Description

Title of Invention: MULTISPECIFIC ANTIBODIES AND USES THEREOF

BACKGROUND

[0001] T cell engagers are multifunctional molecules capable of recruiting a patient's own T cells to malignant cells, resulting in cytotoxic activity directed to the malignant cells. A common design of a T cell engager includes an anti-CD3 antibody and another antibody specific to a tumor-associated antigen (TAA) . Current T cell engagers have limited T cell-mediated response, especially in poorly immunogenic tumor. Further, it is known that T cell agonist antibodies, e.g., CD3 antibodies, may cause severe side effects such as cytokine release syndrome (CRS) . Development of a suitable T cell engager, therefore, requires overcoming the multiple challenges in sufficient tumor targeting, minimized systemic activation, and in vitro and in vivo stability.

[0002] Delta-like ligand 3 (DLL3) is an inhibitory Notch ligand in the Notch ligand family. In contrast to other members of the family, such as DLL1 and DLL4, the human DLL3 protein has a distinct structure that consists of one Delta/Serrate/LAG-2 (DSL) domain, six epithelial growth factor (EGF) like repeats, a 21 amino acid (aa) transmembrane domain (TM) , and a 105 aa intracellular domain (ICD) . DLL3 is highly expressed on the cell surface in SCLC tumors and other tumor types of neuroendocrine origin, including glioblastoma multiforme (GBM) , large cell neuroendocrine lung tumors (LCNEC) , metastatic melanoma, small cell bladder cancer (SCBC) , and neuroendocrine prostate cancer (NEPC) . The DLL3 protein is expressed by more than 80%of SCLC tumors overall, with a high degree of uniformity across neoplastic cells. In contrast, only minimal expression is observed in normal tissues (e.g., neurons, pancreatic islet cells, and pituitary cells) , and it is exclusively cytoplasmic. In preclinical models, DLL3 expression promotes SCLC cell migration and invasion through a mechanism that involves the control of epithelial mesenchymal transition protein Snail. DLL3 is thus a potential TAA for T cell engagers.

[0003] Cadherin 17 (CDH17) , also known as liver-intestine cadherin (LI-cadherin) , is a distinctive member of the cadherin superfamily. Unlike classical cadherins with five cadherin domains and a cytoplasmic tail of more than 100 aa, CDH17 has a long extracellular domain (seven cadherin domains, namely EC1 to EC7) and a short cytoplasmic tail (20 aa residues) . Under physiological conditions, the expression of CDH17 in humans and mice is mainly restricted to epithelial cells in the small intestine and colon, but not in vital organs such as the liver, stomach, heart, lung and brain. Pathophysiologically, the expression of CDH17 is upregulated in gastric cancer

(GC) , colorectal cancer (CRC) , hepatocarcinoma (HCC) and pancreatic cancer (PC) . Moreover, the prevalence of CDH17 overexpression is considered to be high among GI patients, with positive rate over 50-90%depending on tumor types. Thus, CDH17 is considered as an ideal TAA for T cell engagers targeting tumors above.

SUMMARY

[0004] The present disclosure provides T cell engagers in a multi-specific antibody platform. The T cell engagers include an anti-CD3 moiety, an anti-4-1BB moiety and an anti-tumor associated antigen (TAA) moiety. The anti-CD3 and/or the anti-4-1BB moieties have balanced activities to ensure efficacy and safety. For instance, the anti-CD3 moiety can have a T cell activation activity (EC50) in the range of 0.5 to 10 nM, and the anti-4-1BB moiety can have a 4-1BB signaling activation activity (EC50) in the range of 0.05 to 1 nM. Improved anti-CD3 antibodies are also disclosed, which exhibited better in vitro and in vivo stability while retaining the preferable affinity. These new anti-CD3 sequences can be used alone or in T cell engagers. As demonstrated in the experimental examples, these newly developed T cell engagers possess superior anti-tumor activity as compared to benchmark molecules, and are associated with significantly lower CRS risks.

[0005] Accordingly, one embodiment provides a multi-specific antibody, comprising (a) an anti-CD3 antibody or antigen binding fragment thereof, and (b) an anti-4-1BB antibody or antigen binding fragment thereof, wherein: the anti-CD3 antibody or antigen binding fragment thereof has a T cell activation activity (EC50) in the range of 0.5 to 10 nM, which is measured by incubating (i) a multi-specific antibody comprising the anti-CD3 antibody or antigen binding fragment thereof and a second antibody or antigen binding fragment thereof specific to a first tumor associated antigen (TAA) , (ii) T cells expressing CD3 and a first reporter gene regulated by an NFAT (nuclear factor of activated T cell) response element (NFAT-RE) , and (iii) cells expressing the first TAA, and measuring activity of the first reporter gene; and the anti-4-1BB antibody or antigen binding fragment thereof has a 4-1BB signaling activation activity (EC50) in the range of 0.05 to 1 nM, which is measured by incubating (i) a multi-specific antibody comprising the anti-4-1BB antibody or antigen binding fragment thereof and a second antibody or antigen binding fragment thereof specific to a second TAA, (ii) T cells expressing 4-1BB and a second reporter gene regulated by NF-κB, and (iii) cells expressing the second TAA, and measuring activity of the second reporter gene.

[0006] In some embodiments, the anti-CD3 antibody or antigen binding fragment thereof has a T cell activation activity (EC50) in the range of 1 to 10 nM. In some embodiments, the anti-4-1BB antibody or antigen binding fragment thereof has a

4-1BB signaling activation activity (EC50) in the range of 0.1 to 1 nM. In some embodiments, the first TAA and second TAA are delta-like ligand 3 (DLL3) or cadherin 17 (CDH17) .

[0007] In some embodiments, the multi-specific antibody further comprises a third antibody or antigen binding fragment thereof having specificity to a TAA.

[0008] In some embodiments, the anti-4-1BB antibody is a single domain antibody. In some embodiments, the anti-4-1BB antibody is disposed at the C-terminal end of an Fc fragment. In some embodiments, the anti-CD3 fragment is disposed at the N-terminal end of the Fc fragment. In some embodiments, the third antibody or antigen binding fragment thereof has specificity to DLL3 or CHD17.

[0009] Also provided, in one embodiment, is an antibody or antigen-binding fragment thereof having specificity to a cluster of differentiation 3 (CD3) protein, wherein the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions HCDR1, HCDR2, and HCDR3, and a light chain variable region (VL) comprising light chain complementarity determining regions LCDR1, LCDR2, and LCDR3, wherein: the HCDR1 comprises SYNVH (SEQ ID NO: 16) , the HCDR2 comprises RVRX$_1$X$_2$X$_3$DTSYNSALKS (SEQ ID NO: 96) , the HCDR3 comprises GPYGGSSEPLFDY (SEQ ID NO: 18) , the LCDR1 comprises KASQNINKYLN (SEQ ID NO: 19) , the LCDR2 comprises TTNNLQT (SEQ ID NO: 20) , and the LCDR3 comprises LQHNSGYT (SEQ ID NO: 21); and wherein: (1) X$_1$ is any amino acid except N; X$_2$ is any amino acid except N; and X$_3$ is G or A; or (2) X$_1$ is any amino acid except N; X$_2$ is any amino acid; and X$_3$ is A.

[0010] In some embodiments, X$_1$ is Y, P, A, W, V or G; X$_2$ is E, S, T, Q, G, R, I, V, or Y; and X$_3$ is G or A. In some embodiments, X$_1$ is Y, X$_2$ is N, and X$_3$ is A. In some embodiments, (1) X$_1$ is Y, X$_3$ is G, and X$_2$ is E, S, T, Q, G, R, I, V, or Y; (2) X$_1$ is Y, X$_2$ is N, and X$_3$ is A; or (3) X$_1$ is P, A, W, V or G, X$_2$ is S, and X$_3$ is G.

[0011] In some embodiments, the HCDR2 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 55-69.

[0012] In some embodiments, the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 70-79, 80-84 and 85-88, or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 70-79, 80-84 and 85-88. In some embodiments, the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 2 and 89 or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 2 and 89.

[0013] In some embodiments, the VH comprises the amino acid sequence of any one of SEQ ID NOs: 70-79 and 80-84, and the VL comprises the amino acid sequence of SEQ ID NO: 2. In some embodiments, the VH comprises the amino acid sequence of any one of SEQ ID NOs: 85-88, and the VL comprises the amino acid sequence of SEQ ID NO: 89.

[0014] A multi-specific antibody is also provided, comprising: (a) a first antigen-binding moiety comprising the antibody or antigen-binding fragment thereof of the present disclosure and (b) a second antigen-binding moiety having specificity to a human 4-1BB protein. In some embodiments, the multi-specific antibody further comprises a third antigen-binding moiety.

[0015] Methods and uses for treating a disease or condition such as cancer are also provided. In some embodiments, the cancer is selected from the group consisting of ovarian cancer, prostate cancer, cancer of the urinary tract, pancreatic cancer, lung cancer, breast cancer, bladder cancer, colorectal cancer, endometrial cancer, esophageal cancer, head and neck cancer, kidney cancer, leukemia, liver cancer, lymphoma, melanoma, and thyroid cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 demonstrates that various DLL3-TOPAs with specificity to distinct DLL3 domains all exhibited potent cytotoxicity in SHP77 cells.

[0017] FIG. 2 demonstrates that the 310P3C5-TOPAbody yielded superior tumor inhibition rates and T cell infiltration in tumors when compared to benchmark AMG757.

[0018] FIG. 3 shows that the cell-based CD3 binding activity of the antibodies was minimally affected by substitution of the NG site in the CDR region with SG/TG/RG/NA.

[0019] FIG. 4 shows that the 155z16F-series mutated CD3 antibodies showed a variety of different CD3 functional and binding activities.

[0020] FIG. 5 shows that substitution of the YNG site in the CDR region of the CD3 155z16 antibody with PSG/ASG/VSG/WSG/GSG resulted in a higher level of cell-based binding and functional activity than that observed for 155z16 SG.

[0021] FIG. 6 shows that all trispecific antibodies with the engineered CD3 antibody sequences had comparable cell lysis activity and TNFα secretion.

[0022] FIG. 7 shows that all the engineered antibody sequences had remarkable improvement in stability in human serum up to 14 days.

[0023] FIG. 8 showed that the treatment with TG-and SG-modified TOPA molecules led to a significant reduction in tumor size compared to the original molecules.

[0024] FIG. 9 shows that the activation of T cells by DLL3-TOPA antibodies surpassed that induced by benchmark AMG757/RG6524 and combo therapies.

[0025] FIG. 10 shows that DLL3-TOPAbody had minimal cytokine release PBMC only culture.

[0026] FIG. 11 shows that the CDH17-TOPAbodies displayed dose-dependent activity in inducing CD3 and 4-1BB downstream signaling incubated with tumor cells of CDH17 expression.

[0027] FIG. 12 shows that the CDH17-TOPAbodies displayed dose-dependent T cell activation measured by proinflammatory cytokine release and cytotoxicity.

[0028] FIG. 13 shows that the CDH17-TOPAbodies had superior cytotoxicity against tumor cells, especially in low E: T ratios.

[0029] FIG. 14 shows that the CDH17-TOPAbodies had minimal cytokine release PBMC only culture.

[0030] FIG. 15 shows that the CDH17-TOPAbodies showed potent anti-tumor efficacy in immunologically cold tumor.

[0031] FIG. 16 shows that the CDH17-TOPAbodies elicited a long-term anti-tumor response.

DETAILED DESCRIPTION

[0032] Definitions

[0033] 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.

[0034] As used herein, an “antibody” or “antigen-binding moiety” 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.

[0035] A full-length antibody comprises two heavy chains and two light chains. The variable regions of the light and heavy chains are responsible for antigen binding. The variable domains of the heavy chain and light chain may be referred to as “VH” and “VL” , respectively. The variable regions in both chains generally contain three highly variable loops called the complementarity determining regions (CDRs) (light chain (LC) CDRs including LC-CDR1, LC-CDR2, and LC-CDR3, heavy chain (HC)

CDRs including HC-CDR1, HC-CDR2, and HC-CDR3) . CDR boundaries for the antibodies and antigen-binding fragments disclosed herein may be defined or identified by the conventions of Kabat, Chothia, or Al-Lazikani (Al-Lazikani 1997; Chothia 1985; Chothia 1987; Chothia 1989; Kabat 1987; Kabat 1991) . The three CDRs of the heavy or light chains 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. The constant regions of the heavy and light chains are not involved in antigen binding, but exhibit various effector functions. Antibodies are assigned to classes based on the amino acid sequence of the constant region of their heavy chain. The five major classes or isotypes of antibodies are IgA, IgD, IgE, IgG, and IgM, which are characterized by the presence of α, δ, ε, γ, and μ heavy chains, respectively. Several of the major antibody classes are divided into subclasses such as lgG1 (γ1 heavy chain) , lgG2 (γ2 heavy chain) , lgG3 (γ3 heavy chain) , lgG4 (γ4 heavy chain) , lgA1 (α1 heavy chain) , or lgA2 (α2 heavy chain) .

[0036] The term “half antibody” as used herein refers to one immunoglobulin heavy chain associated with one immunoglobulin light chain. One skilled in the art will readily appreciate that a half-antibody may encompass a fragment thereof and may also have an antigen binding domain consisting of a single variable domain, e.g., originating from a camelidae.

[0037] The term “single chain half antibody” as used herein refers to a single chain polypeptide comprising a VL domain, optionally a CL domain, a tether, a VH domain, optionally a CH1 domain, a hinge domain, a CH2 domain and a CH3 domain, wherein said domains are positioned relative to each other in an N-terminal to C-terminal direction as follows: VL-tether-VH-hinge-CH2-CH3, VL-tether-VH-partial hinge-CH2-CH3, VL-tether-VH-hinge variant -CH2-CH3, or VL-CL-tether-VH-CH1-hinge-CH2-CH3.

[0038] The expression “single domain antibodies” (sdAbs) or “single variable domain (SVD) antibodies” generally refers to antibodies in which a single variable domain (VH or VL) can confer antigen binding. In other words, the single variable domain does not need to interact with another variable domain in order to recognize the target antigen. Examples of single domain antibodies include those derived from camelids (lamas and camels) and cartilaginous fish (e.g., nurse sharks) and those derived from recombinant methods from humans and mouse antibodies (Nature (1989) 341: 544-546; Dev Comp Immunol (2006) 30: 43-56; Trend Biochem Sci (2001) 26: 230-235; Trends Biotechnol (2003) : 21: 484-490; WO 2005/035572; WO 03/035694; Febs Lett (1994) 339: 285-290; WO00/29004; WO 02/051870) . When the sdAb contains only a heavy chain, it can be exchangably used with “VHH” or “single heavy chain variable domain antibody” or “nanobody” .

[0039] 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.

[0040] A “Fab” with regard to an antibody refers to a monovalent antigen-binding fragment of the antibody 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 antibody at the residues proximal to the N-terminus of the disulfide bond between the heavy chains of the hinge region.

[0041] A “Fab’ ” refers to a Fab fragment that includes a portion of the hinge region, which can be obtained by pepsin digestion of an antibody at the residues proximal to the C-terminus of the disulfide bond between the heavy chains of the hinge region and thus is different from Fab in a small number of residues (including one or more cysteines) in the hinge region.

[0042] A “F (ab) $_2$” refers to a dimer of Fab’ that comprises two light chains and part of the two heavy chains.

[0043] 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.

[0044] 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.

[0045] Antibodies, antigen-binding moieties, 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.

[0046] Light chains are classified as either kappa or lambda (K, λ) . Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

[0047] Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VK) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CK) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen-binding site or amino-terminus of the antibody. The N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 and CK domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

[0048] As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the VK domain and VH domain, or subset of the complementarity determining regions (CDRs) , of an antibody combine to form the variable region that defines a three dimensional antigen-binding site. This quaternary antibody structure forms the antigen-binding site present at the end of each arm of the Y. More specifically, the antigen-binding site is defined by three CDRs on each of the VH and VK chains (i.e. CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2 and CDR-L3) . In some instances, e.g., certain immunoglobulin molecules derived from camelid species or engineered based on camelid immunoglobulins, a complete immunoglobulin molecule may consist of heavy chains only, with no light chains. See, e.g., Hamers-Casterman et al., Nature 363: 446-448 (1993) .

[0049] In naturally occurring antibodies, the six "complementarity determining regions" or "CDRs" present in each antigen-binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen-binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen-binding domains, referred to as "framework" regions, show less inter-molecular variability. The framework regions largely adopt a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β -sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been precisely defined (see "Sequences of Proteins of Immunological Interest, " Kabat, E., et al., U.S. Department of Health and Human Services, (1983) ; and Chothia and Lesk, J. MoI. Biol., 196: 901-917 (1987)) .

[0050] In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "complementarity determining region" ("CDR") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat et al., U. S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) and by Chothia et al., J. MoI. Biol. 196: 901-917 (1987) , which are incorporated herein by reference in their entireties. The CDR definitions according

to Kabat and Chothia include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth in the table below as a comparison. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

	Kabat	Chothia
CDR-H1	31-35	26-32
CDR-H2	50-65	52-58
CDR-H3	95-102	95-102
CDR-L1	24-34	26-32
CDR-L2	50-56	50-52
CDR-L3	89-97	91-96

[0051] Kabat et al. also defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., U. S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983) .

[0052] In addition to table above, the Kabat number system describes the CDR regions as follows: CDR-H1 begins at approximately amino acid 31 (i.e., approximately 9 residues after the first cysteine residue) , includes approximately 5-7 amino acids, and ends at the next tryptophan residue. CDR-H2 begins at the fifteenth residue after the end of CDR-H1, includes approximately 16-19 amino acids, and ends at the next arginine or lysine residue. CDR-H3 begins at approximately the thirty third amino acid residue after the end of CDR-H2; includes 3-25 amino acids; and ends at the sequence W-G-X-G, where X is any amino acid. CDR-L1 begins at approximately residue 24 (i.e., following a cysteine residue) ; includes approximately 10-17 residues; and ends at the next tryptophan residue. CDR-L2 begins at approximately the sixteenth residue after the end of CDR-L1 and includes approximately 7 residues. CDR-L3 begins at approximately the thirty third residue after the end of CDR-L2 (i.e., following a cysteine residue) ; includes approximately 7-11 residues and ends at the sequence F or W-G-X-G, where X is any amino acid.

[0053] Antibodies disclosed herein may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In another embodiment, the variable region may be condricthoid in origin (e.g., from sharks) .

[0054] As used herein, the term "heavy chain constant region" includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain constant region comprises at least one of: a CH1 domain, a hinge (e.g., upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. For example, an antigen-binding polypeptide for use in the disclosure may comprise a polypeptide chain comprising a CH1 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain; a polypeptide chain comprising a CH1 domain and a CH3 domain; a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain, or a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, a CH2 domain, and a CH3 domain. In another embodiment, a polypeptide of the disclosure comprises a polypeptide chain comprising a CH3 domain. Further, an antibody for use in the disclosure may lack at least a portion of a CH2 domain (e.g., all or part of a CH2 domain) . As set forth above, it will be understood by one of ordinary skill in the art that the heavy chain constant region may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

[0055] The heavy chain constant region of an antibody disclosed herein may be derived from different immunoglobulin molecules. For example, a heavy chain constant region of a polypeptide may comprise a CH1 domain derived from an IgG_1 molecule and a hinge region derived from an IgG_3 molecule. In another example, a heavy chain constant region can comprise a hinge region derived, in part, from an IgG_1 molecule and, in part, from an IgG_3 molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG_1 molecule and, in part, from an IgG_4 molecule.

[0056] As used herein, the term "light chain constant region" includes amino acid sequences derived from antibody light chain. Preferably, the light chain constant region comprises at least one of a constant kappa domain or constant lambda domain.

