(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
09 March 2023 (09.03.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/030258 A1

(51) International Patent Classification:
C07K 16/28 (2006.01) *C12N 15/13* (2006.01)
A61K 39/395 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/CN2022/115536

(22) International Filing Date:
29 August 2022 (29.08.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PCT/CN2021/115621
31 August 2021 (31.08.2021) CN

(71) Applicant: **LANOVA MEDICINES LIMITED** [CN/CN]; 2889 Jinke Road, Building 10, Room 318, Chamtime Plaza, Shanghai 201203 (CN).

(72) Inventors: **LI, Runsheng**; No. 177, Group 6, Rennan Village, Kangqiao Town, Pudong New District, Shanghai 201315 (CN). **HUANG, Wentao**; No. 177, Group 6, Rennan Village, Kangqiao Town, Pudong New District, Shanghai 201315 (CN).

(74) Agent: **SINO-CREATIVITY INTELLECTUAL PROPERTY LAW FIRM**; Room 25A3-1, East Wing, Hanwei Plaza No.7 Guanghua Road, Chaoyang District, Beijing 100020 (CN).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: ANTI-4-1BB NANOBODIES

(57) Abstract: Provided are anti-4-1BB nanobodies and bispecific or multispecific antibodies that incorporate the nanobodies. Methods of using the antibodies for treating and diagnosing diseases such as cancer are also provided.

WO 2023/030258 A1

ANTI-4-1BB NANOBODIES

BACKGROUND

[0001] 4-1BB (CD137, tumor necrosis factor receptor superfamily 9) is a member of TNF-receptor superfamily (TNFRSF) and is a costimulatory molecule which is expressed following the activation of immune cells, both innate and adaptive immune cells. 4-1BB plays an important role in modulating the activity of various immune cells. 4-1BB agonists enhance immune cell proliferation, survival, secretion of cytokines and cytolytic activity CD8 T cells. Many other studies showed that activation of 4-1BB enhances immune response to eliminate tumors in mice. Therefore, it was suggested that 4-1BB is a promising target molecule in cancer immunology.

[0002] A single-domain antibody (sdAb), also known as a nanobody, is an antibody fragment consisting of a single monomeric variable antibody domain. Nanobodies produced from camelids and certain other animals are also referred to as VHH fragments. Like a whole antibody, a nanobody is able to bind selectively to a specific antigen. With a molecular weight of only 12–15 kDa, single domain antibodies are much smaller than common antibodies (150–160 kDa). Single domain antibodies, given their small sizes and one-chain nature, can be particularly suitable for inclusion as a fragment in other proteins, such as chimeric antigen receptors (CAR) and bispecific antibodies.

SUMMARY

[0003] Provided are anti-human 4-1BB nanobodies which are suitable for inclusion in bispecific or trispecific antibodies. Accordingly, in one embodiment of the present disclosure, provided is a single domain antibody or a polypeptide comprising the single domain antibody, wherein the single domain antibody has binding specificity to the human 4-1BB protein and comprises a complementarity determining region 1 (CDR1), a CDR2 and a CDR3, wherein the CDR1, CDR2 and CDR3 comprise, respectively, the amino acid sequences of (a) the amino acid sequences of SEQ ID NO:18, 58 and 32; (b) the amino acid sequences of SEQ ID NO:18, 59 and 38; (c) the amino acid sequences of SEQ ID NO:17, 24 and 31; (d) the amino acid sequences of SEQ ID NO:18, 25 and 32; (e) the amino acid sequences of SEQ ID NO:18, 26 and 33; (f) the amino acid sequences of SEQ ID NO:18, 27 and 34; (g) the amino acid sequences of SEQ ID NO:18, 28 and 35; (h) the amino acid

sequences of SEQ ID NO:19, 28 and 35; (i) the amino acid sequences of SEQ ID NO:20, 28 and 35; (j) the amino acid sequences of SEQ ID NO:19, 28 and 35; (k) the amino acid sequences of SEQ ID NO:21, 29 and 36; (l) the amino acid sequences of SEQ ID NO:22, 29 and 36; (m) the amino acid sequences of SEQ ID NO:21, 29 and 36; (n) the amino acid sequences of SEQ ID NO:21, 29 and 36; (o) the amino acid sequences of SEQ ID NO:19, 26 and 33; (p) the amino acid sequences of SEQ ID NO:18, 28 and 37; (q) the amino acid sequences of SEQ ID NO:23, 30 and 38; or (r) the amino acid sequences of SEQ ID NO:18, 28 and 39.

[0004] In some embodiments, the CDR1 comprises the amino acid sequence of SEQ ID NO:18, the CDR2 comprises the amino acid sequence of SEQ ID NO:58, and the CDR3 comprises the amino acid sequence of SEQ ID NO:32. In some embodiments, the single domain antibody or a polypeptide comprises the amino acid sequence of any one of SEQ ID NO:40-48 and 60-62.

[0005] In some embodiments, the CDR1 comprises the amino acid sequence of SEQ ID NO:18, the CDR2 comprises the amino acid sequence of SEQ ID NO:59, and the CDR3 comprises the amino acid sequence of SEQ ID NO:38. In some embodiments, the single domain antibody or a polypeptide comprises the amino acid sequence of any one of SEQ ID NO:49-57.

[0006] In some embodiments, the single domain antibody or a polypeptide comprises the amino acid sequence of any one of SEQ ID NO:1-16.

[0007] Also provided is a composition comprising the antibody or the polypeptide and a pharmaceutically acceptable carrier. Still also provided is one or more polynucleotide encoding the antibody or the polypeptide, an isolated cell comprising one or more polynucleotide encoding the antibody or fragment thereof.

[0008] Treatment methods and uses are also provided. In one embodiment, a method of treating cancer in a patient in need thereof is provided, comprising administering to the patient an effective amount of the antibody or the polypeptide of the present disclosure. In some embodiments, the cancer is a solid tumor. In some embodiments, the cancer is selected from the group consisting of bladder cancer, liver cancer, colon cancer, rectal cancer, endometrial cancer, leukemia, lymphoma, pancreatic cancer, small cell lung cancer, non-small cell lung cancer, breast cancer, urethral cancer, head and neck cancer, gastrointestinal

cancer, stomach cancer, oesophageal cancer, ovarian cancer, renal cancer, melanoma, prostate cancer and thyroid cancer. In some embodiments, the method further comprises administering to the patient a second cancer therapeutic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIG. 1A-B show that the anti-41BB nanobodies dose-dependently bound to soluble human and cynomolgus 4-1BB.

[0010] FIG. 2 shows that the anti-41BB nanobodies could block 4-1BB ligand binding to 4-1BB in a concentration-dependent manner.

[0011] FIG. 3A-B show that the anti-41BB nanobodies efficiently induced 4-1BB-mediated NFκB activity in the presence of Fc crosslinking.

[0012] FIG. 4A-B show that the anti-41BB humanized nanobodies from parental clone VV02-1LP-263 bound to cell surface human or cynomolgus 4-1BB in a concentration-dependent manner.

[0013] FIG. 5 shows that the anti-41BB humanized nanobodies from parental clone VV02-1SP (1)-73 bound to cell surface human 4-1BB in a concentration-dependent manner.

[0014] FIG.6 shows that the optimized anti-4-1BB sequences maintained the comparable binding activity to 4-1BB-bearing CHO-K1 cells as the parental sequence.

[0015] FIG.7 shows that the optimized anti-4-1BB based bi-specific antibodies had comparable potency in induction of 4-1BB activation in the presence of target cells as the parental antibody TAA-263-1-3.

DETAILED DESCRIPTION

Definitions

[0016] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "an antibody," is understood to represent one or more antibodies. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0017] A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 98 % or 99 %) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Ausubel *et al.* eds. (2007) Current Protocols in Molecular Biology. Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Biologically equivalent polynucleotides are those having the above-noted specified percent homology and encoding a polypeptide having the same or similar biological activity.

[0018] The term "an equivalent nucleic acid or polynucleotide" refers to a nucleic acid having a nucleotide sequence having a certain degree of homology, or sequence identity, with the nucleotide sequence of the nucleic acid or complement thereof. A homolog of a double stranded nucleic acid is intended to include nucleic acids having a nucleotide sequence which has a certain degree of homology with or with the complement thereof. In one aspect, homologs of nucleic acids are capable of hybridizing to the nucleic acid or complement thereof. Likewise, "an equivalent polypeptide" refers to a polypeptide having a certain degree of homology, or sequence identity, with the amino acid sequence of a reference polypeptide. In some aspects, the sequence identity is at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99%. In some aspects, the equivalent polypeptide or polynucleotide has one, two, three, four or five addition, deletion, substitution and their combinations thereof as compared to the reference polypeptide or polynucleotide. In some aspects, the equivalent sequence retains the activity (*e.g.*, epitope-binding) or structure (*e.g.*, salt-bridge) of the reference sequence.

[0019] As used herein, an "antibody" or "antigen-binding polypeptide" refers to a polypeptide or a polypeptide complex that specifically recognizes and binds to an antigen. An antibody can be a whole antibody and any antigen binding fragment or a single chain thereof. Thus the term "antibody" includes any protein or peptide containing molecule that comprises

at least a portion of an immunoglobulin molecule having biological activity of binding to the antigen. Examples of such include, but are not limited to a complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework (FR) region, or any portion thereof, or at least one portion of a binding protein.

[0020] The terms "antibody fragment" or "antigen-binding fragment", as used herein, is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. The term "antibody fragment" includes aptamers, spiegelmers, and diabodies. The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex.

[0021] A "single-chain variable fragment" or "scFv" refers to a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins. In some aspects, the regions are connected with a short linker peptide of ten to about 25 amino acids. The linker can be rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the V_H with the C-terminus of the V_L, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. ScFv molecules are known in the art and are described, *e.g.*, in US patent 5,892,019.

[0022] The term antibody encompasses various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon (γ, μ, α, δ, ε) with some subclasses among them (*e.g.*, γ l- γ4). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) *e.g.*, IgG_1, IgG_2, IgG_3, IgG_4, IgG_5, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant disclosure. All immunoglobulin classes are clearly within the scope of the present disclosure, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000. The

four chains are typically joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

[0023] Antibodies, antigen-binding polypeptides, variants, or derivatives thereof of the disclosure include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, *e.g.*, Fab, Fab' and $F(ab')_2$, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VK or VH domain, fragments produced by a Fab expression library, and anti- idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to LIGHT antibodies disclosed herein). Immunoglobulin or antibody molecules of the disclosure can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgGl, IgG2, IgG3, IgG4, IgAl and IgA2) or subclass of immunoglobulin molecule.

[0024] By "specifically binds" or "has specificity to," it is generally meant that an antibody binds to an epitope via its antigen-binding domain, and that the binding entails some complementarity between the antigen-binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen-binding domain more readily than it would bind to a random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

[0025] As used herein, the terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the progression of cancer. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone

to have the condition or disorder or those in which the condition or disorder is to be prevented.

[0026] By "subject" or "individual" or "animal" or "patient" or "mammal," is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sport, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, cows, and so on.

[0027] As used herein, phrases such as "to a patient in need of treatment" or "a subject in need of treatment" includes subjects, such as mammalian subjects, that would benefit from administration of an antibody or composition of the present disclosure used, *e.g.*, for detection, for a diagnostic procedure and/or for treatment.

Anti-4-1BB Nanobodies

[0028] The present disclosure provides nanobodies, including humanized ones, against the human 4-1BB protein. These antibodies have high binding affinity to 4-1BB and can effectively block the interaction between 4-1BB and its ligand. As demonstrated in **Example 4**, these antibodies exhibited potent ability to induce 4-1BB-mediated NF-κB activity only in the presence of Fc crosslinking. Therefore, these antibodies are non-agonist antibodies which do not activate 4-1BB signaling on their own. When combined with a second antibody, however, the ensuing bispecific antibody can activate 4-1BB signaling in the presence of the target antigen of the second antibody. In other words, the present nanobodies are particularly suitable for development into bispecific or multi-specific antibodies.

[0029] Accordingly, in one embodiment of the present disclosure, provided is a single domain antibody or a polypeptide comprising the single domain antibody, wherein the single domain antibody includes a CDR1, a CDR2 and a CDR3, which respectively have the CDR1, CDR2 and CDR3 sequences of any one of the antibodies in **Table 1**.

[0030] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-317 (SEQ ID NO:1). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:17, 24 and 31, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:1.

[0031] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-263 (SEQ ID NO:2). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 25 and 32, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:2.

[0032] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-355 (SEQ ID NO:3). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 26 and 33, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:3.

[0033] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-3LP-66 (SEQ ID NO:4). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 27 and 34, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:4.

[0034] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-265 (SEQ ID NO:5). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 28 and 35, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:5.

[0035] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-2LP-13 (SEQ ID NO:6). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:19, 28 and 35, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:6.

