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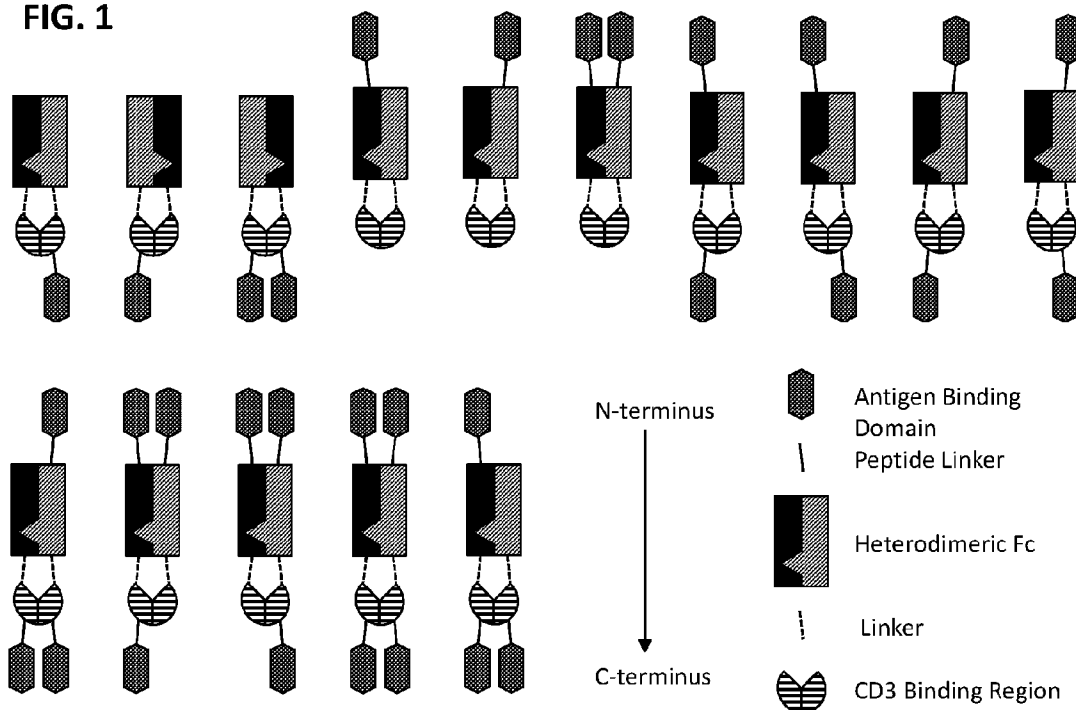
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FIXATION A CD3 CONTRAINTE ET METHODES D'UTILISATION ASSOCIEES

(54) Title: MULTISPECIFIC POLYPEPTIDE CONSTRUCTS HAVING CONSTRAINED CD3 BINDING AND RELATED
METHODS AND USES

FIG. 1



(57) **Abrégé/Abstract:**

The invention relates generally to multispecific polypeptides having constrained CD3 binding. In some embodiments, components of the multispecific polypeptides are connected by a non-cleavable linker. Also provided are methods of making and using these multispecific polypeptides in a variety of therapeutic, diagnostic and prophylactic indications.

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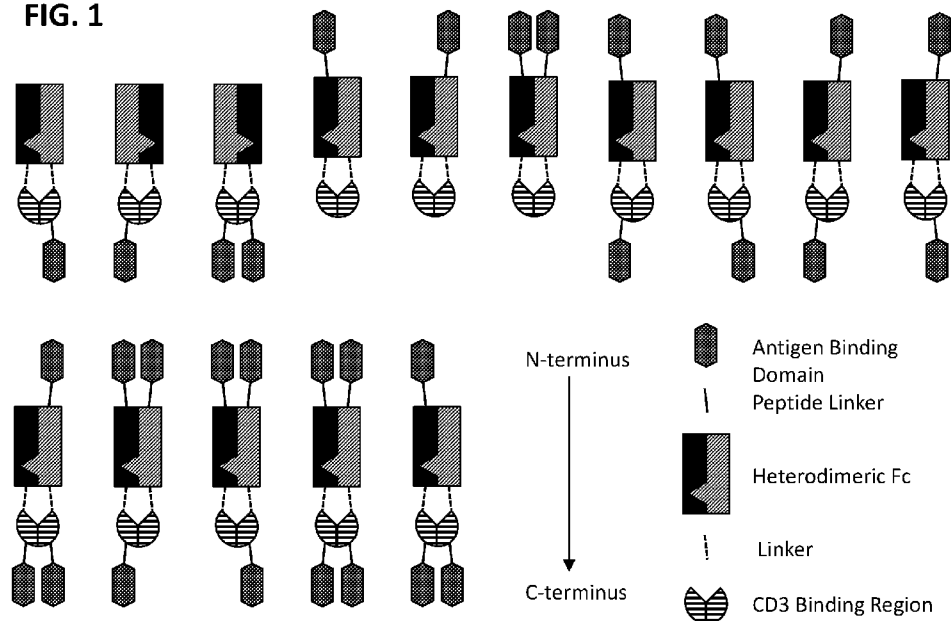
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(54) Title: MULTISPECIFIC POLYPEPTIDE CONSTRUCTS HAVING CONSTRAINED CD3 BINDING AND RELATED METHODS AND USES

FIG. 1



(57) Abstract: The invention relates generally to multispecific polypeptides having constrained CD3 binding. In some embodiments, components of the multispecific polypeptides are connected by a non-cleavable linker. Also provided are methods of making and using these multispecific polypeptides in a variety of therapeutic, diagnostic and prophylactic indications.

[Continued on next page]

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MULTISPECIFIC POLYPEPTIDE CONSTRUCTS HAVING CONSTRAINED CD3 BINDING AND RELATED METHODS AND USES

Cross-Reference to Related Applications

[0001] This application claims priority to U.S. provisional application 62/656,331, filed April 11, 2018, entitled “MULTISPECIFIC POLYPEPTIDE CONSTRUCTS HAVING CONSTRAINED CD3 BINDING AND RELATED METHODS AND USES,” the contents of which are incorporated by reference in their entirety for all purposes.

Incorporation by Reference of Sequence Listing

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 744952000240SeqList.TXT, created April 10, 2019 which is 212 kilobytes in size. The information in the electronic format of the Sequence Listing is incorporated by reference in its entirety.

Field

[0003] The invention relates generally to multispecific polypeptides having constrained CD3 binding. In some embodiments, components of the multispecific polypeptides are connected by a non-cleavable linker. Also provided are methods of making and using these multispecific polypeptides in a variety of therapeutic, diagnostic and prophylactic indications.

Background

[0004] Therapeutic antibodies that cause target cell depletion generally rely on effector functions mediated via interaction with Fc-gamma-receptors (FcγRs) and complement proteins. Effector cells expressing FcγRs are predominately those of the innate immune system. T-cells are not direct effector cells involved in antibody mediated target cell depletion.

[0005] CD3 (Cluster of Differentiation 3) T-cell co-receptor is a multimeric protein composed of four distinct polypeptide chains, referred to as the ε, γ, δ, and ζ chains. The CD3 complex serves as the signaling module of the T cell receptor that associates non-covalently with the antigen-binding α/β chains of T cell receptor (TCR).

[0006] Because direct engagement of CD3 results in T-cell activation, it is a desirable target for a variety of therapeutic and/or diagnostic indications. Accordingly, there exists a need for antibodies and therapeutics that target the CD3/TCR pathway.

Summary

[0007] The present disclosure provides multispecific polypeptide constructs that exhibit constrained CD3 binding. In some embodiments, the multispecific polypeptide construct is composed of a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein the first and second components are coupled by a linker, such as a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA). In some embodiments, the CD3-binding region binds CD3 (CD3ε). In some embodiments, the antigen binding domain is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the first component comprises a first antigen binding domain and the second component comprises a second antigen binding domain, wherein each of the antigen binding domains bind a tumor associated antigen (TAA). In some cases, the first antigen binding domain is positioned at the amino terminus of the multispecific construct and the second antigen binding domain is positioned at the carboxy terminus of the multispecific construct. In some embodiments, the first antigen binding domain is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In particular embodiments of provided multispecific polypeptide constructs, at least one antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct.

[0008] Provided herein is a multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus: a first antigen binding domain that binds to a tumor-associated antigen (TAA); an immunoglobulin Fc region; a linker, such as a non-cleavable linker, a CD3 binding region that binds CD3 (CD3ε); and a second antigen binding domain that binds a tumor-associated antigen (TAA). Also provided is a multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus: an immunoglobulin Fc region; a linker,

such as a non-cleavable linker; a CD3 binding region that binds CD3 (CD3ε); and an antigen binding domain that binds a tumor-associated antigen (TAA). Provided is a multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus: an antigen binding domain that binds to a tumor-associated antigen (TAA); an immunoglobulin Fc region; a linker, such as a non-cleavable linker; and a CD3 binding region that binds CD3 (CD3ε).

[0009] In some of any of the provided embodiments, the linker is a non-cleavable linker. In some embodiments, the linker is a linker that does not contain a substrate recognition site specific to cleavage by a protease.

[0010] In some of any of the provided embodiments, the positioning of the Fc region N-terminal to the CD3 binding region reduces or prevents the ability of the CD3 binding region to bind CD3. In some embodiments, the first component (component #1) and the second component (component #2) of the multispecific polypeptide constructs are linked and binding to CD3 is disallowed, unless the antigen binding domain(s) is bound to its cognate antigen. In some embodiments, component #1 contains at least one antigen binding domain and an Fc region. In some embodiments, component #2 contains at least a CD3 binding region domain and an antigen binding domain, the former of which is capable of binding CD3 (when the multispecific construct is bound to antigen recognized by the antigen binding domain or domains of component #1 or component #2). Thus, linkage of the CD3 binding region to the Fc region as described ensures that the multispecific polypeptide constructs do not bind or otherwise engage CD3 unless the antigen binding domain(s) is bound to its cognate antigen. This is advantageous as it prevents systemic binding of the CD3 binding region to T-cells and instead focuses the CD3 binding region's ability to bind to site of antigen expression. This is beneficial as it diminishes or eliminates a major binding sink of peripheral T-cells, potentially allowing more favorable distribution and localization at site of antigen expression, e.g., tumor cells or the tumor microenvironment.

[0011] When the antigen binding domain(s) is bound to its cognate antigen, the multispecific polypeptide construct, via component #2, is capable of forming an immune synapse between an antigen-expressing cell and a T-cell. This co-engagement mediates antigen dependent T-cell activation, cytotoxicity, cytokine release, degranulation and proliferation. In some embodiments, the multispecific polypeptide constructs are capable of interacting with FcγRs and mediating innate immune effector functions, for example antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis

(ADCP). In some embodiments, the multispecific polypeptide constructs are capable of interacting with complement proteins, namely C1q, and mediating complement-dependent cytotoxicity.

[0012] In some embodiments, the cognate antigen recognized by the antigen binding domain(s) of a provided multispecific polypeptide construct is a tumor associated antigen (TAA).

[0013] Thus, among the provided embodiments, the multispecific polypeptide construct is composed of a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein the first and second components are coupled by a linker, such as a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA). In some embodiments, the CD3-binding region binds CD3 (CD3ε). In some embodiments, the antigen binding domain is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the first component comprises a first antigen binding domain and the second component comprises a second antigen binding domain, wherein each of the antigen binding domains bind a tumor associated antigen (TAA). In some cases, the first antigen binding domain is positioned at the amino terminus of the multispecific construct and the second antigen binding domain is positioned at the carboxy terminus of the multispecific construct. In some embodiments, the first antigen binding domain is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In particular embodiments of provided multispecific polypeptide constructs, at least one antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct.

[0014] In some embodiments, the CD3 binding region is an antibody or an antigen binding fragment. In particular embodiments, the antibody or antigen binding fragment is a two chain polypeptide containing a variable heavy (VH) and a variable light (VL) chain. In some embodiments, the antibody or antigen-binding fragment is an Fv. In particular embodiments, the Fv is a disulfide-stabilized Fv (dsFv) containing an interchain disulfide bond between the VH and VL chains.

[0015] Provided herein is a multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein the CD3 binding region is an anti-CD3 antibody or antigen binding fragment that is an Fv antibody fragment comprising a variable heavy chain region (VH) and a variable light chain region (VL); the Fc is a heterodimeric Fc comprising a first Fc polypeptide and a second Fc polypeptide and the VH and VL of the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc; the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and the first component comprises a first antigen binding domain and the second component comprises a second antigen binding domain, wherein each of the antigen binding domains bind a tumor associated antigen (TAA).. In some embodiments, the CD3-binding region binds CD3 (CD3ε). In some embodiments, the first antigen binding domain is positioned amino-terminally relative to the Fc region of the multispecific construct and the second antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific construct. In some embodiments, the multispecific construct comprises in order, from N-terminus to C-terminus: the first antigen binding domain that binds to a tumor-associated antigen (TAA); the immunoglobulin Fc region; the non-cleavable linker; the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε); and the second antigen binding domain that binds a tumor-associated antigen (TAA).

[0016] Provided herein is a multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein: the CD3 binding region is an anti-CD3 antibody or antigen binding fragment that is a disulfide-stabilized Fv antibody fragment (dsFv) comprising a variable heavy chain (VH) and a variable light chain (VL); the Fc is a heterodimeric Fc comprising a first Fc polypeptide and a second Fc polypeptide and the VH and VL of the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc; the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA). In particular embodiments of provided multispecific polypeptide constructs, at least one antigen binding domain is positioned carboxy-terminally relative to

the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the CD3-binding region binds CD3 (CD3ε).

[0017] Provided herein is a multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein: the CD3 binding region is an anti-CD3 antibody or antigen binding fragment that is an Fv antibody fragment comprising a variable heavy chain (VH) and a variable light chain (VL); the Fc is a heterodimeric Fc comprising a first Fc polypeptide and a second Fc polypeptide and the VH and VL of the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc; the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA), wherein the antigen-binding domain is a single chain antibody fragment, such as an sdAb or an scFv. In particular embodiments of provided multispecific polypeptide constructs, at least one antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the CD3-binding region binds CD3 (CD3ε).

[0018] In embodiments provided herein, the multispecific construct comprises in order, from N-terminus to C-terminus: the first antigen binding domain that binds to a tumor-associated antigen (TAA); the immunoglobulin Fc region; the non-cleavable linker; the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε); and the second antigen binding domain that binds a tumor-associated antigen (TAA).

[0019] In embodiments provided herein, the multispecific construct comprises in order, from N-terminus to C-terminus: the immunoglobulin Fc region; the non-cleavable linker; the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε); and an antigen binding domain that binds a tumor-associated antigen (TAA).

[0020] In embodiments provided herein, the multispecific construct comprises in order, from N-terminus to C-terminus: the antigen binding domain that binds to a tumor-associated antigen (TAA); the immunoglobulin Fc region; the non-cleavable linker; and the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε).

[0021] Provided herein is a multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus: a first antigen binding domain that binds to a tumor-associated antigen (TAA); an immunoglobulin Fc

region; a linker, such as a non-cleavable linker; a CD3 binding region that binds CD3 (CD3ε); and a second antigen binding domain that binds a tumor-associated antigen (TAA). Also provided is a multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus: an immunoglobulin Fc region; a linker, such as a non-cleavable linker; a CD3 binding region that binds CD3 (CD3ε); and an antigen binding domain that binds a tumor-associated antigen (TAA). Provided is a multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus: an antigen binding domain that binds to a tumor-associated antigen (TAA); an immunoglobulin Fc region; a linker, such as a non-cleavable linker; and a CD3 binding region that binds CD3 (CD3ε).

[0022] In some aspects, the antigen binding domain, or independently each of the antigen binding domains, is selected from an antibody or antigen binding fragment, a natural cognate binding partner, an Anticalin (engineered lipocalin), a Darpin, a Fynomer, a Centyrin (engineered fibronectin III domain), a cystine-knot domain, an Affilin, an Affibody, or an engineered CH3 domain. In some embodiments, the natural cognate binding partner comprises an extracellular domain or binding fragment thereof of the native cognate binding partner of the TAA, or a variant thereof that exhibits binding activity to the TAA.

[0023] In some embodiments, the antigen-binding domain(s) includes one or more copies of an antibody or an antigen-binding fragment thereof. In some embodiments, the antigen-binding domain(s) includes one or more copies of an antibody or an antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody. In some embodiments, the antigen-binding domain(s) include one or more copies of one or more single domain antibody (sdAb) fragments, for example V_HH, V_{NAR}, engineered V_H or V_K domains. V_HHs can be generated from camelid heavy chain only antibodies. V_{NAR}S can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric V_H and V_K domains, including interface engineering and selection of specific germline families.

[0024] In some embodiments, the one or more antigen binding domains independently bind an antigen that is a tumor associated antigen (TAA). In some examples, the antigen binding domain, or independently each of the antigen binding domains, binds to a tumor antigen selected from among 1-92-LFA-3, 5T4, Alpha-4 integrin, Alpha-V integrin,

alpha4beta1 integrin, alpha4beta7 integrin, AGR2, Anti-Lewis-Y, Apelin J receptor, APRIL, B7-H3, B7-H4, BAFF, BTLA, C5 complement, C-242, CA9, CA19-9, (Lewis a), Carbonic anhydrase 9, CD2, CD3, CD6, CD9, CD11a, CD19, CD20, CD22, CD24, CD25, CD27, CD28, CD30, CD33, CD38, CD40, CD40L, CD41, CD44, CD44v6, CD47, CD51, CD52, CD56, CD64, CD70, CD71, CD74, CD80, CD81, CD86, CD95, CD117, CD123, CD125, CD132, (IL-2RG), CD133, CD137, CD138, CD166, CD172A, CD248, CDH6, CEACAM5 (CEA), CEACAM6 (NCA-90), CLAUDIN-3, CLAUDIN-4, cMet, Collagen, Cripto, CSFR, CSFR-1, CTLA-4, CTGF, CXCL10, CXCL13, CXCR1, CXCR2, CXCR4, CYR61, DL44, DLK1, DLL3, DLL4, DPP-4, DSG1, EDA, EDB, EGFR, EGFRviii, Endothelin B receptor (ETBR), ENPP3, EpCAM, EPHA2, EPHB2, ERBB3, F protein of RSV, FAP, FGF-2, FGF8, FGFR1, FGFR2, FGFR3, FGFR4, FLT-3, Folate receptor alpha (FR α), GAL3ST1, G-CSF, G-CSFR, GD2, GITR, GLUT1, GLUT4, GM-CSF, GM-CSFR, GP IIb/IIIa receptors, Gp130, GPIIB/IIIA, GPNMB, GRP78, HER2/neu, HER3, HER4, HGF, hGH, HVEM, Hyaluronidase, ICOS, IFNalpha, IFNbeta, IFNgamma, IgE, IgE Receptor (Fc ϵ RI), IGF, IGF1R, IL1B, IL1R, IL2, IL11, IL12, IL12p40, IL-12R, IL-12Rbeta1, IL13, IL13R, IL15, IL17, IL18, IL21, IL23, IL23R, IL27/IL27R (wsx1), IL29, IL-31R, IL31/IL31R, IL2R, IL4, IL4R, IL6, IL6R, Insulin Receptor, Jagged Ligands, Jagged 1, Jagged 2, KISS1-R, LAG-3, LIF-R, Lewis X, LIGHT, LRP4, LRRC26, Ly6G6D, LyPD1, MCSP, Mesothelin, MRP4, MUC1, Mucin-16 (MUC16, CA-125), Na/K ATPase, NGF, Nicastrin, Notch Receptors, Notch 1, Notch 2, Notch 3, Notch 4, NOV, OSM-R, OX-40, PAR2, PDGF-AA, PDGF-BB, PDGFRalpha, PDGFRbeta, PD-1, PD-L1, PD-L2, Phosphatidyl-serine, P1GF, PSCA, PSMA, PSGR, RAAG12, RAGE, SLC44A4, Sphingosine 1 Phosphate, STEAP1, STEAP2, TAG-72, TAPA1, TEM-8, TGFbeta, TIGIT, TIM-3, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TMEM31, TNFalpha, TNFR, TNFRS12A, TRAIL-R1, TRAIL-R2, Transferrin, Transferrin receptor, TRK-A, TRK-B, uPAR, VAP1, VCAM-1, VEGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, VISTA, WISP-1, WISP-2, and WISP-3.

[0025] In some embodiments, the Fc region is a homodimeric Fc region. In some embodiments, the immunoglobulin Fc region of the first component is an IgG isotype selected from the group consisting of IgG1 isotype, IgG2 isotype, IgG3 isotype, and IgG4 subclass. In some examples, the Fc region is an Fc region of a human IgG1, a human IgG2, a human IgG3, or a human IgG4, or is an immunologically active fragment thereof. In some embodiments, the Fc region comprises a polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:1. In some cases, the Fc region comprises a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:2. In some of any such embodiments, the Fc region comprises a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 4 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:4. In some examples, the Fc region comprises a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:5. In some examples, the Fc region comprises a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 6 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:6.

[0026] In some embodiments, the immunoglobulin Fc region is a polypeptide comprising an amino acid sequence that is derived from an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-6.

[0027] In some embodiments, the immunoglobulin Fc region is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-6. In some embodiments, the immunoglobulin Fc region is a polypeptide comprising an amino acid sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-6.

[0028] In some embodiments, the Fc region is a heterodimeric Fc region.

[0029] In some embodiments, the Fc region is a heterodimer containing a first Fc polypeptide and a second Fc polypeptide wherein one or both of the first and second Fc polypeptides of the heterodimeric Fc region are a variant Fc polypeptide comprising at least one modification to induce heterodimerization compared to an Fc region of human IgG1, human IgG2 or human IgG4. In some embodiments, the at least one modification is in or compared to an Fc region of human IgG1. In some embodiments, the at least one modification is in or compared to the Fc polypeptide set forth in SEQ ID NO:1 or an immunologically active fragment thereof. In some cases, one or both Fc polypeptides of the heterodimeric Fc region comprises at least one modification to induce heterodimerization compared to a polypeptide of a homodimeric Fc region, optionally compared to the Fc polypeptide set forth in SEQ ID NO:1 or an immunologically active fragment thereof. In

some embodiments, each of the Fc polypeptides of the heterodimeric Fc independently comprise at least one amino acid modification. In some cases, the at least one modification is selected from a steric modification(s), a knob-into-hole modification(s), a charge mutation(s) to increase electrostatic complementarity of the polypeptides, a modification(s) to alter the isoelectric point (pI variant), or combinations thereof.

[0030] In some examples, the amino acid modification is a charge mutation to increase electrostatic complementarity of the polypeptides. In some embodiments, the first and/or second Fc polypeptides comprise a modification in complementary positions, wherein the modification is replacement with an amino acid having an opposite charge to the complementary amino acid of the other polypeptide. In some embodiments, the first or second polypeptide comprise a modification in complementary positions, wherein the modification is replacement with an amino acid having an opposite charge to the complementary amino acid of the other polypeptide. In some embodiments, at least the first or second Fc polypeptides each comprise a modification in complementary positions, wherein the modification is replacement with an amino acid having an opposite charge to the complementary amino acid of the other polypeptide. In some embodiments, the first and second Fc polypeptides each comprise a modification in complementary positions, wherein the modification is replacement with an amino acid having an opposite charge to the complementary amino acid of the other polypeptide.

[0031] In some examples, the amino acid modification is a knob-into-hole modification.

[0032] In some embodiments, the first Fc polypeptide of the heterodimeric Fc comprises the modification selected from among Thr366Ser, Leu368Ala, Tyr407Val, and combinations thereof and the second Fc polypeptide of the heterodimeric Fc comprises the modification T366W. In some cases, the first and second Fc polypeptides further comprise a modification of a non-cysteine residue to a cysteine residue, wherein the modification of the first polypeptide is at one of a position Ser354 and Y349 and the modification of the second Fc polypeptide is at the other of the position Ser354 and Y349. In some embodiments, the first Fc polypeptide comprises the modifications T366W/S354C and the second Fc polypeptide comprises the modifications T366S/L368A/Y407V/Y349C. In some embodiments, the first Fc polypeptide comprises the modifications L368D/K370S and the second Fc polypeptide comprises the modifications S364K/E357Q.

[0033] In some embodiments, the first Fc polypeptide comprises the modifications L368D/K370S and the second Fc polypeptide comprises the modifications S364K/E357Q.

[0034] In some embodiments, at least one of the first and second polypeptide comprises the modifications Q295E/N384D/Q418E/N421D.

[0035] In some embodiments, one of the first or second Fc polypeptide of the heterodimeric Fc further comprises a modification at residue Ile253. In some instances, the modification is Ile253Arg. In some embodiments, one of the first or second Fc polypeptide of the heterodimeric Fc further comprises a modification at residue His435. In some instances, the modification is His435Arg.

[0036] In some embodiments, the Fc region, such as the first and/or second Fc polypeptide comprises a polypeptide that lacks Lys447.

[0037] In some of any of the provided embodiments, the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:82, 86 or 201, and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:83, 87, 90, 92, 202 or 205. In some embodiments, the first Fc polypeptide and the second Fc polypeptide comprises sequences selected from the group consisting of SEQ ID NOS: 82 and 83, respectively; SEQ ID NOS: 86 and 87, respectively; SEQ ID NOS: 201 and 202, respectively; SEQ ID NOS: 82 and 90, respectively; SEQ ID NOS: 86 and 92, respectively; and SEQ ID NOS: 201 and 205, respectively.

[0038] In some embodiments, the immunoglobulin Fc region is a polypeptide comprising an amino acid sequence that is derived from an amino acid sequence selected from the group consisting of SEQ ID NOS: 1-6 comprising one or modifications. In some embodiments, the immunoglobulin Fc region is a polypeptide comprising an amino acid sequence that is derived from an amino acid sequence selected from the group consisting of SEQ ID NOS: 1-6 comprising one or modifications to prevent glycosylation, to alter Fc receptor interactions, to reduce Fc receptor binding, to enhance the interaction with CD32A, to reduce the complement protein C1q binding, to extend the half-life, to enhance FcRn binding, to alter antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), to induce heterodimerization, to prevent dimerization, to stabilize the homodimerization at the CH3:CH3 interface, and combinations thereof.

[0039] In some embodiments, modifications within the Fc region reduce binding to Fc-receptor-gamma receptors while having minimal impact on binding to the neonatal Fc receptor (FcRn). In some embodiments, the mutated or modified Fc polypeptide includes the following mutations: Met252Tyr and Met428Leu or Met252Tyr and Met428Val (M252Y, M428L, or M252Y, M428V) using the Kabat numbering system.

[0040] In some embodiments, the Fc region comprises a polypeptide comprising at least one modification to enhance FcRn binding. In some examples, the modification is at a position selected from the group consisting of Met252, Ser254, Thr256, Met428, Asn434, and combinations thereof. In some cases, the modification is at a position selected from the group consisting of Met252Y, Ser254T, Thr256E, Met428L, Met428V, Asn434S, and combinations thereof. In some particular embodiments, the modification is at position Met252 and at position Met428. In some cases, the modification is Met252Y and Met428L. In some cases, the modification is Met252Y and Met428V.

[0041] In some embodiments, the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 94, 96 or 207, and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 98, 100, or 209. In some embodiments, the first Fc polypeptide and the second Fc polypeptide comprises sequences selected from the group consisting of SEQ ID NOS: 94 and 98, respectively; SEQ ID NOS: 96 and 100, respectively; and SEQ ID NOS: 207 and 209, respectively.

[0042] In some embodiments, the Fc region comprises a polypeptide comprising at least one modification to enhance Fc γ R binding. In some cases, the modification is modification at Ser239 or Ile332. In some embodiments, the glycosylation of the Fc region is modified to enhance Fc γ R binding as compared to an unmodified Fc region. In some examples, the Fc region lacks or has reduced fucose content.

[0043] In some embodiments, the Fc region comprises a polypeptide comprising at least one amino acid modification that reduces effector function and/or reduces binding to an effector molecule selected from an Fc gamma receptor or C1q. In some embodiments, the one or more amino acid modification is deletion of one or more of Glu233, Leu234 or Leu235.

[0044] In some embodiments, the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:82, 86, 94 or 96, and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:83, 87, 90, 92, 98 or 100. In some embodiments, the Fc region comprises a polypeptide comprising at least one amino acid modification that reduces effector function and/or reduces binding to an effector molecule selected from an Fc gamma receptor or C1q. In some examples, the one or more amino acid modification is deletion of one or more of Glu233, Leu234 or Leu235. In some aspects, the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 84, 88, 95 or 97 and

the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 85, 89, 91, 93, 99 or 101.

[0045] In some embodiments, the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 84, 88, 95, 97, 203 or 208 and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 85, 89, 91, 93, 99, 101, 204, 206 or 210. In some embodiments, the first Fc polypeptide and the second Fc polypeptide comprises sequences selected from the group consisting of SEQ ID NOS: 84 and 85, respectively; SEQ ID NOS: 88 and 89, respectively; SEQ ID NOS: 203 and 204, respectively; SEQ ID NOS: 95 and 99, respectively; SEQ ID NOS: 97 and 101, respectively; SEQ ID NOS: 208 and 210, respectively; SEQ ID NOS: 84 and 91, respectively; SEQ ID NOS: 88 and 93, respectively; and SEQ ID NOS: 203 and 206, respectively.

[0046] In some embodiments, the CD3 binding region is an anti-CD3 antibody or antigen-binding fragment. In some embodiments, the anti-CD3 antibody or antigen binding fragment comprises a variable heavy chain region (VH) and a variable light chain region (VL). In some of any such embodiments, the CD3 binding region is monovalent.

[0047] In some embodiments, the anti-CD3 antibody or antigen binding fragment is not a single chain antibody, optionally is not a single chain variable fragment (scFv). In some embodiments, the Fc is a heterodimeric Fc and the VH and VL that comprise the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc.

[0048] In some embodiments, the CD3 binding region is not able to, or is not substantially able to, bind or engage CD3 unless at least one of the antigen binding domains is bound to its TAA. In some aspects, the CD3 binding region is not able to, or is not substantially able, to bind or engage CD3 unless at least two of the antigen binding domains is bound to their TAA(s).

[0049] In some embodiments, the multispecific polypeptide construct contains a linker that is a polypeptide linker. In some embodiments, the linker is a polypeptide of up to 25 amino acids in length. In some cases, the linker is a polypeptide of from or from about 2 to 24 amino acids, 2 to 20 amino acids, 2 to 18 amino acids, 2 to 14 amino acids, 2 to 12 amino acids, 2 to 10 amino acids, 2 to 8 amino acids, 2 to 6 amino acids, 6 to 24 amino acids, 6 to 20 amino acids, 6 to 18 amino acids, 6 to 14 amino acids, 6 to 12 amino acids, 6 to 10 amino acids, 6 to 8 amino acids, 8 to 24 amino acids, 8 to 20 amino acids, 8 to 18 amino acids, 8 to 14 amino acids, 8 to 12 amino acids, 8 to 10 amino acids, 10 to 24 amino acids, 10 to 20

amino acids, 10 to 18 amino acids, 10 to 14 amino acids, 10 to 12 amino acids, 12 to 24 amino acids, 12 to 20 amino acids, 12 to 18 amino acids, 12 to 14 amino acids, 14 to 24 amino acids, 14 to 20 amino acids, 14 to 18 amino acids, 18 to 24 amino acids, 18 to 20 amino acids or 20 to 24 amino acids. In some embodiments, the linker is a polypeptide that is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length.

[0050] In some embodiments, the linker is 3 to 18 amino acids in length. In some embodiments, the linker is 12 to 18 amino acids in length. In some embodiments, the linker is 15 to 18 amino acids in length. In some embodiments, the linker is a polypeptide that is 18 amino acids in length.

[0051] In some embodiments, the non-cleavable linker does not contain a substrate recognition site that is specifically recognized for cleavage by a protease. In some embodiments, the protease is produced by an immune effector cell, by a tumor, or by cells present in the tumor microenvironment. In some embodiments, the protease is produced by an immune effector cell and the immune effector cell is an activated T cell, a natural killer (NK) cell, or an NK T cell. In some embodiments, the protease is selected from among matriptase, a matrix metalloprotease (MMP), granzyme B, and combinations thereof. In some embodiments, the protease is granzyme B.