[0057] A "light chain-heavy chain pair" refers to the collection of a light chain and heavy chain that can form a dimer through a disulfide bond between the CL domain of the light chain and the CH1 domain of the heavy chain.

[0058] As previously indicated, the subunit structures and three dimensional configuration of the constant regions of the various immunoglobulin classes are well known. As

used herein, the term "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain and the term "CH1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

[0059] The "CH1 domain" (also referred to as "C1" of "H1" domain) usually extends from about amino acid 118 to about amino acid 215 (EU numbering system) .

[0060] As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain, a region in IgG corresponding to Glu216 to Pro230 of human IgG1, EU numbering system (Burton, Molec. Immunol. 22: 161-206 (1985)) . Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-Sbonds in the same positions. This hinge region is flexible, thus allowing the two N-terminal antigen-binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux et al., J. Immunol 161: 4083 (1998)) .

[0061] As used herein the term "CH2 domain" includes the portion of a heavy chain molecule that extends, e.g., from about residue 244 to residue 360 of an antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering system; and residues 231-340, EU numbering system; see Kabat et al., U. S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) . The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It is also well documented that the CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 residues.

[0062] The "CH3 domain" (also referred to as "C3" domain) comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (i.e. from about amino acid residue 341 to the C-terminal end of an antibody sequence, typically at amino acid residue 446 or 447 of an IgG, EU numbering system) .

[0063] The term "Fc region" , "Fc domain" or "fragment crystallizable region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native-sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy-chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly en-

gineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. Suitable native-sequence Fc regions for use in the antibodies described herein include human IgG1, IgG2 (IgG2A, IgG2B) , IgG3 and IgG4.

[0064] As used herein the term "disulfide bond" includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the CH1 and CK regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system) .

[0065] As used herein, the term "chimeric antibody" will be held to mean any antibody wherein the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant disclosure) is obtained from a second species. In certain embodiments the target binding region or site will be from a non-human source (e.g. mouse or primate) and the constant region is human.

[0066] "Humanized antibody" is used herein to describe an antibody that comprises heavy and light chain variable region sequences from a non-human species (e.g. a mouse) but in which at least a portion of the VH and/or VL sequence has been altered to be more "human-like, " i.e., more similar to human germline variable sequences. A "humanized antibody" is an antibody or a variant, derivative, analog, or fragment thereof, which immuno-specifically binds to an antigen of interest and which comprises a framework (FR) region having substantially the amino acid sequence of a human antibody and a complementary determining region (CDR) having substantially the amino acid sequence of a non-human antibody. As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99%identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', F (ab')$_2$, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. In an embodiment, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc) , typically that of a human immunoglobulin. In some embodiments, a humanized antibody contains the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1,

hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or humanized heavy chain.

[0067] The term “epitope” as used herein refers to the specific group of atoms or amino acids on an antigen to which an antibody or antibody moiety binds. Two antibodies or antibody moieties may bind the same epitope within an antigen if they exhibit competitive binding for the antigen.

[0068] 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. ”

[0069] 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.

[0070] 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.

[0071] As used herein, phrases such as “to a patient in need of treatment” or “asubject 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.

[0072] T-Cell Engagers of Improved Stability and Efficacy

[0073] CD3 (cluster of differentiation 3) is a protein complex and T cell co-receptor that is involved in activating both the cytotoxic T cell ($CD8^+$ naive T cells) and T helper cells ($CD4^+$naive T cells) . CD3 is composed of four distinct chains. Given its complex extracellular structure, anti-CD3 agonist antibodies are difficult to develop. For instance, certain protein engineering may improve the affinity of an anti-CD3 antibody, but that improvement may be at the cost of in vivo stability or toxicity. Indeed, current therapeutic uses of anti-CD3 antibodies are frequently associated with off-target toxicities due to CD3 activation in the absence of targeted tumor cells.

[0074] Anti-CD3 antibodies can be used to construct T cell engagers that recruit T cells to a target tumor cell. In some examples, such T cell engagers can further include an anti-4-1BB moiety. In such a T cell engager, the activities of the anti-CD3 moiety and the anti-4-1BB moiety may need to be tuned as a whole, not just in isolation. Trough screening of a great number of anti-CD3 and anti-4-1BB antibodies, and the associated T cell engager, the instant inventors have discovered certain preferred activity ranges.

[0075] In one embodiment, a multi-specific antibody is provided which can be used as a T cell engager. The multi-specific antibody, in some embodiments, includes an anti-CD3 antibody or antigen binding fragment thereof (an anti-CD3 moiety) and an anti-4-1BB antibody or antigen binding fragment thereof (an anti-4-1BB moiety) .

[0076] In one embodiment, the anti-CD3 moiety has a T cell activation activity (EC50) in the range of 0.1 to 20 nM. In one embodiment, the anti-CD3 moiety has a T cell activation activity (EC50) in the range of 0.2 to 15 nM, 0.5 to 10 nM, 0.5 to 8 nM, 1 to 10 nM, 2 to 8 nM, or 3 to 7 nM, without limitation.

[0077] In one embodiment, the anti-4-1BB moiety has a 4-1BB signaling activation activity (EC50) in the range of 0.01 to 2 nM. In one embodiment, the anti-4-1BB moiety has a 4-1BB signaling activation activity (EC50) in the range of 0.02 to 1.5 nM, 0.05 to 1.2 nM, 0.1 to 1 nM, or 0.2 to 0.8 nM, without limitation.

[0078] The activities of the anti-CD3 moiety and the anti-4-1BB moiety can be measured with various different assays known in the art and in different formats. In some embodiments, the T cell activation activity of an anti-CD3 moiety is measured with an NFAT (nuclear factor of activated T cell) signaling assay. In some embodiments, the 4-1BB signaling activation activity of an anti-4-1BB moiety is measured with an NF-κB-Reporter assay.

[0079] In some examples, the T cell activation activity of an anti-CD3 moiety is measured with an NFAT (nuclear factor of activated T cell) assay. An example NFAT assay

is performed as follows. A T cell engager that includes a candidate anti-CD3 antibody and a TAA-targeting antibody (concentration range: e.g., 1nM to 100nM) is incubated, for a period of time (e.g., 6 hours) , with Jurkat T cells (e.g., from Promega, CAT#J1601) that contain a reporter gene (e.g., luciferase) regulated by the NFAT response element (NFAT-RE) . The Jurkat-CD3-NFAT cell line is genetically modified to stably express CD3 and luciferase downstream of the response element. Luciferase expression is induced upon antibody binding to the CD3 receptor. These effector cells at a density of $2.5X10^4$ cells per well can be cocultured with $2.5X10^4$ target cells (e.g., TAA-expressing tumor cells, such as HEK293-hDLL3) (E/T Ratio=1: 1) in a white 96-well plate. Antibodies are 4-fold serially diluted at a final concentration ranging from 100 nM to 0.006 nM. Following a 6 hours of incubation at 37°C, luminescence can be obtained by adding the substrate of luciferase and measured by a microplate reader.

[0080] In some examples, the 4-1BB signaling activation activity of an anti-4-1BB moiety is measured with a 4-1BB NF-κB luciferase reporter assay. An example 4-1BB NF-κB luciferase reporter assay is performed as follows. A T cell engager that includes a candidate anti-4-1BB antibody and a TAA-targeting antibody (concentration range: e.g., 0.1nM to 100nM) is incubated, for a period of time (e.g., 6 hours) with effector cells and target cells. The H_TNFRSF9 (4-1BB) NF-κB-Reporter Jurkat cells expressing 4-1BB (Genomeditech, cat#GM-C09468) can be used as effector cells and TAA-expressing tumor cells, such as HEK293-hDLL3, can be used as target cells. H_TNFRSF9 (4-1BB) NF-κB-Reporter Jurkat cell line is genetically modified to stably express 4-1BB and luciferase downstream of a response element. Luciferase expression is induced upon antibody binding to the 4-1BB receptor. The effector cells at a density of $2.5X10^4$ cells per well can be cocultured with $2.5X10^4$ target cells (E/T Ratio=1: 1) in a white 96-well plate. Antibodies are serially diluted and added to a white 96-well assay plate, at a final concentration ranging from 100 nM to 0.006 nM. Following a 6 hours incubation at 37°C, luminescence can be obtained by adding the substrate of luciferase and measured by a microplate reader.

[0081] Engineered Anti-CD3 Antibodies

[0082] A candidate anti-CD3 antibody, 155z16 from PCT/CN2022/124968, was considered an excellent candidate anti-CD3 antibody for use in T cell engagers. During development, however, it was discovered that 155z16 was not sufficient stable when incubated at 37°C in human serum. Protein engineering, therefore, was conducted to improve the stability of the antigen, without compromising on its activities.

[0083] The sequences of 155z16 are provided in Table A1. A dipeptide site NG in the VH CDR2 might have contributed to the instability, it was contemplated. Accordingly,

mutations at either or both of these two residues were tested for their effect on improving the stability of the antibody. The tested mutations are provided in Table A2, and their corresponding VH sequences are provided in Table 2.

[0084] Table A1. Variable regions and CDR sequences of 155z16

Name	Sequence	SEQ ID NO:
155z16 VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGK GLEWMG**RVRYNGDTSYNSALKS**RLTISRDTSKNQFSLKLSSVT AADTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	1
155z16 VL	DIQMTQSPSSLSASVGDRVTITC**KASQNINKYLN**WFQQKPGKA PRLLIY**TTNNLQT**GIPSRFSGSGSGTDFTLTISSLQPEDFATY FC**LQHNSGYT**FGQGTKLEIK	2
HCDR1	SYNVH	16
HCDR2	RVRYNGDTSYNSALKS	17
HCDR3	GPYGGSSEPLFDY	18
LCDR1	KASQNINKYLN	19
LCDR2	TTNNLQT	20
LCDR3	LQHNSGYT	21

[0085] Table A2. Engineered VH CDR2 sequences

Name	HCDR2 Sequence	SEQ ID NO:
155z16-NG VH	**RVRYNGDTSYNSALKS**	17
155z16-EG VH	**RVRYEGDTSYNSALKS**	55
155z16-SG VH	**RVRYSGDTSYNSALKS**	56
155z16-TG VH	**RVRYTGDTSYNSALKS**	57
155z16-QG VH	**RVRYQGDTSYNSALKS**	58
155z16-NA VH	**RVRYNADTSYNSALKS**	59
155z16-GG VH	**RVRYGGDTSYNSALKS**	60
155z16-RG VH	**RVRYRGDTSYNSALKS**	61
155z16-IG VH	**RVRYIGDTSYNSALKS**	62
155z16-VG VH	**RVRYVGDTSYNSALKS**	63
155z16-YG VH	**RVRYYGDTSYNSALKS**	64

[0086] These mutated antibody variants had improved stability as compared to the parental antibody, for some of which the inventors considered enhancement of affinity could be beneficial. Two strategies were adopted to enhance the binding affinity of some of the engineered antibodies. Strategy one entailed engineering of the framework, and strategy two entailed substitution of the N-terminal -1Y residue (relative to the NG dipeptide) with a different amino acid (except with N) .

[0087] Both strategies resulted in further engineered antibodies with improved stability and antibody activities, as demonstrated in Examples 4. The engineered antibody

sequences are shown in Tables 4-5. The further mutated VH CDR2 sequences are summarized in Tables B and C.

[0088] The HCDR2 and variable regions were further optimized to increase the activity of the antibodies, as summarized in Table B1-B2 below.

[0089] Table B. Engineered VH CDR2

Name	HCDR2 Sequence	SEQ ID NO:
155z16YSG VH	RVRYSGDTSYNSALKS	56
155z16PSG VH	RVRPSGDTSYNSALKS	65
155z16ASG VH	RVRASGDTSYNSALKS	66
155z16WSG VH	RVRWSGDTSYNSALKS	67
155z16VSG VH	RVRVSGDTSYNSALKS	68
155z16GSG VH	RVRGSGDTSYNSALKS	69

[0090] Table C. Engineered CDR Sequences for Use in Engineered Framework

Name		HCDR2 Sequence	SEQ ID NO:
HCDR1		SYNVH	16
HCDR2	VH	RVRYNGDTSYNSALKS	17
	RG	RVRYRGDTSYNSALKS	61
	NA	RVRYNADTSYNSALKS	59
	SG	RVRYSGDTSYNSALKS	56
	TG	RVRYTGDTSYNSALKS	57
HCDR3		GPYGGSSEPLFDY	18
LCDR1		KASQNINKYLN	19
LCDR2		TTNNLQT	20
LCDR3		LQHNSGYT	21

[0091] In some embodiments, therefore, provided are antibody or antigen-binding fragment thereof having specificity to a human cluster of differentiation 3 (CD3) protein, which has excellent in vitro and in vivo stability and well-balanced activity for clinical uses, either alone, in combination with other agents, or as part of a T cell engager.

[0092] In some embodiments, the anti-CD3 antibody or fragment includes HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 of:

[0093] HCDR1: SYNVH (SEQ ID NO: 16) ,

[0094] HCDR2: RVRX$_1$X$_2$X$_3$DTSYNSALKS (SEQ ID NO: 96) ,

[0095] HCDR3: GPYGGSSEPLFDY (SEQ ID NO: 18) ,

[0096] LCDR1: KASQNINKYLN (SEQ ID NO: 19) ,

[0097] LCDR2: TTNNLQT (SEQ ID NO: 20) , and

[0098] LCDR3: LQHNSGYT (SEQ ID NO: 21) .

[0099] In some embodiments, X_1 is any amino acid except N; X_2 is any amino acid except N; and X_3 is G or A, or X_1 is any amino acid except N; X_2 is any amino acid; and X_3 is A.

[0100] In some embodiments, X_1 is Y, P, A, W, V and G, X_2 is selected from E, S, T, Q, G, R, I, V, and Y, and X_3 is G or A; or X_1 is Y, X_2 is N, and X_3 is A.

[0101] In some embodiments, X_1 is Y, X_3 is G, and X_2 is selected from E, S, T, Q, G, R, I, V, and Y, such as those illustrated in Table A2. In some embodiments, X_1 is Y, X_2 is N, and X_3 is A, such as those illustrated in Table A2. In some embodiments, X_2 is S, and X_3 is G, and X_1 is selected from P, A, W, V and G, such as those illustrated in Table B. In some embodiments, the VH CDR2 includes a sequence selected from SEQ ID NO: 55-69.

[0102] In some embodiments, VH includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 70-79, 80-84 and 85-88, or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 70-79, 80-84 and 85-88. In some embodiments, the VL includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 2 and 89 or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 2 and 89.

[0103] In some embodiments, the VH includes the VH CDR1, VH CDR2, and VH CDR3 as provided herein and includes the fragment regions of SEQ ID NO: 85. In some embodiments, the VL includes the VL CDR1, VL CDR2, and VL CDR3 as provided herein and includes the fragment regions of SEQ ID NO: 89.

[0104] In some embodiments, at least one, or two, or three, or four, or five, or six of the VH CDR1, CDR2, and CDR3 and the VL CDR1, CDR2, and CDR3 of the above are modified by one, two or three amino acid additions, deletions, substitutions, or the combinations thereof.

[0105] The CDRs, heavy chain variable regions, light chain variable regions or single heavy chain variable domains of the present disclosure can be further modified. In some embodiments, the modified heavy chain variable region, light chain variable region or single heavy chain variable domain retains at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%or 99%sequence identity and is still capable of binding to the target site.

[0106] In some embodiments, the modification is substitution at no more than one hot spot position from each of the CDRs. In some embodiments, the modification is substitution at one, two or three such hot spot positions. In one embodiment, the modification is substitution at one of the hot spot positions. Such substitutions, in some embodiments, are conservative substitutions.

[0107] In some embodiments, the anti-CD3 antibodies and antigen-binding fragments thereof, or the anti-CD3 moieties are of a format of a full-length antibody, a half-antibody, a single chain half-antibody, a Fab, a Fab' , a F (ab') 2, or a scFv.

[0108] In some embodiments, the anti-CD3 antibodies and antigen-binding fragments thereof, or the anti-CD3 moieties can be a scFv. The scFv may comprise from N-to C-terminus: VL-tether-VH or VH-tether-VL.

[0109] Anti-4-1BB Antibodies or Antigen-Binding Moieties

[0110] The present disclosure provides multi-specific antibodies containing one or more antigen-binding moieties that have binding specificity to the human 4-1BB protein. In some embodiments, the anti-4-1BB antibodies and antigen-binding fragments thereof, or the anti-4-1BB moieties are of a format of a full-length antibody, a half-antibody, a single chain half-antibody, a Fab, a Fab' , a F (ab') 2, a scFv, or a nanobody/sdAb/ VHH.

[0111] In accordance with one embodiment of the present disclosure, provided are antigen-binding moieties that include a single domain antibody (sdAb) with the CDR regions. The CDRs are summarized in Table D below (Kabat numbering) .

[0112] Table D. CDR sequences of the anti-4-1BB antigen-binding moieties

Name	Sequence		SEQ ID NO:
B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIF**SIMF**WYRQAPGKQREYVS**HITSNG RTNYADSVTG**RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAA**DDATNTRTY**WG QGTLVTVSS		3
	CDR1	SIMF	22
	CDR2	HITSNGRTNYADSVTG	23
	CDR3	DDATNTRTY	24
B125z2 VHH	EVQLVESGGGLVQPGGSLRLSCAASGFAFS**DYAIG**WFRQAPGKEREGVS**CISS SDGSTYYADSVKG**RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAT**DTTTRCPI WSYYVESDY**WGQGTLVTVSS		100
	CDR1	DYAIG	101
	CDR2	CISSSDGSTYYADSVKG	102
	CDR3	DTTTRCPIWSYYVESDY	103

[0113] In some embodiments, at least one, or two, or three, or four, or five, or six of the VH CDR1, CDR2, and CDR3 of the above are modified by one, two or three amino acid additions, deletions, substitutions, or the combinations thereof.

[0114] In one embodiments, the HCDR1 includes the amino acid sequence of SEQ ID NO: 22, the HCDR2 includes the amino acid sequence of SEQ ID NO: 23, the HCDR3 includes the amino acid sequence of SEQ ID NO: 24. In some embodiments, the VH comprises the amino acid sequence of SEQ ID NO: 3 or a peptide having at least 90% (or at least 95%, 96%, 97%, 98%, or 99%) sequence identity to the amino acid sequence of SEQ ID NO: 3.

[0115] In one embodiments, the HCDR1 includes the amino acid sequence of SEQ ID NO: 101, the HCDR2 includes the amino acid sequence of SEQ ID NO: 102, the HCDR3 includes the amino acid sequence of SEQ ID NO: 103. In some embodiments, the VH comprises the amino acid sequence of SEQ ID NO: 100 or a peptide having at least 90% (or at least 95%, 96%, 97%, 98%, or 99%) sequence identity to the amino acid sequence of SEQ ID NO: 100.

[0116] The CDRs, heavy chain variable regions, light chain variable regions or single heavy chain variable domains of the present disclosure can be further modified. In some embodiments, the modified heavy chain variable region, light chain variable region or single heavy chain variable domain retains at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%or 99%sequence identity and is still capable of binding to the target site.

[0117] In some embodiments, the modification is substitution at no more than one hot spot position from each of the CDRs. In some embodiments, the modification is substitution at one, two or three such hot spot positions. In one embodiment, the modification is substitution at one of the hot spot positions. Such substitutions, in some embodiments, are conservative substitutions.