[0036] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-3LP-142 (SEQ ID NO:7). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:20, 28 and 35, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:7.

[0037] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-3LP-83 (SEQ ID NO:8). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:19, 28 and 35, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:8.

[0038] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-417 (SEQ ID NO:9). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:21, 29 and 36, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:9.

[0039] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-422 (SEQ ID NO:10). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:22, 29 and 36, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:10.

[0040] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-238 (SEQ ID NO:11). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:21, 29 and 36, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:11.

[0041] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1LP-288 (SEQ ID NO:12). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:21, 29 and 36, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:12.

[0042] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1SP(1)-168 (SEQ ID NO:13). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:19, 26 and 33, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:13.

[0043] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1SP (1)-55 (SEQ ID NO:14). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 28 and 37, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:14.

[0044] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1SP (1)-73 (SEQ ID NO:15). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:23, 30 and 38, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:15.

[0045] In some embodiments, the CDR1, CDR2, and CDR3 are those of antibody VV02-1SP (1)-358 (SEQ ID NO:16). In some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 28 and 39, respectively. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:16.

[0046] Humanized antibodies are also provided, such as those provided in SEQ ID NO:40-48 and 60-62 for antibody VV02-1LP-263 and SEQ ID NO:49-57 for antibody VV02-1SP (1)-73. For VV02-1LP-263 (CDR1 in SEQ ID NO:18, CDR2 in SEQ ID NO:25 and CDR3 in SEQ ID NO:32), the CDR2 can incorporate either or both of the mutations (D54G and D61E, Kabat numbering). Accordingly, in some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 58 and 32, respectively. In some embodiments, the antibody includes any one of SEQ ID NO:40-48 and 60-62. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to any one of SEQ ID NO:40-48 or 60-62.

[0047] Among these humanized sequences, 263 huNb-1-3_1 (SEQ ID NO:60), 263 huNb-1-3_2 (SEQ ID NO:61), and 263 hnNb-1-3_3 (SEQ ID NO:62) were further optimized ones, which were demonstrated (**Example 7** and **FIG. 6-7**) to be suitable for further clinical development. In some embodiments, the antibody includes SEQ ID NO:60. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:60. In some embodiments, the antibody includes SEQ ID NO:61. In some embodiments, the antibody

includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:61. In some embodiments, the antibody includes SEQ ID NO:62. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO:62.

[0048] For VV02-1SP (1)-73 (CDR1 in SEQ ID NO:23, CDR2 in SEQ ID NO:30 and CDR3 in SEQ ID NO:38), a mutation (N31S, Kabat numbering) or either of two mutations (D54S and D61E, Kabat numbering) can be incorporated to the CDR1 and CDR2, respectively. Accordingly, in some embodiments, the CDR1, CDR2 and CDR3 include the amino acid sequences of SEQ ID NO:18, 59 and 38, respectively. In some embodiments, the antibody includes any one of SEQ ID NO:49-57. In some embodiments, the antibody includes the recited CDR1, CDR2 and CDR3 and has at least 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to any one of SEQ ID NO:49-57.

[0049] Also provided, in some embodiments, is an nanobody or polypeptide that includes the nanobody, which includes a CDR1 having an amino acid sequence selected from SEQ ID NO:17-23, a CDR2 having an amino acid sequence selected from SEQ ID NO:24-30 or 58-59, and a CDR3 having an amino acid sequence selected from SEQ ID NO:31-39.

[0050] Also provided, in some embodiments, is an nanobody or polypeptide that includes the nanobody, which includes a CDR1 having an amino acid sequence selected from SEQ ID NO:21-22, a CDR2 having the amino acid sequence of SEQ ID NO:29, and a CDR3 having the amino acid sequence of SEQ ID NO:36.

[0051] Also provided, in some embodiments, are anti-4-1BB antibodies and antigen binding fragments that compete with any of the antibodies disclosed herein in binding to human 4-1BB. Also provided, in some embodiments, are anti-4-1BB antibodies and antigen binding fragments that bind to the same epitope as any of the antibodies disclosed herein. Also provided, in some embodiments, are anti-4-1BB antibodies and antigen binding fragments that included the CDR1, CDR2, and CDR3 of the antibodies disclosed herein.

[0052] Also provided are compositions that include the antibody or the polypeptide and a pharmaceutically acceptable carrier.

[0053] It will also be understood by one of ordinary skill in the art that antibodies as disclosed herein may be modified such that they vary in amino acid sequence from the

naturally occurring binding polypeptide from which they were derived. For example, a polypeptide or amino acid sequence derived from a designated protein may be similar, *e.g.*, have a certain percent identity to the starting sequence, *e.g.*, it may be 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% identical to the starting sequence. In some embodiments, the modified antibody or fragment retains the designate CDR sequences.

[0054] In certain embodiments, the antibody comprises an amino acid sequence or one or more moieties not normally associated with an antibody. Exemplary modifications are described in more detail below. For example, an antibody of the disclosure may comprise a flexible linker sequence, or may be modified to add a functional moiety (*e.g.*, PEG, a drug, a toxin, or a label).

[0055] Also provided are bispecific and multispecific antibodies that includes one, two, three or four units of the single domain anti-4-1BB antibody as disclosed herein, and one or more other specificities (not 4-1BB).

Bispecific and Multispecific Antibodies, and Chimeric Antigen Receptors (CAR)

[0056] As provided, the anti-4-1BB antibodies disclosed here are particularly useful for preparing bispecific and multispecific antibodies, as well as chimeric antigen receptors (CAR). This is at least because of these antibodies' enhanced therapeutic index and their small sizes.

[0057] Accordingly, in one embodiment, provided is a bispecific antibody that includes an anti-4-1BB nanobody of the present disclosure, or an antigen-binding fragment thereof, and a second antibody or antigen-binding fragment having binding specificity to a target antigen that is not 4-1BB. In some embodiment, a third or fourth specificity is further included.

[0058] The target antigen that is not 4-1BB, in some embodiments, is a tumor antigen. An abundance of tumor antigens are known in the art and new tumor antigens can be readily identified by screening. Non-limiting examples of tumor antigens include ABL, ALK, B4GALNT1 , BAFF, BCL2, BRAF, BTK, CD19, CD20, CD30, CD38, CD52, CD73, Claudin 18.2, CTLA-4, EGFR, FOLR1, FLT3, HDAC, HER2, IDH2, IL-1β, IL-6, IL-6R, JAK1/2, JAK3, KIT, LAG-3, MEK, Nectin 4, ROR1, mTOR, PARP, PD-1, PDGFR, PDGFRα, PD-L1, PI3Kδ, PIGF, PTCH, RAF, RANKL, Smoothened, VEGF, VEGFR, and VEGFR2. Other examples are Her2, EpCAM, CD33, CD47, CD133, CEA, gpA33, Mucins,

TAG-72, CIX, PSMA, GD2, GD3, GM2, Integrin, αVβ3, α5β1, ERBB2, ERBB3, MET, IGF1R, EPHA3, TRAILR1, TRAILR2, RANKL, FAP and Tenascin.

[0059] Also provided, are chimeric antigen receptor (CAR) that includes a nanobody of the present disclosure. In the CAR, the nanobody can serve as the antigen recognition domain. In addition, in some embodiments, the CAR also includes an extracellular hinge region, a transmembrane domain, and an intracellular T-cell signaling domain.

[0060] The hinge, also called a spacer, is a small structural domain that sits between the antigen recognition region and the cell's outer membrane. A suitable hinge enhances the flexibility of the scFv receptor head, reducing the spatial constraints between the CAR and its target antigen. Example hinge sequences are based on membrane-proximal regions from immune molecules such as IgG, CD8, and CD28.

[0061] The transmembrane domain is a structural component, consisting of a hydrophobic alpha helix that spans the cell membrane. It anchors the CAR to the plasma membrane, bridging the extracellular hinge and antigen recognition domains with the intracellular signaling region. Typically, the transmembrane domain from a membrane-proximal component of the endodomain can be used, such as the CD28 transmembrane domain.

[0062] The intracellular T-cell signaling domain lies in the receptor's endodomain, inside the cell. After an antigen is bound to the external antigen recognition domain, CAR receptors cluster together and transmit an activation signal. Then the internal cytoplasmic end of the receptor perpetuates signaling inside the T cell. To mimic this process, CD3-zeta's cytoplasmic domain is commonly used as the main CAR endodomain component.

[0063] T cells also require co-stimulatory molecules in addition to CD3 signaling in order to persist after activation. In some embodiments, the endodomains of CAR receptor also includes one or more chimeric domains from co-stimulatory proteins, such as CD28, CD27, CD134 (OX40), and CD137 (4-1BB).

Polynucleotides Encoding the Antibodies and Methods of Preparing the Antibodies

[0064] The present disclosure also provides isolated polynucleotides or nucleic acid molecules encoding the antibodies, variants or derivatives thereof of the disclosure. The polynucleotides of the present disclosure may encode the entire heavy and light chain

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

[0065] Methods of making antibodies are well known in the art and described herein. In certain embodiments, both the variable and constant regions of the antigen-binding polypeptides of the present disclosure are fully human. Fully human antibodies can be made using techniques described in the art and as described herein. For example, fully human antibodies against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Exemplary techniques that can be used to make such antibodies are described in U.S. patents: 6,150,584; 6,458,592; 6,420,140 which are incorporated by reference in their entireties.

Cancer Treatment

[0066] As described herein, the antibodies, bispecific antibodies, polypeptides, variants or derivatives of the present disclosure may be used in certain treatment and diagnostic methods.

[0067] The present disclosure is further directed to antibody-based therapies which involve administering the antibodies of the disclosure to a patient such as an animal, a mammal, and a human for treating one or more of the disorders or conditions described herein. Therapeutic compounds of the disclosure include, but are not limited to, antibodies of the disclosure (including variants and derivatives thereof as described herein) and nucleic acids or polynucleotides encoding antibodies of the disclosure (including variants and derivatives thereof as described herein).

[0068] In some embodiments, provided are methods for treating a cancer in a patient in need thereof. The method, in one embodiment, entails administering to the patient an effective amount of an antibody of the present disclosure. In some embodiments, at least one of the cancer cells (e.g., stromal cells) in the patient over-express a tumor antigen.

[0069] Cellular therapies, such as chimeric antigen receptor (CAR) T-cell or NK cell therapies, are also provided in the present disclosure. A suitable cell can be used, that is put in

contact with an antibody or CAR of the present disclosure (or alternatively engineered to express an antibody or CAR of the present disclosure). Upon such contact or engineering, the cell can then be introduced to a cancer patient in need of a treatment. The cancer patient may have a cancer of any of the types as disclosed herein. The cell (e.g., T cell or NK cell) can be, for instance, a tumor-infiltrating T lymphocyte, a CD4+ T cell, a CD8+ T cell, or the combination thereof, without limitation.

[0070] In some embodiments, the cell was isolated from the cancer patient him- or her-self. In some embodiments, the cell was provided by a donor or from a cell bank. When the cell is isolated from the cancer patient, undesired immune reactions can be minimized.

[0071] Additional diseases or conditions associated with increased cell survival, that may be treated, prevented, diagnosed and/or prognosed with the antibodies or variants, or derivatives thereof of the disclosure include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (*e.g.*, acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (*e.g.*, chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (*e.g.*, Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyo sarcoma, colon carcinoma, pancreatic cancer, breast cancer, thyroid cancer, endometrial cancer, melanoma, prostate cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma and retinoblastoma.

Diagnostic Methods

[0072] Over-expression of 4-1BB is observed in certain tumor samples, and patients having 4-1BB-over-expressing cells are likely responsive to treatments with the anti-4-1BB antibodies of the present disclosure. Accordingly, the antibodies of the present disclosure can also be used for diagnostic and prognostic purposes.

[0073] A sample that preferably includes a cell can be obtained from a patient, which can be a cancer patient or a patient desiring diagnosis. The cell be a cell of a tumor tissue or a tumor block, a blood sample, a urine sample or any sample from the patient. Upon optional pre-treatment of the sample, the sample can be incubated with an antibody of the present disclosure under conditions allowing the antibody to interact with a 4-1BB protein potentially present in the sample. Methods such as ELISA can be used, taking advantage of the anti-4-1BB antibody, to detect the presence of the 4-1BB protein in the sample.

[0074] Presence of the 4-1BB protein in the sample (optionally with the amount or concentration) can be used for diagnosis of cancer, as an indication that the patient is suitable for a treatment with the antibody, or as an indication that the patient has (or has not) responded to a cancer treatment. For a prognostic method, the detection can be done at once, twice or more, at certain stages, upon initiation of a cancer treatment to indicate the progress of the treatment.

Compositions

[0075] The present disclosure also provides pharmaceutical compositions. Such compositions comprise an effective amount of an antibody, and an acceptable carrier. In some embodiments, the composition further includes a second anticancer agent (e.g., an immune checkpoint inhibitor).