[0052] In some embodiments, the linker comprises the amino acid sequence GS, GGS, GGGGS (SEQ ID NO:149), GGGGGS (SEQ ID NO:135) and combinations thereof. In some embodiments, the linker comprises the amino acid sequence (GGS)_n, wherein n is 1 to 10. In some embodiments, the linker comprises the amino acid sequence (GGGGS)_n (SEQ ID NO: 173), wherein n is 1 to 10. In some embodiments, the linker comprises (GGGGGS)_n (SEQ ID NO:172), wherein n is 1 to 4.

[0053] In some embodiments, the linker is or comprises GGS. In some embodiments, the linker is or comprises GGGGS (SEQ ID NO: 149). In some embodiments, the linker is or comprises GGGGGS (SEQ ID NO: 135). In some embodiments, the linker is or comprises GGSGGS (“(GGS)₂”) (SEQ ID NO: 10). In some embodiments, the linker is or comprises GGSGGSGGS (“(GGS)₃”) (SEQ ID NO: 11). In some embodiments, the linker is or comprises GGSGGSGGSGGS (“(GGS)₄”) (SEQ ID NO: 12). In some embodiments, the linker is or comprises GGSGGSGGSGGSGGS (“(GGS)₅”) (SEQ ID NO: 13). In some embodiments, the linker is or comprises GGGGSGGGGSGGGGGS (SEQ ID NO: 119). In some embodiments, the linker is or comprises GGSGGGGSGGGGSGGGGGS (SEQ ID NO: 147). In some embodiments, the linker is or comprises GGGGSGGGGSGGGGGS (SEQ ID NO:170). In some embodiments, the linker is or comprises GGGGG (SEQ ID NO:192).

[0054] In some embodiments, the antigen binding domain and the immunoglobulin Fc region of the first component (which in some cases is the first antigen binding domain) are operably linked via one or more further amino acid linkers (referred to herein as an intra-component linker). The intra-component peptide linker of the first component (also called LP1) can be a peptide linker such as any as described in Section II.3. The intra-component linker present in the first component, i.e. linking the Fc region and an antigen binding domain, can be of various lengths, for example 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length. In some embodiments, these intra-component linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. In some embodiments, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, *i.e.*, (GGG)₂ (SEQ ID NO: 10); GGSGGSGGS, *i.e.*, (GGG)₃ (SEQ ID NO: 11); GGSGGSGGSGGS, *i.e.*, (GGG)₄ (SEQ ID NO: 12); and GGSGGSGGSGGSGGS, *i.e.*, (GGG)₅ (SEQ ID NO: 13).

[0055] In some embodiments, the multispecific polypeptide construct comprises at least (i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment thereof; and (ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment thereof, wherein one or both of the first and second polypeptide comprise at least one antigen-binding domain that binds to a tumor associated antigen (TAA). In some embodiments, the VH of the anti-CD3 antibody or antigen-binding fragment is on the same polypeptide as the at least one antigen-binding domain that binds to a tumor associated antigen (TAA). In some embodiments, the polypeptide comprising the VL of the anti-CD3 antibody or antigen-binding fragment does not contain the at least one antigen-binding domain that binds to a tumor associated antigen (TAA). In particular embodiments of provided multispecific polypeptide constructs, at least one antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct.

[0056] In some embodiments, the second component includes one or more copies of the CD3 binding region.

[0057] In some embodiments, the CD3 binding region is an anti-CD3 antibody or antigen-binding fragment that includes one or more copies of an antibody or an antigen-binding fragment thereof that is able to bind or engage CD3, such as CD3ε. In some

embodiments, the anti-CD3 binding domain includes one or more copies of an antibody or an antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody.

[0058] In some embodiments, the anti-CD3 binding domain includes an Fv antibody fragment that binds CD3 ϵ (referred to herein as an anti-CD3 ϵ Fv fragment).

[0059] In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes an amino acid sequence selected from the group of SEQ ID NO: 32-81, 191, 196-200, 211, and 212. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 32-81, 191, 196-200, 211, and 212. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence selected from the group of SEQ ID NO: 32-62, 196-198, and 211 and an amino acid sequence selected from the group consisting of SEQ ID NO: 63-81, 191, 199, 200, and 212. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 32-62, 196-198, and 211 and an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 63-81, 191, 199, 200, and 212.

[0060] In some embodiments, the anti-CD3 ϵ Fv antibody fragment is a disulfide stabilized anti-CD3 binding Fv fragment (dsFv).

[0061] In some embodiments, the first component includes one or more copies of an antigen-binding domain. In certain embodiments, the first component contains at least two antigen binding domains, such as two antigen binding domains. In some embodiments, the at least two antigen binding domains of the first component bind to the same TAA. In some cases, the at least two antigen binding domains of the first component bind to different epitopes of the same TAA. In some instances, the at least two antigen binding domains of the first component bind to the same epitope of the same TAA. In some embodiments, the at least two antigen binding domain of the first component bind to different TAAs.

[0062] In some embodiments, the second component includes one or more copies of an antigen-binding domain. In certain embodiments, the second component contains at least two

antigen binding domains, such as two antigen binding domains. In some embodiments, the at least two antigen binding domains of the second component bind to the same TAA. In some cases, the at least two antigen binding domains of the second component bind to different epitopes of the same TAA. In some instances, the at least two antigen binding domains of the second component binds to a same epitope of the same TAA. In some embodiments, the at least two antigen binding domains of the second component bind to different TAAs.

[0063] In some embodiments, the first component contains a first antigen binding domain and the antigen binding domain of the second component is a second antigen binding domain. In some embodiments, the multispecific antigen binding domain comprises at least a first antigen binding domain and a second antigen binding domain, wherein the first antigen binding domain and second antigen binding domain bind to the same TAA. In some cases, the first antigen binding domain and the second antigen binding domain bind different epitopes of the same TAA. In some instances, the first antigen binding domain and the second antigen binding domain bind the same epitope of the same TAA. In some embodiments, the multispecific antigen binding domain comprises at least a first antigen binding domain and a second antigen binding domain wherein the first antigen binding domain and the second antigen binding domain bind different TAAs.

[0064] In some embodiments, the antigen binding domain of the second component (which in some cases is the second antigen binding domain) and the CD3 binding region are operably linked via one or more further amino acid linkers (referred to herein as an intra-component linker). The intra-component peptide linker of the second component (also called LP2) can be a peptide linker such as any as described in Section II.3. The intra-component linker of present in the second component, i.e. linking the CD3 binding region and an antigen binding domain, can be of various lengths, for example 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length. In some embodiments, the intra-component linker of the second component is composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. In some embodiments, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, *i.e.*, (GGS)₂ (SEQ ID NO: 10); GGSGGSGGS, *i.e.*, (GGS)₃ (SEQ ID NO: 11); GGSGGSGGSGGS, *i.e.*, (GGS)₄ (SEQ ID NO: 12); and GGSGGSGGSGGSGGS, *i.e.*, (GGS)₅ (SEQ ID NO: 13).

[0065] Provided herein is a multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising a heterodimeric Fc region and a second component comprising an anti-CD3 antibody or antigen-binding fragment comprising a variable heavy chain region (VH) and a variable light chain region (VL),

wherein: the VH and VL that comprise the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc; the first and second components are coupled by a linker, wherein the heterodimeric Fc region is positioned N-terminal to the anti-CD3 antibody or antigen-binding fragment; and one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA).

[0066] In some embodiments, the linker is a polypeptide of up to 50 amino acids in length. In some embodiments, the linker is a polypeptide of up to 25 amino acids in length. In some embodiments, the linker is a polypeptide of up to 15 amino acids in length.

[0067] In any of the provide embodiments, the one or more antigen binding domain that binds TAA results in monovalent, bivalent, trivalent, or tetravalent binding to the TAA. In some embodiments, the one or more antigen binding domains that bind TAA independently are selected from an sdAb, an scFv or a Fab. In some embodiments, the one or more antigen binding domains that binds a TAA is a single chain molecule, such as a single chain antibody fragment containing a VH and a VL, for example an sdAb or an scFv. In some embodiments the one or more antigen binding domains that binds a TAA is a sdAb, such as a V_{HH} or a VH_{NAR} . In some embodiments, at least one of the antigen binding domains is a Fab containing a first chain comprising a VH-CH1 (Fd) and a second chain comprising a VL-CL.

[0068] In some embodiments, the antigen binding domain that binds the TAA is attached to the VH of the anti-CD3 binding domain. In some embodiments, the antigen binding domain that binds the TAA is attached to the same side (e.g., knob or hole) of the heterodimeric Fc to which the VH of the anti-CD3 binding domain is attached. In some embodiments, the antigen binding domain that binds the TAA is a sdAb attached to the VH of the anti-CD3 binding domain. In some embodiments, the antigen binding domain that binds the TAA is a sdAb attached to same side (e.g., knob or hole) of the heterodimeric Fc domain to which the VH of the anti-CD3 binding domain is attached. In some embodiments, the antigen binding domain that binds the TAA is a V_{HH} or a VH_{NAR} that is attached to the VH of the anti-CD3 binding domain. In some embodiments, the antigen binding domain that binds the TAA is a V_{HH} or a VH_{NAR} that is attached to the same side (e.g., knob or hole) of the heterodimeric Fc domain to which the VH of the anti-CD3 binding domain is attached. In some embodiments, the antigen binding domain that binds the TAA is a V_{HH} attached to the VH of the anti-CD3 binding domain. In some embodiments, the antigen binding domain that binds the TAA is a V_{HH} attached to the same side (e.g., knob or hole) of the heterodimeric Fc domain to which the VH of the anti-CD3 binding domain is attached. In some embodiments,

the antigen binding domain that binds the TAA is a VH_{NAR} attached to the VH of the anti-CD3 binding domain. In some embodiments, the antigen binding domain that binds the TAA is a VH_{NAR} attached to the same side (e.g., knob or hole) or the Fc domain to which the VH of the anti-CD3 binding domain is attached.

[0069] In some embodiments, the multispecific polypeptide construct comprises at least (i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment; and (ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment, wherein one or both of the first and second polypeptide comprise at least one antigen-binding domain that binds to a tumor associated antigen (TAA). In some instances, only one of the first or second polypeptide comprises the at least one antigen-binding domain that binds a TAA.

[0070] In some embodiments, the at least one of the antigen binding domain(s) is a Fab. In some embodiments, the multispecific polypeptide construct comprises: (i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment; (ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment, and (iii) a third polypeptide comprising a VH-CH1 (Fd) or VL-CL of a Fab antibody fragment that binds to a tumor-associated antigen, wherein the first and/or second polypeptide further comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment. In some cases, only one of the first or second polypeptide comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment. In some embodiments, both the first or second polypeptide comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment. In some cases, the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of one of the first or second polypeptide of the multispecific polypeptide construct. In some embodiments, the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment is positioned amino-terminally relative to the Fc region of the first polypeptide or second polypeptide and carboxy-terminally relative to the CD3 binding region of the other of the first or second polypeptide.

[0071] In some embodiments, the at least one antigen binding domain is positioned amino-terminally relative to the Fc region and/or is positioned carboxy-terminally relative to the CD3 binding region of one of the first or second polypeptide of the multispecific

polypeptide construct. In some cases, the at least one antigen binding domain is positioned amino-terminally relative to the Fc region of the multispecific construct and the second antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific construct. In particular embodiments of provided multispecific polypeptide constructs, at least one antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the at least one antigen binding domain is a sdAb. In some embodiments the at least one antigen binding domain that is a sdAb is positioned carboxy-terminally to the CD3 binding region of the multispecific construct. In some embodiments the at least one antigen binding domain that is a sdAb is positioned amino-terminally to the Fc region of the multispecific construct. In some embodiments the at least one antigen binding domain is a V_HH. In some embodiments, the at least one antigen binding domain that is a V_HH is positioned carboxy-terminally to the CD3 binding region of the multispecific construct. In some embodiments the at least one antigen binding domain that is a V_HH is positioned amino-terminally to the Fc region of the multispecific construct.

[0072] In some embodiments, the multispecific polypeptide construct comprises a first linking peptide (LP1) between the first antigen binding domain and the immunoglobulin Fc polypeptide region (Fc region). In some embodiments, the multispecific polypeptide construct comprises a second linking peptide (LP2) between the anti-CD3 binding domain (CD3 binding region) and the second antigen binding domain. In some embodiments, the multispecific polypeptide construct comprises a first linking peptide (LP1) between the first antigen binding domain and the immunoglobulin Fc polypeptide region (Fc region) and a second linking peptide (LP2) between the anti-CD3 binding domain (CD3 binding region) and the second antigen binding domain.

[0073] In some embodiments, the multispecific polypeptide construct has the structural arrangement from N-terminus to C-terminus as follows: first antigen binding domain – LP1 – immunoglobulin Fc polypeptide linker region (Fc region) – linker – anti-CD3 binding domain – LP2 – second antigen binding domain. In some embodiments, the multispecific polypeptide construct has the structural arrangement from N-terminus to C-terminus as follows: second antigen binding domain – LP2 – immunoglobulin Fc polypeptide linker region (Fc region) – linker – anti-CD3 binding domain (CD3 binding region) – LP1 – first antigen binding domain.

[0074] In some embodiments, the two linking peptides LP1 and LP2 are not identical to each other. In some cases, LP1 or LP2 is independently a peptide of about 1 to 20 amino

acids in length. In some examples, LP1 or LP2 independently comprise a peptide that is or comprises any Gly-Ser linker as set forth in SEQ ID NOs: 10-13, 119, 135, 147, 149.

[0075] In some embodiments, the multispecific construct is a construct having any of the structural arrangement shown in Figure 1. In some embodiments, the construct is a bispecific construct that has a structural arrangement from N-terminus to C-terminus as follows. The N-terminal end of the bispecific construct includes a first antigen binding domain that binds a tumor associated antigen (TAA). The first binding domain binds a first epitope on the TAA target. Coupled to the first antigen binding domain is a central immunoglobulin Fc polypeptide region that regulates Fc γ R interactions and/or FcRn interaction. In some embodiments, the central immunoglobulin Fc polypeptide region is heterodimeric. The immunoglobulin Fc polypeptide region is coupled to a linker located at a position C-terminal to the end of the immunoglobulin Fc polypeptide region. The linker is attached to an anti-CD3 binding sequence located C-terminal to the Fc region, which, in some cases, is at the distal end of the second component. The C-terminus of the bispecific construct includes a second antigen binding domain that binds a TAA. In some embodiments, the second antigen binding domain binds the same TAA as the first antigen binding domain located within the first component. In some embodiments, the second antigen binding domain binds a second epitope on the TAA, wherein the second epitope is non-competitive with the first epitope on the TAA. In some embodiments, the second antigen binding domain binds a distinct TAA from that of the first antigen binding domain.

[0076] In some of any of the provided embodiments, the anti-CD3 antibody or antigen binding fragment is an Fv antibody fragment. In some embodiments, the Fv antibody fragment comprises a disulfide stabilized anti-CD3 binding Fv fragment (dsFv). In some embodiments, the anti-CD3 binding sequence is an Fv antibody fragment that is engineered to include a disulfide linkage between the variable heavy chain (VH) and variable light chain (VL) regions, thereby producing a disulfide stabilized anti-CD3 binding Fv fragment (dsFv). In some embodiments, the VH and VL domains that comprise the anti-CD3 Fv are operably linked to opposite members of a heterodimeric Fc region. In these embodiments, the anti-CD3 Fv binds CD3 in a monovalent fashion. In aspects as provided, the anti-CD3 dsFv does not engage CD3 unless the multispecific polypeptide construct is bound to a cognate antigen.

[0077] In some embodiments, each of the first antigen binding domain and the second antigen binding domain of the bispecific construct includes one or more copies of an antibody or an antigen-binding fragment thereof. In some embodiments, each of the first antigen

binding domain and the second antigen binding domain of the bispecific construct includes one or more copies of an antibody or an antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody. In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, is an antibody or antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody. In some embodiments, the each of the first antigen binding domain and the second antigen binding domain of the bispecific construct includes one or more copies of one or more single domain antibody (sdAb) fragments, for example V_HH, V_{NAR}, engineered V_H or V_K domains. V_HHs can be generated from natural camelid heavy chain only antibodies, genetically modified rodents that produce heavy chain only antibodies, or naïve/synthetic camelid or humanized camelid single domain antibody libraries. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric V_H and V_K domains, including interface engineering and selection of specific germline families.

[0078] In some embodiments, the antibody or antigen-binding fragment is an sdAb. In some cases, the sdAb is a human or humanized sdAb. In some aspects, the sdAb is V_HH, V_{NAR}, an engineered V_H domain or an engineered V_K domain. In some examples, the antibody or antigen-binding fragment thereof is an scFv. In some cases, the antibody or antigen-binding fragment thereof is a Fab.

[0079] In some of any of the provided embodiments, the anti-CD3 antibody or antigen-binding fragment comprises a V_H CDR1 comprising the amino acid sequence TYAMN (SEQ ID NO: 16); a V_H CD2 comprising the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17); a V_H CDR3 comprising the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a V_L CDR1 comprising the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a V_L CDR2 comprising the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a V_L CDR3 comprising the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0080] In some of any of the provided embodiments, the anti-CD3 antibody or antigen-binding fragment comprises a V_H CDR1 comprising the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a V_H CD2 comprising the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a V_H CDR3 comprising the amino acid sequence

HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 comprising the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 comprising the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 comprising the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0081] In some of any of the provided embodiments, the anti-CD3 antibody or antigen-binding fragment comprises a VH CDR1 sequence that includes at least the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that includes at least the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNHWV (SEQ ID NO: 225)

[0082] In some of any of the provided embodiments, the anti-CD3 antibody or antigen-binding fragment comprises: a VH having the amino acid sequence of any of SEQ ID NOS: 14, 32-62, 196-198, and 211 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 14, 32-62, 196-198, and 211; and a VL having the amino acid sequence of any of SEQ ID NOS: 15, 63-81, 191, 199, 200, and 212 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 15, 63-81, 191, 199, 200, and 212.

[0083] In some embodiments, the anti-CD3 antibody or antigen-binding fragment is an Fv. In some embodiments, the anti-CD3 Fv comprises: a VH having the amino acid sequence of any of SEQ ID NOS: 14, 32-43, 45-47, 48, 196 and 211 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 14, 32-43, 45-47, 48, 196 and 211; and a VL having the amino acid sequence of any of SEQ ID NOS: 15, 63, 65-71, 73, 75, 77, and 199 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 15, 63, 65-71, 73, 75, 77, and 199. In some cases, the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 14 and the amino acid sequence of SEQ ID NO: 15. In other cases, the anti-CD3 Fv comprises the amino acid sequence of SEQ ID NO: 196 and the amino acid sequence of SEQ ID NO: 199.

[0084] In some embodiments, the VH and VL chain regions of the CD3 binding domain each independently comprise at least one amino acid modification. In some embodiments,

the at least one amino acid modification of the VH and VL chain regions of the CD3 binding domain increase the stability of the CD3 binding domain. In some embodiments, the at least one amino acid modification of the VH and VL chain regions of the CD3 binding domain increase the ability of the CD3 binding domain to bind CD3. In some embodiments, the at least one amino acid modification of the VH and VL chain regions of the CD3 binding domain increase the stability of the CD3 binding domain by creating a disulfide linkage between the VH and VL chain regions.

[0085] In some embodiments, the CD3 binding region has a disulfide stabilized linkage between the VH and VL regions. In some embodiments, the anti-CD3 antibody or antigen-binding fragment is disulfide stabilized Fv (dsFv). In some embodiments, the disulfide stabilized anti-CD3 Fv comprises an anti-CD3 VH with the mutation 44 to Cys and an anti-CD3 VL with the mutation 100 to Cys by Kabat numbering. In some embodiments, the disulfide stabilized anti-CD3 Fv comprises an anti-CD3 VH with the mutation G44C and an anti-CD3 VL with the mutation G100C by Kabat numbering. In some embodiments, the disulfide stabilized anti-CD3 Fv comprises an anti-CD3 VH with the mutation at position 105 to Cys and an anti-CD3 VL with the mutation position 43 to Cys by Kabat numbering.

[0086] In some embodiments, the anti-CD3 dsFv comprises: a VH having the amino acid sequence of any of SEQ ID NOS: 44, 49-62, 197 and 198 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 44, 49-62, 197 and 198; and a VL having the amino acid sequence of any of SEQ ID NOS: 64, 72, 74, 76, 78-81, 191, 200 and 212 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 64, 72, 74, 76, 78-81, 191, 200 and 212. In some cases, the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 44 and the amino acid sequence of SEQ ID NO: 72. In some embodiments, the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 198 and the amino acid sequence of SEQ ID NO: 200. In some embodiments, the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 197 and the amino acid sequence of SEQ ID NO: 200.

[0087] In some embodiments, the multispecific construct also includes an agent conjugated to the multispecific construct. In some embodiments, the agent is a therapeutic agent. In some embodiments, the agent is a detectable moiety. In some embodiments, the detectable moiety is a diagnostic agent. In some embodiments, the agent is conjugated to the multispecific construct via a linker. In some embodiments, the linker is a non-cleavable linker.

[0088] In some embodiments, the multispecific construct described herein is used in conjunction with one or more additional agents or a combination of additional agents. Suitable additional agents include current pharmaceutical and/or surgical therapies for an intended application, such as, for example, cancer. For example, the multispecific construct can be used in conjunction with an additional chemotherapeutic or anti-neoplastic agent.

[0089] In some embodiments, the multispecific construct and additional agent are formulated into a single therapeutic composition, and the multispecific construct and additional agent are administered simultaneously. In some embodiments, the multispecific construct and additional agent are separate from each other, *e.g.*, each is formulated into a separate therapeutic composition, and the multispecific construct and the additional agent are administered simultaneously, or the multispecific construct and the additional agent are administered at different times during a treatment regimen. For example, the multispecific construct is administered prior to the administration of the additional agent, the multispecific construct is administered subsequent to the administration of the additional agent, or the multispecific construct and the additional agent are administered in an alternating fashion. As described herein, the multispecific construct and additional agent are administered in single doses or in multiple doses.

[0090] In some embodiments, the multispecific construct naturally contains one or more disulfide bonds. In some embodiments, the multispecific construct can be engineered to include one or more disulfide bonds.

[0091] The disclosure also provides an isolated nucleic acid molecule or polynucleotide encoding at least a portion of a multispecific construct described herein and/or one or more nucleic acid molecules encoding a multispecific construct described herein, such as for example, at least a first nucleic acid encoding at least a portion of the first component of the multispecific construct and a second nucleic acid encoding at least a portion of the second component of the multispecific construct, as well as vectors that include these isolated nucleic acid sequences.

[0092] Among the provided embodiments is a polynucleotide(s) encoding any of the provided multispecific polypeptide constructs. Also provided is a polynucleotide encoding a polypeptide chain of any of the provided multispecific polypeptide constructs. Further provided is a polynucleotide, comprising a first nucleic acid sequence encoding a first polypeptide of any of the provided multispecific constructs and a second nucleic acid sequence encoding a second polypeptide of the multispecific construct, wherein the first and second nucleic acid sequence are separated by an internal ribosome entry site (IRES), or a

nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping. In some cases, the first nucleic acid sequence and second nucleic acid sequence are operably linked to the same promoter. In some embodiments, the multispecific polypeptide construct comprises a third polypeptide chain, and the polynucleotide further comprises a third nucleic acid encoding the third polypeptide of the multispecific construct. In some embodiments, the third nucleic acid is separated from the first and/or second polypeptide by an internal ribosome entry site (IRES), or a nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping and/or the third nucleic acid sequence is operably linked to the same promoter as the first and/or second nucleic acid sequence. In some examples, the nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping is selected from a T2A, a P2A, a E2A or a F2A (SEQ ID NOS: 159-164, or encoded by the sequence set forth in SEQ ID NO: 165)

[0093] Provided herein is a vector comprising any of the provided polynucleotides. In some embodiments, the vector is an expression vector. In some examples, the vector is a viral vector or a eukaryotic vector, optionally wherein the eukaryotic vector is a mammalian vector.

[0094] Provided is a cell, comprising any of the provided polynucleotides or vectors. In some cases, the cell is recombinant or isolated. In some examples, the cell is a mammalian cell. In some examples, the cell is a HEK293 or CHO cell.

[0095] The disclosure provides methods of producing a multispecific construct by culturing a cell under conditions that lead to expression of the multispecific construct, wherein the cell comprises such a nucleic acid molecule(s). In some embodiments, the cell comprises such a vector.

[0096] Provided herein is a method of producing a multispecific polypeptide construct, the method comprising introducing into a cell any of the provided polynucleotides or vectors and culturing the cell under conditions to that lead to expression of the multispecific construct to produce the multispecific polypeptide construct. Also provided is a method of producing a multispecific polypeptide construct, the method comprising culturing any of the provided cells under conditions in which the multispecific polypeptide is expressed or produced by the cell. In some cases, the cell is a mammalian cell. In some examples, the cell is a HEK293 or CHO cell. In some embodiments, the method further includes isolating or purifying the multispecific polypeptide construct from the cell. In some cases, the multispecific polypeptide construct is a heterodimer.

[0097] Provided herein is a multispecific polypeptide construct produced by any of the provided methods.

[0098] Provided herein is a method of stimulating or inducing an immune response, the method comprising contacting a target cell and a T cell with the any of the provided multispecific polypeptide constructs or pharmaceutical compositions, said target cell expressing a tumor associated antigen recognized by the multispecific polypeptide construct. In some embodiments, the target cell is a tumor cell expressing the tumor associated antigen (TAA).

[0099] In some embodiments, the contacting is carried out ex vivo or in vitro. In some embodiments, the contacting is carried out in vivo in a subject.

[0100] Provided is a method of stimulating or inducing an immune response in a subject, the method comprising administering, to a subject in need thereof, a therapeutically effective amount of any of the provided multispecific conjugates or pharmaceutical compositions. In some cases, the method increases cell-mediated immunity. In some embodiments, the method increases T-cell activity. In some embodiments, the method increases cytolytic T-cell (CTL) activity. In some examples, the immune response is increased against a tumor or cancer. In some embodiments, the method treats a disease or condition in the subject.

[0101] The present disclosure also provides methods of treating, preventing, delaying the progression of or otherwise ameliorating a symptom of one or more pathologies or alleviating a symptom associated with such pathologies, by administering a multispecific polypeptide construct of the disclosure to a subject in which such treatment or prevention is desired. Provided herein is a method of treating a disease or condition in a subject, the method comprising administering, to a subject in need thereof, a therapeutically effective amount of any of the provided multispecific conjugates or pharmaceutical compositions. In some embodiments, the disease or condition is a tumor or a cancer.

[0102] In some embodiments of any of the provided method, the subject, such as the subject to be treated is, *e.g.*, human or other mammal. In some embodiments of any of the provided method, the subject is a human. In some embodiments, the subject is a non-human mammal, such as a non-human primate, companion animal (*e.g.*, cat, dog, horse), farm animal, work animal, or zoo animal. In some embodiments, the subject is a rodent.

[0103] A multispecific polypeptide construct of the disclosure used in any of the embodiments of these methods and uses can be administered at any stage of the disease. For example, such a multispecific polypeptide construct can be administered to a patient suffering

cancer of any stage, from early to metastatic. The terms subject and patient are used interchangeably herein.

[0104] A multispecific polypeptide construct of the disclosure used in any of the embodiments of these methods and uses can be used in a treatment regimen comprising neoadjuvant therapy.

[0105] A multispecific polypeptide construct of the disclosure used in any of the embodiments of these methods and uses can be administered either alone or in combination with one or more additional agents, including small molecule inhibitors, other antibody-based therapies, polypeptide or peptide-based therapies, nucleic acid-based therapies and/or other biologics. In some embodiments, a multispecific polypeptide construct is administered in combination with one or more additional agents such as, by way of non-limiting example, a chemotherapeutic agent, such as an alkylating agent, an anti-metabolite, an anti-microtubule agent, a topoisomerase inhibitor, a cytotoxic antibiotic, and any other nucleic acid damaging agent. In some embodiments, the additional agent is a taxane, such as paclitaxel (*e.g.*, Abraxane®). In some embodiments, the additional agent is an anti-metabolite, such as gemcitabine. In some embodiments, the additional agent is an alkylating agent, such as platinum-based chemotherapy, such as carboplatin or cisplatin. In some embodiments, the additional agent is a targeted agent, such as a kinase inhibitor, *e.g.*, sorafenib or erlotinib. In some embodiments, the additional agent is a targeted agent, such as another antibody, *e.g.*, a monoclonal antibody (*e.g.*, bevacizumab), a bispecific antibody, or a multispecific antibody. In some embodiments, the additional agent is a proteasome inhibitor, such as bortezomib or carfilzomib. In some embodiments, the additional agent is an immune modulating agent, such as lenalidomide or IL-2. In some embodiments, the additional agent is radiation. In some embodiments, the additional agent is an agent considered standard of care by those skilled in the art. In some embodiments, the additional agent is a chemotherapeutic agent well known to those skilled in the art. In some embodiments, the multispecific polypeptide construct and the additional agent(s) are formulated in a single composition. In some embodiments, the multispecific polypeptide construct and the additional agent(s) are administered as two or more separate compositions. In some embodiments, the multispecific polypeptide construct and the additional agent(s) are administered simultaneously. In some embodiments, the multispecific polypeptide construct and the additional agent(s) are administered sequentially.

[0106] In some embodiments, the additional agent(s) is a chemotherapeutic agent, such as a chemotherapeutic agent selected from the group consisting of docetaxel, paclitaxel,

abraxane (*i.e.*, albumin-conjugated paclitaxel), doxorubicin, oxaliplatin, carboplatin, cisplatin, irinotecan, and gemcitabine.

[0107] In some embodiments, the additional agent(s) is a checkpoint inhibitor, a kinase inhibitor, an agent targeting inhibitors in the tumor microenvironment, and/or a T cell or NK agonist. In some embodiments, the additional agent(s) is radiation therapy, alone or in combination with another additional agent(s) such as a chemotherapeutic or anti-neoplastic agent. In some embodiments, the additional agent(s) is a vaccine, an oncovirus, and/or a DC-activating agent such as, by way of non-limiting example, a toll-like receptor (TLR) agonist and/or α -CD40. In some embodiments, the additional agent(s) is a tumor-targeted antibody designed to kill the tumor via ADCC or via direct conjugation to a toxin (*e.g.*, an antibody drug conjugate (ADC)).

[0108] In some embodiments, the checkpoint inhibitor is an inhibitor of a target selected from the group consisting of CTLA-4, LAG-3, PD-1, PDL1, TIGIT, TIM-3, B7H3, B7H4, and Vista. In some embodiments, the kinase inhibitor is selected from the group consisting of B-RAFi, MEKi, and Btk inhibitors, such as ibrutinib. In some embodiments, the kinase inhibitor is crizotinib. In some embodiments, the tumor microenvironment inhibitor is selected from the group consisting of an IDO inhibitor, an α -CSF1R inhibitor, an α -CCR4 inhibitor, a TGF-beta, a myeloid-derived suppressor cell, or a T-regulatory cell. In some embodiments, the agonist is selected from the group consisting of OX40, GITR, CD137, CD28, ICOS, CD27, and HVEM. In some embodiments, the checkpoint inhibitor is an antibody that binds a target selected from CTLA-4, PD-1, and/or PD-L1. In some embodiments, the checkpoint inhibitor is an anti-CTLA4 antibody, an anti-PD-1 antibody, and an anti-PD-L1 antibody, and/or combinations thereof. In some embodiments, the checkpoint inhibitor is an anti-CTLA4 antibody such as, *e.g.*, Yervoy™. In some embodiments, the checkpoint inhibitor is an anti-PD-1 antibody such as, *e.g.*, Opdivo™ and/or Keytruda™.