[0118] Anti-Tumor Antigen Antibodies or Antigen-Binding Moieties

[0119] Tumor antigen includes tumor specific antigens and tumor associated antigens. “Tumor specific antigens (TSA) ” are unique to tumor cancer cells. “Tumor Associated Antigen (TAA) ” are relatively restricted to tumor cells. TSAs and TAAs typically are portions of intracellular molecules expressed on the cell surface as part of the major histocompatibility complex. TAA in its broadest sense relates to factors which are primarily, if not exclusively expressed in tumors and thus can act as potential immune-therapeutic targets for antibody-based therapy. The primary and preferably exclusive expression of TAAs in tumor tissue ensures that the therapeutic antibody mediated immune reaction will be localized to the tumor only so that adverse events and effects on pharmacokinetic behavior are observed at least not to the same extent as for therapeutic antibodies which target antigens that are expressed both in tumor and normal tissues.

[0120] Exemplary tumor antigens include, but not limited to alphafetoprotein (AFP) , melanoma associated antigen -MAGE-1, MAGE-2, MAGE-3, carcinoembryonic antigen (CEA) , Tyrosinase (Tyr) , midkin (MK) , BAGE, CASP-8, β-catenin, CA-125, CDK-1, ESO-1, gp75, gp100, MART-1, mucins (MUC-1) , MUM-1, p53, PAP, PSA, PSMA, ras, trp-1, HER-2, TRP-2, IL13R alpha, AIM-3, or NY-ESO1, C9orf112, SART1, BRAP, RTN4, GLEA2, TNKS2, KIAA0376, ING4, HSPH1, C13orf24, RBPSUH, C6orf153, NKTR, NSEP1, U2AF1L, CYNL2, TPR, SOX2, or GOLGA, alpha-actinin-4, Bage-1, BCR-ABL, Bcr-Abl fusion protein, beta-catenin, CA 125, CA 15-3 (CA 27.29\BCAA) , CA 195, CA 242, CA-50, CAM43, Casp-8, cdc27, cdk4, cd-

kn2a, CEA, coa-1, dek-can fusion protein, EBNA, EF2, Epstein Barr virus antigens, ETV6-AML1 fusion protein, HLA-A2, HLA-All, hsp70-2, KIAA0205, Mart2, Mum-1, 2, and 3, neo-PAP, myosin class I, 0S-9, pml-RARa fusion protein, Date Recue/Date Received 2021-04-16 PTPRK, K-ras, N-ras, triosephosphate isomerase, Gage 3, 4, 5, 6, 7, GnTV, Herv-K-mel, Lage-1, NA-88, NY-Eso-1/Lage-2, SP17, SSX-2, TRP2-Int2, gp100 (Pmel 17) , tyrosinase, TRP-1, TRP-2, MAGE-1, MAGE-3, RAGE, GAGE-1, GAGE-2, p15 (58) , RAGE, SCP-1, Hom/Me1-40, PRAME, p53, H-Ras, HER-2/neu, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, human papillomavirus (HPV) antigens E6 and E7, TSP-180, MAGE-4, MAGE-5, MAGE-6, p185erbB2, p180erbB-3, c-met, nm-23H1, PSA, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, 13-Catenin, Mum-1, p16, TAGE, PSMA (prostate-specific membrane antigen) , B cell maturation antigen (BCMA) , CT7, telomerase, 43-9F, 5T4, 791Tgp72, 13HCG, BCA225, BTAA, CD68\KP1, CO-029, FGF-5, G250, Ga733 (EpCAM) , HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB\70K, NY-CO-1, RCAS1, SDCCAG16, TA-90, TAAL6, TAG72, TLP, or TPS.

[0121] Other tumor antigens include, without limitation, AIM2 (absent in melanoma 2) , BMI1 (BMI1 polycomb ring finger oncogene) , COX-2 (cyclooxygenase-2) , TRP-1 (tyrosine related protein 2) TRP-2 (tyrosine related protein 2) , GP100 (glycoprotein 100) , EGFRvIII (epidermal growth factor receptor variant III) , EZH2 (enhancer of zeste homolog 2) , LICAM (human L1 cell adhesion molecule) , Livin, Livinβ, MRP-3 (multidrug resistance protein 3) , Nestin, OLIG2 (oligodendrocyte transcription factor) , SOX2 (SRY-related HMG-box 2) , ART1 (antigen recognized by T cells 1) , ART4 (antigen recognized by T cells 4) , SART1 (squamous cell carcinoma antigen recognized by T cells 1) , SART2, SART3, B-cyclin, b-catenin, Gli1 (glioma-associated oncogene homlog 1) , Cav-1 (caveolin-1) , cathepsin B, CD74 (cluster of Differentiation 74) , E-cadherin (epithelial calcium-dependent adhesion) , EphA2/Eck (EPH receptor A2/epithelial kinase) , Fra-1/Fosl 1 (fos-related antigen 1) , GAGE-1 (G antigen 1) , Ganglioside/GD2, GnT-V, β1, 6-N (acetylglucosaminyltransferase-V) , Her2/neu (human epidermal growth factor receptor 2) , Ki67 (nuclear proliferation-associated antigen of antibody Ki67) , Ku70/80 (human Ku heterodimer proteins subunits) , IL-13Ra2 (interleukin-13 receptor subunit alpha-2) , MAGE-A(melanoma-associated antigen 1) , MAGE-A3 (melanoma-associated antigen 3) , NY-ESO-1 (New York oesophageal squamos cell carcinoma 1) , MART-1 (melanoma antigen recognized by T cells) , PROX1 (prospero homeobox protein 1) , PSCA (prostate stem cell antigen) , SOX10 (SRY-related HMG-box 10) , SOX11, Survivin, UPAR (urokinase-type plasminogen activator receptor, alpha folate receptor (FRα) , αvβ6 integrin, B cell maturation antigen (BCMA) , B7-H3 (CD276) , B7-H6, carbonic anhydrase IX (CAIX) , CD16, CD19, CD20, CD22, CD30, CD33, CD37,

CD38, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD133, CD138, CD171, carcinoembryonic antigen (CEA) , claudin 6, (CLDN6) , claudin 18 isoform 2 (CLDN18.2) , C-type lectin-like molecule-1 (CLL-1) , CD2 subset 1 (CS-1) , chondroitin sulfate proteoglycan 4 (CSPG4) , cutaneous T cell lymphoma-associated antigen 1 (CTAGE1) , delta like canonical Notch ligand 3 (DLL3) , epidermal growth factor receptor (EGFR) , epidermal growth factor receptor variant III (EGFRvIII) , epithelial glycoprotein 2 (EGP2) , epithelial glycoprotein 40 (EGP40) , epithelial cell adhesion molecule (EPCAM) , ephrin type-Areceptor 2 (EPHA2) , erb-b2 receptor tyrosine kinase 4 (ERBB4) , fibroblast activation protein (FAP) , Fc Receptor Like 5 (FCRL5) , fetal acetylcholinesterase receptor (AchR) , ganglioside G2 (GD2) , ganglioside G3 (GD3) , Glypican-3 (GPC3) , EGFR family including ErbB2 (HER2) , HER2 p95, IL-10Rα, IL-13Rα2, Kappa, cancer/testis antigen 2 (LAGE-1A) , Lambda, Lewis-Y (LeY) , L1 cell adhesion molecule (L1-CAM) , melanoma antigen gene (MAGE) -A1, MAGE-A3, MAGE-A4, MAGE-A6, MAGEA10, melanoma antigen recognized by T cells 1 (MelanA or MART1) , Mesothelin (MSLN) , MUC1, MUC16, MHC class I chain related proteins A (MICA) , MHC class I chain related proteins B (MICB) , neural cell adhesion molecule (NCAM) , polysialic acid; placenta-specific 1 (PLAC1) , preferentially expressed antigen in melanoma (PRAME) , prostate stem cell antigen (PSCA) , prostate-specific membrane antigen (PSMA) , receptor tyrosine kinase-like orphan receptor 1 (ROR1) , synovial sarcoma, X breakpoint 2 (SSX2) , Survivin, tumor associated glycoprotein 72 (TAG72) , tumor endothelial marker 1 (TEM1/CD248) , tumor endothelial marker 7-related (TEM7R) , trophoblast glycoprotein (TPBG) , UL16-binding protein (ULBP) 1, ULBP2, ULBP3, ULBP4, ULBP5, ULBP6, vascular endothelial growth factor receptor 2 (VEGFR2) , and Wilms tumor 1 (WT-1) .

[0122] Anti-DLL3 Antibodies or Antigen-Binding Moieties

[0123] The present disclosure provides multi-specific antibodies containing one or more antigen-binding moieties that have binding specificity to the human DLL3 protein. In some embodiments, the anti-DLL3 antibodies and antigen-binding fragments thereof, or the anti-DLL3 moieties are of a format of a full-length antibody, a half-antibody, a single chain half-antibody, a Fab, a Fab' , a F (ab') 2, a scFv, or a nanobody/sdAb/VHH.

[0124] In accordance with one embodiment of the present disclosure, provided are antibody or antigen-binding fragments that include the heavy chain and light chain variable domains with the CDR regions. The CDRs are summarized in Table E below (Kabat numbering) .

[0125] Table E. Variable region and CDR sequences of the anti-DLL3 antibodies.

Name	Sequence	SEQ ID NO:
36B7F3 VH	QVQLQQSGPELVKPGASVKISCRASGYTFT**DHHLN**WVKQRPGQGLEWIG**WIFPGSGSDYYNERFKD**KATLTVDKSSSTAYMLLSSLTSEDSAVYFCER**WDY**WGQGTTLTVSS	4
36B7F3 VL	QIVLTQSPTIMSASPGEKVTMTC**SASSSISYMH**WYQQKPGTSPKRWIY**DTSTLAS**GVPARFSGGGSGTSYSLTISSMEAEDAATYYC**HQRSSYPLT**FGAGTKLELK	5
HCDR1	DHHLN	25
HCDR2	WIFPGSGSDYYNERFKD	26
HCDR3	WDY	27
LCDR1	SASSSISYMH	28
LCDR2	DTSTLAS	29
LCDR3	HQRSSYPLT	30
362H3D3 VH	EVQLQQSGPELLKPGASVKISCKASGYTFT**DYTMH**WVKQSHGRSLEWIG**GIHPNYGGTSYNEKFKD**KATLTVDKSSSTAYMEFRSLTSEDSAVYYCAR**WGYYGGSYWYFDV**WGTGTTVTVSS	6
362H3D3 VL	DIQMTQSPASLSVSVGETVTITC**RASENIYSNLA**WYQQKQGKSPQLLVY**AATNLAD**GVPSRFSGSGSGTQYSLKINSLQSEDFGSYYC**QHFWGTPYT**FGGGTKLEIK	7
HCDR1	DYTMH	31
HCDR2	GIHPNYGGTSYNEKFKD	32
HCDR3	WGYYGGSYWYFDV	33
LCDR1	RASENIYSNLA	34
LCDR2	AATNLAD	35
LCDR3	QHFWGTPYT	36
310P3C5 VH	EVQLQQSGPELVKPGASVKISCKASGYTFT**DFYMN**WVKQTHGKSLEWIG**DANPNNGGTTHNPRFKG**QATLTVDKSSNTAYMELRSLTSEDSAVYYCAR**GGLPGDFDY**WGQGTTLTVSS	8
310P3C5 VL	DIQMTQSSSSFSVSLGDRVTITC**KASEDIYNRLA**WYQQKPGNAPRLLIS**GVTSLET**GVPSRFSGSGSGKDYTLSITSLQTEDVATYYC**QQYWSFPWT**FGGGTKLEIK	9
HCDR1	DFYMN	37
HCDR2	DANPNNGGTTHNPRFKG	38
HCDR3	GGLPGDFDY	39
LCDR1	KASEDIYNRLA	40

LCDR2	GVTSLET	41
LCDR3	QQYWSTPWT	42
310P3C5-z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFT**DFYMN**WVKQAPGQGLEWIG**DANPNNGGTTHNPRFKG**RATLTVDKSASTAYMELRSLRSEDTAVYYCAR**GGLPGDFDY**WGQGTTVTVSS	10
310P3C5-z15 VL	DIQMTQSPSSLSASVGDRVTITC**KASEDIYNRLA**WYQQKPGKAPKLLIS**GVTSLET**GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC**QQYWSTPWT**FGGGTKVEIK	11
HCDR1	DFYMN	37
HCDR2	DANPNNGGTTHNPRFKG	38
HCDR3	GGLPGDFDY	39
LCDR1	KASEDIYNRLA	40
LCDR2	GVTSLET	41
LCDR3	QQYWSTPWT	42
129H2B9 VH	EVNLEESGGGLVQPGGSMKLSCVVSGFTFS**NYWMS**WVRQSPEKGLEWVA**EIRLKSDNYATHYAESVKG**KFTISRDDSKSRLYLQMNSLRPEDTGIYYCTS**NRFAY**WGQGTLVTVSA	12
129H2B9 VL	NIVMTQSPKSVSMSVGERVTLSC**KASENVGTYVY**WYQQKPEQSPKMMIY**GASNRYT**GVPDRFTGSGSATDFTLTIRSVQAEDLADYHC**GQSYSYPFT**FGSGTKLEIK	13
HCDR1	NYWMS	43
HCDR2	EIRLKSDNYATHYAESVKG	44
HCDR3	NRFAY	45
LCDR1	KASENVGTYVY	46
LCDR2	GASNRYT	47
LCDR3	GQSYSYPFT	48
148C3A7 VH	EVQLQQSGPELIKPGTSVKMSCKASGYTFT**TYLMH**WVKQKPGQGLEWIG**YINPYNDVTEYHEKFKD**KATLTSDKSSSTAYMELSSLTSEDSAVYYCAR**LGGYGPGWYLDV**WGAGTTVTVSS	14
148C3A7 VL	DIQMTQSPASLSASVGETVTITC**RASGSIHNYLA**WYLHKQGKSPQLLVY**NAKTLAD**GVPSRFSGSGSGTQYSLKINSLQPEDFGSYYC**HHFWSTPWT**FGGGTKLEIK	15
HCDR1	TYLMH	49
HCDR2	YINPYNDVTEYHEKFKD	50
HCDR3	LGGYGPGWYLDV	51
LCDR1	RASGSIHNYLA	52
LCDR2	NAKTLAD	53
LCDR3	HHFWSTPWT	54

[0126] In some embodiments, the VH CDR1, CDR2, and CDR3 are selected from any set of VH CDR1, CDR2, and CDR3 shown in Table E, and the VL CDR1, CDR2, and CDR3 are selected from any set of VL CDR1, CDR2, and CDR3 shown in Table E. In some

embodiments, the VH CDR1, CDR2, and CDR3 and the VL CDR1, CDR2, and CDR3 are selected from those derived from the same antigen-binding moieties.

[0127] In some embodiments, at least one, or two, or three, or four, or five, or six of the VH CDR1, CDR2, and CDR3 and the VL CDR1, CDR2, and CDR3 of the above are modified by one, two or three amino acid additions, deletions, substitutions, or the combinations thereof.

[0128] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 4, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 4, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 5, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 5.

[0129] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO:6, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 6, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 7, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 7.

[0130] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 8, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 8, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 9, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 9.

[0131] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties are humanized and comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 10, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 10, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 11, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 11.

[0132] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 12, and a light chain

variable region comprising an amino acid sequence of SEQ ID NO: 13, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 13.

[0133] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 14, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 14, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 15, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to an amino acid sequence of SEQ ID NO: 15.

[0134] Anti-CDH17 Antibodies or Antigen-Binding Moieties

[0135] The present disclosure provides multi-specific antibodies containing one or more antigen-binding moieties that have binding specificity to the human cadherin 17 (CDH17) protein. In some embodiments, the anti-CDH17 antibodies and antigen-binding fragments thereof, or the anti-CDH17 moieties are of a format of a full-length antibody, a half-antibody, a single chain half-antibody, a Fab, a Fab' , a F (ab') 2, a scFv, or a nanobody/sdAb/VHH.

[0136] In accordance with one embodiment of the present disclosure, provided are antibody or antigen-binding fragments that include the heavy chain and light chain variable domains as shown in Table 7, in which the CDR regions are highlighted (according to Kabat numbering) .

[0137] In some embodiments, the VH CDR1, CDR2, and CDR3 are selected from any set of VH CDR1, CDR2, and CDR3 of any of the anti-CDH17 VH in Table 7, and the VL CDR1, CDR2, and CDR3 are selected from any of the anti-CDH17 VL shown in Table 7. In some embodiments, the VH CDR1, CDR2, and CDR3 and the VL CDR1, CDR2, and CDR3 are selected from those derived from the same antigen-binding moieties.

[0138] In some embodiments, at least one, or two, or three, or four, or five, or six of the VH CDR1, CDR2, and CDR3 and the VL CDR1, CDR2, and CDR3 of the above are modified by one, two or three amino acid additions, deletions, substitutions, or the combinations thereof.

[0139] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties to CDH17 includes a heavy chain variable region (VH) that includes the amino acid sequence of SEQ ID NO: 104, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 104 while retaining the CDRs of SEQ ID NO: 104, and a light chain variable region (VL) that includes the amino acid sequence of SEQ ID NO: 105, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 105 while retaining the CDRs of SEQ ID NO: 105.

[0140] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties to CDH17 includes a heavy chain variable region (VH) that includes the amino acid sequence of SEQ ID NO: 106, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 106 while retaining the CDRs of SEQ ID NO: 106, and a light chain variable region (VL) that includes the amino acid sequence of SEQ ID NO: 107, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 107 while retaining the CDRs of SEQ ID NO: 107.

[0141] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties to CDH17 includes a heavy chain variable region (VH) that includes the amino acid sequence of SEQ ID NO: 108, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 108 while retaining the CDRs of SEQ ID NO: 108, and a light chain variable region (VL) that includes the amino acid sequence of SEQ ID NO: 109, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 109 while retaining the CDRs of SEQ ID NO: 109.

[0142] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties to CDH17 includes a heavy chain variable region (VH) that includes the amino acid sequence of SEQ ID NO: 110, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 110 while retaining the CDRs of SEQ ID NO: 110, and a light chain variable region (VL) that includes the amino acid sequence of SEQ ID NO: 111, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 111 while retaining the CDRs of SEQ ID NO: 111.

[0143] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties to CDH17 includes a heavy chain variable region (VH) that includes the amino acid sequence of SEQ ID NO: 110, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 110 while retaining the CDRs of SEQ ID NO: 110, and a light chain variable region (VL) that includes the amino acid sequence of SEQ ID NO: 112, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 112 while retaining the CDRs of SEQ ID NO: 112.

[0144] In certain embodiments, the antibodies and the fragments thereof, or the antigen-binding moieties to CDH17 includes a heavy chain variable region (VH) that includes the amino acid sequence of SEQ ID NO: 110, or a peptide having at least 90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 110 while retaining the CDRs of SEQ ID NO: 110, and a light chain variable region (VL) that includes the amino acid sequence of SEQ ID NO: 113, or a peptide having at least

90%, at least 95%, or at least 98%sequence identity to the amino acid sequence of SEQ ID NO: 113 while retaining the CDRs of SEQ ID NO: 113.

[0145] According to specific embodiments, the antibody is humanized. Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab' , F (ab') $_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc) , typically that of a human immunoglobulin (Jones et al., Nature, 321: 522-525 (1986) ; Riechmann et al., Nature, 332: 323-329 (1988) ; and Presta, Curr. Op. Struct. Biol., 2: 593-596 (1992)) .

[0146] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321: 522-525 (1986) ; Riechmann et al., Nature 332: 323-327 (1988) ; Verhoeyen et al., Science, 239: 1534-1536 (1988)) , by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) , wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0147] The CDRs, heavy chain variable regions, light chain variable regions or single heavy chain variable domains of the present disclosure can be further modified. In some em-

bodiments, the modified heavy chain variable region, light chain variable region or single heavy chain variable domain retains at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%or 99%sequence identity and is still capable of binding to the target site.

[0148] In some embodiments, the modification is substitution at no more than one hot spot position from each of the CDRs. In some embodiments, the modification is substitution at one, two or three such hot spot positions. In one embodiment, the modification is substitution at one of the hot spot positions. Such substitutions, in some embodiments, are conservative substitutions.