[0076] In a specific embodiment, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Further, a “pharmaceutically acceptable carrier” will generally be a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

[0077] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents such as acetates, citrates or phosphates. Antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; and agents for the adjustment of tonicity such as sodium chloride or dextrose are also envisioned. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin, incorporated herein by reference. Such compositions will contain a therapeutically effective amount of the antigen-binding polypeptide, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0078] In an embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically

sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

EXAMPLES

Example 1: Generation of Nanobodies against Human 4-1BB

[0079] This example describes the generations of nanobodies against the human 4-1BB protein.

[0080] Llamas were immunized with recombinant ECD of human 4-1BB fused to a human immunoglobulin Fc domain. Llamas whose sera contained sufficient titers of anti-4-1BB antibody were selected for generation of phage libraries. Briefly, lymphocytes were isolated from peripheral blood collected from immunized llamas. The lymphocyte RNA was extracted and cDNA encoding VHH domains was amplified by PCR and used for the construction of M13 phage-display-based nanobody libraries. Several rounds of panning were applied to screen the phage libraries expressing anti-4-1BB nanobodies.

[0081] All the positive clones were screened through ELISA and FACS assays prior to sequencing. Based on the sequence diversity, 16 unique clones were selected, the sequences of which are shown in **Table. 1**. The anti-4-1BB nanobodies fused with human IgG1 Fc fragment with N297A mutation at the C-terminus were characterized for their specificity and activity through a series of functional assays including binding, ligand competition and 4-1BB signal activation which resulted in the identification of lead nanobodies for further humanization.

Table 1. Sequences of the selected 16 unique clones.

Antibody	Sequence	SEQ ID NO:
VV02-1LP-317	EVDLVESGGGLVQPGGSLRLSCAASGFTFS**RSAMS**WARQAPGKGFEWVS**G IYSGGSTYYVDSVEG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAT**WGS QQIGVWHEDDY**WGQGTQVTVSS	1

VV02-1LP-263	EVQLVESGGGLVQPGGSLRLSCAASGFTFS**SSAMS**WARQTPGKGFEWVS**G** **IYSDGSTYYTDSVKD**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAT**WGT** **LRFGVWAEYDH**WGQGTQVTVSS	2
VV02-1LP-355	QVQLVESGGGLVQPGGSLRLSCAASGFTFS**SSAMS**WARQAPGKDFEWVS**Y** **IYSDGNTYYADSVKG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAT**WHT** **LRVGVWDEYDY**WGQGTQVTVSS	3
VV02-3LP-66	QLQLVESGGGLVQPGGSLRLSCAASGFTFS**SSAMS**WARQAPGKEFEWVS**Y** **IYSDGNTYYTDSVKG**RFTVSRDNAKNTVYLQMNSLKPEDTAVYYCAT**WNS** **LQVGVWDEYDY**WGQGTQVTVSS	4
VV02-1LP-265	EVQLVESGGGLVQPGGSLRLSCAASGFTFS**SSAMS**WARQVPGKGFEWVA**Y** **IYSDGSTYYADSVKG**RFTISRDNAKDTVYLHMNSLKFEDMAVYYCAT**WRS** **QQVGRWDEYDH**WGQGTQVTVSS	5
VV02-2LP-13	AVQLVESGGGLVQPGGSLRLSCAASGFTFS**SYAMS**WARQVPGKGFEWVA**Y** **IYSDGSTYYADSVKG**RFTISRDNAKDTVYLHMNSLKFEDMAVYYCAT**WRS** **QQVGRWDEYDH**WGQGTQVTVSS	6
VV02-3LP-142	AVQLVESGGGLVQRGGSLKLSCVGSGFDFS**DHAMS**WARQVPGKGFEWVA**Y** **IYSDGSTYYADSVKG**RFTISRDNAKDTVYLHMNSLKFEDMAVYYCAT**WRS** **QQVGRWDEYDH**WGQGTQVTVSS	7
VV02-3LP-83	EVDLVESGGGLVQPGGSLRLSCAASGFTFR**SYAMS**WARQVPGKGFEWVA**Y** **IYSDGSTYYADSVKG**RFTISRDNAKDTVYLHMNSLKFEDMAVYYCAT**WRS** **QQVGRWDEYDH**WGQGTQVTVSS	8
VV02-1LP-417	QLQLVESGGGLVQPGGSLRLSCAASGFALD**YSAIG**WFRQAPGKEREGVL**C** **ISSSGDVTIYADSVKG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA**PR** **ICSTYSSDDY**WGQGTQVTVSS	9
VV02-1LP-422	QLQLVESGGGLVQPGGSLRLSCAASGFTLA**DYAIG**WFRQAPGKEREGVL**C** **ISSSGDVTIYADSVKG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA**PR** **ICSTYSSDDY**WGQGTQVTVSS	10
VV02-1LP-238	QVQLVESGGGLVQAGGALRLSCAASGFTLD**YSAIG**WFRQAPGKEREGVL**C** **ISSSGDVTIYADSVKG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA**PR** **ICSTYSSDDY**WGQGTQVTVSS	11
VV02-1LP-288	EVQVVESGGGLVQPGGSLRLSCAASGSSLD**YSAIG**WFRQAPGKEREGVL**C** **ISSSGDVTIYADSVKG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA**PR** **ICSTYSSDDY**WGQGTQVTVSS	12
VV02-1SP (1)-168	QVQLVESGGGLVQPGGSLRLSCAASGFTFS**SYAMS**WARQAPGKDFEWVS**Y** **IYSDGNTYYADSVKG**RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAT**WHT** **LRVGVWDEYDY**WGQGTQVTVSS	13
VV02-1SP (1)-55	EVQLVESGGGLVQPGGSLRLSCAASGFTFS**SSAMS**WARQVPGKGFEWVA**Y** **IYSDGSTYYADSVKG**RFTISRDNAKDTVYLHMNSLKFEDMAVYYCAT**WRS** **QQVGRWDEYDY**WGQGIQVTVSS	14
VV02-1SP (1)-73	EVDLVESGGGLVQPGGSLRLSCAVSGFTFS**NSAMS**WARQAPGKEFEWVS**S** **IYSDGKTYYVDSVKG**RFTISRDNAKNTVYLQMSSLKPEDTAVYYCAT**WKT** **LRVGVWDESDY**WGQGTQVTVSS	15
VV02-1SP (1)-358	EVQLVESGGGLVQPGGSLMISCAASGFTFS**SSAMS**WARQVPGKGFEWVA**Y** **IYSDGSTYYADSVKG**RFTISRDNAKDTVYLHMNSLKFEDMAVYYCAT**WRS** **QQVGRWDKYDY**WGQGTQVTVSS	16

[0082] Their CDR sequences are summarized in **Table 1A** below.

Table 1A. Sequences of the selected 16 unique clones.

Antibody	CDR1	SEQ ID NO:	CDR2	SEQ ID NO:	CDR3	SEQ ID NO:
VV02-1LP-317	RSAMS	17	GIYSGGSTYYVDSVEG	24	WGSQQIGVWHEDDY	31
VV02-1LP-263	SSAMS	18	GIYSDGSTYYTDSVKD	25	WGTLRFGVWAEYDH	32
VV02-1LP-355	SSAMS	18	YIYSDGNTYYADSVKG	26	WHTLRVGVWDEYDY	33
VV02-3LP-66	SSAMS	18	YIYSDGNTYYTDSVKG	27	WNSLQVGVWDEYDY	34
VV02-1LP-265	SSAMS	18	YIYSDGSTYYADSVKG	28	WRSQQVGRWDEYDH	35
VV02-2LP-13	SYAMS	19	YIYSDGSTYYADSVKG	28	WRSQQVGRWDEYDH	35
VV02-3LP-142	DHAMS	20	YIYSDGSTYYADSVKG	28	WRSQQVGRWDEYDH	35
VV02-3LP-83	SYAMS	19	YIYSDGSTYYADSVKG	28	WRSQQVGRWDEYDH	35
VV02-1LP-417	YSAIG	21	CISSSGDVTIYADSVKG	29	PRICSTYSSDDY	36
VV02-1LP-422	DYAIG	22	CISSSGDVTIYADSVKG	29	PRICSTYSSDDY	36
VV02-1LP-238	YSAIG	21	CISSSGDVTIYADSVKG	29	PRICSTYSSDDY	36
VV02-1LP-288	YSAIG	21	CISSSGDVTIYADSVKG	29	PRICSTYSSDDY	36
VV02-1SP (1)-168	SYAMS	19	YIYSDGNTYYADSVKG	26	WHTLRVGVWDEYDY	33
VV02-1SP (1)-55	SSAMS	18	YIYSDGSTYYADSVKG	28	WRSQQVGRWDEYDY	37
VV02-1SP (1)-73	NSAMS	23	SIYSDGKTYYVDSVKG	30	WKTLRVGVWDESDY	38
VV02-1SP (1)-358	SSAMS	18	YIYSDGSTYYADSVKG	28	WRSQQVGRWDKYDY	39

[0083] Antibodies VV02-1LP-417, VV02-1LP-422, VV02-1LP-238, and VV02-1LP-288 appear to share very similar CDRs, while the remaining ones also have homologous CDRs.

Example 2. Soluble 4-1BB binding properties of anti-4-1BB nanobodies

[0084] This example tested the binding properties of the resulting anti-4-1BB nanobodies to human and cynomolgus 4-1BB proteins by ELISA assay.

[0085] His-tagged 4-1BB was coated at 2 μg/ml overnight and then blocked by 2% BSA in PBS. Serially diluted anti-4-1BB nanobodies were incubated with the coated antigen for 1h at room temperature with a reference antibody "Ref mAb". The resulting plates were washed with PBS/T and incubated with goat anti-human IgG-HRP for 1h at room temperature. The

plates were developed with TMB substrate and analyzed by spectrophotometer at OD 450nm. The results of the ELISA assays are showed in **FIG.1** and **Table. 2**, which shows EC_{50} of binding to human and cynomolgus 4-1BB protein.

Table 2. Binding EC_{50} (nM) on human and Cyno 4-1BB protein.

Antibody	hu4-1BB EC_{50}(nM)	Cyno 4-1BB EC_{50} (nM)	Antibody	hu4-1BB EC_{50} (nM)	Cyno 4-1BB EC_{50} (nM)
VV02-1LP-317	>30	29.32	VV02-1LP-422	0.37	6.94
VV02-1LP-263	>30	0.04	VV02-1LP-238	9.12	26.46
VV02-1LP-355	23.97	0.03	VV02-1LP-288	>30	>30
VV02-3LP-66	>30	1.95	VV02-1SP (1)-168	>30	0.06
VV02-1LP-265	>30	3.86	VV02-1SP (1)-55	>30	1.87
VV02-2LP-13	>30	4.19	VV02-1SP (1)-73	>30	0.11
VV02-3LP-142	>30	18.24	VV02-1SP (1)-358	>30	0.11
VV02-3LP-83	>30	2.03	Ref mAb	18.77	0.51
VV02-1LP-417	11.11	>30			

Example 3. Blocking 4-1BB/4-1BB ligand interaction by anti-41BB nanobodies

[0086] In this example, the anti-41BB nanobodies were examined for their ability to block the binding of 4-1BB to its ligand.

[0087] CHO-K1 cells overexpressing human 4-1BB were incubated with biotinylated human 4-1BB ligand (0.3 μg/ml) in the presence of serial diluted anti-41BB nanobodies for 1h at 4°C with Ref mAb as the reference antibody. Then, cells were incubated with Alexa Fluor 633-conjugated streptavidin. Binding was measured with an Agilent flow cytometer. As shown in **FIG. 2** and **Table 3,** all of the tested nanobodies could block 4-1BB ligand binding to 4-1BB in a concentration-dependent manner while Ref mAb had no such effect.

Table 3. Competition IC_{50} (nM) on 4-1BB/4-1BB ligand interaction

Antibody	IC_{50} (nM)	Antibody	IC_{50} (nM)
VV02-1LP-317	1.416	VV02-1LP-422	1.484
VV02-1LP-263	0.566	VV02-1LP-238	1.876
VV02-1LP-355	0.4346	VV02-1LP-288	2.573
VV02-3LP-66	0.5733	VV02-1SP (1)-168	0.3873
VV02-1LP-265	0.6417	VV02-1SP (1)-55	0.5729
VV02-2LP-13	0.9945	VV02-1SP (1)-73	0.5725
VV02-3LP-142	0.9233	VV02-1SP (1)-358	0.9879
VV02-3LP-83	0.6824	Ref mAb	2.657
VV02-1LP-417	2.053		

Example 4. Functional property of anti-41BB nanobodies in a Jurkat-41BB NFκB reporter assay

[0088] This example evaluated the functional property of the anti-41BB nanobodies in a 4-1BB reporter assay.