[0109] In some embodiments, the inhibitor is a CTLA-4 inhibitor. In some embodiments, the inhibitor is a LAG-3 inhibitor. In some embodiments, the inhibitor is a PD-1 inhibitor. In some embodiments, the inhibitor is a PDL1 inhibitor. In some embodiments, the inhibitor is a TIGIT inhibitor. In some embodiments, the inhibitor is a TIM-3 inhibitor. In some embodiments, the inhibitor is a B7H3 inhibitor. In some embodiments, the inhibitor is a B7H4 inhibitor. In some embodiments, the inhibitor is a Vista inhibitor. In some embodiments, the inhibitor is a B-RAFi inhibitor. In some embodiments, the inhibitor is a MEKi inhibitor. In some embodiments, the inhibitor is a Btk inhibitor. In some embodiments,

the inhibitor is ibrutinib. In some embodiments, the inhibitor is crizotinib. In some embodiments, the inhibitor is an IDO inhibitor. In some embodiments, the inhibitor is an α -CSF1R inhibitor. In some embodiments, the inhibitor is an α -CCR4 inhibitor. In some embodiments, the inhibitor is a TGF-beta. In some embodiments, the inhibitor is a myeloid-derived suppressor cell. In some embodiments, the inhibitor is a T-regulatory cell.

[0110] In some embodiments, the agonist is OX40. In some embodiments, the agonist is GITR. In some embodiments, the agonist is CD137. In some embodiments, the agonist is CD28. In some embodiments, the agonist is ICOS. In some embodiments, the agonist is CD27. In some embodiments, the agonist is HVEM.

[0111] In some embodiments, the multispecific polypeptide construct is administered during and/or after treatment in combination with one or more additional agents such as, for example, a chemotherapeutic agent, an anti-inflammatory agent, and/or an immunosuppressive agent. In some embodiments, the multispecific polypeptide construct and the additional agent are formulated into a single therapeutic composition, and the multispecific polypeptide construct and additional agent are administered simultaneously. Alternatively, the multispecific polypeptide construct and additional agent are separate from each other, *e.g.*, each is formulated into a separate therapeutic composition, and the multispecific polypeptide construct and the additional agent are administered simultaneously, or the multispecific polypeptide construct and the additional agent are administered at different times during a treatment regimen. For example, the multispecific polypeptide construct is administered prior to the administration of the additional agent, the multispecific polypeptide construct is administered subsequent to the administration of the additional agent, or the multispecific polypeptide construct and the additional agent are administered in an alternating fashion. As described herein, the multispecific polypeptide construct and additional agent are administered in single doses or in multiple doses.

[0112] In some embodiments, the multispecific polypeptide construct and the additional agent(s) are administered simultaneously. For example, the multispecific polypeptide construct and the additional agent(s) can be formulated in a single composition or administered as two or more separate compositions. In some embodiments, the multispecific polypeptide construct and the additional agent(s) are administered sequentially, or the multispecific polypeptide construct and the additional agent are administered at different times during a treatment regimen.

[0113] In addition to the elements described above, the multispecific polypeptide construct can contain additional elements such as, for example, amino acid sequence N- or C-

terminal of the multispecific polypeptide construct. For example, a multispecific polypeptide construct can include a targeting moiety to facilitate delivery to a cell or tissue of interest. Multispecific polypeptide construct can be conjugated to an agent, such as a therapeutic agent, a detectable moiety or a diagnostic agent. Examples of agents are disclosed herein.

[0114] The multispecific polypeptide construct can also include any of the conjugated agents, linkers and other components described herein in conjunction with a multispecific polypeptide construct of the disclosure.

[0115] The disclosure also pertains to immunoconjugates comprising a multispecific polypeptide construct conjugated to a cytotoxic agent such as a toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate). Suitable cytotoxic agents for use in targeting diseased T cells such as in a T cell-derived lymphoma include, for example, dolastatins and derivatives thereof (*e.g.* auristatin E, AFP, MMAD, MMAF, MMAE). In some embodiments, the agent is a dolastatin. In some embodiments, the agent is an auristatin or derivative thereof. In some embodiments, the agent is a maytansinoid or maytansinoid derivative. In some embodiments, the agent is DM1 or DM4. In some embodiments, the agent is a duocarmycin or derivative thereof. In some embodiments, the agent is a calicheamicin or derivative thereof. In some embodiments, the agent is a pyrrolobenzodiazepine.

[0116] In some embodiments, the linker between the multispecific polypeptide construct and the cytotoxic agent is cleavable. In some embodiments, the linker is non-cleavable. In some embodiments, two or more linkers are present. The two or more linkers are all the same, *e.g.*, cleavable or non-cleavable, or the two or more linkers are different, *e.g.*, at least one cleavable and at least one non-cleavable.

[0117] The multispecific polypeptide constructs and conjugates thereof are useful in methods for treating a variety of disorders and/or diseases. Non-limiting examples of disease include: all types of cancers (breast, lung, colorectal, prostate, melanomas, head and neck, pancreatic, etc.), rheumatoid arthritis, Crohn's disease, SLE, cardiovascular damage, ischemia, etc. For example, indications would include leukemias, including T-cell acute lymphoblastic leukemia (T-ALL), lymphoblastic diseases including multiple myeloma, and solid tumors, including lung, colorectal, prostate, pancreatic, and breast, including triple negative breast cancer. For example, indications include bone disease or metastasis in cancer, regardless of primary tumor origin; breast cancer, including by way of non-limiting example, ER/PR+ breast cancer, Her2+ breast cancer, triple-negative breast cancer; colorectal cancer; endometrial cancer; gastric cancer; glioblastoma; head and neck cancer, such as esophageal

cancer; lung cancer, such as by way of non-limiting example, non-small cell lung cancer; multiple myeloma ovarian cancer; pancreatic cancer; prostate cancer; sarcoma, such as osteosarcoma; renal cancer, such as by way of nonlimiting example, renal cell carcinoma; and/or skin cancer, such as by way of nonlimiting example, squamous cell cancer, basal cell carcinoma, or melanoma. In some embodiments, the cancer is a squamous cell cancer. In some embodiments, the cancer is a skin squamous cell carcinoma. In some embodiments, the cancer is an esophageal squamous cell carcinoma. In some embodiments, the cancer is a head and neck squamous cell carcinoma. In some embodiments, the cancer is a lung squamous cell carcinoma.

[0118] Also provided is a pharmaceutical composition comprising any of the multispecific polypeptide constructs provided herein and a pharmaceutically acceptable carrier. In some cases, the pharmaceutical composition is sterile. Pharmaceutical compositions according to the disclosure can include a multispecific polypeptide construct of the disclosure and a carrier. These pharmaceutical compositions can be included in kits, such as, for example, diagnostic kits.

[0119] One skilled in the art will appreciate that the antibodies of the disclosure have a variety of uses. For example, the proteins of the disclosure are used as therapeutic agents for a variety of disorders. The antibodies of the disclosure are also used as reagents in diagnostic kits or as diagnostic tools, or these antibodies can be used in competition assays to generate therapeutic reagents.

BRIEF DESCRIPTION OF DRAWINGS

[0120] **FIG. 1** is a schematic of the basic components of the multispecific polypeptide constructs of the present disclosure having constrained CD3 binding. The antigen binding domain(s) are positioned at the amino and/or carboxy termini. The Fc region, such as a heterodimeric Fc region, is positioned N-terminal to the CD3 binding region. This positioning of the Fc in close proximity to the CD3 binding region obstructs CD3 binding. The linker can be a non-cleavable linker as provided herein.

[0121] **FIG. 2A** and **B** is a schematic of various FR α -targeting constrained CD3 constructs composed of two polypeptides, Chain 1 and Chain 2. As shown in **FIG. 2A**, Chain 1 contains a FR α sdAb (antigen binding domain), linked to a heterodimeric Fc “hole,” linked via a non-cleavable linker (ranging from 3 amino acids in cx1356 to 18 amino acids in cx681) to anti-CD3 VL domain, linked to a second FR α sdAb; and Chain 2 contains a FR α sdAb, linked to a complementary heterodimeric Fc “knob”, linked via the same non-cleavable linker

as above to anti-CD3 VH domain, linked to second FR α sdAb. When co-expressed the CD3 binding domain is properly assembled via the association of the VL:VH on the hole and knob, respectively (**FIG. 2B**). An exemplary anti-CD3 is a disulfide-stabilized Fv (dsFv) containing a variable light (VL) chain with the mutation G100C and a variable heavy (VH) chain with a mutation G44C.

[0122] FIG. 3A is a schematic of various B7H3-targeting constrained CD3 constructs composed of two polypeptides, Chain 1 and Chain 2. Chain 1 contains either a heterodimeric Fc “hole,” linked via a non-cleavable linker to an anti-CD3 VL domain modified at G100C (top); a B7H3-targeting sdAb linked to a heterodimeric Fc “hole,” linked via a non-cleavable linker to an anti-CD3 VL domain (middle); or an B7H3-targeting sdAb linked to a heterodimeric Fc “hole,” linked via a non-cleavable linker to an anti-CD3 VL domain modified at G100C (bottom). Chain 2 contains either a B7H3-targeted sdAb, linked to a complementary heterodimeric Fc “knob,” linked via the linker as above to an anti-CD3 VH domain modified at G44C linked to second B7H3 sdAb (top); a B7H3-targeted sdAb, linked to a complementary heterodimeric Fc “knob,” linked via the linker as above to an anti-CD3 VH domain (middle); or a B7H3-targeted sdAb, linked to a complementary heterodimeric Fc “knob,” linked via the linker as above to an anti-CD3 VH domain modified by G44C (bottom). When co-expressed the CD3 binding domain is properly assembled via the association of the VL:VH on the hole and knob, respectively. Where denoted the VH:VL interaction is stabilized by an engineered disulfide bond between the modified residues G44C in the VH domain and G100C in the VL domain.

[0123] FIG. 3B is a schematic of various B7H3-targeting constrained CD3 constructs composed of two polypeptides, Chain 1 and Chain 2. Chain 1 contains a heterodimeric Fc “hole,” linked via a non-cleavable linker to an anti-CD3 VL domain modified at G100C linked to a co-stimulatory receptor targeting sdAb. Chain 2 contains either a B7H3-targeted sdAb, linked to a complementary heterodimeric Fc “knob,” linked via the linker as above to an anti-CD3 VH domain modified at G44C linked to second B7H3-targeted sdAb (top); a heterodimeric Fc “knob,” linked via the linker as above to an anti-CD3 VH domain modified at G44C linked to a B7H3-targeted sdAb (middle); or a B7H3-targeted sdAb, linked to a complementary heterodimeric Fc “knob,” linked via the linker as above to an anti-CD3 VH domain modified by G44C (bottom). When co-expressed the CD3 binding domain is properly assembled via the association of the VL:VH on the hole and knob, respectively. VH:VL interaction is stabilized by an engineered disulfide bond between the modified residues G44C in the VH domain and G100C in the VL domain. The resulting constructs are engage B7H3

either in bivalent (top) or monovalent (middle and bottom) manner. All the constructs herein express contain a co-stimulatory receptor targeting sdAb.

[0124] FIG. 3C is a schematic of various B7H3-targeting constrained CD3 constructs composed of three polypeptides, Chain 1, Chain 2 and Chain 3, wherein the B7H3 targeting domain is a FAB. Chain 1 contains a BH73-targeting VH, an IgG Constant Heavy 1 (CH1) linked via a hinge to a first member of a heterodimeric Fc (Fc-Het-1), linked via the linker as above to an anti-CD3 VL domain that either lacks (top) or contains the modification of G100C (bottom). Chain 2 contains a BH73-targeting VH, an IgG Constant Heavy 1 (CH1) linked via a hinge to a second member of a heterodimeric Fc (Fc-Het-2), linked via the linker as above to an anti-CD3 VH domain that either lacks (top) or contains the modification of G44C (bottom). Chain 3 contains a complementary B7H3-targeting VL domain linked to human Ig Constant Light (CL) region. When co-expressed the CD3 binding domain is properly assembled via the association of the VL:VH on the complimentary heterodimeric Fc regions. Where denoted the VH:VL interaction is stabilized by an engineered disulfide bond between the modified residues G44C in the VH domain and G100C in the VL domain.

[0125] FIG. 4A is a schematic of various DLL3-targeting constrained CD3 constructs composed of two polypeptides, Chain 1 and Chain 2. Chain 1 contains a heterodimeric Fc “hole”, linked via a non-cleavable linker to an anti-CD3 VL domain modified at G100C linked to a co-stimulatory receptor targeting sdAb. Chain 2 contains either a DLL3-targeted sdAb, linked to a complementary heterodimeric Fc “knob”, linked via the linker as above to an anti-CD3 VH domain modified at G44C linked to second DLL3-targeted sdAb (top); a heterodimeric Fc “knob”, linked via the linker as above to an anti-CD3 VH domain modified at G44C linked to a DLL3-targeted sdAb (middle); or a DLL3-targeted sdAb, linked to a complementary heterodimeric Fc “knob”, linked via the linker as above to an anti-CD3 VH domain modified by G44C (bottom). When co-expressed the CD3 binding domain is properly assembled via the association of the VL:VH on the hole and knob, respectively. VH:VL interaction is stabilized by an engineered disulfide bond between the modified residues G44C in the VH domain and G100C in the VL domain. The resulting constructs are engage DLL3 either in bivalent (top) or monovalent (middle and bottom) manner. All the constructs herein express contain a co-stimulatory receptor targeting sdAb.

[0126] FIG. 4B is a schematic of a DLL3-targeting constrained CD3 construct, cx5499 composed of two polypeptides, Chain 1 and Chain 2. cx5499 is identical to cx5352 shown in **FIG. 4A** (top), but lacking co-stimulatory receptor targeting sdAb on the C-terminus of Chain 1. When co-expressed the CD3 binding domain is properly assembled via the

association of the VL:VH on the hole and knob, respectively. VH:VL interaction is stabilized by an engineered disulfide bond between the modified residues G44C in the VH domain and G100C in the VL domain. The resulting constructs are engage DLL3 either in bivalent (top) or monovalent (middle and bottom) manner.

[0127] **FIG. 5A-D** depict cellular binding by representative FR α -targeting constrained CD3 engaging constructs, cx1356 and cx681. **FIG. 5A** and **FIG. 5C** show binding to Ovar5 cells (a FR α positive ovarian cancer cell line). **FIG. 5B** and **FIG. 5D** depict the lack of binding to T-cells. **FIG. 5A** and **FIG. 5B** display histograms of the normalized cell counts vs fluorescence at 100nM of each construct. The secondary anti-human APC antibody only control is shown in the filled black trace, while the positive control anti-CD3 binding is shown in the open trace, and cx1356 and cx681 are shown in the gray shaded traces in **FIG. 5A** and **FIG. 5B**. The full titration of each construct on the various cell types are shown in **FIG. 5C** and **FIG. 5D**.

[0128] **FIG. 6A-F** depict cellular binding by representative B7H3-targeting constrained CD3 engaging constructs. **FIG. 6A, C, and E** show binding to A375 cells (a B7H3 positive human melanoma cell line). **FIG. 6B, D, and F** show the lack of binding to isolated T cells.

[0129] **FIG. 7A-F** depict the impact of linker length on the capacity to agonize CD3 in the presence of FR α positive IGROV1 cells (**FIG. 7A, 7C, 7E**), or FR α negative NCI-H460 cells (**FIG. 7B, 7D, 7F**). **FIG. 7A-B** show the kinetics of CD3 signaling by 2 nM of various constructs on antigen positive and negative cells, respectively. **FIG. 7C-D** show the magnitude of CD3 agonizing capacity by 2 nM of various constructs on antigen positive and negative cells, respectively. **FIG. 7E-F** show the potency of CD3 agonizing capacity of various constructs with differing linker lengths on antigen positive and negative cells, respectively. A Jurkat CD3 NFAT-GFP reporter cell line was used to assess CD3 signaling. Constrained CD3 binding proteins only effectively engage and cluster CD3 on T cells when bound to a second antigen on target cells.

[0130] **FIG. 8A-D** depict the ability of representative B7H3-targeting constrained CD3 engaging constructs to agonize CD3 in a target dependent manner. **FIG. 8A** and **FIG. 8C** depict the capacity to mediate CD3 signaling in the presence of B7H3 positive A375 cells, while **FIG. 8B** and **FIG. 8D** show the inability to mediate CD3 signaling in the presence of B7H3 negative CCRF-CEM cells. A Jurkat CD3 NFAT-GFP reporter cell line was used to assess CD3 agonism.

[0131] **FIG. 9A** depicts the ability of a representative B7H3-targeting constrained CD3 engaging construct (cx3072) to induce T-cell mediated cytotoxicity in a target dependent

manner. Target cells were labeled with cytoID red label and dying cells were visualized by addition of Caspase 3/7 green reagent. Cytotoxicity was assessed by determining the overlap area of red target cells and green dying cells. A B7H3 negative A375 cell line, generated by CRISPR technology was used to test antigen specific T-cell mediated cytotoxicity. cx3072 was unable to elicit T-cell mediated cytotoxicity of these B7H3 deficient cells.

[0132] **FIG. 9B** shows the ability of cx5952 to induce T-cell mediated cytotoxicity in the presence of B7H3 positive A375 cells, but not in the presence of B7H3 negative CCRF-CEM cells.

[0133] **FIG. 10A** and **10B** depict the ability of representative B7H3-targeting constrained CD3 engaging constructs to induce T-cell mediated cytotoxicity in a target dependent manner. **FIG. 10A** depicts the capacity of these constructs to induce T-cell mediated cytotoxicity in the presence of B7H3 positive A375 cells, while **FIG. 10B** depicts the capacity of these constructs to induce T-cell mediated cytotoxicity in the presence of B7H3 negative CCRF-CEM cells. Cytotoxicity was assessed by determining the overlap area of red target cells and green dying cells.

[0134] **FIG. 10C** and **10D** depict the ability of representative B7H3-targeting constrained CD3 engaging constructs to induce T-cell mediated cytotoxicity in a target dependent manner. **FIG. 10C** depicts the capacity of these constructs to induce T-cell mediated cytotoxicity in the presence of B7H3 positive A375 cells, while **FIG. 10C** depicts the capacity of these constructs to induce T-cell mediated cytotoxicity in the presence of B7H3 negative CCRF-CEM cells. Cytotoxicity was assessed by determining the overlap area of red target cells and green dying cells.

[0135] **FIGS. 11A-C** depict the ability of a representative B7H3-targeting constrained CD3 engaging construct (cx5952) to induce T-cell mediated T-cell activation in a target dependent manner. T-cell activation of CD4+ or CD8+ T cells was assessed by expression of the T cell activation markers CD25 (**FIG. 11A**), CD69 (**FIG. 11B**), and CD71 (**FIG. 11C**).

[0136] **FIGS. 11D-11K** depict the ability of representative B7H3-targeting constrained CD3 engaging constructs to induce T-cell activation in a target dependent manner. B7H3-target-dependent CD4+ T-cell activation is shown by expression of the T cell activation markers CD25 (**FIG. 11D**) and CD71 (**FIG. 11F**). B7H3-target-dependent CD8+ T-cell activation is shown by expression of the T cell activation markers CD25 (**FIG. 11H**) and CD71 (**FIG. 11J**). T-cell activation was not observed in the absence of B7H3 positive cells, based on T cell activation marker CD25 as shown in CD4+ T cells (**FIG. 11E**) or CD8+ T

cells (**FIG. 11I**) or based on T cell activation marker CD71 as shown in CD4+ T cells (**FIG. 11G**) or CD8+ T cells (**FIG. 11K**)

[0137] **FIG. 12A** depicts the ability of representative B7H3-targeting constrained CD3 engaging constructs to induce IFN γ production in a target dependent manner. **FIG. 12A** shows the production of IFN γ from T-cells cultured with B7H3 positive A375 cells and in the presence of B7H3 negative CCRF-CEM cells in the presence of the representative B7H3-targeting CD3 engaging constructs.

[0138] **FIG. 12B** depicts the ability of representative B7H3-targeting constrained CD3 engaging constructs to induce IFN γ production in a target dependent manner. **FIG. 12B** shows the production of IFN γ from T-cells cultured with B7H3 positive A375 cells and in the presence of B7H3 negative CCRF-CEM cells in the presence of the representative B7H3-targeting CD3 engaging constructs.

[0139] **FIG. 13A** and **13B** depict cellular binding of representative B7H3-targeting constrained CD3 engaging constructs. cx5187 and cx5823 each contain two B7H3 binding domains while complex cx5873 and cx5965 each contain one B7H3 binding domain. **FIG. 13A** shows binding to B7H3 positive A375 cells. **FIG. 13B** shows the lack of binding to B7H3 negative CCRF-CEM cells and isolated T-cells.

[0140] **FIG. 13C** and **FIG. 13D** depict the ability of representative B7H3-targeting constrained CD3 engaging constructs to agonize CD3 in a target dependent manner. **FIG. 13C** shows that engaging B7H3 positive A375 cells with a construct that is bivalent and bi-epitopic to B7H3 (cx5187) induced more potent CD3 signaling than constructs that are monovalent to B7H3 (cx5873 and cx5965). **FIG. 13D** shows the lack of activation of T-cells in the presence of B7H3 negative CCRF-CEM cells. A Jurkat CD3 NFAT-GFP reporter cell line was used to assess CD3 agonism.

[0141] **FIG. 14A** and **FIG. 14B** depict the ability of representative B7H3-targeting constrained CD3 engaging constructs to induce T-cell mediated cytotoxicity in a target dependent manner. **FIG 14A** shows that targeting B7H3 positive A375 cells with a construct that is bivalent and bi-epitopic to B7H3 (cx5187) induced more potent T-cell mediated cytotoxicity than constructs that are monovalent to B7H3 (cx5873 and cx5965). **FIG 14B** depicts the lack of T-cell mediated cytotoxicity against B7H3 negative CCRF-CEM cells.

[0142] **FIG. 15A-D** depict the ability of representative B7H3-targeting constrained CD3 engaging molecules to activate T-cells in the presence of B7H3 positive A375cells, but not in the presence of B7H3 negative CCRF-CEM cells. **FIG. 15A** and **15B** show that targeting

B7H3 positive A375 cells with a construct that is bivalent and bi-epitopic to B7H3 (cx5187), induced more potent CD25 expression on CD4+ and CD8+ T-cells than constructs that are monovalent to B7H3 (cx5873 and cx5965). **FIG. 15C** and **15D** show the lack of CD25 expression on CD4+ and CD8+ T-cells in the presence of B7H3 negative CCRF-CEM cells.

[0143] **FIG. 16A and 16B** demonstrate the ability of representative B7H3-targeting constrained CD3 engaging constructs to elicit T-cell mediated cytotoxicity in the presence of B7H3-positive A375 cells (**FIGS. 16A**) but not in the presence of CCRF-CEM B7H3-negative cells (**FIGS. 16B**).

[0144] **FIGS. 16C-16J** demonstrate the ability of representative B7H3-targeting constrained CD3 engaging constructs to elicit T cell activation in the presence of B7H3-positive A375 cells but not in the presence of CCRF-CEM B7H3-negative cells, as assessed by: expression of CD25 on CD4+ T cells (**FIGS. 16C and 16D**, respectively), CD25 expression on CD8+ T cells (**FIGS. 16E and 16F**, respectively), CD71 expression on CD4+ T cells (**FIGS. 16G and 16H**, respectively), CD71 expression on CD8+ T cells (**FIGS. 16I and 16J**, respectively).

[0145] **FIGS. 16K and 16L** demonstrate the ability of representative B7H3-targeting constrained CD3 engaging constructs to elicit T cell cytokine production in the presence of B7H3-positive A375 cells (**FIG. 16K**) but not in the presence of CCRF-CEM B7H3-negative cells (**FIG. 16L**).

[0146] **FIG. 17A and 17B** demonstrate that representative monovalent (cx5800 and cx5801) and bivalent (cx5352) DLL3-targeting constrained CD3 engaging constructs bound to a DLL3 expressing cell line, SHP-77 (**FIG. 17A**), but not to isolated T-cells (**FIG. 17B**). Binding was assessed by flow cytometry.

[0147] **FIG. 17C** depicts the ability of representative DLL3-targeting constrained CD3 engaging constructs to agonize CD3 signaling in the presence of DLL3 positive SHP-77 cells. Engaging DLL3 positive cells with a construct that is bivalent and bi-epitopic to DLL3 (cx5352) induced more potent T-cell activation than constructs that are monovalent to DLL3 (cx5800 and cx5801). A Jurkat CD3 NFAT-Luciferase reporter cell line was used to assess CD3 signaling.

[0148] **FIG. 18A-18E** demonstrate the ability of a representative DLL3-targeting constrained CD3 engaging construct, cx5499 to elicit T-cell mediated cytotoxicity and T-cell activation in the presence of DLL3-positive SHP-77 cells. **FIG. 18A** demonstrates the ability of the representative DLL3-targeting constrained CD3 engaging constructs to elicit T-cell mediated cytotoxicity in the presence of DLL3-positive SHP-77 cells. **FIGS. 18C-18D**

demonstrate the ability of representative DLL3-targeting constrained CD3 engaging constructs to elicit T cell activation in the presence of DLL3-positive SHP-77 cells, as assessed by: expression of CD25 on CD4+ T cells (**FIG. 18B**), CD69 expression on CD4+ T cells (**FIG. 18C**), CD25 expression on CD8+ T cells (**FIG. 18D**) and CD69 expression on CD8+ T cells (**FIG. 18E**).

DETAILED DESCRIPTION

[0149] The present disclosure provides constrained T-cell engaging fusion proteins in the form of multispecific polypeptide constructs that bind at least CD3 and a second antigen. The multispecific polypeptide constructs provided herein include at least a first component that includes one or more copies of an antigen-binding domain that binds an antigen operably linked to an immunoglobulin Fc region, a second component that includes one or more copies of at least a binding domain that binds CD3 (referred to herein as an anti-CD3 binding domain or a CD3 binding region, which are terms that are used interchangeably herein), and a linker, such as a polypeptide linker, that joins the first component and the second component. In some embodiments, the antigen is a tumor associated antigen (TAA). In some embodiments, the linker is a non-cleavable linker. In some embodiments, the linker does not contain a substrate recognition site that is specifically recognized by a protease, such as a protease that is granzyme B, an MMP or matriptase.

[0150] The provided multispecific polypeptide constructs include a configuration in which the first component containing the Fc region is N-terminal to the second component containing the CD3 binding region. In such an embodiment, the first and second components are joined via a linker that is C-terminal to the end of the Fc region. In some embodiments the antigen binding domain(s) is positioned on the amino-terminal (N-term) region of the multispecific polypeptide construct. In some embodiments, the antigen binding domain(s) is positioned on the carboxy-terminal (C-term) region of the multispecific polypeptide construct. In some embodiments, the antigen binding domain(s) is positioned on both the N- and C-terminal regions of the multispecific polypeptide construct. Various configurations of a multispecific polypeptide construct as provided herein are shown in **FIG. 1**.

[0151] The provided multispecific polypeptide constructs exhibit constrained T-cell engaging activity because such constructs only substantially bind to CD3 once an antigen is bound via the antigen-binding domain. This is exemplified in the Examples and Figures provided herein, which demonstrate the ability of constrained CD3-engaging proteins to efficiently bind TAA positive cells, while having little to no binding of T cells. This unique

property allows constrained CD3-engaging proteins to distribute to sites where TAA is present without binding to peripheral T cells. This format is distinct from other CD3 engaging multispecific constructs, in that constitutive CD3 binding is disallowed or eliminated, providing a significant benefit by avoiding peripheral T-cell binding and permitting superior distribution to the site(s) where antigen is present as recognized by the antigen binding domain. Furthermore, other CD3 engaging constructs mediate antigen-dependent T-cell activation. However, the multispecific polypeptide constructs provided herein mediate both antigen dependent T-cell binding and activation.

[0152] The constrained T-cell engaging activity of the provided multispecific polypeptide constructs is due, in some aspects, to the positioning of the Fc region N-terminal to the CD3-binding region. In some embodiments, such positioning reduces, attenuates, dampens and/or prevents CD3 binding by the CD3 binding region. In the absence of antigen binding by the antigen binding domain, the multispecific polypeptide constructs provided herein demonstrate reduced or eliminated CD3 binding and T-cell activating capacity. In some embodiments, in the presence of an antigen binding event mediated by the antigen binding domain(s) of the multispecific polypeptide constructs, the capacity to bind CD3 by the CD3 binding region is greatly enhanced. In some embodiments, in the presence of an antigen binding event mediated by the antigen binding domains(s) of the multispecific polypeptide constructs, the capacity to activate T-cells is greatly enhanced. Engagement of its cognate antigen by the antigen binding domain(s) within the multispecific polypeptide construct leads to subsequent T-cell engagement and mediates antigen-dependent T-cell activation, such as cytotoxicity, cytokine release, degranulation and proliferation. In some embodiments, the provided multispecific polypeptide constructs can be used to increase an immune response, such as to enhance T-cell activity, including cytolytic (or cytotoxic) T-cell activity. The modulation of the immune response can, in some aspects, treat a disease or condition in a subject.

[0153] In some embodiments, the one or more antigen binding domains bind an antigen on a tumor cell or a cell of the tumor microenvironment. In some aspects, the provided multispecific polypeptide constructs can be used to increase immune responses, such as T-cell activity, e.g. cytotoxicity activity, against a tumor or cancer. In some embodiments, the provided multispecific polypeptide constructs can be used to treat a tumor or cancer in the subject.

[0154] In some embodiments, the multispecific polypeptide constructs of the disclosure ensure that there will be no binding of T-cells via CD3 in peripheral blood, as the CD3

binding region of these constructs is constrained or otherwise blocked and/or inhibited by the presence of the Fc region. Thus, the multispecific polypeptide constructs of the disclosure provide a number of advantages. In some aspects, these constructs limit the sink effect caused by binding all T-cells. In some aspects, these constructs reduce systemic toxicity.

[0155] In some embodiments, the provided multispecific polypeptide constructs of the disclosure allow for controlled biodistribution to a desired site in a subject, such as, for example, a site of tumor-associated antigen (TAA) expression. Sites of TAA expression include, for example, tumor and the surrounding tumor microenvironment.

[0156] In some embodiments, the multispecific polypeptide constructs of the disclosure exhibit specificity for CD3 and one or more other antigen. In some embodiments, the multispecific polypeptide constructs can contain more than one antigen binding domain able to bind one or more TAA, such as 2, 3 or 4 antigen binding domains, see e.g. **FIG. 1**. In some embodiments, the one or more antigen binding domains bind the same antigen. In some embodiments, the multispecific polypeptide constructs include more than one antigen binding domains that bind distinct epitopes on the same antigen. In some embodiments, the multispecific polypeptide constructs include more than one antigen binding domains that bind one or more distinct antigens. In some embodiments, the multispecific polypeptide constructs include more than one antigen binding domains that bind distinct epitopes on the same antigens as well as include additional antigen binding domains that bind to one or more distinct antigens. In some aspects, the provided multispecific polypeptide constructs are bispecific polypeptide constructs, such that they are able to bind to CD3 and to another antigen, such as a TAA, via binding of the antigen-binding domain of the multispecific polypeptide construct. In some examples, the provided multispecific polypeptide constructs are bispecific polypeptide constructs that provide multivalent engagement of one or more TAA through the use of a first antigen-binding domain and a second antigen-binding domain. For example, in some embodiments, the bispecific polypeptide constructions include a first antigen-binding single domain antibody (sdAb) and a second antigen-binding sdAb.

[0157] In some embodiments, the multispecific polypeptide constructs provided herein exist in two states in terms of capacity to bind CD3 and subsequently activate T-cells: (1) the “inactive” state occurs when there is no binding of any or all of the antigen binding domain(s), such that the CD3 binding is constrained and T-cell interaction is obviated, and (2) the “active” state occurs upon antigen binding by any or all of the antigen binding domain(s), such that the CD3 binding region is able to bind CD3 and the T-cell interaction is allowed.

[0158] In some embodiments, the Fc region is linked to the CD3 binding domain via a linker or linkers. In some embodiments, the Fc region is linked to the CD3 binding region via a non-cleavable linker or linkers, such as any as described.

[0159] In some embodiments, the Fc region is a homodimeric Fc region. In some embodiments, the Fc region is a heterodimeric Fc region. In some embodiments, the Fc region is a monomeric Fc region. In some embodiments, the Fc region of the multispecific polypeptide constructs are capable of interacting with FcγRs and mediating innate immune effector functions, for example, antibody dependent cellular toxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP). In some embodiments, the Fc region of the multispecific polypeptide constructs are capable of interacting with complement proteins, namely C1q, and mediating complement dependent cytotoxicity. Thus, in some aspects, the multispecific polypeptide constructs of the disclosure allow for multiple immune effector mechanisms, including innate immune effectors and T-cells.