[0149] A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine) , acidic side chains (e.g., aspartic acid, glutamic acid) , uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine) , nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan) , beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine) . Thus, a nonessential amino acid residue in an immunoglobulin polypeptide is preferably replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[0150] 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.

[0151] Antibodies may be produced by a process of affinity maturation in which a modified antibody is generated that has an improvement in the affinity of the antibody for antigen, compared to an unmodified parent antibody. Affinity-matured antibodies may be produced by procedures known in the art, e.g., Marks et al., Rio/Technology 10: 779-783 (1992) ; Barbas et al. Proc Nat. Acad. Sci. USA 91 : 3809-3813 (1994) ; Schier et al. Gene 169: 147-155 (1995) ; Yelton et al. J. Immunol. 155: 1994-2004 (1995) ; Jackson et al., J. Immunol. 154 (7) : 331 0-15 9 (1995) ; and Hawkins et al, J. Mol. Biol. 226: 889-896 (1992) .

[0152] Multi-specific antibodies

[0153] An antibody provided herein is a multispecific antibody, e.g., a bispecific antibody or a tri-specific antibody. The term “multispecific antibody” as used herein refers to an

antibody comprising an antigen-binding domain that has polyepitopic specificity (i.e., is capable of binding to two, or more, different epitopes on one molecule or is capable of binding to epitopes on two, or more, different molecules) .

[0154] In some embodiments, multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different antigen binding sites (such as a bispecific antibody) or at least three different antigen binding sites (such as a tri-specific antibody) .

[0155] In certain embodiments, a multispecific antibody provided herein may be a tri-specific antibody. The term “tri-specific antibody” as used herein refers to a multi-specific antibody comprising an antigen-binding domain that is capable of binding to three different epitopes on one molecule or is capable of binding to epitopes on three different molecules. A tri-specific antibody may also be referred to herein as having “triple specificity” or as being “triple specific” . Exemplary tri-specific antibodies may bind both CD3 and any other antigen.

[0156] In certain embodiments, one of the binding specificities is for CD3, the second is for 4-1BB, and the third is for a tumor antigen. Tri-specific antibodies may also be used to localize cytotoxic agents to cells which express the tumor antigen.

[0157] In certain embodiments, one of the binding specificities is for CD3, the second is for 4-1BB, and the third is for a tumor specific antigen (TSA) or a tumor specific antigen (TSA) . Tri-specific antibodies may also be used to localize cytotoxic agents to cells which express the TAA or the TSA.

[0158] Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, Nature 305: 537 (1983) , WO 93/08829, and Traunecker et al, EMBO J. 10: 3655 (1991)) , and “knob-in-hole” engineering (see, e.g., U. S. Patent No. 5, 731, 168, WO2009/089004, US2009/0182127, US2011/0287009, Marvin and Zhu, Acta Pharmacol. Sin. (2005) 26 (6) : 649-658, and Kontermann (2005) Acta Pharmacol. Sin., 26: 1-9) .

[0159] The present disclosure provides multi-specific antibody platform comprising an anti-CD3 moiety, an anti-4-1BB moiety and a third antigen-binding moiety that can be an anti-TAA or TSA moiety.

[0160] In certain embodiments, the anti-CD3 moiety provided herein can be a format of a Fab’ or a scFv. In certain embodiments, the scFv comprises from N-to C-terminus: VL-tether-VH, or VH-tether-VL.

[0161] The Fab’ or scFv can be fused to N-terminus of a Fc domain. In certain embodiments, when the anti-CD3 moiety is a Fab’ fused to a Fc domain, such moiety forms a half antibody. In certain embodiments, when the anti-CD3 moiety is a scFv fused to a Fc domain, such moiety forms a single chain half antibody.

[0162] In certain embodiments, the anti-4-1BB moiety provided herein can be a format of a single domain antibody (sdAb) /VHH/nanobody. The 4-1BB moiety can be fused to C-terminus of a Fc domain.

[0163] In certain embodiments, the anti-tumor antigen moiety provided herein can be a format of a Fab' or a scFv. In certain embodiments, the scFv comprises from N-to C-terminus: VL-tether-VH, or VH-tether-VL.

[0164] The Fab' or scFv can be fused to N-terminus of a Fc domain. In certain embodiments, when the anti-tumor antigen moiety is a Fab' fused to a Fc domain, such moiety forms a half antibody. In certain embodiments, when the anti-tumor antigen moiety is a scFv fused to a Fc domain, such moiety forms a single chain half antibody.

[0165] In certain embodiments, the anti-CD3 moiety provided herein is of a format of scFv, the anti-4-1BB moiety provided herein is of a format of nanobody, and the anti-tumor antigen moiety provided herein is of a format of Fab' . The anti-CD3 moiety can be fused to N-terminus of a Fc domain, the anti-tumor antigen moiety can be fused to N-terminus of a pairing Fc domain, and the anti-4-1BB moiety can be fused to C-terminus of the Fc domain and C-terminus of the pairing Fc domain.

[0166] In certain embodiments, the anti-CD3 moiety provided herein is of a format of Fab' , the anti-4-1BB moiety provided herein is of a format of nanobody, and the anti-tumor antigen moiety provided herein is of a format of scFv. The anti-CD3 moiety can be fused to N-terminus of a Fc domain, the anti-tumor antigen moiety can be fused to N-terminus of a pairing Fc domain, and the anti-4-1BB moiety can be fused to C-terminus of the Fc domain and C-terminus of the pairing Fc domain.

[0167] In certain embodiments, the anti-CD3 moiety is fused to the Fc domain via a linker. In certain embodiments, the anti-antitumor moiety is fused to the Fc domain via a linker. In certain embodiments, the anti-4-1BB moiety is fused to the Fc domain via a linker.

[0168] In certain embodiments, the anti-tumor antigen moiety of the present disclosure is anti-DLL3 moiety provided herein. In certain embodiments, the anti-tumor antigen moiety of the present disclosure is anti-CDH17 moiety provided herein.

[0169] Linkers within the scope of the present disclosure are characterized in terms of amino acid content, length, rigidity and secondary structure. Linkers within the scope of the present disclosure separate a function polypeptide and another functional polypeptide and allow proper folding and functioning of each domain. In this manner, a linker can be tailored to the particular functional polypeptide and the other functional polypeptide. According to one aspect, functional independence of the structural and fused (heterologous) domains is maximized by a suitable linker to limit steric interference between domains during the export and assembly processes of the bacterial cell.

[0170] Linkers within the scope of the present disclosure include amino acid residues. The amino acid residues may be any of the naturally occurring amino acid residues. Amino acid residues may also be synthetic amino acids known to those of skill in the art. Representative amino acids which may be used in linkers include Glycine, Alanine, Valine, Leucine, Isoleucine, Serine, Cysteine, Selenocysteine, Threonine, Methionine, Proline, Phenylalanine, Tyrosine, Tryptophan, Histidine, Lysine, Arginine, Aspartate, Glutamate, Asparagine, and Glutamine.

[0171] In certain embodiments, a scFv is fused to a Fc domain via a linker. In certain embodiments, the linker includes a hinge variant of "EPKSADKTHT" (SEQ ID NO: 97) .

[0172] In some embodiments, the linker has a length of about three to about fifty amino acids, for example, at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 24, 48 amino acids.

[0173] In some embodiments at least 50%of the amino acids comprised by the linker sequence are glycine or serine residues, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or more are glycine or serine residues. In some embodiments, the linker sequence consists of glycine and serine residues. In some embodiments, the linker may be (GS) n or (GGGGS) n (SEQ ID NO: 90) , wherein the n is 0-8. In some embodiments, the linker comprises an amino acid sequence of GGGGSGGGGSGGGGS (SEQ ID NO: 91) . In some embodiments, the linker comprises an amino acid sequence of GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 92) .

[0174] In certain embodiments, a nanobody is fused to a Fc domain via a linker. The linker comprises an amino acid sequence of GGGGSGGGGSGGGGS (SEQ ID NO: 91) .

[0175] In certain embodiments, the tether in the scFv that connecting the VH and VL is a linker comprising an amino acid sequence of GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 92) .

[0176] Usually, two identical Fc regions form a homodimer. However, two different Fc regions can form a heterodimer with mutations to the one or two individual chain via, for example, a knob-into-hole (KIH) , a disulfide bond (-S-S-) , or via hydrophobic interaction, electrostatic interaction, hydrophilic interaction, or increased flexibility. The Fc region pairing, can be achieved by forming a heterodimer via knob-into-hole (KIH) , hydrophobic interaction, electrostatic interaction, hydrophilic interaction, or increased flexibility.

[0177] In some embodiments, the Fc domain provided herein comprises a knob mutation and the pairing Fc domain comprises hole mutation (s) , or vice versa.

[0178] The term "knob-into-hole" or "KIH" technology as used herein refers to the technology directing the pairing of two polypeptides together in vitro or in vivo by introducing a protuberance (knob) into one polypeptide and a cavity (hole) into the other

polypeptide at an interface in which they interact. For example, KIHs have been introduced in the Fc: Fc binding interfaces, CL: CH1 interfaces or VH/VL interfaces of antibodies (see, e.g., US 201 1/0287009, US2007/0178552, WO 96/02701 1, WO 98/050431, Zhu et al, 1997, Protein Science 6: 781-788, and WO2012/106587) . In some embodiments, KIHs drive the pairing of two different heavy chains together during the manufacture of multispecific antibodies. For example, multispecific antibodies having KIH in their Fc regions can further comprise single variable domains linked to each Fc region, or further comprise different heavy chain variable domains that pair with similar or different light chain variable domains. KIH technology can also be used to pair two different receptor extracellular domains together or any other polypeptide sequences that comprises different target recognition sequences (e.g., including affibodies, peptibodies and other Fc fusions) .

[0179] The term “knob mutation” as used herein refers to a mutation that introduces a protuberance (knob) into a polypeptide at an interface in which the polypeptide interacts with another polypeptide. In some embodiments, the other polypeptide has a hole mutation.

[0180] The term “hole mutation” as used herein refers to a mutation that introduces a cavity (hole) into a polypeptide at an interface in which the polypeptide interacts with another polypeptide. In some embodiments, the other polypeptide has a knob mutation.

[0181] In some embodiments, a knob mutation in a Fc domain comprises T366W (EU numbering) . In some embodiments, a hole mutation in a Fc domain comprises T366S, L368A, and Y407V (EU numbering) .

[0182] Exemplary sequences of the mutated KIH Fc regions are summarized in Table F below.

[0183] Table F. IgG Fc region sequences

Fc variant	**Sequence**	**SEQ ID NO:**
IgG Fc (hole: T366S/L368	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV	98
A/Y407V)	SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**S**C**A**VKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	
IgG Fc(knob: T366W)	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**CLVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	99

[0184] In certain embodiments, the Fc domain and the pairing Fc domain form a heterodimer having a knob-into-hole (KIH) : The Fc domain of SEQ ID NO: 98 (or a variant thereof having at least about 80%, 85%, 87%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%identity thereof) can be paired with the pairing Fc domain of SEQ ID NO: 99 (or a variant thereof having at least about 80%, 85%, 87%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%identity thereof) .

[0185] In certain embodiments, the anti-tumor antigen moiety is fused to a Fc domain with a hole mutation, and the anti-CD3 moiety is fused to a pairing Fc domain with a knob mutation.

[0186] In certain embodiments, the anti-tumor antigen moiety is fused to a Fc domain with a knob mutation, and the anti-CD3 moiety is fused to a pairing Fc domain with a hole mutation.

[0187] In certain embodiments, the multispecific antibody provided herein comprises an anti-CD3 moiety, an anti-4-1BB moiety, an anti-DLL3 or anti-CDH17 moiety. The anti-CD3 moiety is a scFv, the anti-4-1BB moiety is a nanobody, and the anti-DLL3 or anti-CDH17 moiety is a Fab' . The anti-CD3 moiety is fused to N-terminus of a Fc domain, the anti-DLL3 or anti-CDH17 moiety is fused to N-terminus of a pairing Fc domain, and the anti-4-1BB moiety is fused to C-terminus of the Fc domain and to C-terminus of the pairing Fc domain (see the format of FIG. 1A) .

[0188] In certain embodiments, the anti-CD3 moiety is fused to the N-terminus of the Fc domain via a linker 1, the anti-4-1BB moiety is fused to the C-terminus of the Fc domain and the C-terminus of the pairing Fc domain via a linker 2, respectively. In certain embodiments, the Fc domain comprises an amino acid sequence of SEQ ID NO: 94, and the pairing Fc domain comprises an amino acid sequence of SEQ ID NO: 93. In certain embodiments, linker 1 includes a hinge variant of "EPKSADK-THT" (SEQ ID NO: 97) . In certain embodiments, linker 2 comprises an amino acid sequence of SEQ ID NO: 91.

[0189] Multispecific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1) ; cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4, 676, 980, and Brennan et al, Science, 229: 81 (1985)) ; using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny et al, J. Immunol, 148 (5) : 1547-1553 (1992)) ; using "diabody" technology for making bispecific antibody fragments (see, e.g., Hollinger et al, Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993)) ; and using single-chain Fv (sFv) dimers (see, e.g., Gruber et al, J. Immunol, 152: 5368 (1994)) ; and preparing trispecific antibodies as described, e.g., in Tutt et al. J. Immunol. 147: 60 (1991) .

[0190] In some embodiments, the Fc region is an Fc domain, namely, an Fc region possessing some or all effector functions, including for example complement-dependent cytotoxicity (CDC) and antibody-dependent cell mediated cytotoxicity (ADCC) functions. In some embodiments, the Fc domain is derived from IgG1 or IgG3.

[0191] In some embodiments, one or more amino acid modifications may be introduced into the Fc domain, thereby generating an Fc domain variant. The Fc domain variant may comprise a human Fc domain sequence (e.g., derived from human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions. In some embodiments, the Fc domain variant change one or more functional and/or pharmacokinetic properties of the antibody.

[0192] The Fc region can also be engineered to enhance or eliminate effector function. IgG antibodies can induce direct anti-tumor effects by way of indirect anti-tumor effects via the Fc-mediated effector functions that engage other immune cells or killer mechanisms. “Effector functions” or “antibody effector functions” as used herein refer to biological activities attributable to the binding of Fc region of an antibody to its effectors such as C1 complex and Fc receptor (FcγRIIa or FcγRIIIa) . Exemplary effector functions include: complement dependent cytotoxicity (CDC) induced by interaction of antibodies and C1q on the C1 complex; antibody-dependent cell-mediated cytotoxicity (ADCC) induced by binding of Fc region of an antibody to Fc receptor on an effector cell; and antibody dependent cell mediated phagocytosis (ADCP) , where nonspecific cytotoxic cells that express Fcγ receptors (FcγRs) recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell.

[0193] In some embodiments, the Fc regions provided herein are maintained or improved with effector function, such as ADCC and/or CDC.

[0194] Among the four IgG subclasses, IgG1 and IgG3 induce the strongest Fc-effector functions. However, since IgG1 has the longest half-life and is more stable than IgG3, most therapeutic antibodies with Fc-mediated functions are of IgG1 isotype.

[0195] IgG2 and IgG4 isotypes have significantly lower binding affinity to FcγRs. Recent evidence suggests that the IgG2 isotype is not completely devoid of effector function, whereas the IgG4 isotype can undergo in vivo Fab arm exchange leading to bispecific antibody and off-target effects.

[0196] In some embodiments, the Fc domain is derived from human IgG1. In some embodiments, the human IgG1 derived Fc domain does not comprise L234A mutation and/or a L235A mutation. In some embodiments, the human IgG1 derived Fc domain comprises a L234A mutation and/or a L235A mutation. In some embodiments, the Fc domain is derived from human IgG3. In some embodiments, the Fc domain is derived from human IgG2 or IgG4. In some embodiments, the Fc domain is derived from

human IgG4. In some embodiments, the human IgG4 derived Fc domain comprises a S228P, F234A, and/or a L235A mutation. In some embodiments, the human IgG4 derived Fc domain does not comprise a S228P, F234A, and/or a L235A mutation.

[0197] In some embodiments, alterations are made in the Fc domain that result in altered (i.e., either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC) , e.g., as described in US Patent No. 6, 194, 551, WO 99/51642, and Idusogie et al. J. Immunol. 164: 4178-4184 (2000) .

[0198] In some embodiments, the multispecific construct comprises a variant Fc domain comprising one or more amino acid substitutions which alters half-life and/or changes binding to the neonatal Fc receptor (FcRn) . Antibodies with increased half-lives and improved binding to the neonatal Fc receptor (FcRn) , which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117: 587 (1976) and Kim et al., J. Immunol. 24: 249 (1994)) , are described in US2005/0014934A1 (Hinton et al.) . Those antibodies comprise an Fc region with one or more substitutions therein which alters binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues, e.g., substitution of Fc region residue 434 (US Patent No. 7, 371, 826) .

[0199] 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) .

[0200] Antibodies, variants, or derivatives thereof of the disclosure include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from binding to the epitope. For example, but not by way of limitation, the antibodies can be modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the antibodies may contain one or more non-classical amino acids.

[0201] In some embodiments, the antibodies may be conjugated to therapeutic agents, prodrugs, peptides, proteins, enzymes, viruses, lipids, biological response modifiers, pharmaceutical agents, or PEG.

[0202] The antibodies may be conjugated or fused to a therapeutic agent, which may include detectable labels such as radioactive labels, an immunomodulator, a hormone, an enzyme, an oligonucleotide, a photoactive therapeutic or diagnostic agent, a cytotoxic

agent, which may be a drug or a toxin, an ultrasound enhancing agent, a non-radioactive label, a combination thereof and other such agents known in the art.

[0203] Polynucleotides Encoding the Antibodies and Methods of Preparing the Antibodies

[0204] 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.

[0205] The humanized antibody may be designed to minimize unwanted immunological response toward rodent anti-human antibodies, which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients. The humanized antibody may have one or more amino acid residues introduced into it from a source that is non-human. These non-human residues are often referred to as "import" residues, which are typically taken from a variable domain. Humanization may be performed by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. For example, see U.S. Patent No. 4,816,567, the contents of which are herein incorporated by reference. The humanized antibody may be a human antibody in which some hypervariable region residues, and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to those described in U.S. Pat. Nos. 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; and 4,816,567.

[0206] Treatment

[0207] As described herein, the antibodies, variants or derivatives of the present disclosure may be used in certain treatment methods.

[0208] 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) .

[0209] 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.

[0210] In some embodiments, provided are uses of the antibodies of the present disclosure in the manufacture of a medicament for treating a cancer in a patient in need thereof.

[0211] In some embodiments, provided are the antibodies of the present disclosure for use in the treatment of a cancer in a patient in need thereof.

[0212] Unless stated otherwise, the terms “cancer” and “tumor” are used interchangeably herein. These terms in particular relate but are not limited to cancer and tumors selected from the group comprising basal cell carcinoma; bladder cancer; bone cancer such as osteosarcoma; central nervous system tumors such as cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma, pineal parenchymal tumors of intermediate differentiation, primitive neuroectodermal tumors, pineoblastoma and spinal cord tumors; Burkitt's lymphoma; breast cancer; cervical cancer; chronic myelogenous leukemia; colon cancer; rectal cancer; colorectal cancer; esophageal cancer; Ewing family of tumors; extrahepatic bile duct cancer; gallbladder cancer; gastrointestinal stromal tumor (GIST) ; glioma; head and neck cancer; islet cell tumors; Kaposi sarcoma; leukemia; liver cancer; lymphoma; Hodgkin's lymphoma; non-Hodgkin's lymphoma; T-cell lymphoma; mesothelioma; multiple myeloma/plasma cell neoplasm; myeloid leukemia; multiple myeloma; nasopharyngeal cancer; neuroblastoma; small cell lung cancer; non-small cell lung cancer; oropharyngeal cancer; osteosarcoma; ovarian cancer; pancreatic cancer; parathyroid cancer; penile cancer; pharyngeal cancer; phaeochromocytoma; pituitary tumor; prostate cancer; renal cell cancer; respiratory tract carcinoma; retinoblastoma; skin cancer (melanoma) ; small intestine cancer; soft tissue sarcoma; squamous cell carcinoma; squamous neck cancer; stomach (gastric) cancer; testicular cancer; throat cancer; thyroid cancer; transitional cell cancer of the renal pelvis and ureter; urethral cancer; uterine cancer; vaginal cancer; vulvar cancer and Wilms tumor.