[0089] In this assay, the effector cells were 4-1BB NFκB-reporter Jurkat cell line which stably expresses human 4-1BB and has an NFκB luciferase reporter construct integrated into the genome. Following 4-1BB activation, endogenous NFκB transcription factors bind to the DNA response elements to induce transcription of the luciferase gene, whose protein product is then quantified by measuring the luminescence signal. 4-1BB NFκB-reporter Jurkat cell line was cocultured with target cell line CHO-K1 or FcγRIIB-CHO-K1 cell line which overexpressed human Fcγ receptor II B. Antibodies were serially diluted and added to a white 96-well assay plate. After 16 hours of incubation at 37 °C, luminescence was obtained by adding the substrate of luciferase and measured by a microplate reader.

[0090] As shown in **FIG. 3** and **Table 4**, the tested 4-1BB nanobodies induced 4-1BB-mediated NF-κB activity in the presence of Fc crosslinking with an EC_{50} range from 0.002 nM to 0.081 nM. However, when Fc crosslinking was absent, the tested antibodies were not able to activate NF-κB signal.

Table 4. EC_{50} (nM) in Jurkat-41BB NFκB reporter assay

Antibody	EC_{50}(nM)	Antibody	EC_{50}(nM)
VV02-1LP-317	0.031	VV02-1LP-422	0.013
VV02-1LP-263	0.017	VV02-1LP-238	0.008
VV02-1LP-355	0.016	VV02-1LP-288	0.052
VV02-3LP-66	0.035	VV02-1SP (1)-168	0.002
VV02-1LP-265	0.024	VV02-1SP (1)-55	0.023
VV02-2LP-13	0.036	VV02-1SP (1)-73	0.010
VV02-3LP-142	0.014	VV02-1SP (1)-358	0.004
VV02-3LP-83	0.014	Ref mAb	0.080
VV02-1LP-417	0.081		

Example 5. Anti-41BB nanobody humanization

[0091] Humanization of lead nanobodies (VV02-1LP-263 and VV02-1SP (1)-73) was conducted by CDR grafting and back-mutation strategy. The humanized sequences of which are shown in **Table. 5.**

Table 5. Sequences of humanized lead nanobodies.

Antibody	Sequence	SEQ ID NO:
VV02-1LP-263 huNb_1_1	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGLEWVSG IYS**G**GSTYYT**E**SVKDRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKWGT LRFGVWAEYDHWGQGTLVTVSS	40
VV02-1LP-263 huNb_1_2	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGLEWVSG IYSGGSTYYTESVKDRFTISRDNSKNTVYLQMNSLKPEDTAVYYCATWGT LRFGVWAEYDHWGQGTLVTVSS	41
VV02-1LP-263 huNb_1_3	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQTPGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNSKNTVYLQMNSLKPEDTAVYYCATWGT LRFGVWAEYDHWGQGTQVTVSS	42
VV02-1LP-263 huNb_2_1	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKWGT LRFGVWAEYDHWGQGTLVTVSS	43
VV02-1LP-263 huNb_2_2	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGLEWVSG IYSGGSTYYTESVKDRFTISRDNAKNTLYLQMNSLRPEDTAVYYCATWGT LRFGVWAEYDHWGQGTLVTVSS	44
VV02-1LP-263 huNb_2_3	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNAKNTVYLQMNSLKPEDTAVYYCATWGT LRFGVWAEYDHWGQGTLVTVSS	45
VV02-1LP-263 huNb_3_1	QVQLVESGGGLVKPGGSLRLSCAASGFTFSSSAMSWARQAPGKGLEWVSG IYSGGSTYYTESVKDRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARWGT LRFGVWAEYDHWGQGTLVTVSS	46
VV02-1LP-263 huNb_3_2	QVQLVESGGGLVKPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNAKNSLYLQMNSLRPEDTAVYYCATWGT LRFGVWAEYDHWGQGTQVTVSS	47
VV02-1LP-263 huNb_3_3	QVQLVESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNAKNSVYLQMNSLKPEDTAVYYCATWGT LRFGVWAEYDHWGQGTQVTVSS	48
VV02-1SP (1)-73 huNb_1_1	EVQLLESGGGLVQPGGSLRLSCAASGFTFS**S**SAMSWARQAPGKGLEWVSS IYS**S**GKTYYV**E**SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKWKT LRVGVWDESDYWGQGTLVTVSS	49
VV02-1SP (1)-73 huNb_1_2	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKELEWVSS IYSSGKTYYVESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCATWKT LRVGVWDESDYWGQGTLVTVSS	50
VV02-1SP (1)-73 huNb_1_3	EVDLVESGGGLVQPGGSLRLSCAVSGFTFSSSAMSWARQAPGKEFEWVSS IYSSGKTYYVESVKGRFTISRDNAKNTVYLQMSSLRPEDTAVYYCATWKT LRVGVWDESDYWGQGTLVTVSS	51
VV02-1SP (1)-73 huNb_2_1	EVQLVESGGGLIQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVSS IYSSGKTYYVESVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARWKT LRVGVWDESDYWGQGTLVTVSS	52

VV02-1SP (1)-73 huNb_2_2	EVQLVESGGGLIQPGGSLRLSCAASGFTFSSSAMSWARQAPGKEFEWVSS IYSSGKTYYVESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCATWKT LRVGVWDESDYWGQGTQVTVSS	53
VV02-1SP (1)-73 huNb_2_3	EVQLVESGGGLVQPGGSLRLSCAVSGFTFSSSAMSWARQAPGKEFEWVSS IYSSGKTYYVESVKGRFTISRDNAKNTVYLQMSSLRPEDTAVYYCATWKT LRVGVWDESDYWGQGTQVTVSS	54
VV02-1SP (1)-73 huNb_3_1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVAS IYSSGKTYYVESVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARWKT LRVGVWDESDYWGQGTLVTVSS	55
VV02-1SP (1)-73 huNb_3_2	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQAPGKGFEWVSS IYSSGKTYYVESVKGRFTISRDNAKNSLYLQMSSLRAEDTAVYYCATWKT LRVGVWDESDYWGQGTLVTVSS	56
VV02-1SP (1)-73 huNb_3_3	EVQLVESGGGLVQPGGSLRLSCAVSGFTFSSSAMSWARQAPGKEFEWVSS IYSSGKTYYVESVKGRFTISRDNAKNSVYLQMSSLRPEDTAVYYCATWKT LRVGVWDESDYWGQGTLVTVSS	57

[0092] For VV02-1LP-263, two mutations (D54G and D61E, Kabat numbering) were introduced to CDR2 to improve developability. For VV02-1SP (1)-73, a mutation (N31S, Kabat numbering) and two mutations (D54S and D61E, Kabat numbering) were introduced to the CDR1 and CDR2, respectively, to improve developability. The sequence comparisons are shown in **Table 5A** below.

Table 5A. Updated CDRs

Antibody	CDR1	SEQ ID NO:	CDR2	SEQ ID NO:	CDR3	SEQ ID NO:
VV02-1LP-263	SSAMS	18	GIYSDGSTYYTDSVKD	25	WGTLRFGVWAEYDH	32
VV02-1LP-263 Humanized	SSAMS	18	GIYS**G**GSTYYT**E**SVKD	58	WGTLRFGVWAEYDH	32
VV02-1SP (1)-73	NSAMS	23	SIYSDGKTYYVDSVKG	30	WKTLRVGVWDESDY	38
VV02-1SP (1)-73 Humanized	**S**SAMS	18	SIYS**S**GKTYYV**E**SVKG	59	WKTLRVGVWDESDY	38

Example 6. Cell surface 4-1BB binding properties of anti-4-1BB humanized nanobodies

[0093] To evaluate the antigen binding property in a cell-based setting, the 4-1BB humanized nanobodies were analyzed for their binding to 4-1BB overexpressed on CHO-K1 cells by FACS.

[0094] Briefly, CHO-K1 cells overexpressing human 4-1BB or cynomolgus 4-1BB were incubated with the serial diluted anti-4-1BB nanobodies for 30 min at 4°C. Then, the cells were incubated with Alexa Fluor 633-conjugated anti-human Fc secondary antibody. Binding was measured with an Agilent flow cytometer. The results showed that all the tested

humanized nanobodies exhibited typical sigmoidal binding behavior against cell surface human 4-1BB and cyno 4-1BB (**FIG. 4 & FIG. 5**). The binding EC_{50} are shown in **Table 6 & Table 7** accordingly.

Table 6. Binding EC_{50} (nM) on cell surface 4-1BB of humanized nanobodies from parental clone VV02-1LP-263.

	CHO-K1/hu4-1BB		CHO-K1/Cyno-41BB	
	EC_{50} (nM)	Max (MFI)	EC_{50} (nM)	Max (MFI)
VV02-1LP-263_2	0.47	47120	0.64	105589
VV02-1LP-263 huNb_1_2	0.17	23533	0.69	99611
VV02-1LP-263 huNb_1_3	0.11	42059	0.33	133804
VV02-1LP-263 huNb_2_2	0.19	23409	0.64	86388
VV02-1LP-263 huNb_2_3	0.11	39070	0.37	129678
VV02-1LP-263 huNb_3_2	0.14	38041	0.70	128178
VV02-1LP-263 huNb_3_3	0.12	40328	0.70	149247

Table 7. Binding EC_{50} (nM) on cell surface 4-1BB of humanized nanobodies from parental clone VV02-1SP (1)-73.

	CHO-K1/hu4-1BB	
	EC50 (nM)	Max (MFI)
VV02-1SP (1)-73 huNb_1_1	-	233.1
VV02-1SP (1)-73 huNb_1_2	0.80	5243
VV02-1SP (1)-73 huNb_1_3	0.61	9809
VV02-1SP (1)-73 huNb_2_2	0.37	9270
VV02-1SP (1)-73 huNb_2_3	0.49	9238
VV02-1SP (1)-73 huNb_3_1	27.39	3700
VV02-1SP (1)-73 huNb_3_2	0.52	8562
VV02-1SP (1)-73 huNb_3_3	0.66	9627

Example 7. Sequence optimization of anti-4-1BB nanobodies by site-mutation

[0095] To further increase developability of the candidates, anti-4-1BB nanobody VV02-1LP-263 huNb_1_3 was selected to be optimized by site-mutation. The optimized sequences are shown in **Table 8.**

Table 8. Optimized sequences of VV02-1LP-263 huNb_1_3

Name	Sequence	SEQ ID NO:
263 huNb-1-3_1	EVQL**V**ESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQ**A**PGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNSKNTVYLQMNSLKPEDTAVYYCATWGT LRFGVWAEYDHWGQGTQVTVSS	60
263 huNb-1-3_2	EVQL**V**ESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQ**A**PGKGFEWVSG IYSGGSTYYTESVKDRFTISRDN**A**KNTVYLQMNSLKPEDTAVYYCATWGT LRFGVWAEYDHWGQGT**L**VTVSS	61
263 hnNb-1-3_3	EVQL**V**ESGGGLVQPGGSLRLSCAASGFTFSSSAMSWARQTPGKGFEWVSG IYSGGSTYYTESVKDRFTISRDNSKNT**L**YLQMNSL**R**PEDTAVYYCATWGT LRFGVWAEYDHWGQGTQVTVSS	62

[0096] To evaluate the feasibility of optimized anti-4-1BB sequences to construct bi-specific antibody, the optimized anti-4-1BB was fused to the heavy chain C terminus of a IgG1 antibody targeting a tumor associated antigen (TAA) with N297A mutation in Fc fragment via G4S linker. Then, the light chain and heavy chain expression vectors were co-transfected into CHO-K1 cells. After transient transfection, the bispecific antibodies were purified from the medium by protein A affinity chromatography. The well qualified antibodies were applied to in vitro characterization including cell-based 4-1BB binding, 4-1BB activation and developability assessment.

Cell-based 4-1BB binding

[0097] The optimized 4-1BB nanobodies-based bispecific antibodies were analyzed for their binding to 4-1BB overexpressed on CHO-K1 cells according to the protocol described above. As shown in **FIG. 6**, the optimized anti-4-1BB sequences maintained comparable binding activity to 4-1BB-bearing CHO-K1 cells as the parental sequence.

TAA-dependent 4-1BB activation

[0098] To evaluate the ability of anti-TAA-4-1BB bispecific antibody to activate the 4-1BB signaling, a classical reporter gene assay was used. In this assay, the engineered Jurkat cells which stably express 4-1BB and have an NK-kB luciferase reporter construct integrated into the genome were used as effector cells. CHO-K1 cells engineered to overexpress TAA or the blank CHO-K1 cells were used as target cells. These two types of cells were co-incubated overnight with different concentrations antibodies at 37 °C in 5% CO2 incubator. Then, the substrate of luciferase was added, and the luminescence intensity was determined by a microplate reader.

[0099] As shown in **FIG.7**, the optimized anti-4-1BB based bi-specific antibodies had comparable potency in induction of 4-1BB activation in the presence of target cells as the parental antibody TAA-263-1-3.