[0160] In some embodiments, the multispecific polypeptide constructs of the disclosure allow for T-cell and NK cell mediated cytotoxicity to occur simultaneously. In some cases, such activity can occur in a multispecific polypeptide construct in which is contained a first antigen binding domain, *e.g.*, a first anti-TAA antigen binding domain, and a second antigen binding domain, *e.g.*, a second anti-TAA antigen binding domain, that can target distinct and/or non-competing epitopes on a given TAA.

[0161] It is contemplated that the constrained CD3 engaging constructs are amenable for use with any TAA-binding domain, allowing better therapeutic exposure within the tumor or tumor-microenvironment by avoiding interactions with peripheral T-cells and mediating potent TAA-dependent T-cell cytotoxicity. In some embodiments, the second portion or component contains a CD3 binding region that is monovalent to CD3, such that there will be no activation of T-cell unless there is TAA present.

[0162] In some aspects, the multispecific polypeptide constructs of the disclosure provide a number of advantages over current bispecific therapeutics. The multispecific polypeptide constructs of the disclosure are smaller than a conventional therapeutic antibody, *e.g.*, 150kDa vs. 125kDa, which will allow for better target, *e.g.* tumor, penetration. In some aspects, the size of the entire multispecific polypeptide construct provides long half-life for the construct. In some aspects, the multispecific polypeptide constructs of the disclosure exhibit reduced systemic toxicity or toxicity of any area outside the tumor and/or tumor microenvironment, since CD3 binding by the CD3 binding region depends on TAA engagement before CD3 engagement will occur.

[0163] All publications and patent documents cited herein are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an admission that any is pertinent prior art, nor does it constitute any admission as to the contents or date of the same. The invention having now been described by way of written description, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples below are for purposes of illustration and not limitation of the claims that follow.

I. Definitions

[0164] Unless otherwise defined, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. The term “a” entity or “an” entity refers to one or more of that entity. For example, a compound refers to one or more compounds. As such, the terms “a”, “an”, “one or more” and “at least one” can be used interchangeably. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (*e.g.*, electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. *See e.g.*, Sambrook *et al.* *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0165] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0166] As used herein, the term “antibody” refers to immunoglobulin molecules and antigen-binding portions of immunoglobulin (Ig) molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. By “specifically bind” or “immunoreacts with” or “immunospecifically bind” is meant that the antibody reacts with one or more antigenic determinants of the desired antigen and does not react with other polypeptides or binds at much lower affinity ($K_d > 10^{-6}$). Antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, fully human, domain antibody, single chain, Fab, and F(ab')₂ fragments, Fvs, scFvs, and a Fab expression library. Typically, an “antigen-binding fragment” contains at least one CDR of an immunoglobulin heavy and/or light chain that binds to at least one epitope of the antigen of interest. In this regard, an antigen-binding fragment may comprise 1, 2, 3, 4, 5, or all 6 CDRs of a variable heavy chain (VH) and variable light chain (VL) sequence from antibodies that bind the antigen, such as generally six CDRs for an antibody containing a VH and a VL (“CDR1,” “CDR2” and “CDR3” for each of a heavy and light chain), or three CDRs for an antibody containing a single variable domain. Antigen binding fragments include single domain antibodies, such as those only containing a VH or only containing a VL, including, for example, V_HH, V_{NAR}, engineered V_H or V_K domains.

[0167] The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kDa) and one “heavy” chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. In general, antibody molecules obtained from humans relate to any of the classes IgG, IgM, IgA, IgE and IgD, which differ from one another by the nature of the heavy chain present in the molecule. Certain classes have subclasses as well, such as IgG₁, IgG₂, IgG₃, IgG₄, and others. Furthermore, in humans, the light chain may be a kappa chain or a lambda chain.

[0168] The term “monoclonal antibody” (mAb) or “monoclonal antibody composition”, as used herein, refers to a population of antibody molecules that contain only one molecular species of antibody molecule consisting of a unique light chain gene product and a unique heavy chain gene product. In particular, the complementarity determining regions (CDRs) of the monoclonal antibody are identical in all the molecules of the population. MAbs contain an antigen binding site capable of immunoreacting with a particular epitope of the antigen characterized by a unique binding affinity for it.

[0169] The term “antigen-binding site” or “binding portion” refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable (“V”) regions of the heavy (“H”) and light (“L”) chains. Three highly divergent stretches within the V regions of the heavy and light chains, referred to as “hypervariable regions,” are interposed between more conserved flanking stretches known as “framework regions,” or “FRs”. Thus, the term “FR” refers to amino acid sequences that are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as “complementarity-determining regions,” or “CDRs.” The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk J. Mol. Biol. 196:901-917 (1987), Chothia *et al.* Nature 342:878-883 (1989).

[0170] As used herein, the term “epitope” includes any specific portion of an antigen targeted by an antibody, antibody fragment or other binding domain. The term “epitope” includes any protein region to which specific binding is directed. The term “epitope” includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. For example, antibodies may be raised against N-terminal, central, or C-terminal peptides of a polypeptide. In addition, antibodies may be raised against linear or discontinuous epitopes of a polypeptide. An antibody is said to specifically bind an antigen when the dissociation constant is $\leq 1 \mu\text{M}$; for example, in some embodiments $\leq 100 \text{ nM}$ and in some embodiments, $\leq 10 \text{ nM}$ and does not display binding to other proteins either closely related or distinct.

[0171] As used herein, the terms “specific binding,” “immunological binding,” and “immunological binding properties” refer to the non-covalent interactions of the type that occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified

using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and geometric parameters that equally influence the rate in both directions. Thus, both the “on rate constant” (K_{on}) and the “off rate constant” (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. (*See* Nature 361:186-87 (1993)). The ratio of K_{off}/K_{on} enables the cancellation of all parameters not related to affinity and is equal to the dissociation constant K_d . (*See, generally*, Davies et al. (1990) Annual Rev Biochem 59:439-473). An antibody of the present disclosure is said to specifically bind to EGFR, when the binding constant (K_d) is $\leq 1 \mu\text{M}$, for example, in some embodiments $\leq 100 \text{ nM}$, in some embodiments $\leq 10 \text{ nM}$, and in some embodiments $\leq 100 \text{ pM}$ to about 1 pM , as measured by assays such as radioligand binding assays or similar assays known to those skilled in the art.

[0172] The term “isolated polynucleotide” as used herein shall mean a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the “isolated polynucleotide” (1) is not associated with all or a portion of a polynucleotide in which the “isolated polynucleotide” is found in nature, (2) is operably linked to a polynucleotide that it is not linked to in nature, or (3) does not occur in nature as part of a larger sequence. Polynucleotides in accordance with the disclosure include the nucleic acid molecules encoding the heavy chain immunoglobulin molecules shown herein, and nucleic acid molecules encoding the light chain immunoglobulin molecules shown herein.

[0173] The term “isolated protein” referred to herein means a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin, or source of derivation, the “isolated protein” (1) is not associated with proteins found in nature, (2) is free of other proteins from the same source, *e.g.*, free of murine proteins, (3) is expressed by a cell from a different species, or (4) does not occur in nature.

[0174] The term “polypeptide” is used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein fragments, and analogs are species of the polypeptide genus. Polypeptides in accordance with the disclosure comprise the heavy chain immunoglobulin molecules shown herein, and the light chain immunoglobulin molecules shown herein, as well as antibody molecules formed by combinations comprising the heavy chain immunoglobulin molecules with light chain

immunoglobulin molecules, such as kappa light chain immunoglobulin molecules, and vice versa, as well as fragments and analogs thereof.

[0175] The term “naturally-occurring” as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and that has not been intentionally modified by man in the laboratory or otherwise is naturally-occurring.

[0176] The term “operably linked” as used herein refers to positions of components so described are in a relationship permitting them to function in their intended manner. A control sequence “operably linked” to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

[0177] The term “control sequence” as used herein refers to polynucleotide sequences that are necessary to effect the expression and processing of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term “control sequences” is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. The term “polynucleotide” as referred to herein means nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

[0178] The term “oligonucleotide” referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset generally comprising a length of 200 bases or fewer. In some embodiments, oligonucleotides are 10 to 60 bases in length, for example, in some embodiments, 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, *e.g.*, for probes, although oligonucleotides may be double stranded, *e.g.*, for use in the construction of a gene mutant. Oligonucleotides of the disclosure are either sense or antisense oligonucleotides.

[0179] The term “naturally occurring nucleotides” referred to herein includes deoxyribonucleotides and ribonucleotides. The term “modified nucleotides” referred to herein

includes nucleotides with modified or substituted sugar groups and the like. The term “oligonucleotide linkages” referred to herein includes oligonucleotide linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoronmidate, and the like. *See e.g.*, LaPlanche *et al.* Nucl. Acids Res. 14:9081 (1986); Stec *et al.* J. Am. Chem. Soc. 106:6077 (1984), Stein *et al.* Nucl. Acids Res. 16:3209 (1988), Zon *et al.* Anti Cancer Drug Design 6:539 (1991); Zon *et al.* Oligonucleotides and Analogues: A Practical Approach, pp. 87-108 (F. Eckstein, Ed., Oxford University Press, Oxford England (1991)); Stec *et al.* U.S. Patent No. 5,151,510; Uhlmann and Peyman Chemical Reviews 90:543 (1990). An oligonucleotide can include a label for detection, if desired.

[0180] As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. *See* Immunology - A Synthesis (2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland 7 Mass. (1991)). Stereoisomers (*e.g.*, D- amino acids) of the twenty conventional amino acids, unnatural amino acids such as α -, α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present disclosure. Examples of unconventional amino acids include: 4 hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ -N-methylarginine, and other similar amino acids and imino acids (*e.g.*, 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

[0181] Similarly, unless specified otherwise, the left-hand end of single-stranded polynucleotide sequences is the 5' end the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction sequence regions on the DNA strand having the same sequence as the RNA and that are 5' to the 5' end of the RNA transcript are referred to as “upstream sequences”, sequence regions on the DNA strand having the same sequence as the RNA and that are 3' to the 3' end of the RNA transcript are referred to as “downstream sequences”.

[0182] As applied to polypeptides, the term “substantial identity” means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, for example, in some embodiments,

at least 90 percent sequence identity, in some embodiments, at least 95 percent sequence identity, and in some embodiments, at least 99 percent sequence identity.

[0183] In some embodiments, residue positions that are not identical differ by conservative amino acid substitutions.

[0184] As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present disclosure, providing that the variations in the amino acid sequence maintain at least 75%, for example, in some embodiments, at least 80%, 90%, 95%, and in some embodiments 99%. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic amino acids are aspartate, glutamate; (2) basic amino acids are lysine, arginine, histidine; (3) non-polar amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and (4) uncharged polar amino acids are glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. The hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine. The hydrophobic amino acids include alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine. Other families of amino acids include (i) serine and threonine, which are the aliphatic-hydroxy family; (ii) asparagine and glutamine, which are the amide containing family; (iii) alanine, valine, leucine and isoleucine, which are the aliphatic family; and (iv) phenylalanine, tryptophan, and tyrosine, which are the aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art. In some embodiments, amino- and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that

occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie *et al.* Science 253:164 (1991). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in accordance with the disclosure.

[0185] In some embodiments, amino acid substitutions are those that: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various mutants of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (for example, conservative amino acid substitutions) may be made in the naturally- occurring sequence (for example, in the portion of the polypeptide outside the domain(s) forming intermolecular contacts. A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (*e.g.*, a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et al. Nature 354:105 (1991).

[0186] The term “polypeptide fragment” as used herein refers to a polypeptide that has an amino terminal and/or carboxy-terminal deletion and/or one or more internal deletion(s), but where the remaining amino acid sequence is identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full length cDNA sequence. Fragments typically are at least 5, 6, 8 or 10 amino acids long, for example, in some embodiments, at least 14 amino acids long, in some embodiments, at least 20 amino acids long, usually at least 50 amino acids long, and in some embodiments, at least 70 amino acids long. The term “analog” as used herein refers to polypeptides that are comprised of a segment of at least 25 amino acids that has substantial identity to a portion of a deduced amino acid sequence and that has specific binding to EGFR, under suitable binding conditions. Typically, polypeptide analogs comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally- occurring sequence. Analogs typically are at least

20 amino acids long, for example, in some embodiments, at least 50 amino acids long or longer, and can often be as long as a full-length naturally-occurring polypeptide.

[0187] The term “agent” is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials.

[0188] As used herein, the terms “label” or “labeled” refers to incorporation of a detectable marker, *e.g.*, by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (*e.g.*, streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods). In certain situations, the label or marker can also be therapeutic. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (*e.g.*, ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I), fluorescent labels (*e.g.*, a fluorophore, rhodamine, lanthanide phosphors), enzymatic labels (*e.g.*, horseradish peroxidase, p-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (*e.g.*, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance. The term “pharmaceutical agent or drug” as used herein refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient.

[0189] As used herein, a composition refers to any mixture of two or more products, substances, or compounds, including cells. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

[0190] The term “pharmaceutical composition” refers to a composition suitable for pharmaceutical use in a mammalian subject, often a human. A pharmaceutical composition typically comprises an effective amount of an active agent (*e.g.*, multispecific polypeptide construct) and a carrier, excipient, or diluent. The carrier, excipient, or diluent is typically a pharmaceutically acceptable carrier, excipient or diluent, respectively.

[0191] The terms “treating,” “treatment,” or “therapy” of a disease or disorder as used herein mean slowing, stopping or reversing the disease or disorders progression, as evidenced by decreasing, cessation or elimination of either clinical or diagnostic symptoms, by administration of a pharmaceutical composition of the disclosure either alone or in combination with another compound as described herein. “Treating,” “treatment,” or

“therapy” also means a decrease in the severity of symptoms in an acute or chronic disease or disorder or a decrease in the relapse rate. As used herein in the context of cancer, the terms “treatment” or, “inhibit,” “inhibiting” or “inhibition” of cancer refers to at least one of: a statistically significant decrease in the rate of tumor growth, a cessation of tumor growth, or a reduction in the size, mass, metabolic activity, or volume of the tumor, as measured by standard criteria such as, but not limited to, the Response Evaluation Criteria for Solid Tumors (RECIST), or a statistically significant increase in progression free survival (PFS) or overall survival (OS). “Preventing,” “prophylaxis,” or “prevention” of a disease or disorder refers to administration of a pharmaceutical composition, either alone or in combination with another compound, to prevent the occurrence or onset of a disease or disorder or some or all of the symptoms of a disease or disorder or to lessen the likelihood of the onset of a disease or disorder.

[0192] The terms “effective amount” or “therapeutically effective amount” refer to a quantity and/or concentration of a composition that when administered into a patient either alone (*i.e.*, as a monotherapy) or in combination with additional therapeutic agents, yields a statistically significant decrease in disease progression as, for example, by ameliorating or eliminating symptoms and/or the cause of the disease. An effective amount may be an amount that relieves, lessens, or alleviates at least one symptom or biological response or effect associated with a disease or disorder, prevents progression of the disease or disorder, or improves physical functioning of the patient.

[0193] As used herein, “substantially pure” means an object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present.

[0194] Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, for example, in some embodiments, more than about 85%, 90%, 95%, and 99%. In some embodiments, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

[0195] The term patient includes human and veterinary subjects.

[0196] Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (Parker, S., Ed., McGraw-Hill, San Francisco (1985)).

[0197] The term “about” as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to “about X” includes description of “X”.

II. Multispecific Polypeptide Constructs

[0198] Provided herein is a multispecific polypeptide construct containing a first component containing an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein the first and second components are coupled by a linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA). In some embodiments, the linker is a non-cleavable linker. In some embodiments, the linker does not contain a substrate recognition that is specifically recognized for cleavage by a protease.

[0199] In some embodiments, the multispecific polypeptide construct contains in order, from N-terminus to C-terminus: an immunoglobulin Fc region; a linker; a CD3 binding region that binds CD3 (CD3ε); and an antigen binding domain that binds a tumor-associated antigen (TAA). In some embodiments, the multispecific polypeptide construct contains in order, from N-terminus to C-terminus: an antigen binding domain that binds to a tumor-associated antigen (TAA); an immunoglobulin Fc region; a linker; and a CD3 binding region that binds CD3 (CD3ε). In some embodiments, the multispecific polypeptide construct contains at least a first antigen binding domain that binds a TAA and a second antigen binding domain that binds a TAA. In some embodiments, the multispecific polypeptide construct contains, in order, from N-terminus to C-terminus: a first antigen binding domain that binds to a tumor-associated antigen (TAA); an immunoglobulin Fc region; a linker; a CD3 binding region that binds CD3 (CD3ε); and a second antigen binding domain that binds a tumor-associated antigen (TAA).

[0200] Each of the components of the multispecific polypeptide constructs of the disclosure is described in more detail below.

1. Anti-CD3 Binding Domains:

[0201] The multispecific polypeptide constructs of the disclosure include one or more copies of an anti-CD3 binding domain. The anti-CD3 binding domains of the disclosure activate T cells via engagement of CD3 ϵ on the T cells. The anti-CD3 binding domains of the disclosure agonize, stimulate, activate, and/or otherwise augment CD3-mediated T cell activation. Biological activities of CD3 include, for example, T cell activation and other signaling through interaction between CD3 and the antigen-binding subunits of the T-Cell Receptor (TCR). For example, the anti-CD3 binding domains of the disclosure completely or partially activate T cells via engagement of CD3 ϵ on T cells by partially or completely modulating, *e.g.*, agonizing, stimulating, activating or otherwise augmenting CD3-mediated T cell activation.

[0202] In preferred embodiments, the anti-CD3 binding domains of the disclosure specifically bind the epsilon chain of CD3, also known as CD3 ϵ . The anti-CD3 ϵ binding domains of the disclosure activate T cells via engagement of CD3 ϵ on the T cells. The anti-CD3 ϵ binding domains of the disclosure include monoclonal antibodies, such as, for example, mammalian monoclonal antibodies, primate monoclonal antibodies, fully human monoclonal antibodies, as well as humanized monoclonal antibodies and chimeric antibodies, as well as antigen-binding fragments thereof. In some embodiments, the anti-CD3 ϵ binding domain includes one or more copies of an antibody or an antigen-binding fragment thereof.

[0203] In some embodiments, the anti-CD3 ϵ binding domain includes a combination of a VH CDR1 sequence, a VH CDR2 sequence, and a VH CDR3 sequence, wherein at least one of the VH CDR1 sequence, the VH CDR2 sequence, and the VH CDR3 sequence is selected from a VH CDR1 sequence that includes at least the amino acid sequence TYAMN (SEQ ID NO: 16); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17); and a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18). In some embodiments, the anti-CD3 ϵ binding domain includes a combination of a VH CDR1 sequence, a VH CDR2 sequence, and a VH CDR3 sequence, wherein at least one of the VH CDR1 sequence, the VH CDR2 sequence, and the VH CDR3 sequence is selected from a VH CDR1 sequence that includes at least the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); and a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18).

[0204] In some embodiments, the anti-CD3 ϵ binding domain includes a combination of a VL CDR1 sequence, a VL CDR2 sequence, and a VL CDR3 sequence, wherein at least one of the VL CDR1 sequence, the VL CDR2 sequence, and the VL CDR3 sequence is selected from a VL CDR1 sequence that includes at least the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0205] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that includes at least the amino acid sequence TYAMN (SEQ ID NO: 16); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that includes at least the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0206] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that includes at least the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that includes at least the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0207] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that includes at least the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that includes at least the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNHWV (SEQ ID NO: 225).

[0208] In some embodiments, the anti-CD3 ϵ binding domain includes a combination of a VH CDR1 sequence, a VH CDR2 sequence, and a VH CDR3 sequence, wherein at least one of the VH CDR1 sequence, the VH CDR2 sequence, and the VH CDR3 sequence is selected from a VH CDR1 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence TYAMN (SEQ ID NO: 16); a VH CDR2 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17); and a VH CDR3 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18).

[0209] In some embodiments, the anti-CD3 ϵ binding domain includes a combination of a VH CDR1 sequence, a VH CDR2 sequence, and a VH CDR3 sequence, wherein at least one of the VH CDR1 sequence, the VH CDR2 sequence, and the VH CDR3 sequence is selected from a VH CDR1 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); and a VH CDR3 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18).

[0210] In some embodiments, the anti-CD3 ϵ binding domain includes a combination of a VL CDR1 sequence, a VL CDR2 sequence, and a VL CDR3 sequence, wherein at least one of the VL CDR1 sequence, the VL CDR2 sequence, and the VL CDR3 sequence is selected from a VL CDR1 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 sequence that includes a sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0211] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence TYAMN (SEQ ID NO: 16); a VH CD2 sequence that is

at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17); a VH CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0212] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence ALWYSNLWV (SEQ ID NO: 21).

[0213] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more

identical to the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence ALWYSNHWV (SEQ ID NO: 225).

[0214] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that includes at least the amino acid sequence GFTFSTYAMN (SEQ ID NO: 227); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 228); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGDSYVSWFAY (SEQ ID NO: 224), a VL CDR1 sequence that includes at least the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNHWV (SEQ ID NO: 225).

[0215] In some embodiments, the anti-CD3 ϵ binding domain includes a VH CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GFTFSTYAMN (SEQ ID NO: 227); a VH CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 228); a VH CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence HGNFGDSYVSWFAY (SEQ ID NO: 224), a VL CDR1 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence ALWYSNHWV (SEQ ID NO: 225).

[0216] In some embodiments, the anti-CD3 ϵ binding domain includes a CDR3 that includes at least amino acids VLWYSNRWV (SEQ ID NO:226). In some embodiments, the anti-CD3 ϵ binding domain includes a CDR3 that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acids VLWYSNRWV (SEQ ID NO:226).

[0217] In some embodiments, the anti-CD3 ϵ binding domain includes one or more copies of an antibody or an antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody. In some

embodiments, the anti-CD3 binding domain includes an Fv antibody fragment that binds CD3 ϵ (referred to herein as an anti-CD3 ϵ Fv fragment). In some embodiments, the anti-CD3 ϵ Fv antibody fragment is a disulfide stabilized anti-CD3 binding Fv fragment (dsFv). In some embodiments, the anti-CD3 binding domain is monovalent for binding CD3.

[0218] In some embodiments, the CD3 binding region is not a single chain antibody. For example, in some aspects, the CD3 binding region is not a single chain variable fragment (scFv).

[0219] In some embodiments, the CD3 binding region is an Fv antibody fragment containing a variable heavy chain (Hv, also called VH) and variable light chain (Lv, also called VL), such as any as described. In aspects of such embodiments, the immunoglobulin Fc region is a heterodimeric Fc region containing two different Fc polypeptides capable of heterodimeric association between both polypeptides of the Fc heterodimer, such as any as described in Section II.2. In such embodiments, the variable heavy chain (VH) and variable light chain (VL) of the CD3 binding region are linked on opposite chains of the heterodimeric Fc.

[0220] In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes an amino acid sequence selected from the group of SEQ ID NO: 32-81. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 32-81. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence selected from the group of SEQ ID NO: 32-62 and an amino acid sequence selected from the group consisting of SEQ ID NO: 63-81. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 32-62 and an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 63-81 an amino acid sequence.

[0221] In some embodiments, the anti-CD3 ϵ binding domain thereof includes a combination of a heavy chain variable region amino acid sequence and a light chain variable region amino acid sequence comprising an amino acid sequence selected from the group of SEQ ID NO: 32-81. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a combination of a heavy chain variable region amino acid sequence selected from the group of

SEQ ID NO: 32-62 and a light chain variable region amino acid sequence comprising an amino acid sequence selected from the group of SEQ ID NO: 63-81.

[0222] In some embodiments, the anti-CD3 ϵ binding domain thereof is an Fv fragment that includes a combination of heavy chain variable amino acid sequence and a light chain variable amino acid sequence. In some embodiments, the anti-CD3 ϵ binding domain thereof is an Fv fragment that includes a combination of heavy chain variable amino acid sequence and a light chain variable amino acid sequence comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 14, 15, 32-81, 191, 196-200, 211, and 212. In some embodiments, the anti-CD3 ϵ binding domain thereof is an Fv fragment that includes a combination of heavy chain variable amino acid sequence and a light chain variable amino acid sequence comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 14, 15, 32-81, 191, 196-200, 211, and 212. In some embodiments, the anti-CD3 ϵ binding domain thereof is an Fv fragment that includes a combination of heavy chain variable amino acid sequence selected from the group of SEQ ID NO: 14, 32-62, 196-198, and 211 and light chain variable amino acid sequence selected from the group consisting of SEQ ID NO: 15, 63-81, 191, 199, 200, and 212. In some embodiments, the anti-CD3 ϵ binding domain thereof is an Fv fragment that includes a combination of heavy chain variable amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 14, 32-62, 196-198, and 211 and a light chain variable amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 15, 63-81, 191, 199, 200, and 212.

[0223] In some embodiments, the anti-CD3 ϵ binding domain thereof includes a combination of a heavy chain variable region amino acid sequence and a light chain variable region amino acid sequence comprising an amino acid sequence selected from the group of SEQ ID NO: 32-81, 191, 196-200, 211, and 212. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a combination of a heavy chain variable region amino acid sequence selected from the group of SEQ ID NO: 32-62, 196-198, and 211 and a light chain variable region amino acid sequence comprising an amino acid sequence selected from the group of SEQ ID NO: 63-81, 191, 199, 200, and 212.

[0224] In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 14, 32-43, 45-47, 48, 196 and 211 and an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 15, 63, 65-71, 73, 75, 77, and 199. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence selected from the group of SEQ ID NO: 14, 32-43, 45-47, 48, 196 and 211 and an amino acid sequence selected from the group consisting of SEQ ID NO: 15, 63, 65-71, 73, 75, 77, and 199.

[0225] In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 14. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 15. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 14 and a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 15. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 14. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 15. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 14 and a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 15.

[0226] In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 196. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%,

95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID

NO: 199. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 196 and a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 199. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 196. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 199. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 196 and a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 199.

[0227] In particular embodiments, the Fv is a disulfide stabilized Fv fragment (dsFv) in which the the V_H-V_L heterodimer is stabilized by an interchain disulfide bond. In some embodiments, the interchain disulfide bond is engineered by mutation of position in framework positions of the VH and/or VL chain. In some embodiments, the disulfide stabilized anti-CD3 Fv comprises an anti-CD3 VH with the mutation 44 to Cys and an anti-CD3 VL with the mutation 100 to Cys by Kabat numbering. For example, in some embodiments, the VH chain contains the mutation G44C and the VL chain contains the mutation G100C, each by kabat numbering. In some embodiments, the disulfide stabilized anti-CD3 Fv comprises an anti-CD3 VH with the mutation at position 105 to Cys and an anti-CD3 VL with the mutation position 43 to Cys by Kabat numbering.

[0228] In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 44, 49-62, 197 and 198 and an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to an amino acid sequence selected from the group consisting of SEQ ID NO: 64, 72, 74, 76, 78-81, 191, 200 and 212. In some of any such embodiments, the anti-CD3 Fv is a dsFv that has a VH chain containing the mutation G44C and a VL chain containing the mutation G100C, each by kabat numbering. In some embodiments, the anti-CD3 ϵ Fv antibody fragment includes a combination of an amino acid sequence selected from the group of SEQ ID NO: 44, 49-62,

197 and 198 and an amino acid sequence selected from the group consisting of SEQ ID NO: 64, 72, 74, 76, 78-81, 191, 200 and 212.

[0229] In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 44. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 72. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 44 and a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 72. In some of any such embodiments, the anti-CD3 Fv is a dsFv that has a VH chain containing the mutation G44C and a VL chain containing the mutation G100C, each by kabat numbering. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 44. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 72. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 44 and a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 72.

[0230] In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 198. In some embodiments, the anti-CD3 ϵ binding domain includes a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 200. In some embodiments, the anti-CD3 ϵ binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 198 and a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid

sequence of SEQ ID NO: 200. In some of any such embodiments, the anti-CD3 Fv is a dsFv that has a VH chain containing the mutation G44C and a VL chain containing the mutation G100C, each by kabat numbering. In some embodiments, the anti-CD3ε binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 198. In some embodiments, the anti-CD3ε binding domain includes a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 200. In some embodiments, the anti-CD3ε binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 198 and a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 200.

[0231] In some embodiments, the anti-CD3ε binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 197. In some embodiments, the anti-CD3ε binding domain includes a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 200. In some embodiments, the anti-CD3ε binding domain thereof includes a variable heavy chain (VH) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 197 and a variable light chain (VL) comprising an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the amino acid sequence of SEQ ID NO: 200. In some of any such embodiments, the anti-CD3 Fv is a dsFv that has a VH chain containing the mutation G44C and a VL chain containing the mutation G100C, each by kabat numbering. In some embodiments, the anti-CD3ε binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 197. In some embodiments, the anti-CD3ε binding domain includes a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 200. In some embodiments, the anti-CD3ε binding domain thereof includes a variable heavy chain (VH) comprising the amino acid sequence of SEQ ID NO: 197 and a variable light chain (VL) comprising the amino acid sequence of SEQ ID NO: 200.

2. Immunoglobulin Fc polypeptides:

[0232] The first component of the multispecific polypeptide constructs of the disclosure includes an immunoglobulin Fc region. In some embodiments, the immunoglobulin Fc region

is an IgG isotype selected from the group consisting of IgG1 isotype, IgG2 isotype, IgG3 isotype, and IgG4 subclass. In some embodiments, the Fc region is a human Fc. In some embodiments, the immunoglobulin Fc region is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-6. In some embodiments, the immunoglobulin Fc region contains an Fc chain that is an immunologically active fragment of any of SEQ ID Nos: 1-6. In some embodiments, the immunoglobulin Fc region contains an Fc polypeptide chain that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of any of SEQ ID NOs: 1-6 or an immunologically active fragment thereof.

[0233] In some embodiments, the multispecific polypeptide construct is a dimer formed by polypeptides, each containing an Fc. In some specific embodiments, identical or substantially identical polypeptides will be dimerized to create a homodimer. In some embodiments, the dimer is a homodimer in which the two polypeptides of the multispecific polypeptide construct are the same. In other cases, the Fc region is formed by Fc domains that are mutated or modified to promote heterodimerization in which different polypeptides can be dimerized to yield a heterodimer. Thus, in some embodiments, the dimer is a heterodimer in which two polypeptide chains of the multispecific polypeptide construct are different. Exemplary modifications to promote heterodimerization are known, including any as described below.

[0234] In general, the Fc region is responsible for effector functions, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC), in addition to the antigen-binding capacity, which is the main function of immunoglobulins. Additionally, the FcRn sequence present in the Fc region plays the role of regulating the IgG level in serum by increasing the *in vivo* half-life by conjugation to an *in vivo* FcRn receptor. In some embodiments, such functions can be altered, such as reduced or enhanced, in an Fc for use with the provided multispecific polypeptide constructs.

[0235] In some embodiments, the Fc region of the provided multispecific polypeptide constructs exhibit one or more effector functions. In some cases, the Fc region is capable of providing Fc-mediated effector functions, such as for example, ADCC (*e.g.*, release of granzyme B by NK cells), ADCP, and/or CDC. Thus, in some embodiments in which the multispecific polypeptide constructs contain a cleavable linker, cleavage of the linker can produce two components that each have biological activity: the CD3-binding region that is able to bind and engage CD3 on a T cell and the Fc region linked to the TAA-antigen binding domain that can exhibit target-specific effector function. In particular embodiments provided

herein, the multispecific polypeptide constructs contain a non-cleavable linker and may, in some aspects, not exhibit an independent Fc-mediated effector function.

