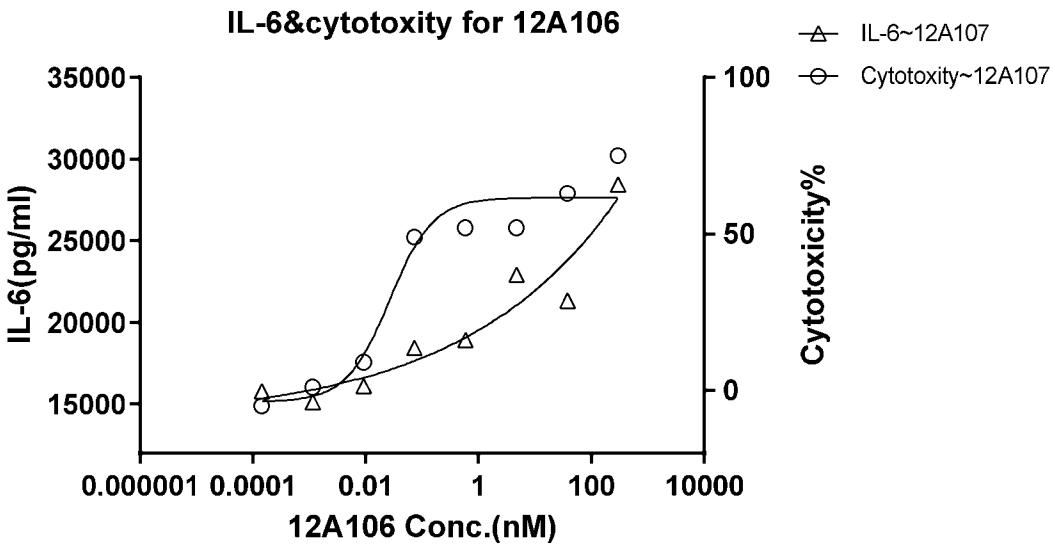
	IL-6~12A98	Cytotoxity~12A98
EC50	0.5810	0.1396

FIG 45B



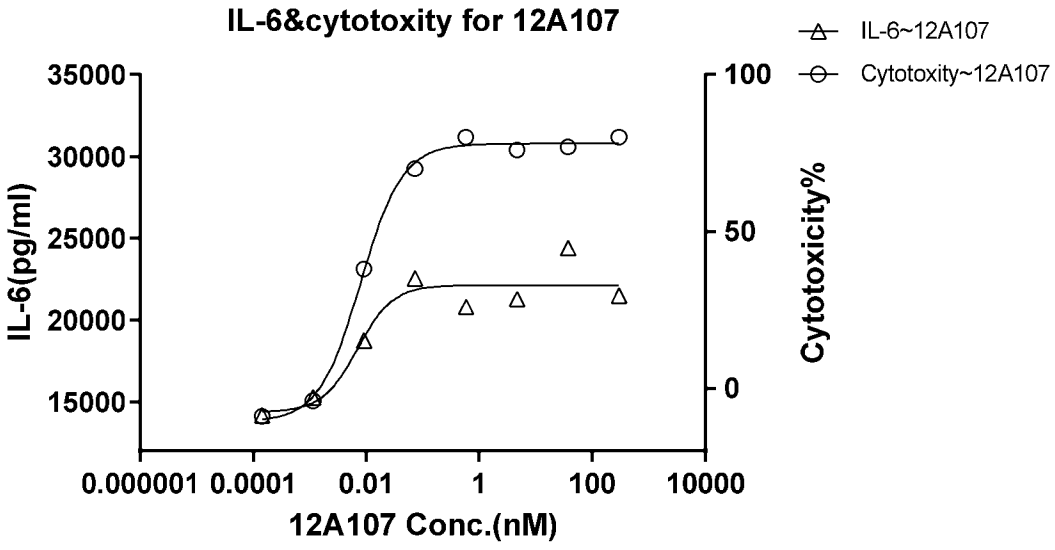
	IL-6~12A99	Cytotoxity~12A99
EC50	3.557	0.1037

FIG 45C



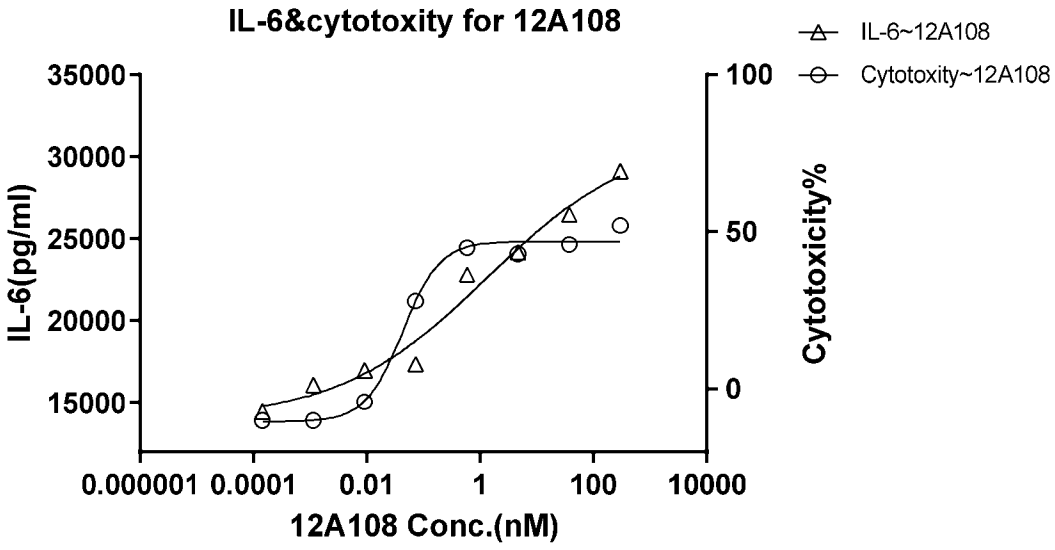
	IL-6~12A107	Cytotoxity~12A107
EC50	~ 2.060e+021	0.02667