Thermal stability assessment

[0100] A forced degradation test was conducted to assess the thermal stability of TAA-4-1BB bispecific antibodies. The antibodies were incubated in the buffer containing 20 mM His, 6% Sucrose+0.02% PS80 pH6.0, at 40 °C for 2 weeks. Representative quality attributes especially aggregation and fragmentation were monitored at 2 weeks using SEC-HPLC. Bispecific antibody derived from the parental anti-4-1BB sequence exhibited poor thermal stability as evidenced by aggregation and fragmentation during incubation. In contrast, optimized 4-1BB sequence-based new TAA-4-1BB bispecific antibodies showed acceptable thermal stability at 40 °C for 2 weeks, indicating potential reasonable developability to move forward (**Table 9**).

Table 9. SEC-HPLC characterization of TAA-4-1BB bispecific antibody in forced degradation test

Sample	Condition	Conc. (mg/ml)	SEC (%)		
			LMW (%)	Main (%)	HMW (%)
TAA-263-1-3_1	T0	6.26	1.66	97.56	0.78
	40°C 2W	5.88	1.59	96.15	2.26
TAA-263-1-3_2	T0	6.68	1.1	98.08	0.83
	40°C 2W	5.26	1.19	96.69	2.12
TAA-263-1-3_3	T0	7.13	1.89	97.25	0.85
	40°C 2W	5.67	1.78	96.15	2.07

* * *

[0101] The present disclosure is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the disclosure, and any compositions or methods which are functionally equivalent are within the scope of this disclosure. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present disclosure without departing from the spirit or scope of the disclosure. Thus, it is intended that the present disclosure cover the modifications and variations of this disclosure provided they come within the scope of the appended claims and their equivalents.

[0102] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

What is claimed is:

1. A single domain antibody or a polypeptide comprising the single domain antibody, wherein the single domain antibody has binding specificity to the human 4-1BB protein and comprises a complementarity determining region 1 (CDR1), a CDR2 and a CDR3, wherein the CDR1, CDR2 and CDR3 comprise, respectively,

(a) the amino acid sequences of SEQ ID NO:18, 58 and 32;

(b) the amino acid sequences of SEQ ID NO:18, 59 and 38;

(c) the amino acid sequences of SEQ ID NO:17, 24 and 31;

(d) the amino acid sequences of SEQ ID NO:18, 25 and 32;

(e) the amino acid sequences of SEQ ID NO:18, 26 and 33;

(f) the amino acid sequences of SEQ ID NO:18, 27 and 34;

(g) the amino acid sequences of SEQ ID NO:18, 28 and 35;

(h) the amino acid sequences of SEQ ID NO:19, 28 and 35;

(i) the amino acid sequences of SEQ ID NO:20, 28 and 35;

(j) the amino acid sequences of SEQ ID NO:19, 28 and 35;

(k) the amino acid sequences of SEQ ID NO:21, 29 and 36;

(l) the amino acid sequences of SEQ ID NO:22, 29 and 36;

(m) the amino acid sequences of SEQ ID NO:21, 29 and 36;

(n) the amino acid sequences of SEQ ID NO:21, 29 and 36;

(o) the amino acid sequences of SEQ ID NO:19, 26 and 33;

(p) the amino acid sequences of SEQ ID NO:18, 28 and 37;

(q) the amino acid sequences of SEQ ID NO:23, 30 and 38; or

(r) the amino acid sequences of SEQ ID NO:18, 28 and 39.

2. The antibody or polypeptide of claim 1, wherein the CDR1 comprises the amino acid sequence of SEQ ID NO:18, the CDR2 comprises the amino acid sequence of SEQ ID NO:58, and the CDR3 comprises the amino acid sequence of SEQ ID NO:32.

3. The antibody or polypeptide of claim 2, which comprises the amino acid sequence of any one of SEQ ID NO:40-48 and 60-62.

4. The antibody or polypeptide of claim 1, wherein the CDR1 comprises the amino acid sequence of SEQ ID NO:18, the CDR2 comprises the amino acid sequence of SEQ ID NO:59, and the CDR3 comprises the amino acid sequence of SEQ ID NO:38.

5. The antibody or polypeptide of claim 4, which comprises the amino acid sequence of any one of SEQ ID NO:49-57.

6. The antibody or polypeptide of claim 1, which comprises the amino acid sequence of any one of SEQ ID NO:1-16.

7. The antibody or polypeptide of any one of claims 1-6, wherein the polypeptide is a chimeric antigen receptor (CAR) or a bispecific antibody having a binding specificity to an antigen different from 4-1BB.

8. A bispecific antibody comprising the antibody of any one of claims 1-6 and a second antibody or antigen-binding fragment having binding specificity to a target antigen that is not 4-1BB.

9. One or more polynucleotide(s) encoding the antibody or polypeptide of any one of claims 1-8.

10. A cell comprising the polynucleotide of claim 9.

11. A composition comprising the antibody or polypeptide of any one of claims 1-8 and a pharmaceutically acceptable carrier.

12. A method of treating cancer in a patient in need thereof, comprising administering to the patient an effective amount of the antibody or polypeptide of any one of claims 1-8.

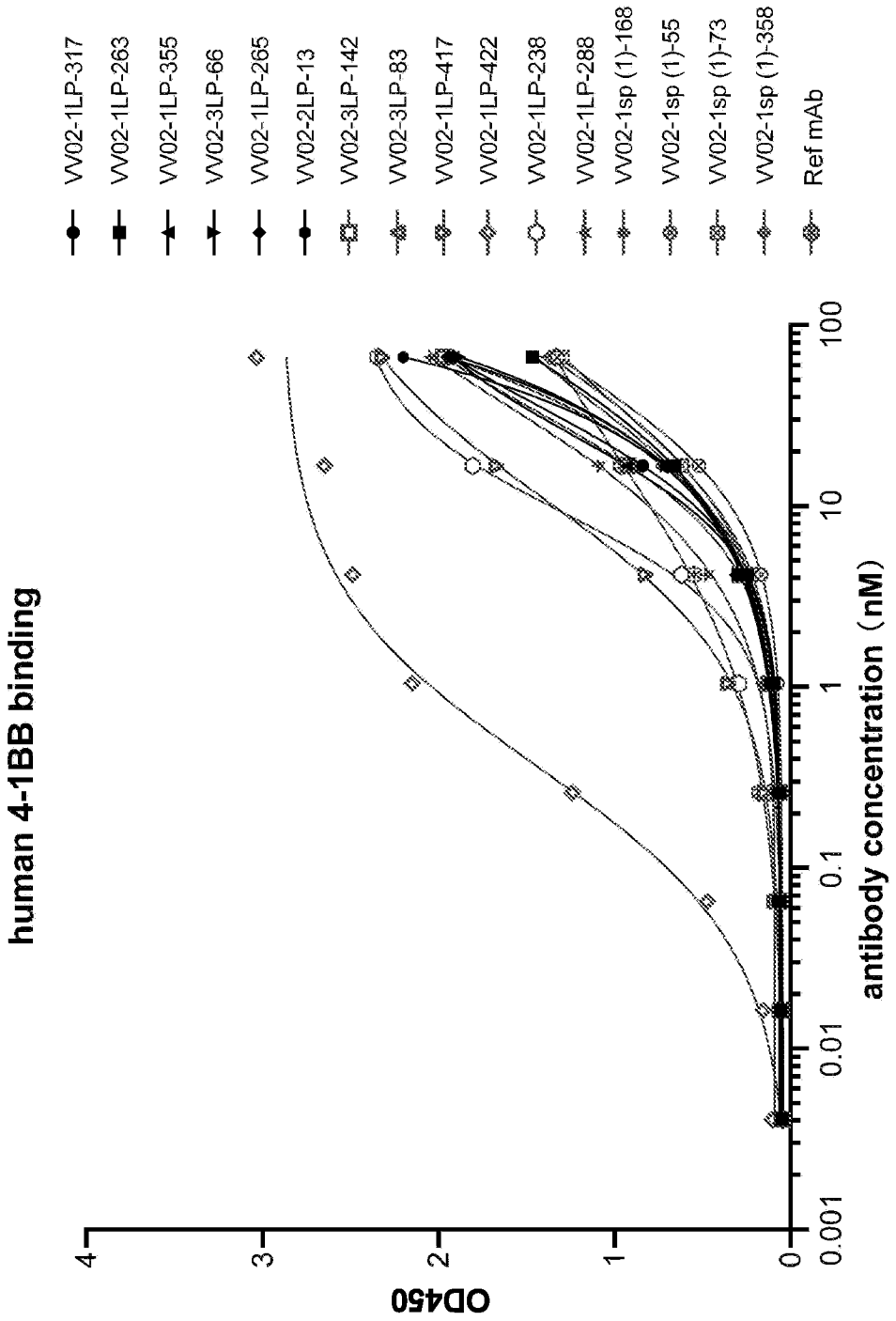
human 4-1BB binding
OD450
antibody concentration（nM）
0.001
0.01
0.1
1
10
100
0
1
2
3
4
VV02-1LP-317
VV02-1LP-263
VV02-1LP-355
VV02-3LP-66
VV02-1LP-265
VV02-2LP-13
VV02-3LP-142
VV02-3LP-83
VV02-1LP-417
VV02-1LP-422
VV02-1LP-238
VV02-1LP-288
VV02-1sp (1)-168
VV02-1sp (1)-55
VV02-1sp (1)-73
VV02-1sp (1)-358
Ref mAb

FIG. 1A

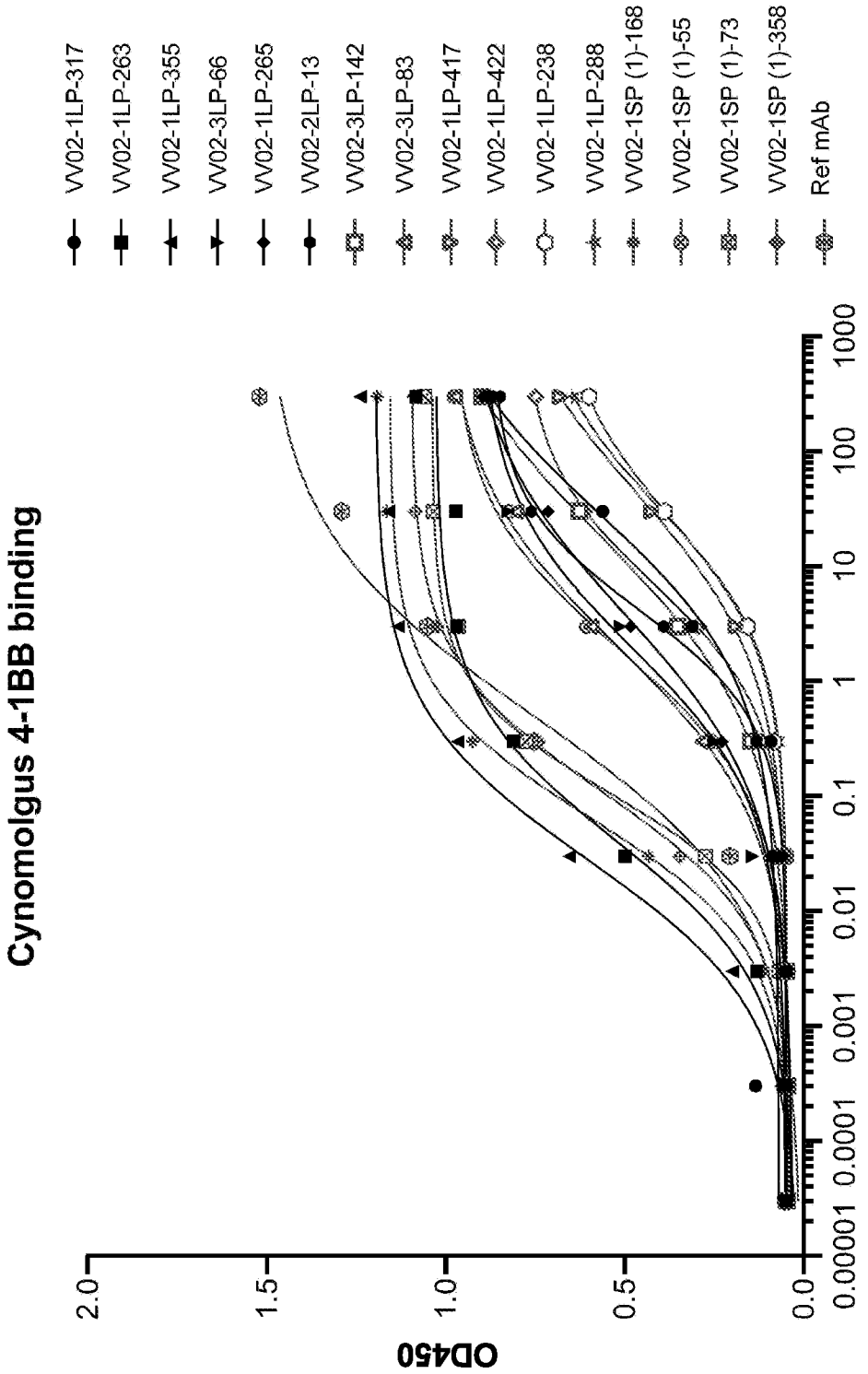
Cynomolgus 4-1BB binding
OD450
2.0
1.5
1.0
0.5
0.0
0.00001
0.0001
0.001
0.01
0.1
1
10
100
1000
antibody concentration (nM)
VV02-1LP-317
VV02-1LP-263
VV02-1LP-355
VV02-3LP-66
VV02-1LP-265
VV02-2LP-13
VV02-3LP-142
VV02-3LP-83
VV02-1LP-417
VV02-1LP-422
VV02-1LP-238
VV02-1LP-288
VV02-1SP (1)-168
VV02-1SP (1)-55
VV02-1SP (1)-73
VV02-1SP (1)-358
Ref mAb