[0213] A specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the particular antibodies, variant or derivative thereof used, the patient’s age, body weight, general health, sex, and diet, and the time of administration, rate of excretion, drug combination, and the severity of the particular disease being treated. Judgment of such factors by medical caregivers is within the ordinary skill in the art. The amount will also depend on the individual patient to be treated, the route of administration, the type of formulation, the characteristics of the

compound used, the severity of the disease, and the desired effect. The amount used can be determined by pharmacological and pharmacokinetic principles well known in the art.

[0214] Methods of administration of the antibodies, variants or include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The antigen-binding polypeptides or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Thus, pharmaceutical compositions containing the antigen-binding polypeptides of the disclosure may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch) , bucally, or as an oral or nasal spray.

[0215] The term “parenteral” as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intra-articular injection and infusion.

[0216] Administration can be systemic or local. In addition, it may be desirable to introduce the antibodies of the disclosure into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

[0217] It may be desirable to administer the antigen-binding polypeptides or compositions of the disclosure locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction, with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the disclosure, care must be taken to use materials to which the protein does not absorb.

[0218] Compositions

[0219] 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) .

[0220] 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. Phar-

macopeia 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.

[0221] 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.

[0222] 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.

[0223] The compounds of the disclosure can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0224] EXAMPLES

[0225] Example 1: 310P3C5-TOPAbody Had Robust in vitro and in vivo T cell Activation Activities

[0226] Cell lysis induced by DLL3-CD3-4-1BB trispecific antibodies

[0227] We generated trispecific antibodies by incorporating different anti-DLL3 antibody sequences into a DLL3-CD3-4-1BB trispecific format. This trispecific antibody is designed to bind to three targets, the DLL3 protein on a tumor cell, and CD3 and 4-1BB on T cells. The antibody's target-binding domains are illustrated in FIG. 1A. It includes a 1+1 format of anti-TAA/CD3 portion, with two anti-4-1BB nanobodies fused to the C-terminus of the Fc fragment. The DLL3 antibody sequences used in the trispecific antibodies are listed below (Table 1) . An example anti-CD3 sequence is 155z16, and two example anti-4-1BB nanobodies are B31z2 and B125z2. The trispecific antibodies tested herein are referred to as TOPAbodies (T cell Omnipotent Precise Agonism Antibodies) , or simply "TOPA. "

[0228] The human DLL3 protein has a distinct extracellular structure that consists of a DSL domain, six EGF like repeats (EGF1 through EGF6) and an unstructured membrane proximal extracellular region (MPER) adjacent the transmembrane domain. The anti-DLL3 antibodies tested herein target the MPER domain, EGF3 domain, or the EGF6 domain (Table 1) .

[0229] Table 1: CD3/4-1BB/DLL3 antibody sequences in the trispecific antibodies

Name	Sequence	DLL3 domain	SEQ ID NO:
155z16 VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVR QPPGKGLEWMG**RVRYNGDTSYNSALKS**RLTISRDTSKN QFSLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	–	1
155z16 VL	DIQMTQSPSSLSASVGDRVTITC**KASQNINKYLN**WFQQ KPGKAPRLLIY**TTNNLQT**GIPSRFSGSGSGTDFTLTIS SLQPEDFATYFC**LQHNSGYT**FGQGTKLEIK	–	2
B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIF**SIMF**WYRQA PGKQREYVS**HITSNGRTNYADSVTG**RFTISRDNSKNTL YLQMNSLRAEDTAVYYCAA**DDATNTRTY**WGQGTLVTVS S	–	3
B125z2 VHH	EVQLVESGGGLVQPGGSLRLSCAASGFAFS**DYAIG**WFR QAPGKEREGVS**CISSSDGSTYYADSVKG**RFTISRDNSK NTLYLQMNSLRAEDTAVYYCAT**DTTTRCPIWSYYVESD Y**WGQGTLVTVSS	–	100
36B7F3 VH	QVQLQQSGPELVKPGASVKISCRASGYTFT**DHHLN**WVK QRPGQGLEWIG**WIFPGSGSDYYNERFKD**KATLTVDKSS STAYMLLSSLTSEDSAVYFCER**WDY**WGQGTTLTVSS	MPER	4

36B7F3 VL	QIVLTQSPTIMSASPGEKVTMTCSASSSISYMHWYQQK PGTSPKRWIYDTSTLASGVPARFSGGGSGTSYSLTISS MEAEDAATYYCHQRSSYPLTFGAGTKLELK	MPER	5
362H3D3 VH	EVQLQQSGPELLKPGASVKISCKASGYTFTDYTMHWVK QSHGRSLEWIGGIHPNYGGTSYNEKFKDKATLTVDKSS STAYMEFRSLTSEDSAVYYCARWGYYGGSYWYFDVWGT GTTVTVSS	MPER	6
362H3D3 VL	DIQMTQSPASLSVSVGETVTITCRASENIYSNLAWYQQ KQGKSPQLLVYAATNLADGVPSRFSGSGSGTQYSLKIN SLQSEDFGSYYCQHFWGTPYTFGGGTKLEIK	MPER	7
310P3C5 VH	EVQLQQSGPELVKPGASVKISCKASGYTFTDFYMNWVK QTHGKSLEWIGDANPNNGGTTHNPRFKGQATLTVDKSS NTAYMELRSLTSEDSAVYYCARGGLPGDFDYWGQGTTL TVSS	EGF6	8
310P3C5 VL	DIQMTQSSSSFSVSLGDRVTITCKASEDIYNRLAWYQQ KPGNAPRLLISGVTSLETGVPSRFSGSGSGKDYTLSIT SLQTEDVATYYCQQYWSFPWTFGGGTKLEIK	EGF6	9
310P3C5-z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFT**DFYMN**WVK QAPGQGLEWIG**DANPNNGGTTHNPRFKG**RATLTVDKSA STAYMELRSLRSEDTAVYYCAR**GGLPGDFDY**WGQGTTV TVSS	EGF6	10
310P3C5-z15 VL	DIQMTQSPSSLSASVGDRVTITC**KASEDIYNRLA**WYQQ KPGKAPKLLIS**GVTSLET**GVPSRFSGSGSGTDFTFTIS SLQPEDIATYYC**QQYWSFPWT**FGGGTKVEIK	EGF6	11
129H2B9 VH	EVNLEESGGGLVQPGGSMKLSCVVSGFTFSNYWMSWVR QSPEKGLEWVAEIRLKSDNYATHYAESVKGKFTISRDD SKSRLYLQMNSLRPEDTGIYYCTSNRFAYWGQGTLVTV SA	EGF3	12
129H2B9 VL	NIVMTQSPKSVSMSVGERVTLSCKASENVGTYVYWYQQ KPEQSPKMMIYGASNRYTGVPDRFTGSGSATDFTLTIR SVQAEDLADYHCGQSYSYPFTFGSGTKLEIK	EGF3	13
148C3A7 VH	EVQLQQSGPELIKPGTSVKMSCKASGYTFTTYLMHWVK QKPGQGLEWIGYINPYNDVTEYHEKFKDKATLTSDKSS STAYMELSSLTSEDSAVYYCARLGGYGPGWYLDVWGAG TTVTVSS	EGF3	14
148C3A7 VL	DIQMTQSPASLSASVGETVTITCRASGSIHNYLAWYLH KQGKSPQLLVYNAKTLADGVPSRFSGSGSGTQYSLKIN SLQPEDFGSYYCHHFWSTPWTFGGGTKLEIK	EGF3	15

[0230] Note: MPER: Membrane Proximal External Region; EGF: Epidermal Growth Factor.

[0231] To evaluate the cytotoxicity mediated by DLL3-CD3-4-1BB trispecific antibodies with DLL3 antibodies targeting different DLL3 epitopes, the lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells was examined by Cytotoxicity Detection KitPLUS (Roche) . AMG757, a BiTE targeting DLL3 on tumor cells and CD3 on T cells with extended half-life, was used as the benchmark in this

study. AMG757 has demonstrated encouraging response durability in patients with relapsed/refractory SCLC in the phase I study DeLLphi-300 (NCT03319940) .

[0232] Briefly, human PBMC at a density of $2X10^5$ cells per well were cultured in a 96-well plate. The SHP77 target cell line, which exhibits low levels of DLL3, was seeded at a density of $2X10^4$ per well. Thus, E: T ratio is 10: 1. Antibodies were 4-fold serially diluted and added to a 96-well assay plate, at a final concentration ranging from 100 nM to 0.0003 nM. Following a 72 hours incubation at 37°C, the supernatants were collected for further analysis. The LDH were examined following the manufacturer's protocol. An ELISA reader was used to measure the absorbance of the samples at 492 nm.

[0233] As shown in FIG. 1B, the 310P3C5-155z16-B31z2 TOPAbody (or simply "310P3C5-TOPAbody") exhibited robust, and the highest T cell cytotoxicity among all candidate TOPAbodies with distinct binding epitopes and AMG757.

[0234] TNFα secretion induced by DLL3-CD3-4-1BB trispecific antibodies

[0235] TNFα secretion induced by the DLL3-CD3-4-1BB trispecific antibodies was examined by LANCE (Perkin Elmer) .

[0236] Briefly, human PBMCs at a density of $2X10^5$ cells per well were cultured in a 96-well plate. The SHP77 target cell line, which exhibits low levels of DLL3, was seeded at a density of $2X10^4$ per well. Thus, E: T ratio is 10: 1. Antibodies were 4-fold serially diluted and added to a 96-well plate, at a final concentration ranging from 100 nM to 0.098 nM. Following a 72 hours incubation at 37°C, the supernatants were collected for further analysis. TNFα was measured by TR-FRET assay, followed by manufacturer's protocol. Lance signal was detected using Envision. Dual emission from 615nM (channel 1) and 665nM (channel 2) was acquired. A standard curve was generated by plotting the LANCE counts (Ch1/Ch2 ratio versus the concentration of standards) . Data were analyzed using a nonlinear regression, 4-parameter logistic equation.

[0237] Like in FIG. 1B, 310P3C5-155z16-B31z2 induced the most potent TNFα secretion among all candidate TOPAbodies (FIG. 1C) .

[0238] Example 2: 310P3C5-TOPAbody Showed Higher in vivo Efficacy than Clinical Benchmark AMG757

[0239] Then we tested the in vivo efficacy of 310P3C5-TOPA in treating B16F10-hDLL3 expressing tumors. B16F10-hDLL3 cells were inoculated into CD3/4-1BB humanized mice. When the average tumor size reached 100 mm^3, 310P3C5-TOPA was administered into the animals at 3 different dosing levels, with a dosing frequency of twice a week. AMG757 was again used as the benchmark. We compared the efficacy of AMG757 to 310P3C5-TOPAbody in the same animal model at equal molar doses.

[0240] The results in FIG. 2A showed that the treatment with 310P3C5-TOPAbody led to a significantly higher inhibition in tumor size as compared to the benchmark AMG757. Consistent with the observed tumor inhibition, the survival curve of the 310P3C5-TOPAbody treated group demonstrated superior outcomes to benchmark AMG757, as illustrated in FIG. 2B.

[0241] To determine the cellular mechanism underlying the trispecific antibody treatment, we profiled tumor-infiltrating and blood immune cells in a separate study with the same administration group and tested the change of immune cell populations by FACS analysis after single dose at 10mpk. As shown in FIG. 2C-E, all the treatments significantly induced lymphocyte infiltration and expansion of T cell subtypes in the tumor sites after 168hr of 1st injection. CD3$^+$ T cells and CD8$^+$ T cells were increased significantly, and T cell infiltration rates were significantly higher in 310P3C5-TOPAbody treated group compared to the AMG757-treated group. Of note, significant increases of CD25 activation in CD4$^+$ cells in blood samples were observed in AMG757-treated group (FIG. 2E-H) . The results indicate that 310P3C5-TOPAbody has a superior safety profile to AMG757 in terms of peripheral immune activation, primarily due to its stringent TAA-dependent activity.

[0242] These data collectively confirmed 310P3C5-TOPAbody's superior anti-tumor efficacy over AMG757, which was supported by a stronger effect in intra-tumor cytotoxic T cell expansion.

[0243] Example 3: CD3 Antibody Engineering To Mitigate Serum Instability

[0244] The anti-CD3 portion of the DLL3-TOPAbody molecules includes the 155z16 sequence. It was observed, however, that 155z16 was not sufficiently stable when incubated at 37 ℃ in human serum (FIG. 7A and 7D) . Sequence analysis revealed the presence of a single NG dipeptide site within the VH CDR2 region of the antibody. Therefore, we endeavored to enhance its serum stability by substituting the asparagine with other amino acids. All the engineered 155z16-Fab antibodies adopted the same light chain (Table 2) .

[0245] Table 2: Engineered sequences based on 155z16

Antibody	Sequence	SEQ ID NO:

155z16-NG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[N]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	1
155z16-EG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[E]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	70
155z16-SG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[S]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	71
155z16-TG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[T]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	72
155z16-QG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[Q]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	73
155z16-NA VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[N]**A**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	74
155z16-GG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[G]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	75
155z16-RG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[R]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	76
155z16-IG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[I]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	77
155z16-VG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[V]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	78
155z16-YG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKG LEWMG**RVRY**[Y]**G**DTSYNSALKSRLTISRDTSKNQFSLKLSSVTAA DTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	79
155z16 VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQKPGKAP RLLIYTTNNLQTGIPSRFSGSGSGTDFTLTISSLQPEDFATYFC LQHNSGYTFGQGTKLEIK	2

[0246] ELISA Testing

[0247] To evaluate the binding activity of the CD3 mutated antibodies, the CD3 VH CDR2 mutated antibodies were subjected to ELISA testing.

[0248] Briefly, microtiter plates were coated with human CD3E or CD3E&3G protein at 1 μg/ml in PBS, 100μl/well at 4℃ overnight, then blocked with 150μl/well of 1%BSA. Five-fold dilutions of the humanized antibodies starting from 20 nM were added to each well and incubated for 1 hour at 37℃. The plates were washed with PBS/Tween and then incubated with anti-Human IgG (H&L) (GOAT) Antibody Peroxidase Conjugated for 30 mins at 37℃. After washing, the plates were developed with TMB

substrate and analyzed by spectrophotometer at OD 450nm. As shown in FIG. 3A-B, most mutated CD3 antibodies maintained the binding activity against CD3E and CD3E&G of the parent antibody.

[0249] Affinity

[0250] The binding of the humanized antibodies to the recombinant CD3E protein (human CD3-his tag) was tested with Biacore using a capture method. The humanized antibodies were captured using Protein A chip. A serial dilution of human CD3-his tag protein was injected over captured antibody for 3 mins at a flow rate of 30 μl/min. The antigen was allowed to associate for 120s and dissociate for 300s. All the experiments were carried out on a Biacore 8K. Data analysis was carried out using Biacore 8K evaluation software. As shown in Table 3, the mutant CD3 antibodies had similar binding kinetics against human CD3E as the parent antibody.

[0251] Table 3. Affinity measured by Biacore

Molecule	Analyte	ka (1/Ms)	kd (1/s)	KD (M)
155z16-Fab-NG	Human CD3E-His	5.87E+04	7.16E-04	1.22E-08
155z16-Fab-EG	Human CD3E-His	1.75E+05	5.36E-03	3.07E-08
155z16-Fab-SG	Human CD3E-His	9.06E+04	1.23E-03	1.36E-08
155z16-Fab-TG	Human CD3E-His	9.25E+04	1.50E-03	1.63E-08
155z16-Fab-QG	Human CD3E-His	9.00E+04	1.65E-03	1.83E-08
155z16-Fab-NA	Human CD3E-His	8.39E+04	1.30E-03	1.55E-08
155z16-Fab-GG	Human CD3E-His	1.02E+05	1.87E-03	1.84E-08
155z16-Fab-RG	Human CD3E-His	8.85E+04	1.94E-03	2.19E-08
155z16-Fab-IG	Human CD3E-His	1.21E+05	2.52E-03	2.08E-08
155z16-Fab-VG	Human CD3E-His	1.13E+05	2.81E-03	2.49E-08
155z16-Fab-YG	Human CD3E-His	9.72E+04	1.68E-03	1.73E-08

[0252] Cell-based binding

[0253] To further evaluate the binding activity of CD3 Fab molecules, the Jurkat cell with endogenous CD3 expressional was used. The mutant CD3 antibodies in Fab form was titrated at a five-fold dilution rate starting from a concentration of 100 nM with staining buffer (DPBS buffer containing 2%BSA) . The antibody dilutions were incubated with $1x10^5$ Jurkat-CD3 cells for 60 minutes (mins) at 4 ℃ in the 96-well microplate to allow the tested antibodies binding to CD3 expressed on cell surface. The binding antibodies were detected with PE fluorophore-conjugated anti-human IgG Fc secondary antibodies (ThermoFisher scientific, Cat. No. 12-4998-82) at a dilution rate of 1: 1000. Cells were analyzed by flow cytometer LSRFortessa Cell Analyzer (BD Biosciences) . Data were analyzed with Flowjo 10.0 software. The graph and

statistics analysis were generated with a four-parameter nonlinear regression curve fit in Graphpad Prism 9 software.

[0254] Based on the data shown in FIG. 3C, the mutant antibodies with SG, TG, RG and NA were further studied .

[0255] Example 4: CD3 Antibody Engineering To Improve Stability And Maintain Efficacy

[0256] Since our findings indicate a slight reduction in CD3-cell based binding when asparagine was mutated, we have devised two strategies to rescue the activity of the CD3 antibodies. The first entailed changing the CD3 mAb framework sequence to enhance binding155z16F-, and the second strategy entailed changing the -1 site (i.e., the adjacent N-terminal residue) of SG (155z16XSG, X means any amino acid except N) .

[0257] Strategy 1: Optimization of framework 155z16F-regions

[0258] 155z16F-Certain mutations were introduced to the framework regions of the VH and VL (Table 4, underlined/not bold) . The activity of these mutant CD3 antibodies 155z16F- (with SG/TG/NA/RG in place of NG, as shown) in scFv form. 155z16F-

[0259] Table 4. Engineered CD3 sequences 155z16F

Antibody	VH	SEQ ID NO:
155z16F-RG	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVRQPPGKGLEWMG**RV RYRGDTSYNSALKS**RLTISRDTSKNQASLKLSSVTAADTAVYYCTR**GPYGG SSEPLFDY**WGQGTMVTVSS	85
155z16F-NA	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVRQPPGKGLEWMG**RV RYNADTSYNSALKS**RLTISRDTSKNQASLKLSSVTAADTAVYYCTR**GPYGG SSEPLFDY**WGQGTMVTVSS	86
155z16F-SG	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVRQPPGKGLEWMG**RV RYSGDTSYNSALKS**RLTISRDTSKNQASLKLSSVTAADTAVYYCTR**GPYGG SSEPLFDY**WGQGTMVTVSS	87
155z16F-TG	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVRQPPGKGLEWMG**RV RYTGDTSYNSALKS**RLTISRDTSKNQASLKLSSVTAADTAVYYCTR**GPYGG SSEPLFDY**WGQGTMVTVSS	88
155z16F-VL	DIQMTQSPSSLSASVGDRVTITC**KASQNINKYLN**WYQQKPGKAPRLLIY**TT NNLQT**GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC**LQHNSGYT**FGQGTK LEIK	89

[0260] To assess the ability of the mutant CD3 antibodies to activate the CD3 signaling pathway, a commercial CD3 NFAT luciferase reporter system was used. In this assay, Jurkat-CD3-NFAT was used as the reporter cell line. Jurkat-CD3-NFAT cell line is genetically modified with CD3 downstream NFAT response element. Luciferase expression is induced upon antibody binding to the CD3 receptor. Briefly, reporter cells at a density of $2.5X10^4$ cells per well were cultured in a white 96-well plate. The test antibodies were 3-fold serially diluted added to a white 96-well assay plate, at a final

concentration starting from 200 nM. Following 6 hours of incubation at 37℃, luminescence was obtained by adding the substrate of luciferase and measured by a microplate reader. Four-parameter logistic curve analysis was performed with GraphPad software.