FIG 45D



	IL-6~12A107	Cytotoxity~12A107
EC50	0.007115	0.008013

FIG 45E



	IL-6~12A108	Cytotoxity~12A108
EC50	1.363	0.04344

FIG 45F

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

C07K16/46(2006.01)i; A61P35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC:C07K,A61P

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

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

VEN;CNABS;CNKI;BAIDU;CNTXT;ENTXTC;BING;PUBMED;NCBI;EMBL;CHINESE PATENT BIOLOGICAL SEQUENCE SEARCH SYSTEM;STN: antibody, Trispecific, Tri-specific, multi-specific, PDL1, PD-L1, kd value, CD3, Bio-Layer Interferometry, BLI, SPR, affinity, FC, CH3, CH2, dicad, disulfide, FcγRIIA, CD32A, FcγRIIA, TAA, reduced, related sequences

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 112119099 A (CDR-LIFE AG) 22 December 2020 (2020-12-22) claims 28-29, 32, 44, 49, 51-55, 58, 60; description, paragraphs 6-7, 18, 35-36, 40, 144-172, 182	1-2, 8-9, 41-42, 47-48, 53-55, 94-107, 110
Y	CN 112119099 A (CDR-LIFE AG) 22 December 2020 (2020-12-22) claims 28-29, 32, 44, 49, 51-55, 58, 60; description, paragraphs 6-7, 18, 35-36, 40, 144-172, 182	3-12, 15-19, 22-30, 33-113, 119-133, 138-142
X	CN 112566937 A (PFIZER INC.) 26 March 2021 (2021-03-26) description, paragraphs 2, 255-257, 324, 406; table 18	114-118
Y	CN 112566937 A (PFIZER INC.) 26 March 2021 (2021-03-26) description, paragraphs 2, 255-257, 324, 406; table 18	3-12, 15-19, 22-30, 33-113, 119-133, 138-143, 147-148
X	CN 101001873 A (APPLIED MOLECULAR EVOLUTION, INC.) 18 July 2007 (2007-07-18) abstract; claims 1-59; description, paragraphs, pages 4-28, 32-34; table 1	114-115

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2024	**05 November 2024**

Name and mailing address of the ISA/CN	Authorized officer
CHINA NATIONAL INTELLECTUAL PROPERTY ADMINISTRATION **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China**	**CHEN,YunHua** Telephone No. **(+86) 010-53962082**

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN 101001873 A (APPLIED MOLECULAR EVOLUTION, INC.) 18 July 2007 (2007-07-18) abstract; claims 1-59; description, paragraphs, pages 4-28, 32-34; table 1	33-39, 125-130
Y	CN 110546160 A (ORIONIS BIOSCI BV ET AL.) 06 December 2019 (2019-12-06) description, paragraphs 293-299	11-12, 133, 141-142
Y	CN 115151564 A (SEAGEN INC.) 04 October 2022 (2022-10-04) description, example 10	11-12, 15-17, 133, 137-138, 140-142
Y	CN 108250296 A (CHANGCHUN GENESCIENCE PHARM CO., LTD.) 06 July 2018 (2018-07-06) description, paragraphs 37-43, 47; example 1	18-19, 22-24, 133, 139-142
Y	CN 111454357 A (SHANGHAI LINGYUE BIOPHARMA CO., LTD.) 28 July 2020 (2020-07-28) table 3	25-30,125,143,147-148
A	CN 114981306 A (NUMAB THERAPEUTICS AG) 30 August 2022 (2022-08-30) the whole document	1-148
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INTERNATIONAL SEARCH REPORT

International application No.
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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/108255

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

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

1. ☑ Claims Nos.: **100-103, 105-132**
because they relate to subject matter not required to be searched by this Authority, namely:

The subject-matter of claims 100-103, 105-132 relates to a method of treating, preventing or alleviating a disease, and therefore claims 100-103, 105-132 do not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). An international search is still carried out based on the manufacture of medicament.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/108255

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
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				WO	2019166650	A9	06 August 2020
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				JP	7384835	B2	21 November 2023
				PT	3797121	T	23 August 2024
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				PE	20210127	A1	19 January 2021
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				EP	4414034	A3	16 October 2024
				EP	3797121	A1	31 March 2021
				EP	3797121	B1	19 June 2024
				DK	3797121	T3	08 July 2024
				PL	3797121	T3	23 September 2024
				SI	3797121	T1	30 September 2024
				IL	278926	A	31 January 2021
				BR	112020022897	A2	23 February 2021
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International application No.
PCT/CN2024/108255

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International application No.
PCT/CN2024/108255

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				IL	286835	B2	01 March 2024
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Information on patent family members

International application No.
PCT/CN2024/108255

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