[0236] In some embodiments, the Fc region includes an Fc polypeptide that is mutated or modified to alter one or more effector functions. Various examples of mutations to Fc polypeptides to alter, such as reduce, effector function are known, including any as described below. In some embodiments, reference to amino acid substitutions in an Fc region is by EU numbering by Kabat (also called Kabat numbering) unless described with reference to a specific SEQ ID NO. EU numbering is known and is according to the most recently updated IMGT Scientific Chart (IMGT®, the international ImMunoGeneTics information system®, http://www.imgt.org/IMGTScientificChart/Numbering/Hu_IGHGnber.html (created: 17 May 2001, last updated: 10 Jan 2013) and the EU index as reported in Kabat, E.A. et al. Sequences of Proteins of Immunological interest. 5th ed. US Department of Health and Human Services, NIH publication No. 91-3242 (1991).

[0237] In some embodiments, provided multispecific polypeptide constructs that contain an Fc region that exhibits reduced effector functions, may be a desirable candidate for applications in which constrained CD3 binding is desired yet certain effector functions (such as CDC and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the multispecific polypeptide constructs and/or cleaved components thereof lack FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Pat. No. 5,500,362 (see, e.g., Hellstrom, I. *et al. Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I *et al.*, *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); U.S. Pat. No. 5,821,337 (see Bruggemann, M. *et al.*, *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assay methods may be employed (see, for example, ACTITM non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, Calif.; and CytoTox 96TM non-radioactive cytotoxicity assay (Promega, Madison, Wis.)). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in an animal model such as that disclosed in Clynes *et al. Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the multispecific

polypeptide construct or cleaved components thereof is unable to bind C1q and hence lacks CDC activity. See, *e.g.*, C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M. S. *et al.*, *Blood* 101:1045-1052 (2003); and Cragg, M. S. and M. J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half-life determinations can also be performed using methods known in the art (see, *e.g.*, Petkova, S. B. *et al.*, *Int'l. Immunol.* 18(12):1759-1769 (2006)).

[0238] In some embodiments, the immunoglobulin Fc region or immunologically active fragment thereof is an IgG isotype. For example, the immunoglobulin Fc region of the fusion protein is of human IgG1 isotype, having an amino acid sequence:

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PAPE[LL]GPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV
DGVEVHNAKT KPREEQYN[ST] YRVVSVLTVL HQDWLNGKEY KCKVSNKALP
APIEKTISKA KGQPREPQVY TLPPSRDELT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH
EALHNHYTQK SLSLSPGK (SEQ ID NO: 1)
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[0239] In some embodiments, the immunoglobulin Fc region or immunologically active fragment thereof comprises a human IgG1 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 1.

[0240] In some embodiments, an IgG1 Fc polypeptide or a variant thereof such as any described below can be made in a G1 m1 or G1 m3 allotype. In some embodiments, the Fc region can contain amino acids of the human G1 m1 allotype, such as residues containing Asp (D) and Leu (L) at positions 356 and 358, *e.g.* as set forth in SEQ ID NO:1. In some cases, an Fc polypeptide can contain amino acid substitutions E356D and M358L to reconstitute residues of allotype G1 m1. In other embodiments, the Fc region can contain amino acids of the human G1 m3 allotype, such as residues Glu (E) and Met (M) at positions 356 and 358 by EU numbering, *e.g.* as set forth in SEQ ID NOS: 194 and 195. In some cases, an Fc polypeptide can contain amino acid substitutions D356E and L358M to reconstitute residues of allotype G1 m3. In some embodiments, the human IgG1 Fc region is modified to alter antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), *e.g.*, the amino acid modifications described in Natsume *et al.*, 2008 *Cancer Res.* 68(10): 3863-72; Idusogie *et al.*, 2001 *J Immunol.* 166(4): 2571-5; Moore

et al., 2010 mAbs, 2(2): 181-189; Lazar et al., 2006 PNAS, 103(11): 4005–4010, Shields et al., 2001 JBC, 276(9): 6591–6604; Stavenhagen et al., 2007 Cancer Res, 67(18): 8882-8890; Stavenhagen et al., 2008 Advan. Enzyme Regul., 48: 152-164; Alegre et al, 1992 J Immunol, 148: 3461-3468; Reviewed in Kaneko and Niwa, 2011 Biodrugs, 25(1):1-11, the contents of each of which are hereby incorporated by reference in their entireties.

[0241] In some embodiments, the Fc region, such as the human IgG1 Fc region is modified to enhance ADCC activity or CDC activity. Examples of mutations that enhance ADCC include modification at Ser239 and Ile332, for example Ser239Asp and Ile332Glu (S239D, I332E). Examples of mutations that enhance CDC include modifications at Lys326 and Glu333. In some embodiments, the Fc region is modified at one or both of these positions, for example Lys326Ala and/or Glu333Ala (K326A and E333A) using the Kabat numbering system.

[0242] In some embodiments, the human IgG1 Fc region fusion proteins of the present disclosure lack or have reduced Fucose attached to the N-linked glycan-chain at N297. There are numerous ways to prevent fucosylation, including but not limited to production in a FUT8 deficient cell line; addition inhibitors to the mammalian cell culture media, for example Castanospermine; and metabolic engineering of the production cell line. In some embodiments, the human IgG1 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent glycosylation of the fusion protein, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D).

[0243] In some embodiments, the Fc region is altered to provide reduced Fc-mediated effector functions, such as via reduced Fc receptor binding, *e.g.* binding to FcγR binding but generally not FcRn binding. In some embodiments, the Fc region of the fusion protein is altered at one or more of the following positions to reduce Fc receptor binding: Leu 234 (L234), Leu235 (L235), Asp265 (D265), Asp270 (D270), Ser298 (S298), Asn297 (N297), Asn325 (N325) or Ala327 (A327). For example, Leu 234Ala (L234A), Leu235Ala (L235A), Asp265Asn (D265N), Asp270Asn (D270N), Ser298Asn (S298N), Asn297Ala (N297A), Asn325Glu (N325E) or Ala327Ser (A327S). In some embodiments, the Fc region of the fusion protein is modified at amino acid Leu235 (Boxed in SEQ ID NO:1 above, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E) or Leu235Ala (L235A). In some embodiments, the Fc region of the fusion protein is modified at amino acid Leu234 (Boxed in SEQ ID NO:1 above, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu234Ala (L234A). In some embodiments, the Fc region of the fusion protein is altered at both amino acid 234 and 235, *e.g.*, Leu234Ala and Leu235Ala (L234A/L235A) or

Leu234Val and Leu235Ala (L234V/L235A). In preferred embodiments, modifications within the Fc region reduce binding to Fc-receptor-gamma receptors while have minimal impact on binding to the neonatal Fc receptor (FcRn).

[0244] In some embodiments, the human IgG Fc region is modified to enhance FcRn binding. Examples of Fc mutations that enhance binding to FcRn are Met252Tyr, Ser254Thr, Thr256Glu (M252Y, S254T, T256E, respectively) (Kabat numbering, Dall'Acqua *et al* 2006, *J. Biol Chem* Vol. 281(33) 23514–23524), Met428Leu and Asn434Ser (M428L, N434S) (Zalevsky *et al* 2010 *Nature Biotech*, Vol. 28(2) 157-159) (EU index of Kabat *et al* 1991 *Sequences of Proteins of Immunological Interest*). In some embodiments, the mutated or modified Fc polypeptide includes the following mutations: Met252Tyr and Met428Leu or Met252Tyr and Met428Val (M252Y, M428L, or M252Y, M428V) using the Kabat numbering system.

[0245] In some embodiments, the Fc region of the fusion protein is lacking an amino acid at one or more of the following positions to reduce Fc receptor binding: Glu233 (E233), Leu234 (L234), or Leu235 (L235). In these embodiments, Fc deletion of these three amino acids reduces the complement protein C1q binding.

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PAPGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV
EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI
EKTISKAKGQ PREPQVYTL PPSRDELTKNQ VSLTCLVKGF YPSDIAVEWE
SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMEAL
HNHYTQKSLS LSPGK (SEQ ID NO: 2)
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[0246] In some embodiments, the Fc region is mutated in one or more of the following positions to reduce Fc receptor binding: Glu233 (E233), Leu234 (L234), or Leu235 (L235). The one or more mutations can include E233P, L234V and/or L235A.

[0247] In some embodiments, the Fc region of the fusion protein is altered at Gly236 (boxed in SEQ ID NO:1 above) to reduce Fc receptor binding. For example, wherein Gly236 is deleted from the fusion protein. In some embodiments, the human IgG1 Fc region is modified at amino acid Gly236 to enhance the interaction with CD32A, *e.g.*, Gly236Ala (G236A).

[0248] In particular embodiments, the mutations of the Fc region to reduce Fc effector function, *e.g.* via reducing Fc receptor binding to FcγR, include mutations from among any of G236R/L328R, E233P/L234V/L235A/G236del/S239K, E233P/L234V/L235A/G236del/S267K, E233P/L234V/L235A/G236del/S239K/A327G, E233P/L234V/L235A/G236del/S267K/A327G or E233P/L234V/L235A/G236del.

[0249] In some embodiments, the human IgG1 Fc region lacks Lys447 (EU index of *Kabat et al 1991 Sequences of Proteins of Immunological Interest*).

[0250] In some embodiments, the fusion or immunologically active fragment thereof comprises a human IgG2 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 2.

[0251] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG2 isotype, having an amino acid sequence:

PAPPVAGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVQFNWYVD
GVEVHNAKTK PREEQF^NSTF RVVSVLTVVH QDWLNGKEYK CKVSNKGLPA
PIEKTISKTK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDISVE
WESNGQPENN YKTTTPMLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE
ALHNHYTQKS LSLSPGK (SEQ ID NO: 3)

[0252] In some embodiments, the fusion or immunologically active fragment thereof comprises a human IgG2 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 3.

[0253] In some embodiments, the human IgG2 Fc region is modified at amino acid Asn297 (Boxed, to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG2 Fc region lacks Lys447 (EU index of *Kabat et al 1991 Sequences of Proteins of Immunological Interest*).

[0254] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG3 isotype, having an amino acid sequence:

PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVQFKWYV
DGVEVHNAKT KPREEQY^NST FRVSVLTVL HQDWLNGKEY KCKVSNKALP
APIEKTISKTK KGQPREPQVY TLPPSREEMT KNQVSLTCLV KGFYPSDIAV
EWESSGQPEN NYNTTPMLD SDGSFFLYSK LTVDKSRWQQ GNIFSCSVMH
EALHN^RFTQK SLSLSPGK (SEQ ID NO: 4)

[0255] In some embodiments, the antibody or immunologically active fragment thereof comprises a human IgG3 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 4.

[0256] In some embodiments, the human IgG3 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG3 Fc region is modified at amino acid 435 to extend the half-life, *e.g.*, Arg435His (R435H). In some embodiments, the human IgG3 Fc region lacks Lys447 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0257] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG4 isotype, having an amino acid sequence:

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PAPEF1LGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
DGVEVHNAKT KPREEQF2ST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP
SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTTPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH
EALHNHYTQK SLSLSLGK (SEQ ID NO: 5)
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[0258] In some embodiments, the antibody or immunologically active fragment thereof comprises a human IgG4 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 5.

[0259] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG4 isotype, having an amino acid sequence:

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PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
DGVEVHNAKT KPREEQF2ST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP
SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTTPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH
EALHNHYTQK SLSLSLGK (SEQ ID NO: 6)
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[0260] In some embodiments, the antibody or immunologically active fragment thereof comprises a human IgG4 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 6.

[0261] In other embodiments, the human IgG4 Fc region is modified at amino acid 235 to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E). In some embodiments, the human IgG4 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some

embodiments, the human IgG4 Fc region lacks Lys447 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0262] In some embodiments, the human IgG Fc region is modified to stabilize the homodimerization at the CH3:CH3 interface by introducing two disulfide bonds by changing Ser354 to Cys (S354C) and Tyr349 to Cys (Y349C) (S354C/Y349C).

[0263] In particular embodiments of multispecific polypeptide constructs provided herein, the human IgG Fc region is modified to induce heterodimerization. Various methods are known for promoting heterodimerization of complementary Fc polypeptides, see e.g. Ridgway et al, *Protein Eng.* 9:617-621 (1996); Merchant et al, *Nat. Biotechnol.* 16(7): 677-81 (1998); Moore et al. (2011) *MAbs*, 3:546-57; Von Kreudenstein et al. *MAbs*, (2013) 5:646-54; Gunasekaran et al. (2010) *J. Biol. Chem.*, 285:19637-46; Leaver-Fay et al. (2016) *Structure*, 24:641-51; Ha et al. (2016) *Frontiers in Immunology*, 7:1; Davis et al. (2010) *Protein Eng Des Sel*, 23:195-202; published international PCT Appl. No. WO 1998/050431, WO 2009/089004, WO2011143545 WO 2014/067011, WO 2012/058768, WO2018027025; published U.S. patent Appl. No. US20140363426, US20150307628, US20180016354, US20150239991; and U.S. patent Nos. US5731168, US7183076, US9701759, US9605084, and US9650446. Methods to promote heterodimerization of Fc chains include mutagenesis of the Fc region, such as by including a set of “knob-into-hole” mutations or including mutations to effect electrostatic steering of the Fc to favor attractive interactions among different polypeptide chains. For example, in some embodiments, the Fc polypeptides of a heterodimer includes a mutation to alter charge polarity across the Fc dimer interface such that coexpression of electrostatically matched Fc chains support favorable attractive interactions thereby promoting desired Fc heterodimer formation, whereas unfavorable repulsive charge interactions suppress unwanted Fc homodimer formation (Guneskaran et al. (2010) *JBC*, 285: 19637-19646). When co-expressed in a cell, association between the chains is possible but the chains do not substantially self-associate due to charge repulsion. Other strategies for generating a heterodimeric Fc include mixing human IgG and IgA CH3 domain segments to create a complementary CH3 heterodimer, which is referred to as a SEED Fc.

[0264] Methods and variants for heterodimerization also include those described in published international PCT App. WO2014/145806, including “knobs and holes” mutations (also called “skew” variants), mutations that relate to “electrostatic steering” or “charge pairs,” and pI variants. Heterodimeric variants also include any as described in U.S. published Appl. No. US2012/0149876 or US2018/011883.

[0265] In some embodiments, to promote heterodimerization both polypeptides of the Fc heterodimer contain paired or complementary amino acid modifications. Exemplary paired amino acid modification of polypeptides of an Fc fusion are set forth in **Table 1**.

Table 1: Paired amino acids of Heterodimeric Fc	
First Fc polypeptide	Second Fc Polypeptide
T366W	T366S/L368W/Y407V
T366W/S354C	T366S/L368A/Y407V/Y349C
S364H/F405A	Y349T/Y349F
T350V/L351Y/F405A/Y407V	T350V/T366L/K392L/T394W
K360D/D399M/Y407A	E345R/Q347R/T366V/K409V
K409D/K392D	D399K/E356K
K360E/K409W	Q347R/D399V/F405T
L360E/K409W/Y349C	Q347R/399V/F405T/S354C
K370E/K409W	E357N/D399V/F405T

[0266] In some embodiments, modifications include introduction of a protuberance (knob) into a first Fc polypeptide and a cavity (hole) into a second Fc polypeptide such that the protuberance is positionable in the cavity to promote complexing of the first and second Fc-containing polypeptides. Amino acids targeted for replacement and/or modification to create protuberances or cavities in a polypeptide are typically interface amino acids that interact or contact with one or more amino acids in the interface of a second polypeptide.

[0267] In some embodiments, a first Fc polypeptide that is modified to contain protuberance (hole) amino acids include replacement of a native or original amino acid with an amino acid that has at least one side chain which projects from the interface of the first Fc polypeptide and is therefore positionable in a compensatory cavity (hole) in an adjacent interface of a second polypeptide. Most often, the replacement amino acid is one which has a larger side chain volume than the original amino acid residue. One of skill in the art knows how to determine and/or assess the properties of amino acid residues to identify those that are ideal replacement amino acids to create a protuberance. In some embodiments, the replacement residues for the formation of a protuberance are naturally occurring amino acid residues and include, for example, arginine (R), phenylalanine (F), tyrosine (Y), or tryptophan (W). In some examples, the original residue identified for replacement is an amino acid residue that has a small side chain such as, for example, alanine, asparagine, aspartic acid, glycine, serine, threonine, or valine.

[0268] In some embodiments, a second Fc polypeptide that is modified to contain a cavity (hole) is one that includes replacement of a native or original amino acid with an amino acid that has at least one side chain that is recessed from the interface of the second

polypeptide and thus is able to accommodate a corresponding protuberance from the interface of a first polypeptide. Most often, the replacement amino acid is one which has a smaller side chain volume than the original amino acid residue. One of skill in the art knows how to determine and/or assess the properties of amino acid residues to identify those that are ideal replacement residues for the formation of a cavity. Generally, the replacement residues for the formation of a cavity are naturally occurring amino acids and include, for example, alanine (A), serine (S), threonine (T) and valine (V). In some examples, the original amino acid identified for replacement is an amino acid that has a large side chain such as, for example, tyrosine, arginine, phenylalanine, or tryptophan.

[0269] The CH3 interface of human IgG1, for example, involves sixteen residues on each domain located on four anti-parallel β -strands which buries 1090 Å² from each surface (see e.g., Deisenhofer et al. (1981) *Biochemistry*, 20:2361-2370; Miller et al., (1990) *J Mol. Biol.*, 216, 965-973; Ridgway et al., (1996) *Prot. Engin.*, 9: 617-621; U.S. Pat. No. 5,731,168). Modifications of a CH3 domain to create protuberances or cavities are described, for example, in U.S. Pat. No. 5,731,168; International Patent Applications WO98/50431 and WO 2005/063816; and Ridgway et al., (1996) *Prot. Engin.*, 9: 617-621. In some examples, modifications of a CH3 domain to create protuberances or cavities are typically targeted to residues located on the two central anti-parallel β -strands. The aim is to minimize the risk that the protuberances which are created can be accommodated by protruding into the surrounding solvent rather than being accommodated by a compensatory cavity in the partner CH3 domain.

[0270] For example, in some embodiments the heterodimeric Fc includes a polypeptide having an amino acid modification within the CH3 domain at Thr366, which when replaced with a more bulky amino acid, e.g., Try (T366W), is able to preferentially pair with a second CH3 domain having amino acid modifications to less bulky amino acids at positions Thr366, Leu368, and Tyr407, e.g., Ser, Ala and Val, respectively (T366S/L368A/Y407V). Heterodimerization via CH3 modifications can be further stabilized by the introduction of a disulfide bond, for example by changing Ser354 to Cys (S354C) and Tyr349 to Cys (Y349C) on opposite CH3 domains (Reviewed in Carter, 2001 *Journal of Immunological Methods*, 248: 7-15).

[0271] In particular embodiments, a multispecific polypeptide construct contains a first and second Fc able to mediate Fc heterodimerization contains a first Fc polypeptide containing mutations T366W and S354C and a second Fc polypeptide containing mutations T366S, L368A, Y407V and Y349C. In some embodiments, the first Fc polypeptide is

selected from an Fc polypeptide comprising the sequence set forth in SEQ ID NO: 201 or 207 and the second Fc polypeptide is selected from an Fc polypeptide comprising the sequence set forth in SEQ ID NO: 202, 205 or 209. In some embodiments, the first Fc polypeptide is or comprises the sequence of amino acids set forth in any of SEQ ID NOS: 82, 86, 94 or 96 and the second Fc polypeptide is or comprises the sequence of amino acids set forth in any of SEQ ID NOS: 83, 87, 90, 92, 98 or 100.

[0272] In some embodiments, the Fc polypeptide exhibits features providing Fc-mediated effector functions. In particular examples, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NOS:201 and a second Fc polypeptide that is or comprises SEQ ID NO: 202 or 205. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 82 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 83 or 90. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 86 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 87 or 92. The first and second Fc polypeptide can be formatted on either polypeptide chain of the construct.

[0273] In some embodiments, one or both of the first and second Fc polypeptides can further include one or more amino acid mutations to further reduce one or more Fc effector functions, such as reduced Fc receptor binding. Exemplary mutations to reduce Fc effector functions include any as described. In some embodiments, the modification can be a deletion of one or more positions Glu233 (E233), Leu234 (L234), or Leu235 (L235), such as a deletion of amino acids Glu233 (E233), Leu234 (L234), and Leu235 (L235). In some embodiments, the first Fc polypeptide is selected from an Fc polypeptide comprising the sequence set forth in SEQ ID NO: 203 or 208 and the second Fc polypeptide is selected from an Fc polypeptide comprising the sequence set forth in SEQ ID NO: 204, 206 or 210. In some embodiments, the first Fc polypeptide is or comprises the sequence of amino acids set forth in any of SEQ ID NOS: 84, 88, 95 or 97 and the second Fc polypeptide is or comprises the sequence of amino acids set forth in any of SEQ ID NOS: 85, 89, 91, 93, 99 or 101.

[0274] In particular examples, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NOS:203 and a second Fc polypeptide that is or comprises SEQ ID NO: 204 or 206. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 84 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 85 or 91. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 88 and the second Fc polypeptide is or comprises the

sequence set forth in SEQ ID NO: 89 or 93. The first and second Fc polypeptide can be formatted on either polypeptide chain of the construct.

[0275] In some embodiments, the first Fc polypeptide or second Fc polypeptide further includes mutations M252Y and/or M428V. In particular examples, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:207 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:209. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:94 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 98. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:96 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 100. In other examples, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:208 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:210. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:95 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 99. In some embodiments, the first Fc polypeptide is or comprises the sequence set forth in SEQ ID NO:97 and the second Fc polypeptide is or comprises the sequence set forth in SEQ ID NO: 101. The first and second Fc polypeptide can be formatted on either polypeptide chain of the construct.

[0276] Additional examples of variants that can facilitate the promotion of heterodimers are any combination or pair of steric variants (e.g. skew variants) of a first Fc polypeptide and a second Fc polypeptide from among: S364K/E357Q and L368D/K370S; L368D/K370S and S364K; L368E/K370S and S364K; T411T/E360E/Q362E and D401K; L368D/K370S and S364K/E357L, K370S and S364K/E357Q and T366S/L368A/Y407V and T366W or 366S/L368A/Y407V/Y349C and T366W/S354C), where each pair represents mutations in the first Fc polypeptide and second Fc polypeptide. In particular embodiments, a provided construct contains a first and second Fc polypeptide containing the pair of mutations L368D/K370S and S364K and E357Q.

[0277] An additional mechanism that can be used in the generation of heterodimers is sometimes referred to as “electrostatic steering” as described in Gunasekaran et al., J. Biol. Chem. 285(25):19637 (2010). This is sometimes referred to herein as “charge pairs”. In this embodiment, electrostatics are used to skew the formation towards heterodimerization. As those in the art will appreciate, these may also have an effect on pI, and thus on purification, and thus could in some cases also be considered pI variants. However, as these were generated to force heterodimerization and were not used as purification tools, they are

classified as “steric variants”. In one embodiment, a first Fc polypeptide can contain mutations D221E/P228E/L368E and a second Fc polypeptide can contain mutations D221R/P228R/K409R. In another embodiment, a first Fc polypeptide can contain mutations C220E/P228E/368E and a second Fc polypeptide can contain mutations C220R/E224R/P228R/K409R.

[0278] In some embodiments, heterodimerization can be facilitated by pI variants. In some aspects, a pI variant can include those that increase the pI of the protein (basic changes). In other aspects, the pI variant can include those that decrease the pI of the protein (acidic changes). In some cases, all combinations of these variants can be done, including combinations in which one Fc polypeptide may be wild type, or a variant that does not display a significantly different pI from wild-type, and the other Fc polypeptide can be either more basic or more acidic. Alternatively, each Fc polypeptide can be changed, one to more basic and one to more acidic. In some embodiments, at least one Fc polypeptide is a negative pI variant Fc containing mutations Q295E/N384D/Q418E/N421D.

[0279] In some embodiments, a combination of steric heterodimerization variants (e.g. knob and hole) and pI or charge pair variants can be used.

[0280] In particular embodiments, the provided constructs contains (a) a first Fc polypeptide comprising the skew variants S364K/E357Q; and b) a second Fc polypeptide containing skew variants L368D/K370S and the pI variants N208D/Q295E/N384D/Q418E/N421D. In some embodiments, one or both of the first and second polypeptide can contain further mutations to reduce Fc effector activity, such as the exemplary mutations E233P/L234V/L235A/G236del/S267K. An example of such a first Fc polypeptide and a second Fc polypeptide able to mediate Fc heterodimerization comprise the sequences set forth in SEQ ID NOs:194 and 195. The first and second Fc polypeptide can be formatted on either polypeptide chain of the construct.

[0281] The resulting multispecific polypeptide constructs can be purified by any suitable method such as, for example, by affinity chromatography over Protein A or Protein G columns. Where two nucleic acid molecules encoding different polypeptides are transformed into cells, formation of homo- and heterodimers will occur. Conditions for expression can be adjusted so that heterodimer formation is favored over homodimer formation.

[0282] Techniques for recovery of heterodimers from homodimers based on a differential affinity of the heterodimers for an affinity reagent are known. In some aspects, such techniques include designing a heterodimer so that one of the Fc polypeptide chains does not bind to the affinity reagent protein A. In some cases, one of the polypeptide chain

can contain one or more amino acid substitution to abrogate or reduce affinity for the protein A reagent in one of the polypeptides of the Fc heterodimer, see e.g. WO2017134440, WO2010151792, Jendeborg et al. (Jendeborg et al., (1997) J. Immunol. Methods, 201(1): 25-34. In some of these embodiments, the Fc region may be modified at the protein-A binding site on one member of the heterodimer so as to prevent protein-A binding and thereby enable more efficient purification of the heterodimeric fusion protein. An exemplary modification within this binding site is Ile253, for example Ile253Arg (I253R). In some embodiments, the modification may be H435R or H435R/Y436F. In some embodiments, an Fc polypeptide of an Fc heterodimer can contain a modification so that it is capable of binding protein A but not protein G (pA+/pG-). Exemplary pA+/pG- amino acid modifications include an Fc containing serine at position 428, serine at position 434 and optionally histidine at position 436, with reference to human IgG1 or comprising these residues at the corresponding positions in human IgG 2, 3, or 4. In some aspects, such amino acid modifications in one IgG Fc polypeptide at positions 428, 434 and optionally 436 reduces or prevents the binding of protein G, enhancing the purification of the protein.

[0283] In some embodiments, any of such modifications to confer differential affinity to an affinity reagent can be combined with any one or more other amino acid modifications described above. For example, the I253R modification may be combined with either the T366S/L368A/Y407V modifications or with the T366W modifications. The T366S/L368A/Y407V modified Fc is capable of forming homodimers as there is no steric occlusion of the dimerization interface as there is in the case of the T336W modified Fc. Therefore, in some embodiments, the I253R modification is combined with the T366S/L368A/Y407V modified Fc to disallow purification any homodimeric Fc that may have formed. Similar modifications can be employed by combining T366S/L368A/Y407V and H453R.

[0284] In some embodiments, the Fc regions of the heterodimeric molecule additionally can contain one or more other Fc mutation, such as any described above. In some embodiments, the heterodimer molecule contains an Fc region with a mutation that reduces effector function.

[0285] In some embodiments, one Fc polypeptide of a heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:201 (e.g. SEQ ID NO:82), 86, 207 (e.g. SEQ ID NO:94), or 96, and the other Fc polypeptide of the heterodimeric Fc contains the sequence of amino acids set forth in any of SEQ ID NOS:201 (e.g. SEQ ID NO:83), 87, 205 (e.g. SEQ ID NO:90), 92, 209 (e.g. SEQ ID NO:98), or 100. In some embodiments, one

Fc polypeptide of a heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 203 (e.g. SEQ ID NO:84), 88, 208 (e.g. SEQ ID NO:95), or 97 and the other Fc polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 204 (e.g. SEQ ID NO:85), 89, 206 (e.g. SEQ ID NO:91), 93, 210 (e.g. SEQ ID NO:99), or 101.

[0286] In some embodiments, the human IgG Fc region is modified to prevent dimerization. In these embodiments, the fusion proteins of the present disclosure are monomeric. For example modification at residue Thr366 to a charged residue, *e.g.* Thr366Lys, Thr366Arg, Thr366Asp, or Thr366Glu (T366K, T366R, T366D, or T366E, respectively), prevents CH3-CH3 dimerization.

[0287] In some embodiments, the Fc region of the fusion protein is altered at one or more of the following positions to reduce Fc receptor binding: Leu 234 (L234), Leu235 (L235), Asp265 (D265), Asp270 (D270), Ser298 (S298), Asn297 (N297), Asn325 (N325) or Ala327 (A327). For example, Leu 234Ala (L234A), Leu235Ala (L235A), Asp265Asn (D265N), Asp270Asn (D270N), Ser298Asn (S298N), Asn297Ala (N297A), Asn325Glu (N325E) or Ala327Ser (A327S). In preferred embodiments, modifications within the Fc region reduce binding to Fc-receptor-gamma receptors while have minimal impact on binding to the neonatal Fc receptor (FcRn).

[0288] In some embodiments, the fusion protein contains a polypeptide derived from an immunoglobulin hinge region. The hinge region can be selected from any of the human IgG subclasses. For example, the fusion protein may contain a modified IgG1 hinge having the sequence of EPKSSDKTHTCPPC (SEQ ID NO: 7), where in the Cys220 that forms a disulfide with the C-terminal cysteine of the light chain is mutated to serine, *e.g.*, Cys220Ser (C220S). In other embodiments, the fusion protein contains a truncated hinge having a sequence DKTHTCPPC (SEQ ID NO: 8).

[0289] In some embodiments, the fusion protein has a modified hinge from IgG4, which is modified to prevent or reduce strand exchange, *e.g.*, Ser228Pro (S228P), having the sequence ESKYGPPCPPC (SEQ ID NO: 9). In some embodiments, the fusion protein contains linker polypeptides. In other embodiments, the fusion protein contains linker and hinge polypeptides.

3. *Linkers*

[0290] The provided multispecific polypeptide constructs contain a linker that joins or couples the first component containing the immunoglobulin Fc region and the second component containing the CD3 binding region. In some embodiments, the linker is a non-cleavable linker. In some embodiments, the linker does not contain a substrate recognition site that is specifically recognized for cleavage by the protease. Thus, linkers in the provided multispecific polypeptide constructs do not include an amino acid sequence that can serve as a substrate for a protease, such as an extracellular protease. For example, the non-cleavable linker does not include a cleavage sequence containing at least one peptide bond which lies within a cleavable peptide sequence of a protease.

[0291] In some embodiments, the linker is positioned at the end of the C-terminal region of the Fc region, such that the Fc region is N-terminal to the CD3 binding region. Because the provided multispecific polypeptide constructs are multimers, such as dimers, the provided constructs include a linker joining the first Fc polypeptide and a first domain (e.g. VH) of the CD3 binding region of the first polypeptide and the second Fc polypeptide and second domain (e.g. VL) of the CD3 binding region of the second polypeptide. Typically, the linkers present in the first and second polypeptides of the multispecific polypeptide construct are the same. Thus, in some embodiments, each domain of the CD3 binding domain is linked via a linker, such as the same linker, to opposite polypeptides of the Fc, such as heterodimeric Fc.

[0292] Various polypeptide linkers for use in fusion proteins are known (see e.g. Chen et al. (2013) Adv. Drug. Deliv. 65:1357-1369; and International PCT publication No. WO 2014/099997, WO2000/24884; U.S. Pat. No. 5,258,498; U.S. Pat. No. 5,525,491; U.S. Pat. No. 5,525,491, U.S. Pat. No. 6,132,992).

[0293] In some embodiments, the linker is chosen so that, when the CD3 binding region is joined to the Fc region of the multispecific polypeptide conjugate, the CD3 binding region is constrained and not able to, or not substantially able to, bind or engage CD3 on the surface of a cell, e.g. T cell, upon contact of the multispecific polypeptide construct with the cell. Various assays can be employed to assess binding or engagement of CD3 by the multispecific polypeptide construct, including assays to assess T cell binding, NFAT activation using a reporter system, cytolytic T cell activity, cytokine production and/or expression of T cell activation markers. Exemplary assays are shown in the provided Examples. Typically, the linker also is one that ensures correct folding of the polypeptide construct, does not exhibit a charge that would be inconsistent with the activity or function of the linked polypeptides or form bonds or other interactions with amino acid residues in one or more of the domains that

would impede or alter activity of the linked polypeptides. In some embodiment, the linker is a polypeptide linker. The polypeptide linker can be a flexible linker or a rigid linker or a combination of both.