FIG. 1B

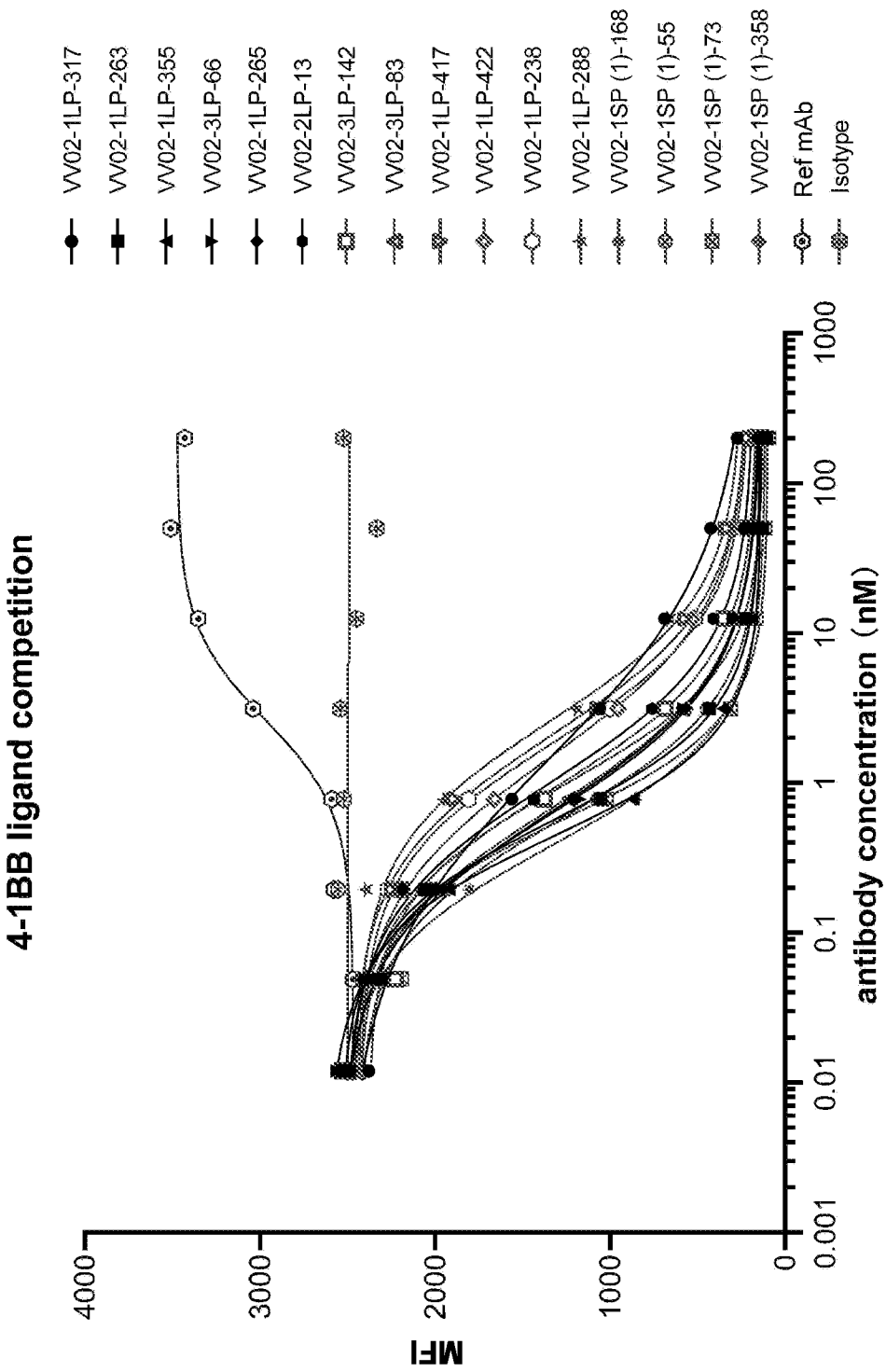
4-1BB ligand competition
MFI
4000
3000
2000
1000
0
0.001
0.01
0.1
1
10
100
1000
antibody concentration（nM）
VV02-1LP-317
VV02-1LP-263
VV02-1LP-355
VV02-3LP-66
VV02-1LP-265
VV02-2LP-13
VV02-3LP-142
VV02-3LP-83
VV02-1LP-417
VV02-1LP-422
VV02-1LP-238
VV02-1LP-288
VV02-1SP (1)-168
VV02-1SP (1)-55
VV02-1SP (1)-73
VV02-1SP (1)-358
Ref mAb
Isotype

FIG. 2

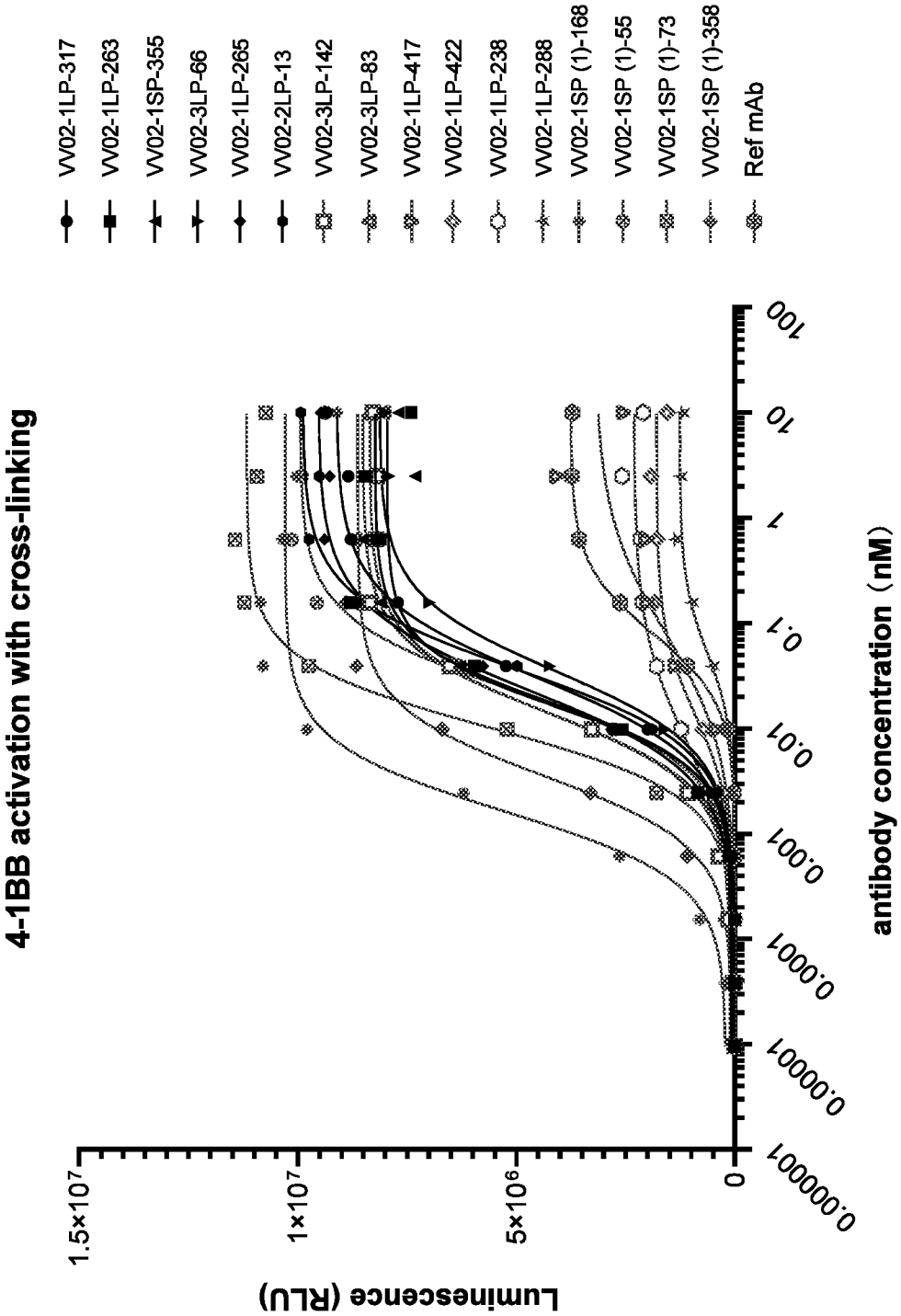
4-1BB activation with cross-linking
VV02-1LP-317
VV02-1LP-263
VV02-1SP-355
VV02-3LP-66
VV02-1LP-265
VV02-2LP-13
VV02-3LP-142
VV02-3LP-83
VV02-1LP-417
VV02-1LP-422
VV02-1LP-238
VV02-1LP-288
VV02-1SP (1)-168
VV02-1SP (1)-55
VV02-1SP (1)-73
VV02-1SP (1)-358
Ref mAb
Luminescence (RLU)
1.5×10^7
1×10^7
5×10^6
0
antibody concentration (nM)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001

FIG. 3A

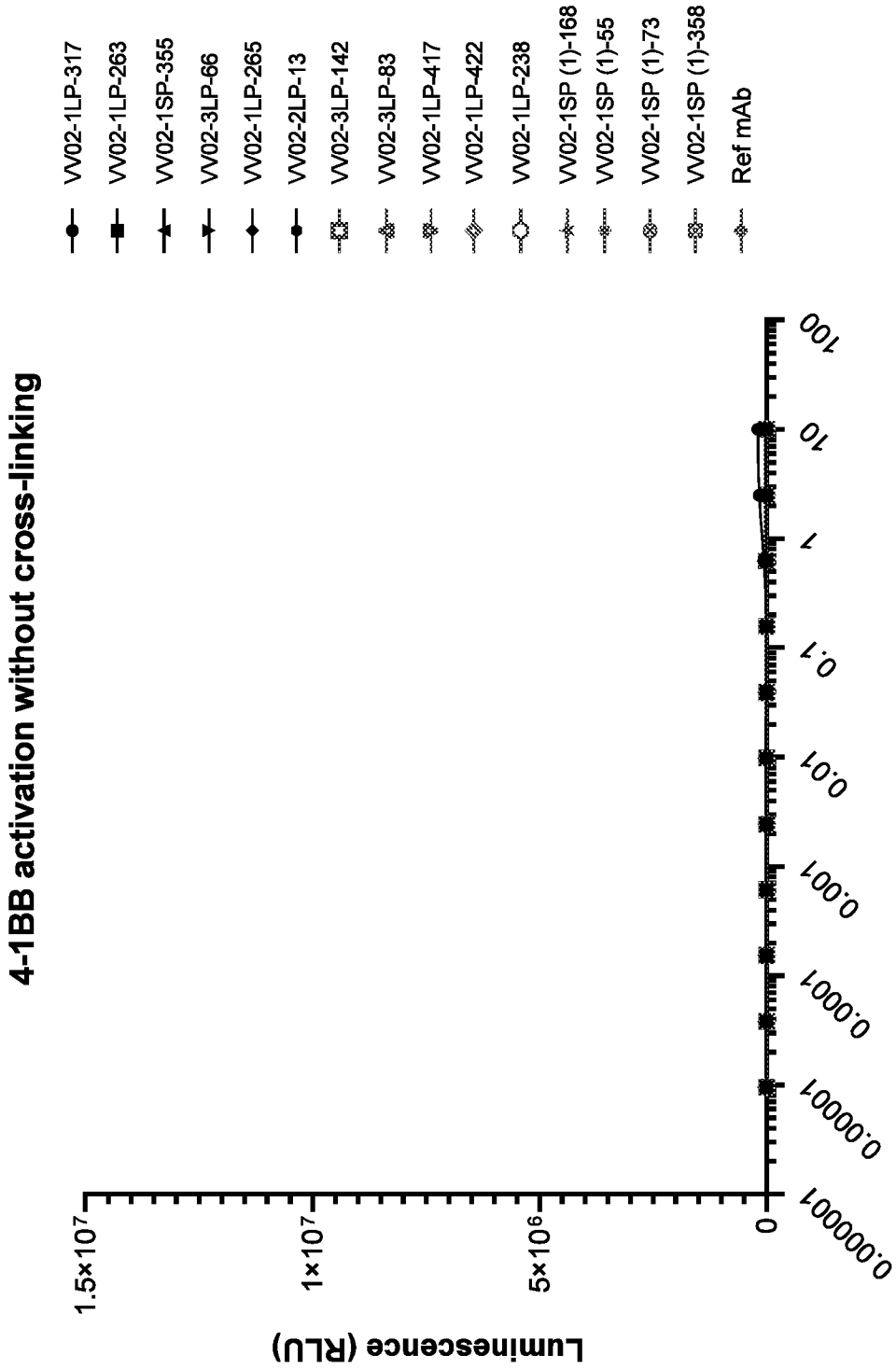
4-1BB activation without cross-linking
VV02-1LP-317
VV02-1LP-263
VV02-1SP-355
VV02-3LP-66
VV02-1LP-265
VV02-2LP-13
VV02-3LP-142
VV02-3LP-83
VV02-1LP-417
VV02-1LP-422
VV02-1LP-238
VV02-1SP (1)-168
VV02-1SP (1)-55
VV02-1SP (1)-73
VV02-1SP (1)-358
Ref mAb
Luminescence (RLU)
1.5×10^7
1×10^7
5×10^6
0
antibody concentration (nM)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001