[0261] As shown in FIG. 4A, the mutated CD3 antibodies showed a variety of different CD3 activities. The CD3 NFAT activity of the indicated molecules is ranked as follows: 155z16F-RG-scFv>155z16F-NA-scFv>155z16F-SG-scFv>155z16F-TG-scFv.

[0262] FACS was used to evaluate the binding activity of CD3 mutated antibodies on human peripheral blood mononuclear cells (PBMC) . SP34 served as the benchmark.

[0263] Briefly, human PBMCs were first incubated with 3-fold serially diluted CD3 mutated 155z16F series starting at 200 nM at 4℃ for 30 mins. After washing by PBS, PE Goat anti-Human IgG Fc Secondary Antibody (eBioscience™, Invitrogen) was added to each well and incubated at 4℃ for 30 mins. Samples were washed twice with FACS buffer. The mean florescence intensity (MFI) of PE was evaluated by BD flowcytometry. As shown in FIG. 4B, the mutated CD3 antibodies bound to human PBMC with a variety of different CD3 binding activities. The ranking of CD3 binding activity for the indicated molecules is as follows: 155z16F-RG-scFv>155z16F-NA-scFv>155z16F-SG-scFv>155z16F-TG-scFv, which is consistent with their CD3 NFAT activity.

[0264] Strategy 2: Changing the -1 site of SG to rescue the activity of 155z16 SG

[0265] In this strategy, we attempted to rescue the CD3 binding and NFAT activity of 155z16 SG by performing saturated mutation on the -1 residue before SG. The original amino acid is tyrosine (YSG) . The CD3 NFAT assay was conducted as described previously.

[0266] Table 5. The engineered CD3 sequences based on 155z16 YSG

Antibody	VH	SEQ ID NO:
155z16YSG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKGLEWMG **RVR**[**YSG**]**DTSYNSALKS**RLTISRDTSKNQFSLKLSSVTAADTAVYYCTR**G** **PYGGSSEPLFDY**WGQGTMVTVSS	71
155z16PSG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKGLEWMG **RVR**[**PSG**]**DTSYNSALKS**RLTISRDTSKNQFSLKLSSVTAADTAVYYCTR**G** **PYGGSSEPLFDY**WGQGTMVTVSS	80

155z16ASG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKGLEWMG **RVRASGDTSYNSALKS**RLTISRDTSKNQFSLKLSSVTAADTAVYYCTR**G PYGGSSEPLFDY**WGQGTMVTVSS	81
155z16WSG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKGLEWMG **RVRWSGDTSYNSALKS**RLTISRDTSKNQFSLKLSSVTAADTAVYYCTR**G PYGGSSEPLFDY**WGQGTMVTVSS	82
155z16VSG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKGLEWMG **RVRVSGDTSYNSALKS**RLTISRDTSKNQFSLKLSSVTAADTAVYYCTR**G PYGGSSEPLFDY**WGQGTMVTVSS	83
155z16GSG VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVRQPPGKGLEWMG **RVRGSGDTSYNSALKS**RLTISRDTSKNQFSLKLSSVTAADTAVYYCTR**G PYGGSSEPLFDY**WGQGTMVTVSS	84
155z16 VL	DIQMTQSPSSLSASVGDRVTITC**KASQNINKYLN**WFQQKPGKAPRLLIY **TTNNLQT**GIPSRFSGSGSGTDFTLTISSLQPEDFATYFC**LQHNSGYT**FG QGTKLEIK	2

[0267] As shown in FIG. 5A, substitution of the YSG site with PSG/ASG/VSG/WSG/ GSG in the CDR region of CD3 antibody resulted in a rescue of CD3 NFAT activity, compared to the parental antibody 155-16 SG (with Y as the original AA) . In line with CD3 NFAT activity, substitution of the YNG site in the CDR region of the CD3 155z16 antibody with PSG/ASG/VSG/WSG/GSG resulted in a higher level of cell-based binding activity to human PBMC than that observed for 155-16 SG (FIG. 5B) .

[0268] Example 5: T cell Activation Activity of DLL3-TOPAbody with CD3 Mutants

[0269] To investigate whether the T cell activation activity of CD3 engineered trispecific antibodies remains the same, we constructed DLL3 trispecific antibodies similar to those described in Example 1 with the newly developed mutant anti-CD3 antibody sequences.

[0270] Table 6: Sequences of engineered DLL3-CD3-4-1BB TOPAbodies

310z15-155z16F-NA-B31z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKS**C**DKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES	93

		NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQ KPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-NA scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-NA scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYNADTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	86
	Fc (knob: T366W)	EPKS**A**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16F-SG-B31z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKS**C**DKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASED□YNRLAWYQQ KPGKAPKLLISGV□SLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEV□HQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-SG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQN□NKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLE□K	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-SG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFS□S**SYNVH**WVR QPPGKGLEWMG**RVRY**SG**DTSYNSALKS**RLTISRDTSKN QASLKLSSVTAAD□AVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	87
	Fc (knob: T366W)	EPKS**A**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFT□SRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16F-TG-B31z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVH□FPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEV□CVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKT□SKAKGQPRE PQVYTLPPSREEM□KNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFT□SRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASED□YNRLAWYQQ KPGKAPKLLISGV□SLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEV□HQGLSSPVTKSFNRGEC	95

Heavy chain 2	CD3 155z16F-TG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-TG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYTGDTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	88
	Fc (knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16PSG-B31z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQ KPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16PSG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQ KPGKAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTIS SLQPEDFATYFCLQHNSGYTFGQGTKLEIK	2
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16PSG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVR QPPGKGLEWMG**RVRPSGDTSYNSALKS**RLTISRDTSKN	80

		QFSLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	
	Fc (knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16ASG-B31z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQ KPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16ASG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQ KPGKAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTIS SLQPEDFATYFCLQHNSGYTFGQGTKLEIK	2
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16ASG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVR QPPGKGLEWMG**RVRASGDTSYNSALKS**RLTISRDTSKN QFSLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	81
	Fc (knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF	94

		LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16VSG-B31z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQ KPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16VSG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQ KPGKAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTIS SLQPEDFATYFCLQHNSGYTFGQGTKLEIK	2
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16VSG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGGSIS**SYNVH**WVR QPPGKGLEWMG**RVRVSGDTSYNSALKS**RLTISRDTSKN QFSLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	83
	Fc (knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**WC** LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16WSG-B31z2-TOPAbody			
	Name	Sequence	SEQ ID NO:
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10
	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQ KPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16WSG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQ KPGKAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTIS SLQPEDFATYFCLQHNSGYTFGQGTKLEIK	2
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16WSG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGGSISSYNVHWVR QPPGKGLEWMGRVRWSGDTSYNSALKSRLTISRDTSKN QFSLKLSSVTAADTAVYYCTRGPYGGSSEPLFDYWGQG TMVTVSS	82
	Fc (knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

310z15-155z16F-SG-B125z2-TOPAbody			
	Name	Sequence	SEQ ID NO:
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVK QAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSA STAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTV TVSS	10

	CH (hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B125z2 VHH	EVQLVESGGGLVQPGGSLRLSCAASGFAFSDYAIGWFRQAPGKEREGVSCISSSDGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATDTTTRCPIWSYYVESDYWGQGTLVTVSS	100
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQKPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-SG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQKPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-SG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVRQPPGKGLEWMG**RVRYSGDTSYNSALKS**RLTISRDTSKNQASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQGTMVTVSS	87
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B125z2 VHH	EVQLVESGGGLVQPGGSLRLSCAASGFAFSDYAIGWFRQAPGKEREGVSCISSSDGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATDTTTRCPIWSYYVESDYWGQGTLVTVSS	100

310z15-155z16F-TG-B125z2-TOPAbody			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	DLL3 310z15 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDFYMNWVKQAPGQGLEWIGDANPNNGGTTHNPRFKGRATLTVDKSASTAYMELRSLRSEDTAVYYCARGGLPGDFDYWGQGTTVTVSS	10
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES	93

		NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B125z2 VHH	EVQLVESGGGLVQPGGSLRLSCAASGFAFSDYAIGWFR QAPGKEREGVSCISSSDGSTYYADSVKGRFTISRDNSK NTLYLQMNSLRAEDTAVYYCATDTTTRCPIWSYYVESD YWGQGTLVTVSS	100
Light chain	DLL3 310z15 VL	DIQMTQSPSSLSASVGDRVTITCKASEDIYNRLAWYQQ KPGKAPKLLISGVTSLETGVPSRFSGSGSGTDFTFTIS SLQPEDIATYYCQQYWSFPWTFGGGTKVEIK	11
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-TG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-TG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYTGDTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	88
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B125z2 VHH	EVQLVESGGGLVQPGGSLRLSCAASGFAFSDYAIGWFR QAPGKEREGVSCISSSDGSTYYADSVKGRFTISRDNSK NTLYLQMNSLRAEDTAVYYCATDTTTRCPIWSYYVESD YWGQGTLVTVSS	100

[0271] CD3 NFAT and 4-1BB NF-κB activity

[0272] The CD3 NFAT and 4-1BB NF-κB activities with the engineered CD3 sequences were measured. The CD3 NFAT activity was examined in Jurkat CD3 and HEK293-hDLL3 or SHP77 co-culture system, while 4-1BB NF-κB activity was determined in Jurkat 4-1BB and HEK293-hDLL3 or SHP77 co-culture system.

[0273] To assess the ability of the candidate molecules to activate the CD3 signaling pathway, a commercial CD3 NFAT luciferase reporter system was used. In this assay, Jurkat-CD3-NFAT was used as the reporter cell line. The Jurkat-CD3-NFAT cell line is genetically modified to stably express CD3 and luciferase downstream of a response element. Luciferase expression is induced upon antibody binding to the CD3 receptor. The HEK293-hDLL3 cell highly expressed human DLL3, in which the expression level is between 10-30 fold, as measured by flow cytometry using 50nM DLL3 antibody 310P3C5 comparing to the non-DLL3 binding control (for example, isotype control) at the same concentration; while SHP77 is relatively low DLL3 ex-

pression cell line, in which the expression level is less than 5 fold, as measured by flow cytometry using 50nM DLL3 antibody 310P3C5 comparing to the isotype control at the same concentration. . Briefly, effector cells at a density of $2.5X10^4$ cells per well were cocultured with $2.5X10^4$ target cells (E/T Ratio=1: 1) in a white 96-well plate. Antibodies were 4-fold serially diluted at a final concentration ranging from 100 nM to 0.006 nM. Following a 6 hours of incubation at 37℃, luminescence was obtained by adding the substrate of luciferase and measured by a microplate reader. Four-parameter logistic curve analysis was performed with GraphPad software.

[0274] Similar to the CD3 NFAT reporter assay, to assess the ability of the candidate antibodies to activate 4-1BB signaling pathway, a commercial 4-1BB NF-κB luciferase reporter system was used. In this assay, H_TNFRSF9 (4-1BB) NF-κB-Reporter Jurkat (Genomeditech, cat#GM-C09468) was used as effector cells and HEK293-hDLL3 or SHP77 cells as target cells. H_TNFRSF9 (4-1BB) NF-κB-Reporter Jurkat cell line is genetically modified to stably express 4-1BB and luciferase downstream of a response element. Luciferase expression is induced upon antibody binding to the 4-1BB receptor. In brief, effector cells at a density of $2.5X10^4$ cells per well were cocultured with $2.5X10^4$ target cells (E/T Ratio=1: 1) in a white 96-well plate. Antibodies were 4-fold serially diluted and added to a white 96-well assay plate, at a final concentration ranging from 100 nM to 0.006 nM. Following a 6 hours incubation at 37℃, luminescence was obtained by adding the substrate of luciferase and measured by a microplate reader. Four-parameter logistic curve analysis was performed with GraphPad software.

[0275] As shown in FIG. 6A-D, both the CD3 NFAT activity (FIG. 5A-B) and the 4-1BB NF-κB activity (FIG. 5C-D) of the CD3-engineered TOPA molecules remained high, compared to the unmodified molecules (310z15-155z16-B31z2-TOPAbody) .

[0276] Cell lysis activity and TNFα secretion

[0277] To investigate whether the T cell activation activity of the engineered TOPA molecules remains intact, cell lysis activity and TNFα secretion were examined in PBMC and SHP77 co-culture system. Briefly, human PBMCs at a density of $2X10^5$ cells per well were cultured in a 96-well plate. The target cell line, SHP77, with low expression of DLL3 was seeded at a density of $2X10^4$. Thus, E: T ratio is 10: 1. Antibodies were 4-fold serially diluted and added to a 96-well plate, at a final concentration ranging from 100 nM to 0.0003 nM. Following a 72 hours incubation at 37℃, the supernatants were collected for further analysis. Cell lysis activity and TNFα secretion was performed as described above in Example 1.

[0278] As shown in FIG6. E-H, all trispecific antibodies with the engineered CD3 sequences demonstrated comparable tumor cell lysis activity (FIG. 6E-F) and TNFα secretion (FIG. 6G-H) to that of the unmodified molecule (310z15-155z16-B31z2-TOPAbody) .

[0279] Example 6: Serum Stability of Engineered DLL3-TOPAbodies

[0280] To test whether the stability of CD3-engineered TOPA molecules in human serum is improved, compared to the original molecule 310z15-155z16-B31z2-TOPAbody (with NG as the liability site) , we incubated CD3-engineered trispecific antibodies in human serum for 14 days. Then, we measured the concentration of testing antibodies using dual-antigen sandwich ELISA (CD3-DLL3) to determine the serum stability of the indicated molecules.

[0281] Notably, in contrast to the unmodified molecule (FIG. 7A) , both 310z15-155z16F-TG-B31z2-TOPAbody (replacing NG with TG) and 310z15-155z16F-SG-B125z2-TOPAbody (replacing NG with SG) (FIG. 7B and FIG. 7C, respectively) exhibited remarkable improvements in serum stability up to 14 days of incubation, with neglectable loss of CD3-DLL3 ELISA binding activity. Consistent with ELISA results, the CD3 NFAT activity of TG-and SG-modified molecules in human serum after 14 days of incubation was largely maintained, compared to that of the original molecules (FIG. 7E, 7F, compared with FIG. 7D) .

[0282] Similarly, as illustrated in FIG. 7G-I, in contrast to the original molecule (310z15-155z16-B31z2-TOPAbody, FIG. 7G) , both PSG (310z15-155z16PSG-B31z2-TOPAbody, FIG. 7H)-and ASG- (310z15-155z16ASG-B31z2-TOPAbody, FIG. 7I) modified molecules showed remarkable improvement in stability in human serum up to 14 days, as showed with minimal change of CD3 NFAT activity.

[0283] Example 7: The TG-and SG-Modified TOPA Molecules Showed Superior Tumor Inhibition Rate With Original Molecule

[0284] Then we tested the in vivo efficacy of TG-and SG-modified TOPA molecules with that of original molecule in treating B16F10-hDLL3 cell-induced tumors. B16F10-hDLL3 cells were inoculated into CD3/4-1BB humanized mice. When the average tumor size reached 100 mm^3, TG-and SG-modified TOPA molecules antibodies at 3 mpk were administered into animal in a dosing frequency of twice in a week for three injections.

[0285] The results in FIG. 8 showed that all treatments of DLL3-CD3-4-1BB trispecific molecules resulted in significant tumor inhibition. And the treatments with TG-and SG-modified TOPA molecules led to an even further reduction in tumor sizes compared to the original molecules. The tumor inhibitor rates at Day 7 were listed below.

Group	Tumor inhibition rate on Day 7	P value
PBS	--	--
310-155z16-B31z2-TOPAbody	83.1 %	****
310z15-155z16-B31z2-TOPAbody	84.7 %	****
310z15-155z16F-SG-B31z2-TOPAbody	85.9 %	****
310z15-155z16F-TG-B31z2-TOPAbody	86.9 %	****
310z15-155z16F-SG-B125z2-TOPAbody	93.7 %	****
310z15-155z16F-TG-B125z2-TOPAbody	88.1 %	****

[0286] ****: <0.0001

[0287] Example 8: Comparison of DLL3-TOPAbody with DLL3 bispecific antibody and Combo Therapy

[0288] Next, we further conducted experiments to compare the activity of DLL3-TOPAbody (310z15-155z16F-TG-B31z2-TOPAbody) with the combination therapy of DLL3-CD3 and DLL3-4-1BB, along with two other clinical benchmarks. The testing methods were described in Example 5. AMG757, a half-life extended bi-specific T-cell engager (BiTE) molecule targeting DLL3 and CD3 simultaneously. AMG757 has demonstrated T-cell-mediated tumor lysis, with manageable safety with encouraging response durability in patients with relapsed/refractory SCLC in the phase I study DeLLphi-300 (NCT03319940) . RG6524 is a DLL3/CD3/CD137 trispecific T cell engager composed of two CD3/CD137 dual specific Fabs and one extra DLL3 Fab, and now is under development for the treatment of solid tumors, small cell lung cancer (SCLC) and neuroendocrine carcinoma (NCT05619744) . Meanwhile, to evaluate the potency of our trispecific antibody in comparison to the combination of DLL3-CD3 bispecific antibody (AMG757) and DLL3-4-1BB bispecific antibody in inducing T cell activation, the DLL3-4-1BB bispecific antibody were generated as illustrated in FIG. 9A.

[0289] As shown in FIG. 9B, DLL3-TOPAbody exhibited a much higher maximal killing activity in comparison to AMG757 and RG6524, suggesting DLL3-TOPAbody could further boost and potentiate T cell killing. In addition, DLL3-TOPAbody also demonstrated the superiority in comparison with the combination of DLL3-CD3 (AMG757) with DLL3/4-1BB BiAb.

[0290] Consistent with T cell killing activity triggered by DLL3-TOPAbody, the secretion of TNFalpha was significantly enhanced by DLL3-TOPAbody compared to all other tested groups (FIG. 9C) .

[0291] Collectively, data supported DLL3-TOPAbody showed superiority in inducing T cell activation and cytotoxicity compared to the clinical benchmarks and the combination therapy of DLL3-CD3 and DLL3-4-1BB.

[0292] Example 9: Minimal Cytokine Release in the Absence of Tumor Antigen by DLL3-TOPAbody

[0293] To investigated whether CD3-engineered trispecific antibodies have any risk in inducing cytokine release in human, we used cytokine release assays in which antibodies were added to PBMC culture at a highest concentration of 400 nM, which corresponds to C_{max} of the predicted efficacious dose in human blood. Subsequently, the level of IL-6 was measured in the supernatant medium. Briefly, human PBMCs at a density of $1x10^5$ cells per well were cultured in a 96-well plate. The antibodies were 4-fold serially diluted and added to a 96-well plate, at a final concentration ranging from 400 nM to 0.39 nM. Following a 72-hour incubation at 37℃, the supernatants were collected and cytokines were detected by Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II from BD.