[0294] In some aspects, the linker is a short, medium or long linker. In some embodiments, the linker is up to 40 amino acids in length. In some embodiments, the linker is up to 25 amino acids in length. In some embodiments, the linker is at least or is at least about 2 amino acids in length. In some aspects, a suitable length is, e.g., a length of at least one and typically fewer than about 40 amino acid residues, such as 2-25 amino acid residues, 5-20 amino acid residues, 5-15 amino acid residues, 8-12 amino acid . In some embodiments, the linker is from or from about 2 to 24 amino acids, 2 to 20 amino acids, 2 to 18 amino acids, 2 to 14 amino acids, 2 to 12 amino acids, 2 to 10 amino acids, 2 to 8 amino acids, 2 to 6 amino acids, 6 to 24 amino acids, 6 to 20 amino acids, 6 to 18 amino acids, 6 to 14 amino acids, 6 to 12 amino acids, 6 to 10 amino acids, 6 to 8 amino acids, 8 to 24 amino acids, 8 to 20 amino acids, 8 to 18 amino acids, 8 to 14 amino acids, 8 to 12 amino acids, 8 to 10 amino acids, 10 to 24 amino acids, 10 to 20 amino acids, 10 to 18 amino acids, 10 to 14 amino acids, 10 to 12 amino acids, 12 to 24 amino acids, 12 to 20 amino acids, 12 to 18 amino acids, 12 to 14 amino acids, 14 to 24 amino acids, 14 to 20 amino acids, 14 to 18 amino acids, 18 to 24 amino acids, 18 to 20 amino acids or 20 to 24 amino acids. In some embodiments, the linker is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length.

[0295] In certain aspects, the longer the linker length, the greater the CD3 binding when the multispecific polypeptide conjugate is bounds to its antigen, e.g. TAA. Thus, in some aspects, the linker is greater than 12 amino acids in length, such as greater than 13, 14, 15, 16, 17 or 18 amino acids in length. In some embodiments, the linker is 12 to 40 amino acids in length, 12 to 30 amino acids, 12 to 24 amino acids, 12 to 18 acids, 12 to 15 amino acids, 15 to 40 amino acids, 15 to 30 amino acids, 15 to 24 amino acids, 15 to 18 amino acids, 18 to 40 amino acids, 18 to 30 amino acids, 18 to 24 amino acids, 24 to 40 amino acids, 24 to 30 amino acids or 30 to 40 amino acids.

[0296] The linkers can be naturally-occurring, synthetic or a combination of both. Particularly suitable linker polypeptides predominantly include amino acid residues selected from Glycine (Gly), Serine (Ser), Alanine (Ala), and Threonine (Thr). For example, the linker may contain at least 75% (calculated on the basis of the total number of residues present in the peptide linker), such as at least 80%, at least 85%, or at least 90% of amino acid residues selected from Gly, Ser, Ala, and Thr. The linker may also consist of Gly, Ser, Ala and/or Thr

[0299] In some embodiments, the linker includes a peptide linker having the amino acid sequence Gly_xXaa-Gly_y-Xaa-Gly_z (SEQ ID NO:174), wherein each Xaa is independently selected from Alanine (Ala), Valine (Val), Leucine (Leu), Isoleucine (Ile), Methionine (Met), Phenylalanine (Phe), Tryptophan (Trp), Proline (Pro), Glycine (Gly), Serine (Ser), Threonine (Thr), Cysteine (Cys), Tyrosine (Tyr), Asparagine (Asn), Glutamine (Gln), Lysine (Lys), Arginine (Arg), Histidine (His), Aspartate (Asp), and Glutamate (Glu), and wherein x, y, and z are each integers in the range from 1-5. In some embodiments, each Xaa is independently selected from the group consisting of Ser, Ala, and Thr. In a specific variation, each of x, y, and z is equal to 3 (thereby yielding a peptide linker having the amino acid sequence Gly-

Gly-Gly-Xaa-Gly-Gly-Gly-Xaa-Gly-Gly-Gly (SEQ ID NO:175), wherein each Xaa is selected as above.

[0300] In some embodiments, the linker is serine-rich linkers based on the repetition of a (SSSSG)_n (SEQ ID NO:185) motif where n is at least 1, though n can be 2, 3, 4, 5, 6, 7, 8 and 9.

[0301] In some cases, it may be desirable to provide some rigidity into the peptide linker. This may be accomplished by including proline residues in the amino acid sequence of the peptide linker. Thus, in some embodiments, a linker comprises at least one proline residue in the amino acid sequence of the peptide linker. For example, a peptide linker can have an amino acid sequence wherein at least 25% (e.g., at least 50% or at least 75%) of the amino acid residues are proline residues. In one particular embodiment, the peptide linker comprises proline residues only.

[0302] In some aspects, a peptide linker comprises at least one cysteine residue, such as one cysteine residue. For example, in some embodiments, a linker comprises at least one cysteine residue and amino acid residues selected from the group consisting of Gly, Ser, Ala, and Thr. In some such embodiments, a linker comprises glycine residues and cysteine residues, such as glycine residues and cysteine residues only. Typically, only one cysteine residue will be included per peptide linker. One example of a specific linker comprising a cysteine residue includes a peptide linker having the amino acid sequence Gly_m-Cys-Gly_n, wherein n and m are each integers from 1-12, e.g., from 3-9, from 4-8, or from 4-7. In a specific variation, such a peptide linker has the amino acid sequence GGGGG-C-GGGGG (SEQ ID NO:177).

[0303] In some embodiments, the linker of the fusion protein is a structured or constrained linker. In particular embodiments, the structured linker contains the sequence (AP)_n or (EAAAK)_n (SEQ ID NO:178), wherein n is 2 to 20, preferably 4 to 10, including but not limited to, AS-(AP)_n-GT (SEQ ID NO:179) or AS-(EAAAK)_n-GT (SEQ ID NO:180), wherein n is 2 to 20, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. In other embodiments, the linker comprises the sequences (GGGGA)_n (SEQ ID NO:181), (PGGGS)_n (SEQ ID NO:182), (AGGGS)_n (SEQ ID NO:183) or GGS-(EGKSSGSGSESKST)_n-GGS (SEQ ID NO:184, wherein n is 2 to 20. In some embodiments, the linker is SSSASASSA (SEQ ID NO:186), GSPGSPG (SEQ ID NO:187), or ATTTGSSPGPT (SEQ ID NO:176). In some embodiments, such linkers, by virtue of their structure, may be more resistant to proteolytic degradation, thereby offering an advantage when injected in vivo.

[0304] In some embodiments, the linker is not a cleavable linker (used interchangeably with non-cleavable linker). In some embodiments, the linker is not cleavable by a protease. In some embodiments, a linker that is not a cleavable linker or that is not cleavable by a protease is one that is generally stable for in vivo delivery or recombinant production. In some aspects, a linker that is not cleavable by a protease includes those that do not contain at least one peptide bond which preferably lies within a cleavable peptide sequence or recognition site of a protease. In particular embodiments, a non-cleavable linker is not a target substrate for a protease, such that it is not preferentially or specifically cleaved by a protease compared to a linker that contains a substrate recognition site for the same protease.

[0305] In some embodiments, the linker does not contain a substrate recognition site or cleavage site for a particular protease, which is the sequence recognized by the active site of a protease that is cleaved by a protease. Typically, for example, for a serine protease, a cleavage sequence is made up of the P1-P4 and P1'-P4' amino acids in a substrate, where cleavage occurs after the P1 position. Typically, a cleavage sequence for a serine protease is six residues in length to match the extended substrate specificity of many proteases, but can be longer or shorter depending upon the protease. Typically, the linker does not include a P1-P1' scissile bond sequence that is recognized by a protease.

[0306] In some aspects, a non-cleavable linker or a linker that does not contain a substrate recognition site that is specifically recognized for cleavage by a protease is one whose cleavage by a protease is substantially less than cleavage of a target substrate of the protease. Typically, a protease exhibits specificity or preference for cleavage of a particular target substrate compared to another non-target substrate. Such a degree of specificity can be determined based on the rate constant of cleavage of a sequence, e.g. linker sequence, which is a measure of preference of a protease for its substrate and the efficiency of the enzyme. Any method to determine the rate of increase of cleavage over time in the presence of various concentrations of substrate can be used to calculate the specificity constant. For example, a substrate is linked to a fluorogenic moiety, which is released upon cleavage by a protease. By determining the rate of cleavage at different protease concentrations the specificity constant for cleavage (k_{cat}/K_m) can be determined for a particular protease towards a particular linker. In some embodiments, a non-cleavable linker, or a linker that does not contain a substrate recognition site that is specifically recognized for cleavage by a protease, is a linker that, if cleaved at all, is cleaved by a protease at a rate of less than $1 \times 10^4 \text{ M}^{-1}\text{S}^{-1}$, or less than $5 \times 10^3 \text{ M}^{-1}\text{S}$, less than $1 \times 10^3 \text{ M}^{-1}\text{S}$, or less than $1 \times 10^2 \text{ M}^{-1}\text{S}$ or less.

[0307] In some embodiments, the linkers in the multispecific constructs provided herein do not contain a substrate recognition site for a protease that include, for example, matrix metalloproteases (MMP), cysteine proteases, serine proteases and plasmin activators. In particular embodiments, the linker does not contain a substrate recognition site for a protease that is a protease that is produced by a tumor, an activated immune effector cell (e.g. a T cell or a NK cell), or a cell in a tumor microenvironment.

[0308] In some embodiments, the linker does not contain a substrate recognition site that is specifically recognized by one or more of the following enzymes or proteases: ADAMS, ADAMTS, *e.g.* ADAM8; ADAM9; ADAM10; ADAM12; ADAM15; ADAM17/TACE; ADAMDEC1; ADAMTS1; ADAMTS4; ADAMTS5; aspartate proteases, *e.g.*, BACE or Renin; aspartic cathepsins, *e.g.*, Cathepsin D or Cathepsin E; Caspases, *e.g.*, Caspase 1, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Caspase 10, or Caspase 14; cysteine cathepsins, *e.g.*, Cathepsin B, Cathepsin C, Cathepsin K, Cathepsin L, Cathepsin S, Cathepsin V/L2, Cathepsin X/Z/P; Cysteine proteinases, *e.g.*, Cruzipain; Legumain; Otubain-2; KLKs, *e.g.*, KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, or KLK14; Metallo proteinases, *e.g.*, Meprin; Neprilysin; PSMA; BMP-1; MMPs, *e.g.*, MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP17, MMP19, MMP20, MMP23, MMP24, MMP26, or MMP27, serine proteases, *e.g.*, activated protein C, Cathepsin A, Cathepsin G, Chymase, coagulation factor proteases (*e.g.*, FVIIa, FIXa, FXa, FXIa, FXIIa), Elastase, Granzyme B, Guanidinobenzoate, HtrA1, Human Neutrophil Elastase, Lactoferrin, Marapsin, NS3/4A, PACE4, Plasmin, PSA, tPA, Thrombin, Trypsin, uPA; Type II Transmembrane Serine Proteases (TTSPs), *e.g.*, DESC1, DPP-4, FAP, Hepsin, Matriptase-2, Matriptase, TMPRSS2, TMPRSS3, or TMPRSS4; and any combination thereof. In some embodiments, the linker does not contain a substrate recognition site that is specifically recognized by granzyme B, a matriptase or an MMP, such as MMP-2.

[0309] In some embodiments, the linker does not comprise an amino acid that is a substrate for Granzyme B. In some embodiments, the linker does not contain an amino acid sequence having the general formula P4 P3 P2 P1 ↓ P1' (SEQ ID NO: 150), wherein P4 is amino acid I, L, Y, M, F, V, or A; P3 is amino acid A, G, S, V, E, D, Q, N, or Y; P2 is amino acid H, P, A, V, G, S, or T; P1 is amino acid D or E; and P1' is amino acid I, L, Y, M, F, V, T, S, G or A. In some embodiments, the linker does not contain an amino acid sequence having the general formula P4 P3 P2 P1 ↓ P1' (SEQ ID NO: 151), wherein P4 is amino acid I

or L; P3 is amino acid E; P2 is amino acid P or A; P1 is amino acid D; and P1' is amino acid I, V, T, S, or G.

[0310] In some embodiments, the linker does not contain the amino acid sequence LEAD (SEQ ID NO: 22), LEPD (SEQ ID NO: 142), or LEAE (SEQ ID NO: 143). In some embodiments, the linker does not contain the amino acid sequence IEPDI (SEQ ID NO: 136), LEADT (SEQ ID NO: 137), IEPDG (SEQ ID NO: 138), IEPDV (SEQ ID NO: 139), IEPDS (SEQ ID NO: 140), IEPDT (SEQ ID NO: 141), IEPDP (SEQ ID NO: 144), LEPDG (SEQ ID NO: 152) or LEADG (SEQ ID NO: 153).

[0311] In some embodiments, the linker does not comprise an amino acid that is a substrate for matriptase. In some embodiments, the linker does not comprise the sequence P1QAR↓(A/V) (SEQ ID NO: 154), wherein P1 is any amino acid. In some embodiments, the linker does not comprise the sequence RQAR(A/V) (SEQ ID NO: 155). In some embodiments, the linker does not comprise the amino acid sequence RQAR (SEQ ID NO: 23). In some embodiments, the linker does not comprise the amino acid sequence RQARV (SEQ ID NO: 156).

[0312] In some embodiments, the linker does not comprise an amino acid that is a substrate for one or more matrix metalloproteases (MMPs). In some embodiments, the MMP is MMP-2. In some embodiments, the linker does not contain a sequence having the general formula P3 P2 P1 ↓ P1' (SEQ ID NO: 157), wherein P3 is P, V or A; P2 is Q or D; P1 is A or N; and P1' is L, I or M. In some embodiments, the linker does not contain the general formula P3 P2 P1 ↓ P1' (SEQ ID NO: 158), wherein P3 is P; P2 is Q or D; P1 is A or N; and P1' is L or I. In some embodiments, the linker does not comprise the amino acid sequence PAGL (SEQ ID NO: 24).

[0313] In some embodiments, the linker is not a linker comprising the amino acid sequence set forth as TGLEADGSPAGLGRQARVG (SEQ ID NO: 25); TGLEADGSRQARVGPAGLG (SEQ ID NO: 26); TGSPAGLEADGSRQARVGS (SEQ ID NO: 27); TGPAGLGLEADGSRQARVG (SEQ ID NO: 28); TGRQARVGLEADGSPAGLG (SEQ ID NO: 29); TGSRQARVGPAGLEADGS (SEQ ID NO: 30); and TGPAGLGSRQARVGLEADGS (SEQ ID NO: 31); GPAGLGLPEPDGSRQARVG (SEQ ID NO: 104); GSGGGGIEPDIGGSGGS (SEQ ID NO: 105); GSGGGGLEADTGGSGGS (SEQ ID NO: 106); GSIEPDIGS (SEQ ID NO: 107); GSLEADTGS (SEQ ID NO: 108); GSGGGGIEPDGGGSGGS (SEQ ID NO: 109); GSGGGGIEPDVGGSGGS (SEQ ID NO: 110); GSGGGGIEPDGGGSGGS (SEQ ID NO: 111); GSGGGGIEPDTGGSGGS (SEQ ID NO: 112); GGSLEPDGSGS (SEQ ID NO: 113); and

GPAGLGLEADGSRQARVG (SEQ ID NO: 114), GGEGGGGSGGSGGGS (SEQ ID NO: 115); GSSAGSEAGGSGQAGVGS (SEQ ID NO: 116); GGSGGGGLEAEGSGGGGS (SEQ ID NO: 117); GGSGGGGIEPDPPGGSGGS (SEQ ID NO: 118); TGGSGGGGIEPDIGGSGGS (SEQ ID NO: 148).

4. Antigen Binding Domains:

[0314] The multispecific polypeptide constructs of the present disclosure include at least one antigen binding domain, such as at least a first antigen binding domain and a second antigen binding domain. In some aspects, the antigen binding domain, or independently each of the antigen binding domains, is selected from an antibody or antigen binding fragment, a natural cognate binding partner, an Anticalin (engineered lipocalin), a Darpin, a Fynomer, a Centyrin (engineered fibronectin III domain), a cystine-knot domain, an Affilin, an Affibody, or an engineered CH3 domain. In some embodiments, the natural cognate binding partner comprises an extracellular domain or binding fragment thereof of the native cognate binding partner of the TAA, or a variant thereof that exhibits binding activity to the TAA.

[0315] In some embodiments, a TAA is a counter-structure that is present primarily on tumor cells of a mammalian subject but generally not found on normal cells of the mammalian subject. A tumor specific antigen need not be exclusive to tumor cells but the percentage of cells of a particular mammal that have the tumor associated antigen is sufficiently high or the levels of the tumor associated antigen on the surface of the tumor are sufficiently high such that it can be targeted by anti-tumor therapeutics, such as multispecific polypeptide constructs as provided, and provide prevention or treatment of the mammal from the effects of the tumor. In some embodiments, in a random statistical sample of cells from a mammal with a tumor, at least 50% of the cells displaying a TAA are cancerous. In other embodiments, at least 60%, 70%, 80%, 85%, 90%, 95%, or 99% of the cells displaying a TAA are cancerous.

[0316] In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, such as the first antigen-binding domain and the second antigen binding domains, includes one or more copies of an antibody or an antigen-binding fragment thereof. In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, includes one or more copies of an antibody or an antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single

domain light chain antibody. In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, such as the first antigen-binding domain and the second antigen binding domains, is a single chain antibody. In some examples, the single chain is an scFv, a scAb, a single domain heavy chain antibody, or a single domain light chain antibody. In some embodiments, each of the first antigen-binding domain and the second antigen binding domains includes one or more single domain antibody (sdAb) fragments, for example V_{HH} , V_{NAR} , engineered V_H or V_K domains. V_{HH} s can be generated from natural camelid heavy chain only antibodies, genetically modified rodents that produce heavy chain only antibodies, or naïve/synthetic camelid or humanized camelid single domain antibody libraries. V_{NAR} s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric V_H and V_K domains, including interface engineering and selection of specific germline families.

[0317] In some embodiments, the antigen binding domain or independently each of the antigen binding domains, such as the first antigen-binding domain and/or the second antigen binding domains, of the multispecific polypeptide constructs contains at least one sdAb or an scFv that binds a TAA. In some embodiments, the at least one scFv or sdAb that binds a TAA is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the multispecific polypeptide construct contains only one scFv or sdAb that binds to a TAA, which can be positioned either amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region. In some embodiments, the multispecific polypeptide construct contains two scFvs or sdAbs that bind to a TAA, positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region. In some embodiments, the multispecific polypeptide construct contains three scFv or sdAb, in which two are positioned amino-terminally relative to the Fc region or carboxy-terminally relative to the CD3 binding region, and the third is positioned at the other end of the multispecific polypeptide construct.

[0318] In some embodiments, the multispecific polypeptide construct is formed from or includes two polypeptides, including a first polypeptide comprising a first Fc polypeptide of a heterodimeric Fc region, a linker, a V_H domain of an anti-CD3 antibody or antigen binding fragment (e.g. Fv), and an scFv or sdAb that binds to a tumor-associated antigen; and a second polypeptide comprising a second Fc polypeptide of the heterodimeric Fc region, the linker, a V_L domain of the anti-CD3 antibody or antigen binding fragment (e.g. Fv) and,

optionally, the same or different scFv or sdAb that binds to a tumor-associated antigen. The scFv or sdAb that binds to a TAA can be positioned amino terminally relative to an Fc polypeptide of the heterodimeric Fc and/or carboxy-terminally relative to a VH or VL chain of the CD3 binding region. In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, of the multispecific polypeptide constructs contains VH and VL sequences assembled as FABs or scFvs. In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, of the multispecific polypeptide constructs contains binding domains as single domain antibodies (sdAbs).

[0319] In some embodiments, the antigen binding domain or independently each of the antigen binding domains, such as the first antigen-binding domain and the second antigen binding domains, contains more than one chain. In some embodiments, the antigen binding domain or independently each of the antigen binding domains, such as the first antigen-binding domain and/or the second antigen binding domains, of the multispecific polypeptide constructs contains VH and VL sequences assembled as FABs.

[0320] In some embodiments, the antigen binding domain or independently each of the antigen binding domains, such as the first antigen-binding domain and/or the second antigen binding domains, of the multispecific polypeptide constructs contains a VH-CH1 (Fd) and a VL-CL of a Fab antibody that binds a TAA. In some embodiments, the Fab antibody containing a VH-CH1 (Fd) and a VL-CL is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct. In some embodiments, the multispecific polypeptide construct contains only one Fab antibody, containing a VH-CH1 (Fd) and VL-CL, that binds to a TAA, which can be positioned either amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region. In some embodiments, the multispecific polypeptide construct contains two Fab antibody fragments, each containing a VH-CH1 (Fd) and VL-CL, that binds to a TAA, in which one is positioned amino-terminally relative to the Fc region and the other is positioned carboxy-terminally relative to the CD3 binding region.

[0321] In some embodiments, the multispecific polypeptide construct is formed from or includes three or more polypeptides, including a first polypeptide comprising a first Fc polypeptide of a heterodimeric Fc region, a linker and a VH-CH1 (Fd) or VL-CL of a Fab antibody fragment that binds to a tumor-associated antigen; a second polypeptide comprising a second Fc polypeptide of the heterodimeric Fc region, the linker and, optionally, the same VH-CH1 (Fd) or VL-CL of the Fab antibody fragment that binds to a tumor-associated

antigen, and a third polypeptide comprising the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment that binds to the TAA.

[0322] In some embodiments, the antigen binding domain, or independently each of the antigen binding domains, is or includes an extracellular domain or binding fragment thereof of the native cognate binding partner of the TAA, or a variant thereof that exhibits binding activity to the TAA.

[0323] In some embodiments, each of the antigen binding domains, such as each of the first antigen-binding domain and the second antigen binding domains, bind the same antigen. In some embodiments, each of the first antigen-binding domain and the second antigen binding domains bind a different antigen. In some embodiments, each of the antigen binding domains, such as each of the first antigen-binding domain and the second antigen binding domains, bind the same tumor associated antigen (TAA). In some embodiments, each of the antigen binding domains, such as each of the first antigen-binding domain and the second antigen binding domains, bind a different TAA. In some embodiments, each of the antigen binding domains, such as each of the first antigen-binding domain and the second antigen binding domains, bind a different epitope on the same TAA. In some embodiments, each of the antigen binding domains, such as each of the first antigen-binding domain and the second antigen binding domains, bind the same epitope on the same TAA.

[0324] In some embodiments, the antigen binding domains results in monovalent, bivalent, trivalent, or tetravalent binding to the TAA. In some embodiments, bivalent binding to the TAA comprises two antigen binding domains that bind the same epitope of the same antigen (e.g. mono-epitopic). In some embodiments, bivalent binding to the TAA comprises two antigen binding domains that bind different epitopes of the same antigen (e.g. bi-epitopic). In some embodiments, monovalent binding to the TAA comprises one antigen binding domain that binds one epitope of the antigen (e.g. mono-epitopic).

[0325] In some embodiments, the TAA is selected from the group consisting of 1-92-LFA-3, 5T4, Alpha-4 integrin, Alpha-V integrin, alpha4beta1 integrin, alpha4beta7 integrin, AGR2, Anti-Lewis-Y, Apelin J receptor, APRIL, B7-H3, B7-H4, BAFF, BTLA, C5 complement, C-242, CA9, CA19-9, (Lewis a), Carbonic anhydrase 9, CD2, CD3, CD6, CD9, CD11a, CD19, CD20, CD22, CD24, CD25, CD27, CD28, CD30, CD33, CD38, CD40, CD40L, CD41, CD44, CD44v6, CD47, CD51, CD52, CD56, CD64, CD70, CD71, CD74, CD80, CD81, CD86, CD95, CD117, CD123, CD125, CD132, (IL-2RG), CD133, CD137, CD138, CD166, CD172A, CD248, CDH6, CEACAM5 (CEA), CEACAM6 (NCA-90), CLAUDIN-3, CLAUDIN-4, cMet, Collagen, Cripto, CSFR, CSFR-1, CTLA-4, CTGF,

CXCL10, CXCL13, CXCR1, CXCR2, CXCR4, CYR61, DL44, DLK1, DLL3, DLL4, DPP-4, DSG1, EDA, EDB, EGFR, EGFRviii, Endothelin B receptor (ETBR), ENPP3, EpCAM, EPHA2, EPHB2, ERBB3, F protein of RSV, FAP, FGF-2, FGF8, FGFR1, FGFR2, FGFR3, FGFR4, FLT-3, Folate receptor alpha (FR α), GAL3ST1, G-CSF, G-CSFR, GD2, GITR, GLUT1, GLUT4, GM-CSF, GM-CSFR, GP IIb/IIIa receptors, Gp130, GPIIB/IIIA, GPNMB, GRP78, HER2/neu, HER3, HER4, HGF, hGH, HVEM, Hyaluronidase, ICOS, IFN α , IFN β , IFN γ , IgE, IgE Receptor (Fc ϵ RI), IGF, IGF1R, IL1B, IL1R, IL2, IL11, IL12, IL12p40, IL-12R, IL-12R β 1, IL13, IL13R, IL15, IL17, IL18, IL21, IL23, IL23R, IL27/IL27R (wsx1), IL29, IL-31R, IL31/IL31R, IL2R, IL4, IL4R, IL6, IL6R, Insulin Receptor, Jagged Ligands, Jagged 1, Jagged 2, KISS1-R, LAG-3, LIF-R, Lewis X, LIGHT, LRP4, LRRC26, Ly6G6D, LyPD1, MCSP, Mesothelin, MRP4, MUC1, Mucin-16 (MUC16, CA-125), Na/K ATPase, NGF, Nicastrin, Notch Receptors, Notch 1, Notch 2, Notch 3, Notch 4, NOV, OSM-R, OX-40, PAR2, PDGF-AA, PDGF-BB, PDGFR α , PDGFR β , PD-1, PD-L1, PD-L2, Phosphatidyl-serine, P1GF, PSCA, PSMA, PSGR, RAAG12, RAGE, SLC44A4, Sphingosine 1 Phosphate, STEAP1, STEAP2, TAG-72, TAPA1, TEM-8, TGF β , TIGIT, TIM-3, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TMEM31, TNF α , TNFR, TNFRS12A, TRAIL-R1, TRAIL-R2, Transferrin, Transferrin receptor, TRK-A, TRK-B, uPAR, VAP1, VCAM-1, VEGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, VISTA, WISP-1, WISP-2, and WISP-3.

[0326] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) folate receptor alpha (FR α). For example, the antigen binding domain contains the binding domain as an sdAb that binds FR α . Exemplary FR α -binding sdAbs are set forth in SEQ ID NOS: 120, 121, and 122.

[0327] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) cMET. For example, the antigen binding domain contains the binding domain as a sdAb that binds cMET. An exemplary cMET-binding sdAb is set forth in SEQ ID NO: 123 (U.S. Pat. No. 9,346,884).

[0328] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) B7H3. For example, the antigen binding domain contains the binding domain as an scFv that binds B7H3. An exemplary B7H3-binding scFv is set forth in SEQ ID NO: 124. In some

embodiments, the antigen binding domain is a sdAb, such as a VHH. Exemplary B7H3-binding sdAbs are set forth in any of SEQ ID NOS: 214-218. In some embodiments, the antigen binding domain is or contains a Fab antibody fragment comprising a VH-CH1 (Fd) and LC. An exemplary B7H3 Fd is set forth in SEQ ID NO: 127 and an exemplary B7H3 LC is set forth in SEQ ID NO: 128 (PCT Publication No, WO2017/030926).

[0329] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) CD20. In some embodiments, such an antigen-binding domain contains a VH set forth in SEQ ID NO: 189 and a VL set forth in SEQ ID NO: 190 or a sequence that exhibits at least at or about 85%, 90%, 95%, 96%, 97%, 98%, 98%, or 99% sequence identity to SEQ ID NO: 189 or SEQ ID NO:190. For example, the antigen binding domain contains the binding domain as an scFv that binds CD20. Exemplary CD20-binding scFvs are set forth in SEQ ID NO: 125 and 213 (U.S. Pub. No. US 2005/0123546).

[0330] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) DLL3. For example, the antigen binding domain contains the binding domain as an scFv that binds DLL3. Exemplary DLL3-binding scFv is set forth in SEQ ID NO: 126 and 188 (U.S. Pub. No. US 2017/0037130). In some embodiments, the antigen binding domain is a sdAb, such as a VHH. Exemplary DLL3-binding sdAbs are set forth in any of SEQ ID NO: 219 or SEQ ID NO:220. In some embodiments, the antigen binding domain is or contains a Fab antibody fragment comprising a Fd and LC that binds DLL3. An exemplary DLL3 Fd is set forth in SEQ ID NO: 133 and an exemplary DLL3 LC is set forth in SEQ ID NO: 134 (U.S. Pat. No. US 8,044,178).

[0331] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) 5T4. An exemplary 5T4 Fd is set forth in SEQ ID NO: 129 and an exemplary 5T4 LC is set forth in SEQ ID NO: 130. In some embodiments, the antibody binding domain comprises a VH-CH1 (Fd) or VL-CL as set forth in SEQ ID NOS: 167 and 168 (U.S. Pat. No. US 8,044,178).

[0332] In some embodiments, at least one antigen binding domain, or independently each antigen binding domain, binds the tumor associated antigen (TAA) gpNMB. In some embodiments, the antigen binding domain is or contains a Fab fragment comprising a Fd and LC chain. An exemplary gpNMB Fd is set forth in SEQ ID NO: 131 and an exemplary gpNMB LC is set forth in SEQ ID NO: 132.

[0333] In some embodiments, the antigen binding domain is linked, directly or indirectly via a linker, to the Fc region and/or to the CD3 binding region. In some embodiments, linkage is via a linker. In some embodiments, the linker is a linking peptide (LP), which can include any flexible or rigid linker as described in Section II.3, although generally peptides linking the antigen binding domain or domains is not a cleavable linker.

[0334] In some embodiments, the multispecific polypeptide construct comprises a first linking peptide (LP1) between the first antigen binding domain and the Fc region. In some embodiments, the multispecific polypeptide construct comprises a second linking peptide (LP2) between the CD3 binding region and the second antigen binding domain. In some embodiments, the multispecific polypeptide construct comprises a first linking peptide (LP1) between the first antigen binding domain and the Fc region and a second linking peptide (LP2) between the CD3 binding region and the second antigen binding domain. In some aspects, the multispecific polypeptide construct has the structural arrangement from N-terminus to C-terminus as follows: first antigen binding domain – LP1- Fc region – linker – CD3 binding region – LP2 – second antigen binding domain. In some embodiments, the two linking peptides are not identical to each other.

[0335] In some embodiments, the LP1 or LP2 is independently a peptide of about 1 to 20 amino acids in length. In some embodiments, the LP1 or LP2 is independently a peptide that is or comprises any Gly-Ser linker as set forth in SEQ ID NOs: 10-13, 119, 135, 147, 149 or GGS.

III. Pharmaceutical Composition

[0336] Provided herein are compositions of any of the provided multispecific polypeptide constructs. It will be appreciated that administration of therapeutic entities in accordance with the disclosure will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed., Mack Publishing Company, Easton, PA (1975)), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as Lipofectin™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in

treatments and therapies in accordance with the present disclosure, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. *See also* Baldrick P. “Pharmaceutical excipient development: the need for preclinical guidance.” *Regul. Toxicol Pharmacol.* 32(2):210-8 (2000), Wang W. “Lyophilization and development of solid protein pharmaceuticals.” *Int. J. Pharm.* 203(1-2):1-60 (2000), Charman WN “Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts.” *J Pharm Sci.* 89(8):967-78 (2000), Powell *et al.* “Compendium of excipients for parenteral formulations” *PDA J Pharm Sci Technol.* 52:238-311 (1998) and the citations therein for additional information related to formulations, excipients and carriers well known to pharmaceutical chemists.