FIG. 3B

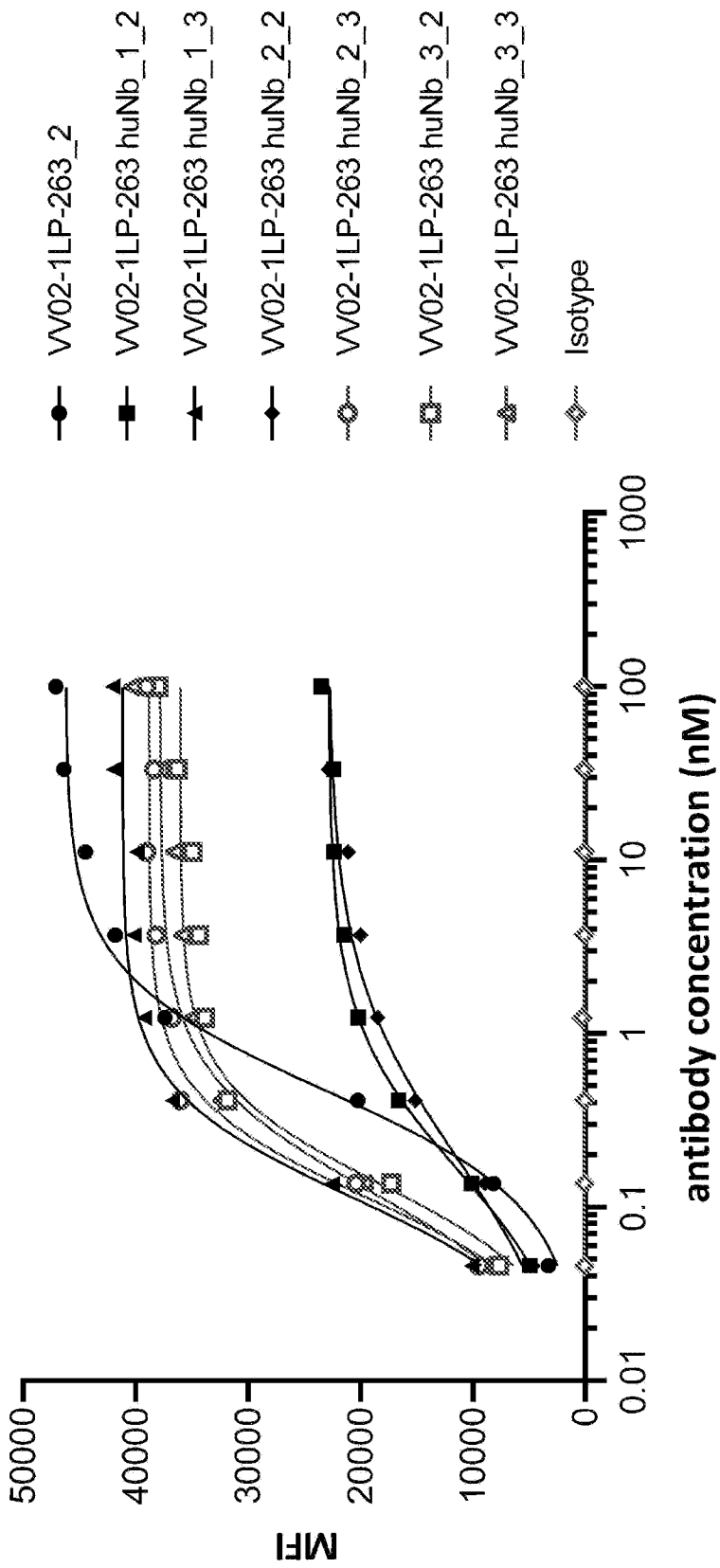
CHO-K1/hu4-1BB binding
MFI
50000
40000
30000
20000
10000
0
0.01
0.1
1
10
100
1000
antibody concentration (nM)
VV02-1LP-263_2
VV02-1LP-263 huNb_1_2
VV02-1LP-263 huNb_1_3
VV02-1LP-263 huNb_2_2
VV02-1LP-263 huNb_2_3
VV02-1LP-263 huNb_3_2
VV02-1LP-263 huNb_3_3
Isotype

FIG. 4A

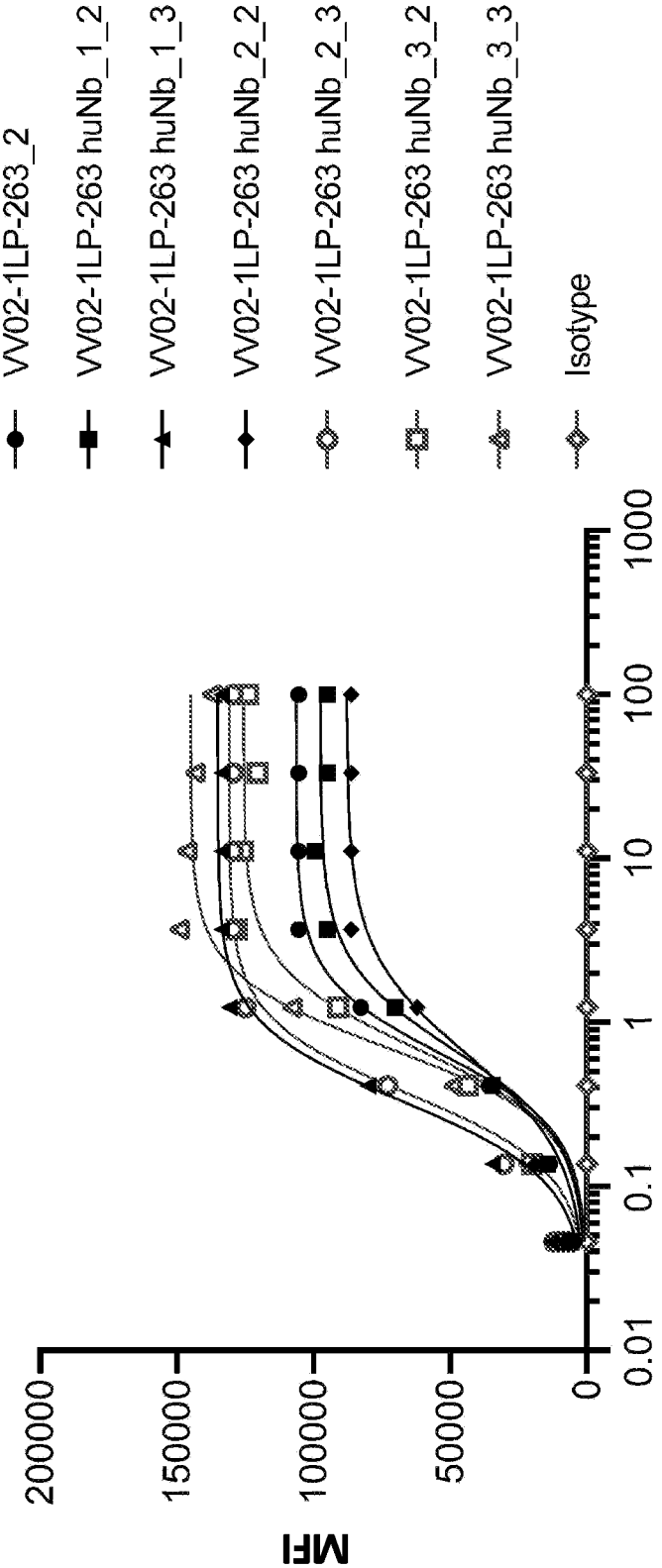
CHO-K1/Cyno 4-1BB binding
MFI
200000
150000
100000
50000
0
0.01
0.1
1
10
100
1000
antibody concentration (nM)
VV02-1LP-263_2
VV02-1LP-263 huNb_1_2
VV02-1LP-263 huNb_1_3
VV02-1LP-263 huNb_2_2
VV02-1LP-263 huNb_2_3
VV02-1LP-263 huNb_3_2
VV02-1LP-263 huNb_3_3
Isotype

FIG. 4B

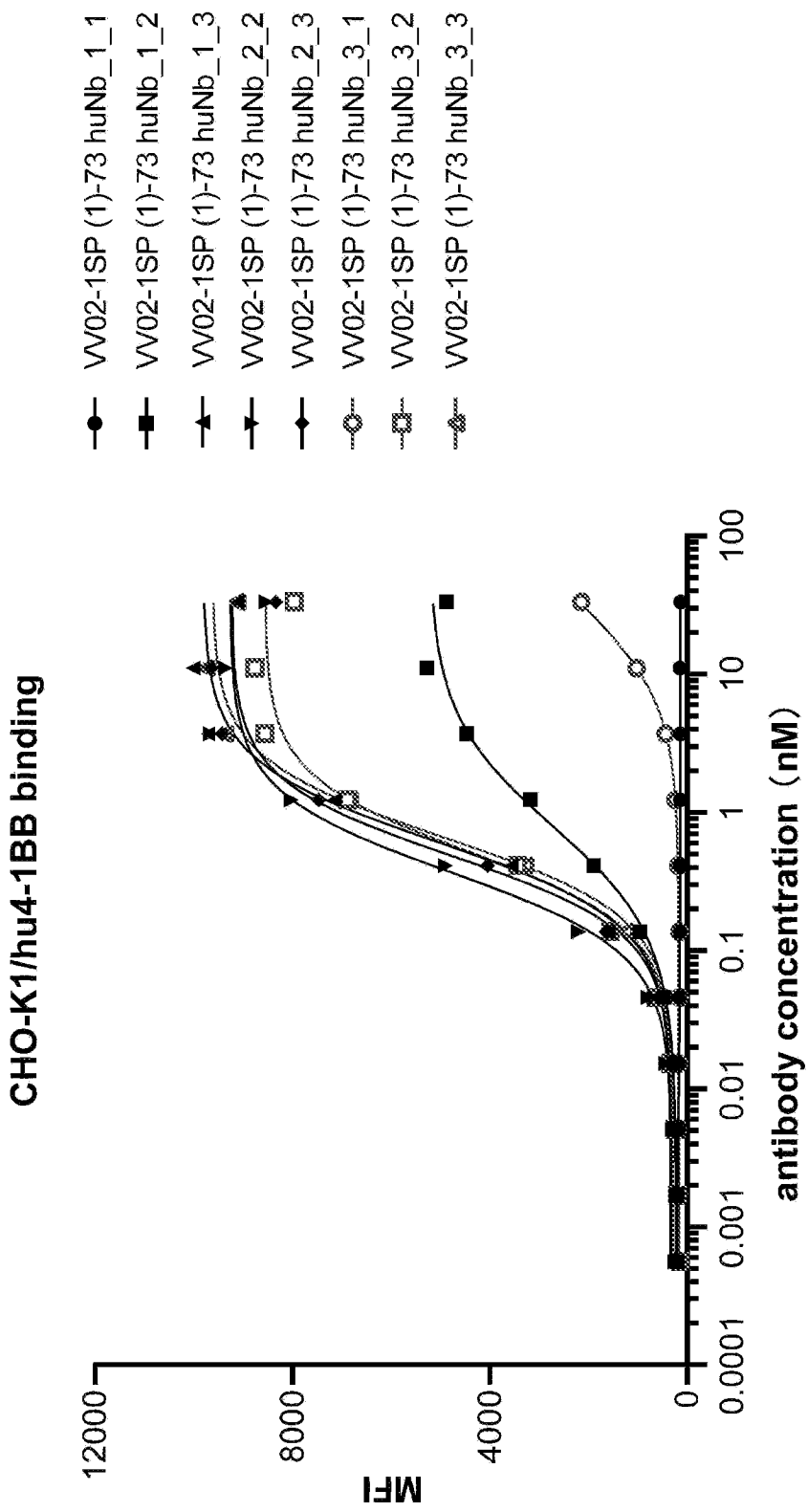
CHO-K1/hu4-1BB binding
VV02-1SP (1)-73 huNb_1_1
VV02-1SP (1)-73 huNb_1_2
VV02-1SP (1)-73 huNb_1_3
VV02-1SP (1)-73 huNb_2_2
VV02-1SP (1)-73 huNb_2_3
VV02-1SP (1)-73 huNb_3_1
VV02-1SP (1)-73 huNb_3_2
VV02-1SP (1)-73 huNb_3_3
MFI
12000
8000
4000
0
0.0001
0.001
0.01
0.1
1
10
100
antibody concentration（nM）