[0294] As shown in FIG. 10, DLL3-TOPAbody (310z15-155z16F-TG-B31z2-TOPAbody) did not induce a significant increase in IL6 which was considered to be the master driver for cytokine release syndrome in the clinic. Compared to the CD3 antibody SP34 and clinically validated antibody AMG757, the amount of IL6 production is neglectable. Overall, these data suggested that TOPAbody with engineered CD3 had a low risk of inducing CRS in the clinic. Moreover, the engineered CD3 had a good potential in the application for multiple TOPAbody programs, such as DLL3-TOPAbody and TOPAbody programs targeting other TAAs.

[0295] Example 10: T cell Activation Activity Mediated by CDH17-TOPAbody

[0296] To further investigate the properties of TOPAbody, we generated trispecific antibodies by incorporating different sequences of anti-CDH17 antibodies into a CDH17-CD3-4-1BB trispecific format, CDH17-TOPAbody. This trispecific antibody is designed to bind to three targets, the CDH17 protein on a tumor cells, and CD3 and 4-1BB on T cells. The configuration of CDH17-TOPAbody is illustrated in FIG. 1A. It includes a 1+1 format of anti-TAA/CD3 portion, with two anti-4-1BB nanobodies fused to the C-terminus of the Fc fragment. The CDH17 antibody with different binding domain sequences used in the trispecific antibodies are listed below (Table 7 and Table 8) .

[0297] To investigate the T cell activation activity of different binding domain of CDH17-CD3-4-1BB trispecific antibodies, we constructed CDH17 trispecific antibodies similar to those described in Example 1 and Example 5 with 155z16 or 155z16F-TG sequences.

[0298] Table 7: Sequences of engineered CDH17-CD3-4-1BB TOPAbodies

Name	Sequence	CDH17 domain	SEQ ID NO:
155z16 VH	EVQLQESGPGLVKPSETLSLTCTVSGGSISSYNVHWVRQPP GKGLEWMGRVRYNGDTSYNSALKSRLTISRDTSKNQFSLKL SSVTAADTAVYYCTRGPYGGSSEPLFDYWGQGTMVTVSS	–	1
155z16 VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQKPG KAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTISSLQPED FATYFCLQHNSGYTFGQGTKLEIK	–	2
155z16F-TG VH	EVQLQESGPGLVKPSETLSLTCTVSGFSISSYNVHWVRQPP GKGLEWMGRVRYTGDTSYNSALKSRLTISRDTSKNQASLKL SSVTAADTAVYYCTRGPYGGSSEPLFDYWGQGTMVTVSS	–	88
155z16F-TG VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQKPG KAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTISSLQPED FATYYCLQHNSGYTFGQGTKLEIK	–	89
B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQAPGK QREYVSHITSNGRTNYADSVTGRFTISRDNSKNTLYLQMNS LRAEDTAVYYCAADDATNTRTYWGQGTLVTVSS	–	3
29H8 VH	QVQLQQSDAELVKPGASVKISCKVSGYTFTDHTIHWMKQRP EQGLEWIGYIYPRDGYTKYNEKFKDKATLTADKSSSTAYIQ LNTLTSEDSAVYFCARFYYGYWYFDVWGTGTTVTVSS	EC1	104
29H8 VL	DIVMTQAAPSVPVTPGESVSISCRSSKSLLRSNGNTYLYWF LQRPGQSPQVLIYRMSNLASGVPDRFSGSGSGTAFTLRISR VEAEDVGVYYCMQHLEYPFTFGAGTKLELK	EC1	105
29H8z3 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDHTIHWVRQAP GQGLEWMGYIYPRDGYTKYNEKFKDRVTMTTDTSTSTAYME LRSLRSDDTAVYYCARFYYGYWYFDVWGQGTTVTVSS	EC1	106
29H8z3 VL	DIVMTQSPLSLPVTPGEPASISCRSSKSLLRSNGNTYLYWF LQKPGQSPQVLIYRMSNLASGVPDRFSGSGSGTDFTLKISR VEAEDVGVYYCMQHLEYPFTFGQGTKLEIK	EC1	107
69E3 VH	QVQLQQPGAELVKPGASVKLSCKASGYTFTTYWMHWVKQRP GQGLEWIGEIDPSDSYANYNQKFKGKATLTVDKSSSTAYMQ LSSLTSEDSAVYYCALYYGYGDWGQGTTLTVSS	EC7	108
69E3 VL	EIVLTQSPTTMAASPGEKIIITCSVSSSISSIHLHWYQQKP GFSPKLLIYRTSNLASGVPTRFSGSGSGTSYSLTIGTMEAE DVATYYCQQGSNLPLTFGAGTRLELK	EC7	109
69E3z12 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWVKQAP GQRLEWIGEIDPSDSYANYNQKFKGKATLTVDKSASTAYME LSSLRSEDTAVYYCALYYGYGDWGQGTTVTVSS	EC7	110
69E3z12 VL	EIVLTQSPGTLSLSPGERATLSCSVSSSISSIHLHWYQQKP GQAPRLLIYRTSNLASGIPDRFSGSGSGTDFTLTISRLEPE DFAVYYCQQGSNLPLTFGQGTRLEIK	EC7	111
69E3z12a27 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWVKQAP GQRLEWIGEIDPSDSYANYNQKFKGKATLTVDKSASTAYME LSSLRSEDTAVYYCALYYGYGDWGQGTTVTVSS	EC7	110
69E3z12a27 VL	EIVLTQSPGTLSLSPGERATLSCSVSSSISSIHLHWYQQKP	EC7	112

	GQAPRLLIY**RTRNRAS**GIPDRFSGSGSGTDFTLTISRLEP EDFAVYYC**QQGSNLPLT**FGQGTRLEIK		
69E3z12a42 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFT**TYWMH**WVKQAP GQRLEWIG**EIDPSDSYANYNQKFKG**KATLTVDKSASTAYME LSSLRSEDTAVYYCAL**YYGYGD**WGQGTTVTVSS	EC7	110
69E3z12a42 VL	EIVLTQSPGTLSLSPGERATLSC**SVSASISRIHLH**WYQQK PGQAPRLLIY**RTRNRAS**GIPDRFSGSGSGTDFTLTISRLE PEDFAVYYC**QQGSNLPLT**FGQGTRLEIK	EC7	113

[0299] Table 8: Sequences of engineered CDH17-TOPAbodies

29H8-155z16-B31z2			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	CDH17 29H8 VH	QVQLQQSDAELVKPGASVKISCKVSGYTFTDHTIHWMK QRPEQGLEWIGYIYPRDGYTKYNEKFKDKATLTADKSS STAYIQLNTLTSEDSAVYFCARFYYGYWYFDVWGTGTT VTVSS	104
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	CDH17 29H8 VL	DIVMTQAAPSVPVTPGESVSISCRSSKSLLRSNGNTYL YWFLQRPGQSPQVLIYRMSNLASGVPDRFSGSGSGTAF TLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKLELK	105
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16 scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQ KPGKAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTIS SLQPEDFATYFCLQHNSGYTFGQGTKLEIK	2
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16 scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGGSISSYNVHWVR QPPGKGLEWMGRVRYNGDTSYNSALKSRLTISRDTSKN QFSLKLSSVTAADTAVYYCTRGPYGGSSEPLFDYWGQG TMVTVSS	1
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94

	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

29H8z3-155z16F-TG-B31z2			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	CDH17 29H8z3 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTDHTIHWVR QAPGQGLEWMGYIYPRDGYTKYNEKFKDRVTMTTDTST STAYMELRSLRSDDTAVYYCARFYYGYWYFDVWGQGTT VTVSS	106
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	CDH17 29H8z3 VL	DIVMTQSPLSLPVTPGEPASISCRSSKSLLRSNGNTYL YWFLQKPGQSPQVLIYRMSNLASGVPDRFSGSGSGTDF TLKISRVEAEDVGVYYCMQHLEYPFTFGQGTKLEIK	107
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-TG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-TG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYTGDTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	88
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

69E3-155z16-B31z2			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	CDH17 69E3 VH	QVQLQQPGAELVKPGASVKLSCKASGYTFTTYWMHWVK QRPGQGLEWIGEIDPSDSYANYNQKFKGKATLTVDKSS STAYMQLSSLTSEDSAVYYCALYYGYGDWGQGTTLTVS S	108
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	CDH17 69E3 VL	EIVLTQSPTTMAASPGEKIIITCSVSSSISSIHLHWYQ QKPGFSPKLLIYRTSNLASGVPTRFSGSGSGTSYSLTI GTMEAEDVATYYCQQGSNLPLTFGAGTRLELK	109
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16 scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWFQQ KPGKAPRLLIYTTNNLQTGIPSRFSGSGSGTDFTLTIS SLQPEDFATYFCLQHNSGYTFGQGTKLEIK	2
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16 scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGGSISSYNVHWVR QPPGKGLEWMGRVRYNGDTSYNSALKSRLTISRDTSKN QFSLKLSSVTAADTAVYYCTRGPYGGSSEPLFDYWGQG TMVTVSS	1
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

69E3z12-155z16F-TG-B31z2			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	CDH17 69E3z12 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWVK QAPGQRLEWIGEIDPSDSYANYNQKFKGKATLTVDKSA	110

		STAYMELSSLRSEDTAVYYCALYYGYGDWGQGTTVTVS S	
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	CDH17 69E3z12 VL	EIVLTQSPGTLSLSPGERATLSCSVSSSISSIHLHWYQ QKPGQAPRLLIYRTSNLASGIPDRFSGSGSGTDFTLTI SRLEPEDFAVYYCQQGSNLPLTFGQGTRLEIK	111
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-TG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-TG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYTGDTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	88
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

69E3z12a27-155z16F-TG-B31z2			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	CDH17 69E3z12a27 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWVK QAPGQRLEWIGEIDPSDSYANYNQKFKGKATLTVDKSA STAYMELSSLRSEDTAVYYCALYYGYGDWGQGTTVTVS S	110
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE	93

		DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	
	Linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	CDH17 69E3z12a27 VL	EIVLTQSPGTLSLSPGERATLSCSVSSSISSIHLHWYQ QKPGQAPRLLIYRTRNRASGIPDRFSGSGSGTDFTLTI SRLEPEDFAVYYCQQGSNLPLTFGQGTRLEIK	112
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-TG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-TG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYTGDTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	88
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**C LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

69E3z12a42-155z16F-TG-B31z2			
	Name	**Sequence**	**SEQ ID NO:**
Heavy chain 1	CDH17 69E3z12a42 VH	QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWVK QAPGQRLEWIGEIDPSDSYANYNQKFKGKATLTVDKSA STAYMELSSLRSEDTAVYYCALYYGYGDWGQGTTVTVS S	110
	CH(hole: T366S/L368A/Y407V)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	93
	Linker	GGGGSGGGGSGGGGS	91

	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3
Light chain	CDH17 69E3z12a42 VL	EIVLTQSPGTLSLSPGERATLSCSVSASISRIHLHWYQ QKPGQAPRLLIYRTRNRASGIPDRFSGSGSGTDFTLTI SRLEPEDFAVYYCQQGSNLPLTFGQGTRLEIK	113
	CL	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	95
Heavy chain 2	CD3 155z16F-TG scFv-VL	DIQMTQSPSSLSASVGDRVTITCKASQNINKYLNWYQQ KPGKAPRLLIYTTNNLQTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCLQHNSGYTFGQGTKLEIK	89
	Linker	GGGGSGGGGSGGGGSGGGGS	92
	CD3 155z16F-TG scFv-VH	EVQLQESGPGLVKPSETLSLTCTVSGFSIS**SYNVH**WVR QPPGKGLEWMG**RVRYTGDTSYNSALKS**RLTISRDTSKN QASLKLSSVTAADTAVYYCTR**GPYGGSSEPLFDY**WGQG TMVTVSS	88
	Fc(knob: T366W)	EPKSADKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPG	94
	linker	GGGGSGGGGSGGGGS	91
	4-1BB B31z2 VHH	QVQLVESGGGLVQPGGSLRLSCSASGDIFSIMFWYRQA PGKQREYVSHITSNGRTNYADSVTGRFTISRDNSKNTL YLQMNSLRAEDTAVYYCAADDATNTRTYWGQGTLVTVS S	3

[0300] CD3 NFAT and 4-1BB NF-κB activity

[0301] The CD3 NFAT and 4-1BB NF-κB activities with different CDH17 binding domain sequences were measured. The CD3 NFAT activity was assessed in a co-culture system consisting of Jurkat CD3 NFAT cells and AsPC-1 (CDH17-High) or Kato III cells (CDH17-Low) , while the 4-1BB NF-κB activity was determined in a co-culture system comprising HEK 4-1BB cells and AsPC-1 or KatoIII cells.

[0302] To assess the ability of the candidate molecules to activate the CD3 signaling pathway, a commercial CD3 NFAT luciferase reporter system was used (Promega, CAT#J1601) . In this assay, Jurkat-CD3-NFAT was used as the reporter cell line. The Jurkat-CD3-NFAT cell line is genetically modified to stably express CD3 and luciferase downstream of a response element. Luciferase expression is induced upon antibody binding to the CD3 receptor. AsPC-1 is a pancreatic adenocarcinoma cell line with high expression level of CDH17 (~$1.5x10^6$ molecules/cell) . KATO-III is a human gastric carcinoma cell line with low expression level of CDH17 (~$1x10^3$ molecules/cell) . Briefly, effector cells at a density of $2.5X10^4$ cells per well were cocultured with $2.5X10^4$ target cells (E/T Ratio=1: 1) in a white 96-well plate. An-

tibodies were 4-fold serially diluted at a final concentration ranging from 100 nM to 0.00038 nM. Following a 5-hour of incubation at 37℃, luminescence was obtained by adding the substrate of luciferase and measured by a microplate reader. Four-parameter logistic curve analysis was performed with GraphPad software.

[0303] Similar to the CD3 NFAT reporter assay, to assess the ability of the candidate antibodies to activate 4-1BB signaling pathway, a commercial 4-1BB NF-κB luciferase reporter system was used. In this assay, HEK-4-1BB NF-κB luciferase reporter system was used (Genomeditech, Cat#GM-C04832) as effector cells and AsPC-1 or KatoIII cells as target cells. HEK-4-1BB NF-κB luciferase reporter cell line is genetically modified to stably express 4-1BB and luciferase downstream of a response element. Luciferase expression is induced upon antibody binding to the 4-1BB receptor. In brief, effector cells at a density of $2.5X10^4$ cells per well were cocultured with $2.5X10^4$ target cells (E/T Ratio=1: 1) in a white 96-well plate. Antibodies were 5-fold serially diluted and added to a white 96-well assay plate, at a final concentration ranging from 100 nM to 0.00005 nM. Following a 5-hour incubation at 37℃, luminescence was obtained by adding the substrate of luciferase and measured by a microplate reader. Four-parameter logistic curve analysis was performed with GraphPad software.

[0304] As shown in FIG. 11A and 11B, all tested CDH17-TOPAbodies demonstrated a dose-dependent response in inducing downstream signaling of CD3. Affinity-matured CDH17-TOPAbody targeting CDH17 EC7 domain, namely 69E3z12a27-155z16F-TG-B31z2 and 69E3z12a42-155z16F-TG-B31z2, showed the most potent activity in inducing CD3 NFAT activity. Consistently, as illustrated in FIG. 11C and 11D, all tested CDH17-TOPAbodies showed dose-dependent activity in inducing 4-1BB downstream signaling. Interestingly, the antibodies targeting the EC1 domain of CDH17, namely 29H8-155z16-B31z2 and 29H8z3-155z16F-B31z2, exhibited the highest activity. Collectively, CDH17-TOPAbodies could induce downstream signaling of CD3 and 4-1BB in both tumor cell lines with high and low expression levels of CDH17.

[0305] Cell lysis activity and TNFα secretion

[0306] To further investigate the potency of CDH17-TOPAbodies in inducing T cell activation, cell lysis activity and TNFα secretion assays were performed in a co-culture system consisting of PBMC and AsPC-1 or SK-CO-1 (with medium level of CDH17 expression, ~$3X10^5$ molecules/cell) or KatoIII cells. Briefly, human PBMCs at a density of $2X10^5$ cells per well were cultured in a 96-well plate. AsPC-1 or SK-CO-1 or KatoIII were seeded at a density of $2X10^4$/well. Thus, E: T ratio is 10: 1. Antibodies were 4-fold serially diluted and added to a 96-well plate, at a final concentration ranging from 100 nM to 0.0015 nM. Following a 72-hour incubation at 37℃, the su-

pernatants were collected for further analysis. Cell lysis activity and TNFα secretion were measured as described above in Example 1.

[0307] As shown in FIG. 12A-F, all tested CDH17-TOPAbodies showed dose-dependent activity in inducing tumor killing and T cell activation in CDH17+ tumor cells, as evidenced by TNFα secretion, suggesting CDH17-TOPAbody could exert its effect on tumors with varying levels of CDH17 expression. Among all tested antibody, CDH17-TOPAbodies that targeting CDH17 EC7 domain demonstrated the strongest effect in tumor cell lysis activity and TNFαsecretion.

[0308] To compare the potency of CDH17-TOPAbody and ARB202, which was a CDH17-CD3 bispecific antibody developed by Arbele Bio undergoing Phase 1 study. Cytotoxicity assay was performed in a co-culture system consisting PBMC and tumor cells with varied CDH17 expression level, as detailed experimental procedures described above.

[0309] As shown in FIG. 12G-J, CDH17-TOPAbody 69E3z12a27-155z16F-TG-B31z2 exhibited superior cytotoxicity than ARB202, particularly in tumor cell with low CDH17 expression level.

[0310] Tumor cytotoxicity at different E: T ratios

[0311] To further investigate T cell killing activity of CDH17-TOPAbodies in tumors with different levels of immune cells infiltration, cell lysis activity was performed in a co-culture system by incubation of PBMC and target cell MDA-MB-468-CDH17 in different E: T ratios, such as 10: 1, 5:1 and 2: 1 respectively. Briefly, human PBMCs at a density of $2X10^4$ cells per well or $1X10^4$ cells per well or $4X10^3$ cells per well were cultured in a 96-well plate. MDA-MB-468-CDH17-GFP were seeded at a density of $2X10^3$/well. ARB202 and CDH17-TOPAbody 69E3z12-155z16F-TG-B31z2 were added to a 96-well plate at a final concentration of 100 nM. The tested cells were incubated at 37°C for 72 hours, then the GFP positive cells were monitored in the Operatta CLS (PE, High Content Analysis system) . The percentage of live cells expressing GFP in the tested samples was normalized to that of the Isotype control group.

[0312] In another study with similar study design, the killing activity of CDH17-TOPAbody 69E3z12a27-155z16F-TG-B31z2 and ARB202 was determined in an even lower E: T ratio. PBMCs were co-incubated with target cell AsPC-1 in different E: T ratios, from 10: 1 to 0.2: 1. AsPC-1 cells were seeded at a density of $2X10^4$/well in a 96-well plate, thus PBMCs were seeded at a density from $2X10^5$ cells per well to $4X10^3$ cells per well. ARB202 and CDH17-TOPAbody 69E3z12a27-155z16F-TG-B31z2 were added to the cell culture starting from 0.0015nM to 25 nM. Following a 72 hours incubation at 37°C, the supernatants were collected for further analysis. LDH level was examined following the manufacturer's protocol.

[0313] As shown in FIG. 13A and FIG. 13B-E, in all conditions tested, CDH17-TOPAbody showed superior killing activity. Especially, CDH17-TOPAbody 69E3z12-155z16F-TG-B31z2 and 69E3z12a27-155z16F-TG-B31z2 had a much potent killing than CDH17-CD3 bsAb ARB202 at a low E: T ratio, suggesting in the poorly infiltrated cold tumors where there were fewer T cells, such as microsatellite-stable CRC tumors, CDH17-TOPAbody might have a better efficacy than CDH17-CD3 bsAb.