[0337] In some embodiments, the multispecific polypeptide constructs, conjugated multispecific polypeptide constructs, and compositions thereof – referred to collectively herein as the Therapeutic(s) and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions suitable for administration. Principles and considerations involved in preparing such compositions, as well as guidance in the choice of components are provided, for example, in Remington’s *Pharmaceutical Sciences: The Science And Practice Of Pharmacy* 19th ed. (Alfonso R. Gennaro, et al., editors) Mack Pub. Co., Easton, Pa.: 1995; *Drug Absorption Enhancement: Concepts, Possibilities, Limitations, And Trends*, Harwood Academic Publishers, Langhorne, Pa., 1994; and *Peptide And Protein Drug Delivery (Advances In Parenteral Sciences, Vol. 4)*, 1991, M. Dekker, New York.

[0338] Such compositions typically comprise the multispecific polypeptide construct or a conjugated thereof and a pharmaceutically acceptable carrier. Where a multispecific polypeptide construct includes a fragment of an antibody, the smallest fragment of the antibody that specifically binds to the target protein can be used. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability of the antibody to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology. (*See, e.g.*, Marasco et al., *Proc. Natl. Acad. Sci. USA*, 90: 7889-7893 (1993)).

[0339] As used herein, the term “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington’s *Pharmaceutical Sciences*, a standard reference text in the field, which is incorporated herein by reference. Suitable examples of such carriers or diluents include, but are not limited to,

water, saline, ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated.

[0340] The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

[0341] A pharmaceutical composition of the disclosure is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (*i.e.*, topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0342] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by

various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be suitable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

[0343] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. Where the composition is lyophilized, sterilization using this method may be conducted either prior to or following lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

[0344] In some embodiments, the pharmaceutical composition is administered to a subject through any route, including orally, transdermally, by inhalation, intravenously, intra-arterially, intramuscularly, direct application to a wound site, application to a surgical site, intraperitoneally, by suppository, subcutaneously, intradermally, transcutaneously, by nebulization, intrapleurally, intraventricularly, intra-articularly, intraocularly, or intraspinally.

[0345] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or

gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0346] For administration by inhalation, the multispecific polypeptide construct are delivered in the form of an aerosol spray from pressured container or dispenser that contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

[0347] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[0348] The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[0349] In one embodiment, the Therapeutics are prepared with carriers that will protect the compound against rapid elimination from the body, such as sustained/controlled release formulations, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

[0350] For example, the Therapeutics can be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions.

[0351] In some embodiments, the pharmaceutical composition comprises a pharmaceutically-acceptable excipient, for example a filler, binder, coating, preservative, lubricant, flavoring agent, sweetening agent, coloring agent, a solvent, a buffering agent, a chelating agent, or stabilizer. Examples of pharmaceutically-acceptable fillers include cellulose, dibasic calcium phosphate, calcium carbonate, microcrystalline cellulose, sucrose,

lactose, glucose, mannitol, sorbitol, maltol, pregelatinized starch, corn starch, or potato starch. Examples of pharmaceutically-acceptable binders include polyvinylpyrrolidone, starch, lactose, xylitol, sorbitol, maltitol, gelatin, sucrose, polyethylene glycol, methyl cellulose, or cellulose. Examples of pharmaceutically-acceptable coatings include hydroxypropyl methylcellulose (HPMC), shellac, corn protein zein, or gelatin. Examples of pharmaceutically-acceptable disintegrants include polyvinylpyrrolidone, carboxymethyl cellulose, or sodium starch glycolate. Examples of pharmaceutically-acceptable lubricants include polyethylene glycol, magnesium stearate, or stearic acid. Examples of pharmaceutically-acceptable preservatives include methyl parabens, ethyl parabens, propyl paraben, benzoic acid, or sorbic acid. Examples of pharmaceutically-acceptable sweetening agents include sucrose, saccharine, aspartame, or sorbitol. Examples of pharmaceutically-acceptable buffering agents include carbonates, citrates, gluconates, acetates, phosphates, or tartrates.

[0352] Sustained-release preparations can be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. In some embodiments, the pharmaceutical composition further comprises an agent for the controlled or sustained release of the product, such as injectable microspheres, bio-erodible particles, polymeric compounds (polylactic acid, polyglycolic acid), beads, or liposomes. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOTTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

[0353] The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) and can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

[0354] It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as

used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the disclosure are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0355] Further provided are kits comprising the pharmaceutical compositions (or articles of manufacture) described herein. The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. The kits described herein may also include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein.

[0356] The formulation can also contain more than one multispecific polypeptide construct as necessary for the particular indication being treated, for example, those with complementary activities that do not adversely affect each other. In some embodiments, or in addition, the composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[0357] In some embodiments, the dosage of the pharmaceutical composition is a single dose or a repeated dose. In some embodiments, the doses are given to a subject once per day, twice per day, three times per day, or four or more times per day. In some embodiments, about 1 or more (such as about 2 or more, about 3 or more, about 4 or more, about 5 or more, about 6 or more, or about 7 or more) doses are given in a week. In some embodiments, multiple doses are given over the course of days, weeks, months, or years. In some embodiments, a course of treatment is about 1 or more doses (such as about 2 or more doses, about 3 or more doses, about 4 or more doses, about 5 or more doses, about 7 or more doses, about 10 or more doses, about 15 or more doses, about 25 or more doses, about 40 or more doses, about 50 or more doses, or about 100 or more doses).

[0358] In some embodiments, the pharmaceutical composition is administered to a subject. Generally, dosages and routes of administration of the pharmaceutical composition are determined according to the size and condition of the subject, according to standard pharmaceutical practice. For example, the therapeutically effective dose can be estimated

initially either in cell culture assays or in animal models such as mice, rats, rabbits, dogs, pigs, or monkeys. An animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. The exact dosage will be determined in light of factors related to the subject requiring treatment. Dosage and administration are adjusted to provide sufficient levels of the active compound or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, time and frequency of administration, drug combination(s), reaction sensitivities, and response to therapy. The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

IV. Methods of Use and Therapeutic Administration

[0359] Also provided are methods for using and uses of the multispecific polypeptide constructs. Such methods and uses include therapeutic methods and uses, for example, involving administration of the molecules or compositions containing the same, to a subject having a disease, condition, or disorder, such as a tumor or cancer. In some embodiments, the molecule and/or composition is administered in an effective amount to effect treatment of the disease or disorder. Uses include uses of the multispecific polypeptide constructs in such methods and treatments, and in the preparation of a medicament in order to carry out such therapeutic methods. In some embodiments, the methods are carried out by administering the multispecific polypeptide constructs, or compositions comprising the same, to the subject having or suspected of having the disease or condition. In some embodiments, the methods thereby treat the disease or condition or disorder in the subject.

[0360] In one embodiment, a multispecific polypeptide construct of the disclosure may be used as therapeutic agents. Such agents will generally be employed to diagnose, prognose, monitor, treat, alleviate, and/or prevent a disease or pathology in a subject. A therapeutic regimen is carried out by identifying a subject, *e.g.*, a human patient or other mammal suffering from (or at risk of developing) a disorder using standard methods. A multispecific polypeptide construct is administered to the subject. A multispecific polypeptide construct is administered to the subject and will generally have an effect due to its binding with the target(s).

[0361] In some embodiments, provided herein is a method of modulating an immune response in a subject by administering a therapeutically effective amount of any of the provided multispecific conjugates or pharmaceutical compositions. In some embodiments, the method of modulating an immune response increases or enhances an immune response in a subject. For example, the increase or enhanced response may be an increase in cell-mediated immunity. In some examples, the method increases T-cell activity, such as cytolytic T-cell (CTL) activity. In some embodiments, the modulated (e.g., increased) immune response is against a tumor or cancer.

[0362] Administration of the multispecific polypeptide construct may activate innate immune cells via engagement of Fc γ Rs through the Fc-region of the multispecific polypeptide construct. Administration of the multispecific polypeptide construct may agonize, stimulate, activate, and/or augment innate immune cell effector functions, including ADCC, cytokine release, degranulation and/or ADCP. In examples where a multispecific polypeptide construct contains a cleavable linker, administration of the multispecific polypeptide construct may activate T-cell once the linker(s) joining the first and second component is cleaved by a protease thereby allowing the anti-CD3 binding portion to bind CD3 ϵ on the T cells. Administration of the multispecific polypeptide construct may agonize, stimulate, activate, and/or augment CD3-mediated T cell activation, cytotoxicity, cytokine release and/or proliferation.

[0363] In some embodiments, the provided methods are for treating a disease or condition in a subject by administering a therapeutically effective amount of any of the provided multispecific conjugates or pharmaceutical compositions. In some embodiments, the disease or condition is a tumor or a cancer. Generally, alleviation or treatment of a disease or disorder involves the lessening of one or more symptoms or medical problems associated with the disease or disorder. For example, in the case of cancer, the therapeutically effective amount of the drug can accomplish one or a combination of the following: reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, to decrease to some extent and/or stop) cancer cell infiltration into peripheral organs; inhibit tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. In some embodiments, a composition of this disclosure can be used to prevent the onset or reoccurrence of the disease or disorder in a subject, *e.g.*, a human or other mammal, such as a non-human primate, companion animal (*e.g.*, cat, dog, horse),

farm animal, work animal, or zoo animal. The terms subject and patient are used interchangeably herein.

[0364] In some embodiments, the pharmaceutical composition can be used to inhibit growth of mammalian cancer cells (such as human cancer cells). A method of treating cancer can include administering an effective amount of any of the pharmaceutical compositions described herein to a subject with cancer. The effective amount of the pharmaceutical composition can be administered to inhibit, halt, or reverse progression of cancers. Human cancer cells can be treated *in vivo*, or *ex vivo*. In *ex vivo* treatment of a human patient, tissue or fluids containing cancer cells are treated outside the body and then the tissue or fluids are reintroduced back into the patient. In some embodiments, the cancer is treated in a human patient *in vivo* by administration of the therapeutic composition into the patient.

[0365] Non-limiting examples of disease include: all types of cancers (breast, lung, colorectal, prostate, melanomas, head and neck, pancreatic, etc.), rheumatoid arthritis, Crohn's disease, SLE, cardiovascular damage, ischemia, etc. For example, indications would include leukemias, including T-cell acute lymphoblastic leukemia (T-ALL), lymphoblastic diseases including multiple myeloma, and solid tumors, including lung, colorectal, prostate, pancreatic, and breast, including triple negative breast cancer. For example, indications include bone disease or metastasis in cancer, regardless of primary tumor origin; breast cancer, including by way of non-limiting example, ER/PR+ breast cancer, Her2+ breast cancer, triple-negative breast cancer; colorectal cancer; endometrial cancer; gastric cancer; glioblastoma; head and neck cancer, such as esophageal cancer; lung cancer, such as by way of non-limiting example, non-small cell lung cancer; multiple myeloma ovarian cancer; pancreatic cancer; prostate cancer; sarcoma, such as osteosarcoma; renal cancer, such as by way of nonlimiting example, renal cell carcinoma; and/or skin cancer, such as by way of nonlimiting example, squamous cell cancer, basal cell carcinoma, or melanoma. In some embodiments, the cancer is a squamous cell cancer. In some embodiments, the cancer is a skin squamous cell carcinoma. In some embodiments, the cancer is an esophageal squamous cell carcinoma. In some embodiments, the cancer is a head and neck squamous cell carcinoma. In some embodiments, the cancer is a lung squamous cell carcinoma.

[0366] A therapeutically effective amount of a multispecific polypeptide construct of the disclosure relates generally to the amount needed to achieve a therapeutic objective. As noted above, this may be a binding interaction between the multispecific polypeptide construct and its target antigen(s) that, in certain cases, agonize, stimulate, activate, and/or augment FcγR-mediated innate immune cell activation or CD3-mediated T cell activation.

The amount required to be administered will furthermore depend on the binding affinity of the multispecific polypeptide construct for its specific antigen(s), and will also depend on the rate at which an administered multispecific polypeptide construct is depleted from the free volume other subject to which it is administered. Common ranges for therapeutically effective dosing of a multispecific polypeptide construct may be, by way of nonlimiting example, from about 0.01 $\mu\text{g/kg}$ body weight to about 10 mg/kg body weight. In some embodiments, the therapeutically effective dosing of a multispecific polypeptide construct of the disclosure may be, by way of nonlimiting example, from about 0.01 mg/kg body weight to about 5-10 mg/kg body weight. Common dosing frequencies may range, for example, from twice daily to once a week.

[0367] Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular disorder. Methods for the screening of multispecific polypeptide construct that possess the desired specificity include, but are not limited to, enzyme linked immunosorbent assay (ELISA) and other immunologically mediated techniques known within the art. A variety of means are known for determining if administration of the provided multispecific polypeptide constructs sufficiently modulates immunological activity by eliminating, sequestering, or inactivating immune cells mediating or capable of mediating an undesired immune response; inducing, generating, or turning on immune cells that mediate or are capable of mediating a protective immune response; changing the physical or functional properties of immune cells; or a combination of these effects. Examples of measurements of the modulation of immunological activity include, but are not limited to, examination of the presence or absence of immune cell populations (using flow cytometry, immunohistochemistry, histology, electron microscopy, polymerase chain reaction (PCR)); measurement of the functional capacity of immune cells including ability or resistance to proliferate or divide in response to a signal (such as using T-cell proliferation assays and pepsin analysis based on ^3H -thymidine incorporation following stimulation with anti-CD3 antibody, anti-T-cell receptor antibody, anti-CD28 antibody, calcium ionophores, PMA (phorbol 12-myristate 13-acetate) antigen presenting cells loaded with a peptide or protein antigen; B cell proliferation assays); measurement of the ability to kill or lyse other cells (such as cytotoxic T cell assays); measurements of the cytokines, chemokines, cell surface molecules, antibodies and other products of the cells (e.g., by flow cytometry, enzyme-linked immunosorbent assays, Western blot analysis, protein microarray analysis, immunoprecipitation analysis); measurement of biochemical markers of activation of immune cells or signaling pathways within immune cells (e.g., Western blot and

immunoprecipitation analysis of tyrosine, serine or threonine phosphorylation, polypeptide cleavage, and formation or dissociation of protein complexes; protein array analysis; DNA transcriptional, profiling using DNA arrays or subtractive hybridization); measurements of cell death by apoptosis, necrosis, or other mechanisms (e.g., annexin V staining, TUNEL assays, gel electrophoresis to measure DNA laddering, histology; fluorogenic caspase assays, Western blot analysis of caspase substrates); measurement of the genes, proteins, and other molecules produced by immune cells (e.g., Northern blot analysis, polymerase chain reaction, DNA microarrays, protein microarrays, 2- dimensional gel electrophoresis, Western blot analysis, enzyme linked immunosorbent assays, flow cytometry); and measurement of clinical symptoms or outcomes such as improvement of autoimmune, neurodegenerative, and other diseases involving self-proteins or self-polypeptides (clinical scores, requirements for use of additional therapies, functional status, imaging studies) for example, by measuring relapse rate or disease severity.

[0368] The multispecific polypeptide construct are also useful in a variety of diagnostic and prophylactic formulations. In one embodiment, a multispecific polypeptide construct is administered to patients that are at risk of developing one or more of the aforementioned disorders. A patient's or organ's predisposition to one or more of the disorders can be determined using genotypic, serological or biochemical markers.

[0369] In another embodiment of the disclosure, a multispecific polypeptide construct is administered to human individuals diagnosed with a clinical indication associated with one or more of the aforementioned disorders. Upon diagnosis, a multispecific polypeptide construct is administered to mitigate or reverse the effects of the clinical indication.

Combination Therapies

[0370] In some embodiments, the multispecific polypeptide constructs, conjugated multispecific polypeptide constructs, and compositions thereof – referred to collectively herein as the Therapeutic(s) – are administered in conjunction with one or more additional agents, or a combination of additional agents. Suitable additional agents include current pharmaceutical and/or surgical therapies for an intended application. For example, the Therapeutic(s) can be used in conjunction with an additional chemotherapeutic or anti-neoplastic agent. For example, the Therapeutic(s) and additional agent are formulated into a single therapeutic composition, and the Therapeutic(s) and additional agent are administered simultaneously. In some embodiments, the Therapeutic(s) and additional agent are separate from each other, *e.g.*, each is formulated into a separate therapeutic composition, and the Therapeutic(s) and the additional agent are administered simultaneously, or the

Therapeutic(s) and the additional agent are administered at different times during a treatment regimen. For example, the Therapeutic(s) is administered prior to the administration of the additional agent, the Therapeutic(s) is administered subsequent to the administration of the additional agent, or the Therapeutic(s) and the additional agent are administered in an alternating fashion. As described herein, the Therapeutic(s) and additional agent are administered in single doses or in multiple doses. In some embodiments, the additional agent is coupled or otherwise attached to the Therapeutic(s). Suitable additional agents are selected according to the purpose of the intended application (*i.e.*, killing, prevention of cell proliferation, hormone therapy or gene therapy). Such agents may include but is not limited to, for example, pharmaceutical agents, toxins, fragments of toxins, alkylating agents, enzymes, antibiotics, antimetabolites, antiproliferative agents, hormones, neurotransmitters, DNA, RNA, siRNA, oligonucleotides, antisense RNA, aptamers, diagnostics, radiopaque dyes, radioactive isotopes, fluorogenic compounds, magnetic labels, nanoparticles, marker compounds, lectins, compounds that alter cell membrane permeability, photochemical compounds, small molecules, liposomes, micelles, gene therapy vectors, viral vectors, and the like. Finally, combinations of agents or combinations of different classes of agents may be used.

[0371] In one embodiment, the multispecific polypeptide constructs are administered in combination therapy, *i.e.*, combined with other agents, *e.g.*, therapeutic agents, that are useful for treating pathological conditions or disorders, such as autoimmune disorders and inflammatory diseases. The term “in combination” in this context means that the agents are given substantially contemporaneously, either simultaneously or sequentially. If given sequentially, at the onset of administration of the second compound, the first of the two compounds is still detectable at effective concentrations at the site of treatment.

[0372] For example, the combination therapy can include one or more multispecific polypeptide constructs of the disclosure co-formulated with, and/or co-administered with, one or more additional therapeutic agents, *e.g.*, one or more cytokine and growth factor inhibitors, immunosuppressants, anti-inflammatory agents, metabolic inhibitors, enzyme inhibitors, and/or cytotoxic or cytostatic agents, as described in more detail below. Furthermore, one or more multispecific polypeptide constructs described herein may be used in combination with two or more of the therapeutic agents described herein. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

[0373] In other embodiments, one or more multispecific polypeptide constructs of the disclosure can be co-formulated with, and/or co-administered with, one or more anti-inflammatory drugs, immunosuppressants, or metabolic or enzymatic inhibitors. Nonlimiting examples of the drugs or inhibitors that can be used in combination with the antibodies described herein, include, but are not limited to, one or more of: nonsteroidal anti-inflammatory drug(s) (NSAIDs), *e.g.*, ibuprofen, tenidap, naproxen, meloxicam, piroxicam, diclofenac, and indomethacin; sulfasalazine; corticosteroids such as prednisolone; cytokine suppressive anti-inflammatory drug(s) (CSAIDs); inhibitors of nucleotide biosynthesis, *e.g.*, inhibitors of purine biosynthesis, folate antagonists (*e.g.*, methotrexate (N-[4-[(2,4-diamino-6-pteridiny)methyl] methylamino] benzoyl]-L-glutamic acid); and inhibitors of pyrimidine biosynthesis, *e.g.*, dihydroorotate dehydrogenase (DHODH) inhibitors. Suitable therapeutic agents for use in combination with the antibodies of the disclosure include NSAIDs, CSAIDs, (DHODH) inhibitors (*e.g.*, leflunomide), and folate antagonists (*e.g.*, methotrexate).

[0374] Examples of additional inhibitors include one or more of: corticosteroids (oral, inhaled and local injection); immunosuppressants, *e.g.*, cyclosporin, tacrolimus (FK-506); and mTOR inhibitors, *e.g.*, sirolimus (rapamycin - RAPAMUNETM or rapamycin derivatives, *e.g.*, soluble rapamycin derivatives (*e.g.*, ester rapamycin derivatives, *e.g.*, CCI-779); agents that interfere with signaling by proinflammatory cytokines such as TNF α or IL-1 (*e.g.* IRAK, NIK, IKK, p38 or MAP kinase inhibitors); COX2 inhibitors, *e.g.*, celecoxib, rofecoxib, and variants thereof; phosphodiesterase inhibitors, *e.g.*, R973401 (phosphodiesterase Type IV inhibitor); phospholipase inhibitors, *e.g.*, inhibitors of cytosolic phospholipase 2 (cPLA2) (*e.g.*, trifluoromethyl ketone analogs); inhibitors of vascular endothelial cell growth factor or growth factor receptor, *e.g.*, VEGF inhibitor and/or VEGF-R inhibitor; and inhibitors of angiogenesis. Suitable therapeutic agents for use in combination with the antibodies of the disclosure are immunosuppressants, *e.g.*, cyclosporin, tacrolimus (FK-506); mTOR inhibitors, *e.g.*, sirolimus (rapamycin) or rapamycin derivatives, *e.g.*, soluble rapamycin derivatives (*e.g.*, ester rapamycin derivatives, *e.g.*, CCI-779); COX2 inhibitors, *e.g.*, celecoxib and variants thereof; and phospholipase inhibitors, *e.g.*, inhibitors of cytosolic phospholipase 2 (cPLA2), *e.g.*, trifluoromethyl ketone analogs. Additional examples of therapeutic agents that can be combined with a multispecific polypeptide construct include one or more of: 6-mercaptopurines (6-MP); azathioprine sulphasalazine; mesalazine; olsalazine; chloroquine/ hydroxychloroquine (PLAQUENIL[®]); pencillamine; aurothiornalate (intramuscular and oral); azathioprine; coichicine; beta-2 adrenoreceptor agonists (salbutamol, terbutaline, salmeteral); xanthines (theophylline, aminophylline); cromoglycate;

nedocromil; ketotifen; ipratropium and oxitropium; mycophenolate mofetil; adenosine agonists; antithrombotic agents; complement inhibitors; and adrenergic agents.

V. Exemplary Embodiments

[0375] Among the provided embodiments are:

1. A multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein:

the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and

one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA).

2. The multispecific polypeptide construct of embodiment 1, wherein the CD3-binding region binds CD3 (CD3ε).

3. The multispecific construct of embodiment 1 or embodiment 2, wherein the antigen binding domain is positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct.

4. The multispecific polypeptide construct of any of embodiments 1-3, wherein the first component comprises a first antigen binding domain and the second component comprises a second antigen binding domain, wherein each of the antigen binding domains bind a tumor associated antigen (TAA).

5. The multispecific polypeptide construct of embodiment 4, wherein the first antigen binding domain is positioned amino-terminally relative to the Fc region of the multispecific construct and the second antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific construct.

6. A multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

a first antigen binding domain that binds to a tumor-associated antigen (TAA);

an immunoglobulin Fc region;

a non-cleavable linker;

a CD3 binding region that binds CD3 (CD3ε); and

a second antigen binding domain that binds a tumor-associated antigen (TAA).

7. A multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

- an immunoglobulin Fc region;
- a non-cleavable linker;
- a CD3 binding region that binds CD3 (CD3 ϵ); and
- an antigen binding domain that binds a tumor-associated antigen (TAA).

8. A multispecific polypeptide construct, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

- an antigen binding domain that binds to a tumor-associated antigen (TAA);
- an immunoglobulin Fc region;
- a non-cleavable linker; and
- a CD3 binding region that binds CD3 (CD3 ϵ).

9. The multispecific polypeptide construct of any of embodiments 1-8, wherein the Fc region is a homodimeric Fc region.

10. The multispecific polypeptide construct of any of embodiments 1-9, wherein the Fc region is an Fc region of a human IgG1, a human IgG2, a human IgG3, or a human IgG4, or is an immunologically active fragment thereof.

11. The multispecific polypeptide construct of any of embodiments 1-10, wherein the Fc region comprises a polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 1 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:1.

12. The multispecific polypeptide construct of any of embodiments 1-10, wherein the Fc region comprises a polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:2;

the Fc region comprises a polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 4 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:4; or

the Fc region comprises a polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 5 or a sequence of amino acids that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:5.

13. The multispecific polypeptide construct of any of embodiments 1-6, 9 and 12, wherein the Fc region is a heterodimeric Fc region.

14. The multispecific polypeptide construct of embodiment 13, wherein one or both Fc polypeptides of the heterodimeric Fc region comprises at least one modification to induce heterodimerization compared to a polypeptide of a homodimeric Fc region, optionally compared to the Fc polypeptide set forth in SEQ ID NO:1 or an immunologically active fragment thereof.

15. The multispecific polypeptide construct of embodiment 14, wherein each of the Fc polypeptides of the heterodimeric Fc independently comprise at least one amino acid modification.

16. The multispecific polypeptide construct of embodiment 15, wherein each of the Fc polypeptides of the heterodimeric Fc comprise a knob-into-hole modification or comprise a charge mutation to increase electrostatic complementarity of the polypeptides.

17. The multispecific polypeptide construct of embodiment 16, wherein the amino acid modification is a knob-into-hole modification.

18. The multispecific polypeptide construct of any of embodiments 13-17, wherein the first Fc polypeptide of the heterodimeric Fc comprises the modification selected from among Thr366Ser, Leu368Ala, Tyr407Val, and combinations thereof and the second Fc polypeptide of the heterodimeric Fc comprises the modification T366W.

19. The multispecific polypeptide construct of embodiment 18, wherein the first and second Fc polypeptides further comprises a modification of a non-cysteine residue to a cysteine residue, wherein the modification of the first polypeptide is at one of the position Ser354 and Y349 and the modification of the second Fc polypeptide is at the other of the position Ser354 and Y349.

20. The multispecific polypeptide construct of embodiment 16, wherein the amino acid modification is a charge mutation to increase electrostatic complementarity of the polypeptides.

21. The multispecific polypeptide construct of any of embodiments 13-16 and 20, wherein the first and/or second Fc polypeptides or each of the first and second Fc polypeptide comprise a modification in complementary positions, wherein the modification is replacement with an amino acid having an opposite charge to the complementary amino acid of the other polypeptide.

22. The multispecific polypeptide construct of any of embodiments 14-21, wherein one of the first or second Fc polypeptide of the heterodimeric Fc further comprises a modification at residue Ile253.

23. The multispecific polypeptide construct of embodiment 22, wherein the modification is Ile253Arg.
24. The multispecific polypeptide construct of any of embodiments 14-23, wherein one of the first or second Fc polypeptide of the heterodimeric Fc further comprises a modification at residue His435.
25. The multispecific polypeptide construct of embodiment 24, wherein the modification is His435Arg.
26. The multispecific polypeptide construct of any of embodiments 1-25, wherein the Fc region comprises a polypeptide that lacks Lys447.
27. The multispecific polypeptide construct of any of embodiments 1-26, wherein the Fc region comprises a polypeptide comprising at least one modification to enhance FcRn binding.
28. The multispecific polypeptide construct of embodiment 27, wherein the modification is at a position selected from the group consisting of Met252, Ser254, Thr256, Met428, Asn434, and combinations thereof.
29. The multispecific polypeptide construct of embodiment 28, wherein the modification is at a position selected from the group consisting of Met252Y, Ser254T, Thr256E, Met428L, Met428V, Asn434S, and combinations thereof.
30. The multispecific polypeptide construct of embodiment 28, wherein the modification is at position Met252 and at position Met428.
31. The multispecific polypeptide construct of embodiment 30, wherein the modification is Met252Y and Met428L.
32. The multispecific polypeptide construct of embodiment 30, wherein the modification is Met252Y and Met428V.
33. The multispecific polypeptide construct of any of embodiments 13-32, wherein the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:82, 86, 94 or 96, and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:83, 87, 90, 92, 98 or 100.
34. The multispecific polypeptide construct of any of embodiments 1-33, wherein the Fc region comprises a polypeptide comprising at least one amino acid modification that reduces effector function and/or reduces binding to an effector molecule selected from an Fc gamma receptor or C1q.

35. The multispecific polypeptide construct of embodiment 34, wherein the one or more amino acid modification is deletion of one or more of Glu233, Leu234 or Leu235.

36. The multispecific polypeptide construct of any of embodiments 13-32, 34 and 35, wherein the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 84, 88, 95 or 97 and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 85, 89, 91, 93, 99 or 101.

37. The multispecific polypeptide construct of any of embodiments 1-32, wherein the Fc region comprises a polypeptide comprising at least one modification to enhance Fc γ R binding.

38. The multispecific polypeptide construct of embodiment 37, wherein the modification is modification at Ser239 or Ile332.

39. The multispecific polypeptide construct of any of embodiments 1-32 and 37, wherein the glycosylation of the Fc region is modified to enhance Fc α R binding as compared to an unmodified Fc region.

40. The multispecific polypeptide construct of embodiment 39, wherein the Fc region lacks or has reduced fucose content.

41. The multispecific polypeptide construct of any of embodiments 1-40, wherein the CD3 binding region is an anti-CD3 antibody or antigen-binding fragment.

42. The multispecific polypeptide construct of embodiment 41, wherein the anti-CD3 antibody or antigen binding fragment comprises a variable heavy chain region (VH) and a variable light chain region (VL) .

43. The multispecific polypeptide construct of any of embodiments 1-42, wherein the CD3 binding region is monovalent.

44. The multispecific polypeptide construct of any of embodiments 41-43, wherein the anti-CD3 antibody or antigen binding fragment is not a single chain antibody, optionally is not a single chain variable fragment (scFv).

45. The multispecific polypeptide construct of embodiment 42 or embodiment 44, wherein the Fc is a heterodimeric Fc and the VH and VL that comprise the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc.

46. The multispecific polypeptide construct of any of embodiments 1-45, wherein the CD3 binding region is not able to, or is not substantially able to, bind or engage CD3 unless at least one of the antigen binding domain is bound to its TAA.

47. The multispecific polypeptide construct of any of embodiments 1-46, wherein the CD3 binding region is not able to, or is not substantially able, to bind or engage CD3 unless at least two of the antigen binding domain is bound to its TAA.

48. The multispecific polypeptide construct of any of embodiments 1-47, wherein the linker is a polypeptide linker.

49. The multispecific polypeptide construct of embodiment 48, wherein the linker is a polypeptide of up to 25 amino acids in length.

50. The multispecific polypeptide construct of embodiment 48 or embodiment 49, wherein the linker is a polypeptide of from or from about 2 to 24 amino acids, 2 to 20 amino acids, 2 to 18 amino acids, 2 to 14 amino acids, 2 to 12 amino acids, 2 to 10 amino acids, 2 to 8 amino acids, 2 to 6 amino acids, 6 to 24 amino acids, 6 to 20 amino acids, 6 to 18 amino acids, 6 to 14 amino acids, 6 to 12 amino acids, 6 to 10 amino acids, 6 to 8 amino acids, 8 to 24 amino acids, 8 to 20 amino acids, 8 to 18 amino acids, 8 to 14 amino acids, 8 to 12 amino acids, 8 to 10 amino acids, 10 to 24 amino acids, 10 to 20 amino acids, 10 to 18 amino acids, 10 to 14 amino acids, 10 to 12 amino acids, 12 to 24 amino acids, 12 to 20 amino acids, 12 to 18 amino acids, 12 to 14 amino acids, 14 to 24 amino acids, 14 to 20 amino acids, 14 to 18 amino acids, 18 to 24 amino acids, 18 to 20 amino acids or 20 to 24 amino acids.

51. The multispecific polypeptide construct of any of embodiments 48-50, wherein the linker is a polypeptide that is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length.

52. The multispecific polypeptide construct of any of embodiments 48-51, wherein the linker is a polypeptide that is 3 to 18 amino acids in length.

53. The multispecific polypeptide construct of any of embodiments 48-51, wherein the linker is a polypeptide that is 12 to 18 amino acids in length.

54. The multispecific polypeptide construction of any of embodiments 48-51, wherein the linker is a polypeptide that is 15 to 18 amino acids in length.

55. A multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising a heterodimeric Fc region and a second component comprising an anti-CD3 antibody or antigen-binding fragment comprising a variable heavy chain region (VH) and a variable light chain region (VL), wherein:

the VH and VL that comprise the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc;

the first and second components are coupled by a non-cleavable linker, wherein the heterodimeric Fc region is positioned N-terminal to the anti-CD3 antibody; and

one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA).

56. The multispecific polypeptide construct of any of embodiments 52-55, wherein the non-cleavable linker does not contain a substrate recognition site that is specifically recognized for cleavage by a protease.