FIG. 5

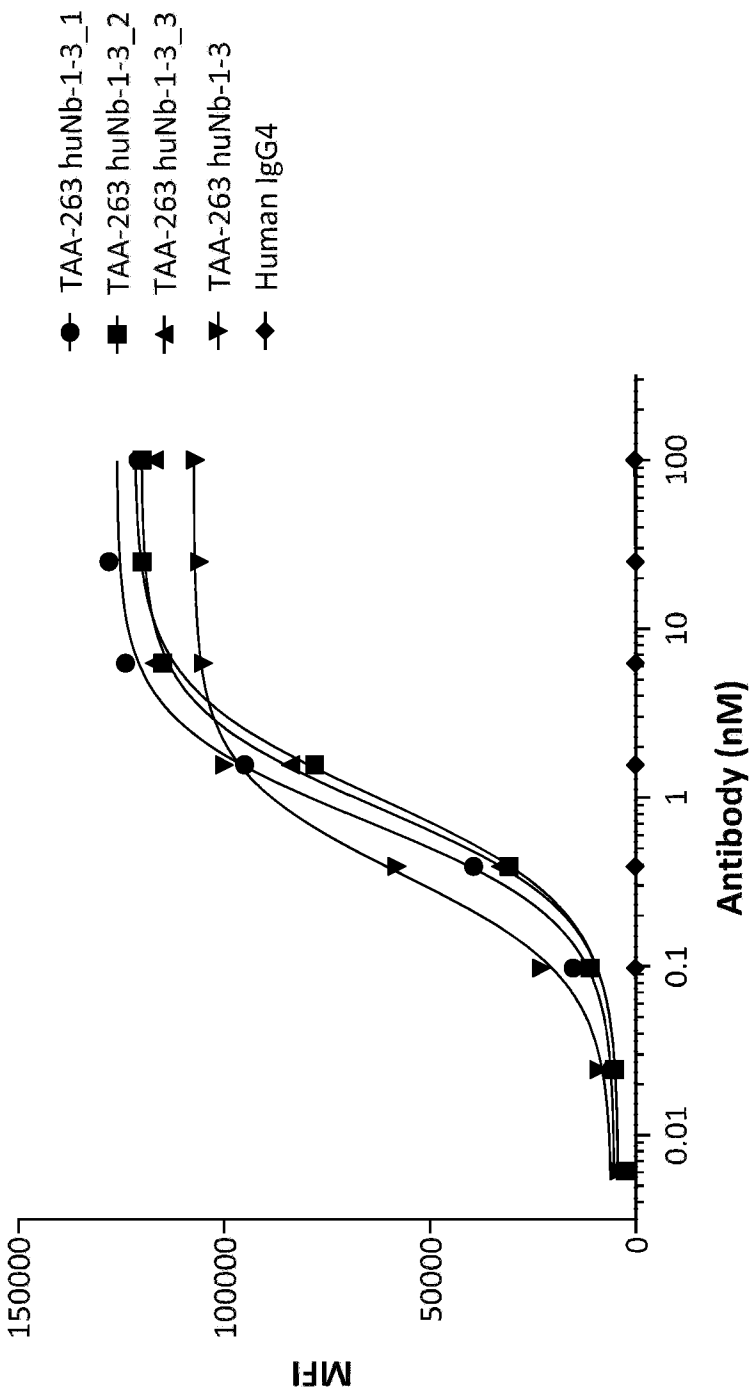
h4-1BB_CHO-K1 binding
TAA-263 huNb-1-3_1
TAA-263 huNb-1-3_2
TAA-263 huNb-1-3_3
TAA-263 huNb-1-3
Human IgG4
MFI
150000
100000
50000
0
0.01
0.1
1
10
100
Antibody (nM)

FIG. 6

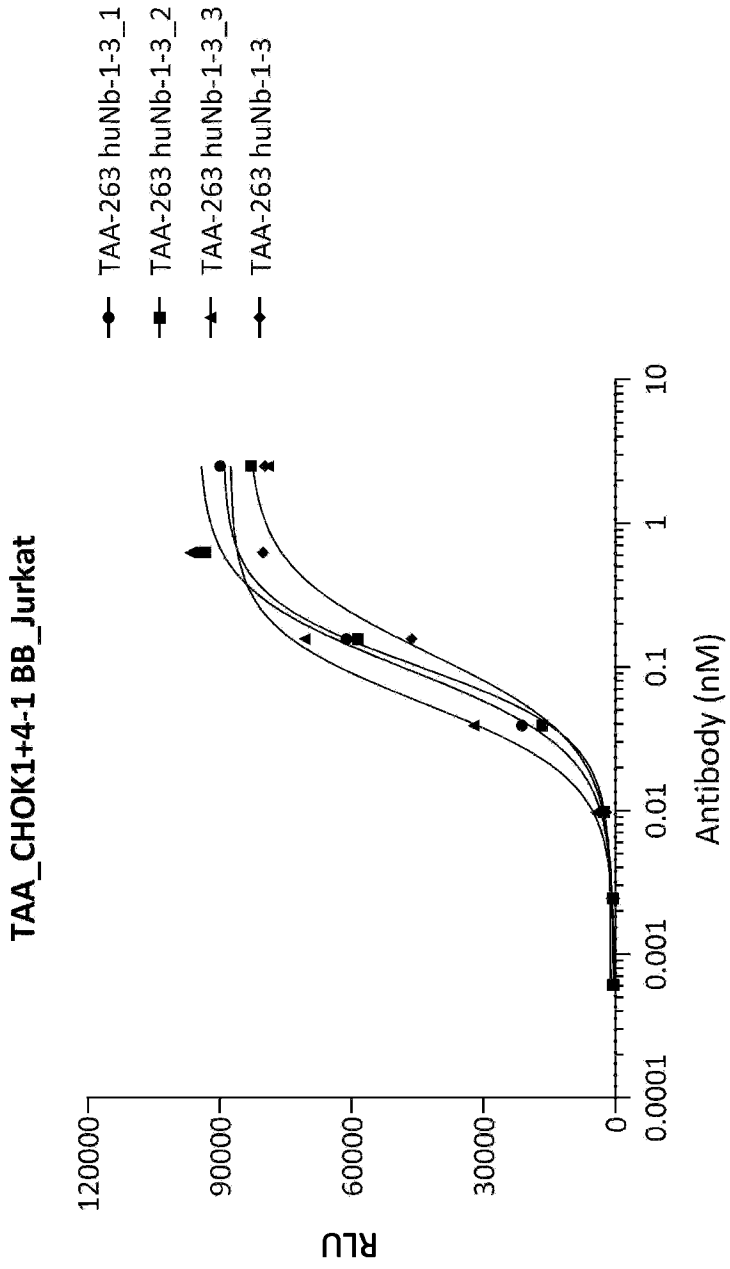
TAA_CHOK1+4-1 BB_Jurkat
TAA-263 huNb-1-3_1
TAA-263 huNb-1-3_2
TAA-263 huNb-1-3_3
TAA-263 huNb-1-3
RLU
120000
90000
60000
30000
0
0.0001
0.001
0.01
0.1
1
10
Antibody (nM)

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2022/115536

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/28(2006.01)i; A61K 39/395(2006.01)i; C12N 15/13(2006.01)i; A61P 35/00(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)

C07K; A61K; C12N; 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)

CNTXT, WPABSC, WPABS, ENTXT, ENTXTC, CNKI, Web of Science, Baidu, incoPat, NCBI, EBI, STNext:CD137, 4-1BB,single domain, VHH, nanobody,antibody, bispecific, sequences blast

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2020362051 A1 (CRESCENDO BIOLOGICS LTD.) 19 November 2020 (2020-11-19) claims 1-38	1-12
A	WO 2019141268 A1 (JIANGSU HENGRUI MEDICINE CO., LTD.) 25 July 2019 (2019-07-25) the whole doucument	1-12
A	CN 110627906 A (SHANGHAI NOVAMAB BIOPHARM CO.) 31 December 2019 (2019-12-31) the whole document	1-12
A	US 2018258177 A1 (EUTILEX CO., LTD.) 13 September 2018 (2018-09-13) the whole document	1-12
A	US 2020377595 A1 (CHUGAI SEIYAKU KABUSHIKI KAISHA) 03 December 2020 (2020-12-03) the whole document	1-12

☑ 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
"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
10 November 2022	**29 November 2022**
Name and mailing address of the ISA/CN **National Intellectual Property Administration, PRC** **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing** **100088, China** Facsimile No. **(86-10)62019451**	Authorized officer **WANG,Hui** Telephone No. **86-(10)-53962103**

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHAI, T. et al. "Generation of a safe and efficacious llama single-domain (vHH) targeting the membrane-proximal region of 4-1BB for engineering therapeutic bispecific antibodies for cancer" *Journal for ImmunoTherapy of Cancer,* Vol. 9, No. 6, 30 June 2021 (2021-06-30), article e002131	1-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2022/115536

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:
      ☑ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.
   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   [1] Actually, the sequences are listed in the form of an Annex C/ST.26 XML file.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2022/115536

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.: **12**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] Claim 12 includes method of treatment of the human/animal body. It does not meet the criteria set out in PCT Rule 39.1 (iv). However, the search has been carried out and based on the manufacturing of a 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/CN2022/115536

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	2020362051	A1	19 November 2020	AU	2018363291	A1	21 May 2020
				KR	20200083574	A	08 July 2020
				IL	274371	A	30 June 2020
				CA	3082297	A1	16 May 2019
				WO	2019092452	A1	16 May 2019
				CN	111683968	A	18 September 2020
				WO	2019092451	A1	16 May 2019
				EP	3710477	A1	23 September 2020
				AU	2018363292	A1	21 May 2020
				KR	20200080304	A	06 July 2020
				JP	2021502810	A	04 February 2021
				IL	274370	A	30 June 2020
				SG	11202003978U	A	28 May 2020
				CA	3082321	A1	16 May 2019
				EP	3710478	A1	23 September 2020
				US	2020362047	A1	19 November 2020
				CN	111699197	A	22 September 2020
				JP	2021502104	A	28 January 2021
				SG	11202003912R	A	28 May 2020
WO	2019141268	A1	25 July 2019	RU	2020127196	A3	24 February 2022
				US	2021363266	A1	25 November 2021
				CN	111065652	A	24 April 2020
				CA	3089260	A1	25 July 2019
				JP	2021510533	A	30 April 2021
				KR	20200113228	A	06 October 2020
				EP	3744734	A1	02 December 2020
				AU	2019208793	A1	03 September 2020
				TW	201932491	A	16 August 2019
				BR	112020014848	A2	08 December 2020
CN	110627906	A	31 December 2019	None			
US	2018258177	A1	13 September 2018	RU	2020120077	A	03 December 2020
				JP	2020036595	A	12 March 2020
				CN	112210010	A	12 January 2021
				KR	20190095919	A	16 August 2019
				CA	3039772	A1	12 July 2018
				BR	112019007714	A2	09 July 2019
				WO	2018127787	A1	12 July 2018
				AU	2018206015	A1	16 May 2019
				SG	11201903021W	A	30 May 2019
				EP	3523332	A1	14 August 2019
				EP	4032911	A1	27 July 2022
				MX	2019004692	A	08 November 2019
				AU	2021202474	A1	20 May 2021
				US	2020172626	A1	04 June 2020
				ES	2905890	T3	12 April 2022
				NZ	753036	A	30 October 2020
				SG	10201914064Q	A	30 March 2020
				JP	6609724	B1	20 November 2019
				US	2019071510	A1	07 March 2019
				CN	110392696	A	29 October 2019

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2022/115536

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
		AU 2019204847 A1	01 August 2019
		KR 20200029618 A	18 March 2020
		US 2021214455 A1	15 July 2021
		RU 2725811 C1	06 July 2020
US 2020377595 A1	03 December 2020	TW 201938194 A	01 October 2019
		WO 2019111871 A1	13 June 2019
		JP 2021508441 A	11 March 2021
		EP 3720963 A1	14 October 2020