[0314] Example 11: Minimal Cytokine Release in the Absence of Tumor Antigen by CDH17-TOPA

[0315] To assess the safety profile of CDH17-TOPAbodies in terms of inducing cytokine release in a PBMC-only system, antibodies were added to PBMC at a highest concentration of 400 nM, which corresponds to Cmax of the predicted efficacious dose in human blood. Subsequently, the levels of IL-6, IL-10, TNF and IFN-γ were measured in the supernatant medium. Briefly, human PBMCs at a density of $1x10^5$ cells per well were cultured in a 96-well plate. The antibodies were 4-fold serially diluted and added to a 96-well plate, at a final concentration ranging from 400 nM to 0.39 nM. Following a 72-hour incubation at 37°C, the supernatants were collected and cytokines were detected by Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II from BD.

[0316] As shown in FIG. 14, CDH17-TOPAbodies did not induce a significant increase in cytokines that are related to cytokine release syndrome, such as IL6. Compared to the CD3 antibody SP34 and clinically validated antibody AMG757, the amount of IL6 production is neglectable. Overall, these data suggested that CDH17-TOPAbody had a low risk in inducing CRS in the clinic.

[0317] Example 12: In vivo efficacy of CDH17-TOPAbodies

[0318] To further evaluate the activity of CDH17-TOPAbodies, we tested the in vivo efficacy of CDH17-TOPAbody in B16F10-hCDH17 syngeneic mouse model. B16F10 that expressing hCDH17 were inoculated into CD3/4-1BB humanized mice. When the average tumor size reached around 100 mm^3, CDH17-TOPAbodies, namely 69E3-155z16-B31z2 and 29H8-155z16-B31z2 were intraperitoneally administered into the animals at 3mg/kg with a dosing frequency of once a week for 4-6 doses. The tumor volume was monitored every 2 days. The study design is show in FIG. 15A.

[0319] As illustrated in FIG. 15B, the tested CDH17-TOPAbodies exhibited significant in vivo tumor inhibition activity, with a TGI of 88.9%and 86.7%respectively on day 11. Consistently, both CDH17-TOPAbodies resulted in a significant extension of animal survival, as showed in FIG. 15C.

[0320] In another experiment with similar study design, the in vivo efficacy of CDH17-TOPAbody 69E3z12a27-155z16-B31z2 was determined in in B16F10-hCDH17

syngeneic mouse model. CDH17-CD3 was compared in parallel. B16F10 that expressing hCDH17 were inoculated into CD3/4-1BB humanized mice. When the average tumor size reached around 100 mm^3, ARB202, CDH17-TOPAbody 69E3z12a27-155z16F-B31z2 were intraperitoneally administered into the animals at equal molar amount, 3.9 mg/kg or 3 mg/kg, with a dosing frequency of once every two weeks for 2 doses. The tumor volume was monitored every 2 days.

[0321] As showed in FIG. 15D, the tested CDH17-TOPAbody exhibited significant in vivo tumor inhibition activity, with a TGI of 84.1%on day 9; while ARB202 had no anti-tumor effect at the dose level tested.

[0322] Example 13: Mechanisms underlying the activity of CDH17-TOPAbodies

[0323] To further dissect the mechanism of CDH17-TOPAbody in inducing T cell activation, in the same animal model described in Example 12, pretreatment with the spingosine-1-phosphate receptor inhibitor FTY720 was performed before the administration of CDH17-TOPAbody, aiming to reduce the frequency of circulating T cells and subsequent recruitement of T cells into the tumor. The experiment procedure was shown in FIG. 16A. Unexpectedly, unlike CD3-based bispecific T cell engagers reported (Belmontes et al., Sci. Transl. Med. 13 2021) , as shown in FIG. 16B, the antitumor response elicited by CDH17-TOPAbody was partially hampered by FTY720, suggesting CDH17-TOPAbody mediated antitumor activity in these models is not only dependent on local expansion of T cell in the tumor site, but also dependent on T cells recruitement from the peripheral.

[0324] Moreover, mice that were treated by CDH17-TOPAbody could reach tumor free after repeated doses, again implying the great treatment potential of CDH17-TOPAbody for anti-tumor therapy. Interestingly, tumor-free mice were resistant to the re-challenge of B16F10-CDH17 and B16F10-WT tumors, which were implanted on Day 56 and Day 96. The results demonstated treated mice could establish a long-term and broad memory response against cancer cells.

[0325] * * *

[0326] 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.

[0327] 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.

[0328] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

Claims

[Claim 1] A multi-specific antibody, comprising (a) an anti-CD3 antibody or antigen binding fragment thereof, and (b) an anti-4-1BB antibody or antigen binding fragment thereof, wherein:
the anti-CD3 antibody or antigen binding fragment thereof has a T cell activation activity (EC50) in the range of 0.5 to 10 nM, which is measured by incubating (i) a multi-specific antibody comprising the anti-CD3 antibody or antigen binding fragment thereof and a second antibody or antigen binding fragment thereof specific to a first tumor associated antigen (TAA) , (ii) T cells expressing CD3 and a first reporter gene regulated by an NFAT (nuclear factor of activated T cell) response element (NFAT-RE) , and (iii) cells expressing the first TAA, and measuring activity of the first reporter gene; and
the anti-4-1BB antibody or antigen binding fragment thereof has a 4-1BB signaling activation activity (EC50) in the range of 0.05 to 1 nM, which is measured by incubating (i) a multi-specific antibody comprising the anti-4-1BB antibody or antigen binding fragment thereof and a second antibody or antigen binding fragment thereof specific to a second TAA, (ii) T cells expressing 4-1BB and a second reporter gene regulated by NF-κB, and (iii) cells expressing the second TAA, and measuring activity of the second reporter gene.

[Claim 2] The multi-specific antibody of claim 1, wherein the anti-CD3 antibody or antigen binding fragment thereof has a T cell activation activity (EC50) in the range of 1 to 10 nM.

[Claim 3] The multi-specific antibody of claim 1 or 2, wherein the anti-4-1BB antibody or antigen binding fragment thereof has a 4-1BB signaling activation activity (EC50) in the range of 0.1 to 1 nM.

[Claim 4] The multi-specific antibody of any preceding claim, wherein the first TAA and second TAA are delta-like ligand 3 (DLL3) .

[Claim 5] The multi-specific antibody of any preceding claim, further comprising a third antibody or antigen binding fragment thereof having specificity to a TAA.

[Claim 6] The multi-specific antibody of any preceding claim, wherein the anti-4-1BB antibody is a single domain antibody.

[Claim 7] The multi-specific antibody of claim 6, wherein the anti-4-1BB antibody is disposed at the C-terminal end of an Fc fragment.

[Claim 8] The multi-specific antibody of claim 7, wherein the anti-CD3 fragment is disposed at the N-terminal end of the Fc fragment.

[Claim 9] The multi-specific antibody of any one of claims 5-8, wherein the third antibody or antigen binding fragment thereof has specificity to DLL3.

[Claim 10] An antibody or antigen-binding fragment thereof having specificity to a cluster of differentiation 3 (CD3) protein, wherein the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions HCDR1, HCDR2, and HCDR3, and a light chain variable region (VL) comprising light chain complementarity determining regions LCDR1, LCDR2, and LCDR3, wherein:
the HCDR1 comprises SYNVH (SEQ ID NO: 16) ,
the HCDR2 comprises RVRX$_1$X$_2$X$_3$DTSYNSALKS (SEQ ID NO: 96) ,
the HCDR3 comprises GPYGGSSEPLFDY (SEQ ID NO: 18) ,
the LCDR1 comprises KASQNINKYLN (SEQ ID NO: 19) ,
the LCDR2 comprises TTNNLQT (SEQ ID NO: 20) , and
the LCDR3 comprises LQHNSGYT (SEQ ID NO: 21) ; and
wherein:
(1) X_1 is any amino acid except N; X_2 is any amino acid except N; and X_3 is G or A; or
(2) X_1 is any amino acid except N; X_2 is any amino acid; and X_3 is A.

[Claim 11] The antibody or antigen-binding fragment thereof of claim 10, wherein X_1 is Y, P, A, W, V or G; X_2 is E, S, T, Q, G, R, I, V, or Y; and X_3 is G or A.

[Claim 12] The antibody or antigen-binding fragment thereof of claim 10, wherein X_1 is Y, X_2 is N, and X_3 is A.

[Claim 13] The antibody or antigen-binding fragment thereof of claim 10, wherein (1) X_1 is Y, X_3 is G, and X_2 is E, S, T, Q, G, R, I, V, or Y; (2) X_1 is Y, X_2 is N, and X_3 is A; or (3) X_1 is P, A, W, V or G, X_2 is S, and X_3 is G.

[Claim 14] The antibody or antigen-binding fragment thereof of claim 10, wherein the HCDR2 comprises an amino acid sequence selected from the group consisting of:
RVRYEGDTSYNSALKS (SEQ ID NO: 55) ,
RVRYSGDTSYNSALKS (SEQ ID NO: 56) ,
RVRYTGDTSYNSALKS (SEQ ID NO: 57) ,
RVRYQGDTSYNSALKS (SEQ ID NO: 58) ,
RVRYNADTSYNSALKS (SEQ ID NO: 59) ,

RVRYGGDTSYNSALKS (SEQ ID NO: 60) ,
RVRYRGDTSYNSALKS (SEQ ID NO: 61) ,
RVRYIGDTSYNSALKS (SEQ ID NO: 62) ,
RVRYVGDTSYNSALKS (SEQ ID NO: 63) ,
RVRYYGDTSYNSALKS (SEQ ID NO: 64) ,
RVRPSGDTSYNSALKS (SEQ ID NO: 65) ,
RVRASGDTSYNSALKS (SEQ ID NO: 66) ,
RVRWSGDTSYNSALKS (SEQ ID NO: 67) ,
RVRVSGDTSYNSALKS (SEQ ID NO: 68) , and
RVRGSGDTSYNSALKS (SEQ ID NO: 69) .

[Claim 15] The antibody or antigen-binding fragment thereof of any one of claims 10-14, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 70-79, 80-84 and 85-88, or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 70-79, 80-84 and 85-88.

[Claim 16] The antibody or antigen-binding fragment thereof of any one of claims 10-15, wherein the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 2 and 89 or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 2 and 89.

[Claim 17] The antibody or antigen-binding fragment thereof of any one of claims 10-16, wherein
(a) the VH comprises the amino acid sequence of any one of SEQ ID NOs: 70-79 and 80-84, and the VL comprises the amino acid sequence of SEQ ID NO: 2; or
(b) the VH comprises the amino acid sequence of any one of SEQ ID NOs: 85-88, and the VL comprises the amino acid sequence of SEQ ID NO: 89.

[Claim 18] The antibody or antigen-binding fragment thereof of any one of claims 10-17, further comprising a heavy chain constant region, a light chain constant region, an Fc region, or a combination thereof.

[Claim 19] A multi-specific antibody comprising:
(a) a first antigen-binding moiety comprising the antibody or antigen-binding fragment thereof of any one of claims 10-18,
(b) a second antigen-binding moiety having specificity to a human 4-1BB protein.

[Claim 20] The multi-specific antibody of claim 19, further comprising a third antigen-binding moiety.

[Claim 21] The multi-specific antibody of claim 20, wherein the third antigen-binding moiety has specificity to a tumor associated antigen (TAA) or a tumor specific antigen (TSA) .

[Claim 22] The multi-specific antibody of any one of claims 19-21, wherein the first antigen-binding moiety, the second antigen-binding moiety, and the third antigen-binding moiety each, independently, is of a format selected from the group consisting of a full-length antibody, a half-antibody, a single chain half-antibody, a Fab, a Fab', a F (ab') 2, a nanobody or a scFv.

[Claim 23] The multi-specific antibody of claim 22, wherein the first antigen-binding moiety is of a format of a scFv.

[Claim 24] The multi-specific antibody of claim 22 or 23, wherein the second antigen-binding moiety is of a format of a nanobody.

[Claim 25] The multi-specific antibody of any one of claims 22-24, wherein the third antigen-binding moiety is of a format of a Fab.

[Claim 26] The multi-specific antibody of any one of claims 22-25, wherein the first antigen-binding moiety is fused to N-terminus of a Fc domain, the third antigen-binding moiety is fused to the N-terminus of a pairing Fc domain, and the second antigen-binding moiety is fused to the C-terminus of the Fc domain and to the C-terminus of the pairing Fc domain, respectively.

[Claim 27] The multi-specific antibody of any one of claims 19-26, wherein the second antigen-binding moiety comprises the CDR1, CDR2 and CDR2 of SEQ ID NO: 3 or 100.

[Claim 28] The multi-specific antibody of claim 27, wherein the second antigen-binding moiety comprises SEQ ID NO: 3 or 100.

[Claim 29] The multi-specific antibody of any one of claims 20-28, wherein the third antigen-binding moiety comprises an antibody or antigen-binding fragment thereof having specificity to a human delta-like ligand 3 (DLL3) protein.

[Claim 30] The multi-specific antibody of claim 29, wherein the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (VH) comprising heavy chain complementarity determining regions HCDR1, HCDR2, and HCDR3, and a light chain variable region (VL) comprising light chain complementarity determining regions LCDR1, LCDR2, and LCDR3, wherein the HCDR1, HCDR2,

HCDR3, LCDR1, LCDR2, and LCDR3 are selected from the group consisting of:
(a) HCDR1: DHHLN (SEQ ID NO: 25) ,
HCDR2: WIFPGSGSDYYNERFKD (SEQ ID NO: 26) ,
HCDR3: WDY (SEQ ID NO: 27) ,
LCDR1: SASSSISYMH (SEQ ID NO: 28) ,
LCDR2: DTSTLAS (SEQ ID NO: 29) , and
LCDR3: HQRSSYPLT (SEQ ID NO: 30) ;
(b) HCDR1: DYTMH (SEQ ID NO: 31) ,
HCDR2: GIHPNYGGTSYNEKFKD (SEQ ID NO: 32) ,
HCDR3: WGYYGGSYWYFDV (SEQ ID NO: 33) ,
LCDR1: RASENIYSNLA (SEQ ID NO: 34) ,
LCDR2: AATNLAD (SEQ ID NO: 35) , and
LCDR3: QHFWGTPYT (SEQ ID NO: 36) ;
(c) HCDR1: DFYMN (SEQ ID NO: 37) ,
HCDR2: DANPNNGGTTHNPRFKG (SEQ ID NO: 38) ,
HCDR3: GGLPGDFDY (SEQ ID NO: 39) ,
LCDR1: KASEDIYNRLA (SEQ ID NO: 40) ,
LCDR2: GVTSLET (SEQ ID NO: 41) , and
LCDR3: QQYWSFPWT (SEQ ID NO: 42) ;
(d) HCDR1: NYWMS (SEQ ID NO: 43) ,
HCDR2: EIRLKSDNYATHYAESVKG (SEQ ID NO: 44) ,
HCDR3: NRFAY (SEQ ID NO: 45) ,
LCDR1: KASENVGTYVY (SEQ ID NO: 46) ,
LCDR2: GASNRYT (SEQ ID NO: 47) , and
LCDR3: GQSYSYPFT (SEQ ID NO: 48) ; and
(e) HCDR1: TYLMH (SEQ ID NO: 49) ,
HCDR2: YINPYNDVTEYHEKFKD (SEQ ID NO: 50) ,
HCDR3: LGGYGPGWYLDV (SEQ ID NO: 51) ,
LCDR1: RASGSIHNYLA (SEQ ID NO: 52) ,
LCDR2: NAKTLAD (SEQ ID NO: 53) , and
LCDR3: HHFWSTPWT (SEQ ID NO: 54) .

[Claim 31] The multi-specific antibody of claim 30, wherein the VH comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, 10, 12 and 14, or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, 10, 12 and 14, and the VL comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 7,

9, 11, 13 and 15, or a peptide having at least 90%sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 7, 9, 11, 13 and 15.

[Claim 32] The multi-specific antibody of claim 31, wherein:
(a) the VH comprises the amino acid sequence of SEQ ID NO: 4, and the VL comprises the amino acid sequence of SEQ ID NO: 5;
(b) the VH comprises the amino acid sequence of SEQ ID NO: 6, and the VL comprises the amino acid sequence of SEQ ID NO: 7;
(c) the VH comprises the amino acid sequence of SEQ ID NO: 8, and the VL comprises the amino acid sequence of SEQ ID NO: 9;
(d) the VH comprises the amino acid sequence of SEQ ID NO: 10 and the VL comprises the amino acid sequence of SEQ ID NO: 11;
(e) the VH comprises the amino acid sequence of SEQ ID NO: 12, and the VL comprises the amino acid sequence of SEQ ID NO: 13; or
(f) the VH comprises the amino acid sequence of SEQ ID NO: 14, and the VL comprises the amino acid sequence of SEQ ID NO: 15.

[Claim 33] The multi-specific antibody of any one of claims 20-28, wherein the third antigen-binding moiety comprises an antibody or antigen-binding fragment thereof having specificity to a human Cadherin 17 (CDH17) protein.
(a) the VH comprises the amino acid sequence of SEQ ID NO: 104, and the VL comprises the amino acid sequence of SEQ ID NO: 105;
(b) the VH comprises the amino acid sequence of SEQ ID NO: 106, and the VL comprises the amino acid sequence of SEQ ID NO: 107;
(c) the VH comprises the amino acid sequence of SEQ ID NO: 108, and the VL comprises the amino acid sequence of SEQ ID NO: 109;
(d) the VH comprises the amino acid sequence of SEQ ID NO: 110 and the VL comprises the amino acid sequence of SEQ ID NO: 111;
(e) the VH comprises the amino acid sequence of SEQ ID NO: 110, and the VL comprises the amino acid sequence of SEQ ID NO: 112; or
(f) the VH comprises the amino acid sequence of SEQ ID NO: 110, and the VL comprises the amino acid sequence of SEQ ID NO: 113.

[Claim 34] A pharmaceutical composition comprising the antibody or antigen-binding fragment thereof of any one of claims 10-18 or the multi-specific antibody of any one of claims 1-9 and 19-33, and a pharmaceutically acceptable carrier.

[Claim 35] One or more polynucleotide (s) encoding the antibody or antigen-binding fragment thereof of any one of claims 10-18 or the multi-specific antibody of any one of claims 1-9 and 19-33.

[Claim 36] A vector comprising the one or more polynucleotide (s) of claim 35.

[Claim 37] A host cell comprising the one or more polynucleotide (s) of claim 34, or the vector of claim 36.

[Claim 38] A method of treating a disease or condition in a subject in need thereof, comprising administering to the subject an effective amount of the antibody or antigen-binding fragment thereof of any one of claims 10-18 or the multi-specific antibody of any one of claims 1-9 and 19-33, the pharmaceutical composition of claim 34, the one or more polynucleotide (s) of claim 35, the vector of claim 36, or the host cell of claim 37.

[Claim 39] Use of the antibody or antigen-binding fragment thereof of any one of claims 10-18 or the multi-specific antibody of any one of claims 1-9 and 19-33, the pharmaceutical composition of claim 34, the one or more polynucleotide (s) of claim 35, the vector of claim 36, or the host cell of claim 37 for the preparation of a medicament for treating a disease or condition.

[Claim 40] The method of claim 38 or the use of claim 39, wherein the disease or condition is cancer.

[Claim 41] The method or use of claim 40, wherein the cancer is selected from the group consisting of ovarian cancer, prostate cancer, cancer of the urinary tract, pancreatic cancer, lung cancer, breast cancer, bladder cancer, colorectal cancer, endometrial cancer, esophageal cancer, head and neck cancer, kidney cancer, leukemia, liver cancer, lymphoma, melanoma, and thyroid cancer.