57. The multispecific polypeptide construct of embodiment 56, wherein the protease is produced by an immune effector cell, by a tumor, or by cells present in the tumor microenvironment.

58. The multispecific polypeptide construct of embodiment 57, wherein the protease is produced by an immune effector cell and the immune effector cell is an activated T cell, a natural killer (NK) cell, or an NK T cell.

59. The multispecific polypeptide construct of any of embodiments 56-58, wherein the protease is selected from among matriptase, a matrix metalloprotease (MMP), Granzyme B, and combinations thereof.

60. The multispecific polypeptide construct of embodiment 59, wherein the protease is granzyme B.

61. The multispecific polypeptide construct of any of embodiments 1-60, wherein the non-cleavable linker comprises GS, GGS, GGGGS (SEQ ID NO:149), GGGGGS (SEQ ID NO:135) and combinations thereof.

62. The multispecific polypeptide construct of any of embodiments 1-61, wherein the non-cleavable linker comprises (GGS)_n, wherein n is 1 to 10.

63. The multispecific polypeptide construct of any of embodiments 1-62, wherein the non-cleavable linker comprises (GGGGS)_n (SEQ ID NO: 173), wherein n is 1 to 10.

64. The multispecific polypeptide construct of any of embodiments 1-62, wherein the non-cleavable linker comprises (GGGGGS)_n (SEQ ID NO:172), wherein n is 1 to 4.

65. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GGS.

66. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GGGGS (SEQ ID NO: 149).

67. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GGGGGS (SEQ ID NO: 135).

68. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises (GGS)₂ (SEQ ID NO: 10).

69. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GSGGSGGS (SEQ ID NO: 11).

70. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GSGGSGGSGGS (SEQ ID NO: 12).

71. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GSGGSGGSGGSGGS (SEQ ID NO: 13).

72. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GGGGSGGGGSGGGGS (SEQ ID NO: 119).

73. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises GSGGGGSGGGGSGGGGS (SEQ ID NO: 147)

74. The multispecific polypeptide construct of any of embodiments 1-64, wherein the non-cleavable linker comprises and GGGGSGGGGSGGGGS (SEQ ID NO:170).

75. The multispecific polypeptide construct of any of embodiments 45-74, wherein the multispecific polypeptide construct comprises at least (i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment; and (ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment, wherein one or both of the first and second polypeptide comprise at least one antigen-binding domain that binds to a tumor associated antigen (TAA).

76. The multispecific polypeptide construct of any of embodiments 1-75, wherein one or more antigen binding domain that binds TAA results in monovalent, bivalent, trivalent, or tetravalent binding to the TAA.

77. The multispecific polypeptide construct of embodiment 75, wherein only one of the first or second polypeptide comprises the at least one antigen-binding domain that binds a TAA.

78. The multispecific polypeptide construct of embodiment 75 or embodiment 77, wherein the at least one antigen binding domain is positioned amino-terminally relative to the Fc region and/or is positioned carboxy-terminally relative to the CD3 binding region of one of the first or second polypeptide of the multispecific polypeptide construct.

79. The multispecific polypeptide construct of embodiment 75 or embodiment 77, wherein the at least one antigen binding domain is positioned amino-terminally relative to the Fc region of the multispecific construct and the second antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific construct.

80. The multispecific polypeptide construct of any of embodiments 1-79, wherein the antigen binding domain, or independently each of the antigen binding domains, comprises an extracellular domain or binding fragment thereof of the native cognate binding partner of the TAA, or a variant thereof that exhibits binding activity to the TAA.

81. The multispecific polypeptide construct of any of embodiments 1-79, wherein the antigen binding domain, or independently each of the antigen binding domains, is an antibody or antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody.

82. The multispecific polypeptide construct of embodiment 81, wherein the antibody or antigen-binding fragment thereof is a Fv, a scFv, a Fab, a single domain antibody (sdAb), a VNAR, or a VHH.

83. The multispecific polypeptide construct of embodiment 81 or embodiment 82, wherein the antibody or antigen-binding fragment is an sdAb.

84. The multispecific polypeptide construct of embodiment 83, wherein the sdAb is a human or humanized sdAb.

85. The multispecific polypeptide construct of embodiment 83 or embodiment 84, wherein the sdAb is VHH, VNAR, an engineered VH domain or an engineered VK domain.

86. The multispecific polypeptide construct of embodiment 81 or embodiment 82, wherein the antibody or antigen-binding fragment thereof is an scFv.

87. The multispecific polypeptide construct of embodiment 81 or embodiment 82, wherein the antibody or antigen-binding fragment thereof is a Fab.

88. The multispecific polypeptide construct of embodiment 87, wherein the multispecific polypeptide construct comprises:

(i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment;

(ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment, and (iii) a third polypeptide comprising a VH-CH1 (Fd) or VL-CL of a Fab antibody fragment that binds to a tumor-associated antigen, wherein the first and/or second polypeptide further comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment.

89. The multispecific polypeptide construct of embodiment 88, wherein only one of the first or second polypeptide comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment.

90. The multispecific polypeptide construct of embodiment 89, wherein both the first or second polypeptide comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment.

91. The multispecific polypeptide construct of embodiment 89 or embodiment 90, wherein the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment is positioned amino-terminally relative to the Fc region and/or at the carboxy-terminally relative to the CD3 binding region of one of the first or second polypeptide of the multispecific polypeptide construct.

92. The multispecific polypeptide construct of any of embodiments 89-91, wherein the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment is positioned amino-terminally relative to the Fc region of the first polypeptide or second polypeptide and at the carboxy-terminally relative to the CD3 binding region of the other of the first or second polypeptide.

93. The multispecific polypeptide construct of any of embodiments 1-92, wherein the antigen binding domain, or independently each of the antigen binding domains, binds to a tumor antigen selected from among 1-92-LFA-3, 5T4, Alpha-4 integrin, Alpha-V integrin, alpha4beta1 integrin, alpha4beta7 integrin, AGR2, Anti-Lewis-Y, Apelin J receptor, APRIL, B7-H3, B7-H4, BAFF, BTLA, C5 complement, C-242, CA9, CA19-9, (Lewis a), Carbonic anhydrase 9, CD2, CD3, CD6, CD9, CD11a, CD19, CD20, CD22, CD24, CD25, CD27, CD28, CD30, CD33, CD38, CD40, CD40L, CD41, CD44, CD44v6, CD47, CD51, CD52, CD56, CD64, CD70, CD71, CD74, CD80, CD81, CD86, CD95, CD117, CD123, CD125, CD132, (IL-2RG), CD133, CD137, CD138, CD166, CD172A, CD248, CDH6, CEACAM5 (CEA), CEACAM6 (NCA-90), CLAUDIN-3, CLAUDIN-4, cMet, Collagen, Cripto, CSFR, CSFR-1, CTLA-4, CTGF, CXCL10, CXCL13, CXCR1, CXCR2, CXCR4, CYR61, DL44, DLK1, DLL3, DLL4, DPP-4, DSG1, EDA, EDB, EGFR, EGFRviii, Endothelin B receptor (ETBR), ENPP3, EpCAM, EPHA2, EPHB2, ERBB3, F protein of RSV, FAP, FGF-2, FGF8, FGFR1, FGFR2, FGFR3, FGFR4, FLT-3, Folate receptor alpha (FR α), GAL3ST1, G-CSF, G-CSFR, GD2, GITR, GLUT1, GLUT4, GM-CSF, GM-CSFR, GP IIb/IIIa receptors, Gp130, GPIIB/IIIA, GPNMB, GRP78, HER2/neu, HER3, HER4, HGF, hGH, HVEM, Hyaluronidase, ICOS, IFN α , IFN β , IFN γ , IgE, IgE Receptor (FceRI), IGF,

IGF1R, IL1B, IL1R, IL2, IL11, IL12, IL12p40, IL-12R, IL-12Rbeta1, IL13, IL13R, IL15, IL17, IL18, IL21, IL23, IL23R, IL27/IL27R (wsx1), IL29, IL-31R, IL31/IL31R, IL2R, IL4, IL4R, IL6, IL6R, Insulin Receptor, Jagged Ligands, Jagged 1, Jagged 2, KISS1-R, LAG-3, LIF-R, Lewis X, LIGHT, LRP4, LRRC26, Ly6G6D, LyPD1, MCSP, Mesothelin, MRP4, MUC1, Mucin-16 (MUC16, CA-125), Na/K ATPase, NGF, Nicastrin, Notch Receptors, Notch 1, Notch 2, Notch 3, Notch 4, NOV, OSM-R, OX-40, PAR2, PDGF-AA, PDGF-BB, PDGFRalpha, PDGFRbeta, PD-1, PD-L1, PD-L2, Phosphatidyl-serine, P1GF, PSCA, PSMA, PSGR, RAAG12, RAGE, SLC44A4, Sphingosine 1 Phosphate, STEAP1, STEAP2, TAG-72, TAPA1, TEM-8, TGFbeta, TIGIT, TIM-3, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TMEM31, TNFalpha, TNFR, TNFRS12A, TRAIL-R1, TRAIL-R2, Transferrin, Transferrin receptor, TRK-A, TRK-B, uPAR, VAP1, VCAM-1, VEGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, VISTA, WISP-1, WISP-2, and WISP-3.

94. The multispecific polypeptide construct of any of embodiments 1-93, wherein multispecific antigen binding domain comprises at least a first antigen binding domain and a second antigen binding domain, wherein the first antigen binding domain and second antigen binding domain bind to the same TAA.

95. The multispecific polypeptide construct of embodiment 94, wherein the first antigen binding domain and the second antigen binding domain binds a different epitope of the same TAA.

96. The multispecific polypeptide construct of embodiment 94, wherein the first antigen binding domain and the second antigen binding domain binds the same epitope of the same TAA.

97. The multispecific polypeptide construct of any of embodiments 1-96, wherein multispecific antigen binding domain comprises at least a first antigen binding domain and a second antigen binding domain wherein the first antigen binding domain and the second antigen binding domain bind a different TAA.

98. The multispecific polypeptide construct of any of embodiments 5-97, wherein the multispecific polypeptide construct comprises a first linking peptide (LP1) between the first antigen binding domain and the Fc region.

99. The multispecific polypeptide construct of any of embodiments 5-98, wherein the multispecific polypeptide construct comprises a second linking peptide (LP2) between the CD3 binding region and the second antigen binding domain.

100. The multispecific polypeptide construct of any of embodiments 5-99, wherein the multispecific polypeptide construct comprises a first linking peptide (LP1) between the

first antigen binding domain and the Fc region and a second linking peptide (LP2) between the CD3 binding region and the second antigen binding domain, and wherein the multispecific polypeptide construct has the structural arrangement from N-terminus to C-terminus as follows: first antigen binding domain – LP1- Fc region –non-cleavable linker – CD3 binding region – LP2 – second antigen binding domain.

101. The multispecific polypeptide construct of embodiment 100, wherein the two linking peptides are identical to each other.

102. The multispecific polypeptide construct of embodiment 100, wherein the two linking peptides are not identical to each other.

103. The multispecific polypeptide construct of any of embodiments 98-102, wherein LP1 or LP2 is independently a peptide of about 1 to 20 amino acids in length.

104. The multispecific polypeptide of embodiment 103, wherein LP1 or LP2 independently comprise a peptide that is or comprises any Gly-Ser linker as set forth in SEQ ID NOs: 10-13, 119, 135, 147, 149 or GGS.

105. The multispecific polypeptide construct of any of embodiments 41-104, wherein the anti-CD3 antibody or antigen binding fragment is an Fv antibody fragment.

106. The multispecific polypeptide construct of embodiment 105, wherein the Fv antibody fragment comprises a disulfide stabilized anti-CD3 binding Fv fragment (dsFv).

107. The multispecific polypeptide construct of any of embodiments 41-106, wherein:

the anti-CD3 antibody or antigen-binding fragment comprises a VH CDR1 comprising the amino acid sequence TYAMN (SEQ ID NO: 16) or SEQ ID NO: 211; a VH CDR2 comprising the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17) or SEQ ID NO: 212; a VH CDR3 comprising the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 comprising the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 comprising the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 comprising the amino acid sequence ALWYSNLWV (SEQ ID NO: 21); or

the anti-CD3 antibody or antigen-binding fragment comprises a VH CDR1 sequence that includes at least the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that includes at least the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that

includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNHWV (SEQ ID NO: 225).

108. The multispecific polypeptide construct of embodiment 106 or embodiment 107, wherein the anti-CD3 dsFv comprises:

a VH having the amino acid sequence of any of SEQ ID NOS: 14 and 32-62 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 14 and 32-62; and

a VL having the amino acid sequence of any of SEQ ID NOS: 15 and 63-81 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 14 and 32-62.

109. The multispecific polypeptide construct of any of embodiments 106-108, wherein the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 14 and the amino acid sequence of SEQ ID NO: 15.

110. The multispecific polypeptide construct of any of embodiments 102-104, wherein the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 44 and the amino acid sequence of SEQ ID NO: 72.

111. The multispecific polypeptide construct of any of embodiments 1-109, wherein the multispecific polypeptide construct is conjugated to an agent.

112. The multispecific polypeptide construct of embodiment 111, wherein the agent is a therapeutic agent, an antineoplastic agent, a toxin or fragment thereof, a detectable moiety or a diagnostic agent.

113. The multispecific polypeptide construct of embodiment 112, wherein the agent is conjugated to the multispecific polypeptide construct via a linker.

114. A polynucleotide(s) encoding the multispecific polypeptide constructs of any of embodiments 1-113.

115. A polynucleotide encoding a polypeptide chain of any of the multispecific polypeptide constructs of any of embodiments 1-113.

116. A polynucleotide, comprising a first nucleic acid sequence encoding a first polypeptide of a multispecific construct of any of embodiments 1-115 and a second nucleic acid sequence encoding a second polypeptide of the multispecific construct, wherein the first and second nucleic acid sequence are separated by an internal ribosome entry site (IRES), or a nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping.

117. The polynucleotide of embodiment 116, wherein the first nucleic acid sequence and second nucleic acid sequence are operably linked to the same promoter.

118. The polynucleotide of embodiment 116 or embodiment 117, wherein the multispecific polypeptide construct comprises a third polypeptide chain, and the polynucleotide further comprises a third nucleic acid encoding the third polypeptide of the multispecific construct.

119. The polynucleotide of embodiment 118, wherein the third nucleic acid is separated from the first and/or second polypeptide by an internal ribosome entry site (IRES), or a nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping and/or the third nucleic acid sequence is operably linked to the same promoter as the first and/or second nucleic acid sequence.

120. The polynucleotide of any of embodiments 116-119, wherein the nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping is selected from a T2A, a P2A, a E2A or a F2A.

121. A vector, comprising the polynucleotide of any of embodiments 114-120.

122. The vector of embodiment 121 that is an expression vector.

123. The vector of embodiment 121 or 122 that is a viral vector or a eukaryotic vector, optionally wherein the eukaryotic vector is a mammalian vector.

124. A cell, comprising polynucleotide or polynucleotides of any of embodiments 114-120, or a vector or vectors of any of embodiments 121-123.

125. The cell of embodiment 124, wherein the cell is recombinant or isolated.

126. The cell of embodiment 125, wherein the cell is a mammalian cell.

127. The cell of embodiment 126, wherein the cell is a HEK293 or CHO cell.

128. A method of producing a multispecific polypeptide construct, the method comprising introducing into a cell a polynucleotide or polynucleotides of any of embodiments 114-120 or a vector or vectors of any of embodiments 121-123 and culturing the cell under conditions to produce the multispecific polypeptide construct.

129. A method of producing a multispecific polypeptide construct, the method comprising culturing the cell of any of embodiments 124-127 under conditions in which the multispecific polypeptide is produced by the cell.

130. The cell of embodiment 128 or 129, wherein the cell is a mammalian cell.

131. The cell of embodiment 130, wherein the cell is a HEK293 or CHO cell.

132. The method of embodiment 128 or embodiment 129, further comprising isolating or purifying the multispecific polypeptide construct from the cell.

133. The method of any of embodiments 128-132, wherein the multispecific polypeptide construct is a heterodimer.

134. A multispecific polypeptide construct produced by the method of any of embodiments 128-133.

135. A pharmaceutical composition comprising the multispecific polypeptide construct of any of embodiments 1-113 or embodiment 134 and a pharmaceutically acceptable carrier.

136. The pharmaceutical composition of embodiment 135 that is sterile.

137. A method of stimulating or inducing an immune response, the method comprising contacting a target cell and a T cell with the multispecific polypeptide construct of any of embodiments 1-113 or embodiment 134 or the pharmaceutical composition of embodiments 109 or embodiment 110, said target cell expressing a tumor associated antigen recognized by the multispecific polypeptide construct.

138. The method of embodiment 137, wherein the target cell is a tumor cell expressing the tumor associated antigen (TAA).

139. The method of embodiment 137 or embodiment 138, wherein the contacting is carried out ex vivo or in vitro.

140. The method of any of embodiments 137-149, wherein the contacting is carried out in vivo in a subject.

141. A method of stimulating or inducing an immune response in a subject, the method comprising administering, to a subject in need thereof, a therapeutically effective amount of the multispecific conjugate of any of embodiments 1-113 or embodiment 134 or the pharmaceutical composition of embodiments 109 or embodiment 110.

142. The method of any of embodiments 137-141, which increases cell-mediated immunity.

143. The method of any of embodiments 137-142, which increases T-cell activity.

144. The method of any of embodiments 137-143, which increases cytolytic T-cell (CTL) activity.

145. The method of any of embodiments 137-144, wherein the immune response is increased against a tumor or cancer.

146. The method of any of embodiments 137-145, wherein the method treats a disease or condition in the subject.

147. A method of treating a disease or condition in a subject, the method comprising administering, to a subject in need thereof, a therapeutically effective amount of the multispecific conjugate of any of embodiments 1-113 or the pharmaceutical composition of embodiments 135 or embodiment 136.

148. The method of embodiment 146 or embodiment 147, wherein the disease or condition is a tumor or a cancer.

149. The method of any of embodiments 140-148, wherein said subject is a human.

VI. EXAMPLES

[0376] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1: Method of Producing Multispecific Constructs With Constrained CD3 Binding

[0377] Example 1 describes the generation and expression of multispecific polypeptide constructs containing a CD3 binding region that exhibits constrained CD3 binding. The multispecific constructs were generated in various configurations, as shown in **FIG. 1, FIGS. 2A-2B, FIGS. 3A-3C and FIGS. 4A-4B**, to contain a heterodimeric Fc region of an immunoglobulin coupled by a linker (e.g. a non-cleavable linker) to the CD3 binding region, and one or more antigen binding domains that binds a tumor associated antigen (TAA) positioned amino-terminally relative to the Fc region and/or carboxy-terminally relative to the CD3 binding region of the multispecific polypeptide construct.

A. Design and Generation of Constructs

[0378] Polynucleotides encoding at least a first polypeptide chain and a second polypeptide chain of the heterodimeric multispecific polypeptide construct were generated and cloned into a plasmid for expression. The first polypeptide chain generally included in order, from the N-terminus to C-terminus, a first Fc polypeptide (e.g. an Fc hole polypeptide); a non-cleavable linker; and a variable light (VL) domain of an anti-CD3 antibody. The second polypeptide chain generally included in order, from the N-terminus to C-terminus, a second Fc polypeptide (e.g. an Fc knob polypeptide); the same non-cleavable linker as the first polypeptide chain; and a variable heavy (VH) domain of an anti-CD3 antibody. The anti-CD3 antibody included either a disulfide- stabilized (dsFv) antibody (anti-CD3 VH with the mutation G44C and VL with the mutation G100C) or contained a non-disulfide stabilized Fv antibody, as set forth in Tables E1.1 and E1.2. Various exemplary Fc polypeptide pairs to facilitate heterodimerization of the polypeptide chains were used as set forth in Tables E1.1 and Table E1.2. One or both of the polypeptide chains additionally encoded one or more TAA antigen binding domain amino-terminal to the Fc domain and/or carboxy-terminal to the CD3 binding region, in various configurations.

Similar constructs can be generated using other heterodimeric Fc configurations, including other knob-into-hole configurations, such as any as described; other CD3-binding regions, including other anti-CD3 antibodies, including dsFv or other monovalent fragments; or other TAA antigen-binding fragments, such as scFv, sdAb or Fab formats can also be used.

[0379] Among generated constructs, the non-cleavable linker included linkers ranging from 3-18 amino acids in size. Examples of non-cleavable linkers used in exemplary generated molecules were GGS (e.g. contained in exemplary construct cx1356), GGSGGS (SEQ ID NO:10, contained in exemplary construct cx1357), GGSGGSGGS (SEQ ID NO:11, contained in exemplary construct cx1358), GGSGGSGGSGGS (SEQ ID NO:12, contained in exemplary construct cx1359), GGSGGSGGSGGSGGS (SEQ ID NO:13, contained in exemplary construct cx1360), and GGGGGSGGGGGSGGGGGS (SEQ ID NO:119, contained in exemplary construct cx5823 and cx5952) or GGSGGGGSGGGGSGGGGGS (SEQ ID NO: 147, contained in exemplary construct cx681).

[0380] Any antigen binding domain that binds to a TAA can be employed in the provided multispecific polypeptide constructs. Exemplary generated proteins contained an antigen binding domain that binds Folate Receptor Alpha (FR α), B7H3 (CD276), or Delta-like 3 (DLL3). The antigen-binding domain can include single chain fragments (e.g. sdAb or scFv) or two chain antigen-binding fragments (Fabs). When the TAA was provided as a single chain fragment, e.g. sdAb or scFv, the TAA antigen binding domain was linked at the N-terminus to one or both polypeptide chains of the Fc heterodimer (e.g. hole and/or knob) by a peptide linker, e.g. PGGGG (SEQ ID NO:102) and/or was linked at the C-terminus to one or both domains (e.g. VH and/or VL) of the CD3 binding region by a peptide linker, e.g. GGGG (SEQ ID NO:103). Other similar peptide linkers can be employed. When the TAA was provided as a Fab antigen-binding fragment the construct was composed of a VH and CH1 linked directly to one or both Fc polypeptides without a linker, as well as a light chain composed of a VL and CL. These TAA binding Fabs can be located on the amino- or carboxy-terminus of the heterodimeric Fc.

[0381] Multispecific polypeptide constructs were generated containing 1, 2, 3 or 4 TAA antigen binding domain, such as to provide for monovalent, bivalent, trivalent, or tetravalent binding, respectively. In some cases, the TAA antigen binding domains were the same (mono-epitopic). In some cases, the TAA antigen binding domains were different, such that the generated multispecific polypeptide constructs exhibited specificity for at least two different TAAs, to different epitopes of the same TAA (bi-epitopic) or the same epitopes of the same TAA (mono-epitopic).

[0382] Among the generated proteins were constructs in which the TAA antigen binding domains were composed as single domain antibodies (sdAbs). Polynucleotides were generated to encode polypeptide chains of exemplary multispecific polypeptide constructs containing non-cleavable linkers. These included constructs designated cx1356, cx1357, cx1358, cx1359, cx1360, and cx681, targeting FR α as depicted in **FIG. 2B**; cx3072, cx5952, cx6079, cx6080, cx6081, cx5823, cx5873, and cx5965, targeting B7H3 as depicted in **FIGS. 3A** and **3B**; and cx5352, cx5499, cx5800, and cx5801 targeting DLL3 as depicted in **FIGS. 4A** and **4B**. Notably, some constructs were generated wherein the VH domain of the dsFv anti-CD3 antibody and the sdAb were both linked to the same side (e.g. hole or knob side) of the Fc heterodimer (e.g. cx3072 and cx5952, shown in **FIG. 3A**). Constructs were engineered without a disulfide stabilized Fv or were engineered with a disulfide linkage stabilizing the VH and VL domains of the anti-CD3 antibody. Some of the exemplary constructs generated additionally contained a sdAb (containing a CDR1, a CDR2 and a CDR3 set forth in SEQ ID Nos: 221, 222 and 223, respectively) targeting 4-1BB co-stimulatory receptor (e.g. cx5823, cx5873, cx5965, cx5352, cx5801, cx5800). A list of exemplary constrained CD3 binding constructs having sdAb TAA domains is given below in **Table E1.1**.

Table E1.1: Exemplary Constrained CD3 engaging constructs

Construct ID	Chain	N-term sdAb (Target)	Fc	Linker	CD3 Binding Domain	C-term sdAb (Target)	Disulfide Stabilized
cx681	1	FR α sdAb1 (SEQ ID NO:120)	IgG1-Knob (SEQ ID NO:82, 86 or 201)	GGSGGGSGGGGS GGGGS (SEQ ID NO:147)	VH34 (SEQ ID NO: 198)	FR α sdAb2 (SEQ ID NO:121)	yes
	2	FR α sdAb1 (SEQ ID NO:120)	IgG1-Hole (SEQ ID NO:83, 87 or 202)	GGSGGGSGGGGS GGGGS (SEQ ID NO:147)	VL21 (SEQ ID NO: 200)	FR α sdAb2 (SEQ ID NO:121)	
cx1356	1	FR α sdAb1 (SEQ ID NO:120)	IgG1-Knob (SEQ ID NO:82, 86 or 201)	GGG	VH34 (SEQ ID NO: 198)	FR α sdAb2 (SEQ ID NO:121)	yes
	2	FR α sdAb1 (SEQ ID NO:120)	IgG1-Hole (SEQ ID NO:83, 87 or 202)	GGG	VL21 (SEQ ID NO: 200)	FR α sdAb2 (SEQ ID NO:121)	
cx1357	1	FR α sdAb1 (SEQ ID NO:120)	IgG1-Knob (SEQ ID NO:82, 86 or 201)	GGSGGS (SEQ ID NO:10)	VH34 (SEQ ID NO: 198)	FR α sdAb2 (SEQ ID NO:121)	yes
	2	FR α sdAb1 (SEQ ID NO:120)	IgG1-Hole (SEQ ID NO:83, 87 or 202)	GGSGGS (SEQ ID NO:10)	VL21 (SEQ ID NO: 200)	FR α sdAb2 (SEQ ID NO:121)	
cx1358	1	FR α sdAb1	IgG1-Knob (SEQ ID NO:11)	GGSGGSGGS (SEQ ID NO:11)	VH34 (SEQ ID NO: 198)	FR α sdAb2 (SEQ ID NO:121)	yes

Table E1.1: Exemplary Constrained CD3 engaging constructs

Construct ID	Chain	N-term sdAb (Target)	Fc	Linker	CD3 Binding Domain	C-term sdAb (Target)	Disulfide Stabilized
		(SEQ ID NO:120)	NO:82, 86 or 201)			NO:121)	
	2	FR α sdAb1 (SEQ ID NO:120)	IgG1-Hole (SEQ ID NO:83, 87 or 202)	GGSGGSGGS (SEQ ID NO:11)	VL21 (SEQ ID NO: 200)	FR α sdAb2 (SEQ ID NO:121)	
cx1359	1	FR α sdAb1 (SEQ ID NO:120)	IgG1-Knob (SEQ ID NO:82, 86 or 201)	GGSGGSGGSGGS(S EQ ID NO:12)	VH34 (SEQ ID NO: 198)	FR α sdAb2 (SEQ ID NO:121)	yes
	2	FR α sdAb1 (SEQ ID NO:120)	IgG1-Hole (SEQ ID NO:83, 87 or 202)	GGSGGSGGSGGS (SEQ ID NO:12)	VL21 (SEQ ID NO: 200)	FR α sdAb2 (SEQ ID NO:121)	
cx1360	1	FR α sdAb1 (SEQ ID NO:120)	IgG1-Knob (SEQ ID NO:82, 86 or 201)	GGSGGSGGSGGSG GS (SEQ ID NO:13)	VH34 (SEQ ID NO: 198)	FR α sdAb2 (SEQ ID NO:121)	yes
	2	FR α sdAb1 (SEQ ID NO:120)	IgG1-Hole (SEQ ID NO:83, 87 or 202)	GGSGGSGGSGGSG GS (SEQ ID NO:13)	VL21 (SEQ ID NO: 200)	FR α sdAb2 (SEQ ID NO:121)	
cx5823	1	B7H3 sdAb 4 (SEQ ID NO: 214)	xELL-Knob (SEQ ID NO:88)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	B7H3 sdAb 5 (SEQ ID NO:216)	yes
	2	None	xELL-Hole (SEQ ID NO:93)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5952	1	B7H3 sdAb 4 (SEQ ID NO:214)	xELL-Knob (SEQ ID NO:88)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	B7H3 sdAb 5 (SEQ ID NO: 216)	yes
	2	None	xELL-Hole (SEQ ID NO:93)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	None	
cx6079	1	B7H3 sdAb 4 (SEQ ID NO:214)	Fc-Het-1 (SEQ ID NO:194)	GGGGGSGGGGSGGG GS (SEQ ID NO:170)	VH32 (SEQ ID NO: 196)	None	no
	2	B7H3 sdAb 4 (SEQ ID NO:214)	Fc-Het-2 (SEQ ID NO:195)	GGGGGSGGGGSGGG GS (SEQ ID NO:170)	VL20 (SEQ ID NO: 199)	None	
cx6080	1	B7H3 sdAb 4 (SEQ ID NO:214)	Fc-Het-1 (SEQ ID NO:194)	GGGGGSGGGGSGGG GS (SEQ ID NO:170)	VH33 (SEQ ID NO: 197)	None	yes
	2	B7H3 sdAb 4 (SEQ ID NO: 214)	Fc-Het-2 (SEQ ID NO:195)	GGGGGSGGGGSGGG GS (SEQ ID NO:170)	VL21 (SEQ ID NO: 200)	None	
cx6081	1	B7H3 sdAb 4 (SEQ ID NO:214)	Fc-Het-1 (SEQ ID NO:194)	GGGGGSGGGGSGGG GS (SEQ ID NO:170)	VH13 (SEQ ID NO: 44)	None	yes
	2	B7H3 sdAb 4 (SEQ ID NO:214)	Fc-Het-2 (SEQ ID NO:195)	GGGGGSGGGGSGGG GS (SEQ ID NO:170)	VL10 (SEQ ID NO: 72)	None	

Table E1.1: Exemplary Constrained CD3 engaging constructs

Construct ID	Chain	N-term sdAb (Target)	Fc	Linker	CD3 Binding Domain	C-term sdAb (Target)	Disulfide Stabilized
cx5841	1	B7H3 sdAb 4 (SEQ ID NO:214)	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	B7H3 sdAb 3 (SEQ ID NO:217)	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5187	1	B7H3 sdAb 4 (SEQ ID NO:214)	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	B7H3 sdAb 3 (SEQ ID NO:217)	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5499	1	DLL3 sdAb1 (SEQ ID NO: 219)	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	DLL3 sdAb 2 (SEQ ID NO: 220)	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	None	
cx3072	1	B7H3 sdAb 2 (SEQ ID NO: 215)	IgG1-Knob (SEQ ID NO:82, 86 or 201)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	B7H3 sdAb 1 (SEQ ID NO:218)	yes
	2	None	IgG1-Knob (SEQ ID NO:83, 87 or 202)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	None	
cx5873	1	None	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	B7H3 sdAb 3 (SEQ ID NO:217)	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5965	1	B7H3 sdAb 4 (SEQ ID NO:214)	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	none	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5352	1	DLL3 sdAb 1 (SEQ ID NO: 219)	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	DLL3 sdAb2 (SEQ ID NO: 220)	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5800	1	DLL3 sdAb 1 (SEQ ID NO:219)	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	None	yes

Table E1.1: Exemplary Constrained CD3 engaging constructs

Construct ID	Chain	N-term sdAb (Target)	Fc	Linker	CD3 Binding Domain	C-term sdAb (Target)	Disulfide Stabilized
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	
cx5801	1	None	xELL-Knob (SEQ ID NO:84, 88 or 203)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VH13 (SEQ ID NO: 44)	DLL3 sdAb 2 (SEQ ID NO: 220)	yes
	2	None	xELL-Hole (SEQ ID NO:91, 93 or 206)	GGGGGSGGGGSG GGGGS (SEQ ID NO:119)	VL10 (SEQ ID NO: 72)	Co-stim Receptor sdAb	

[0383] Exemplary generated constructs also included constructs in which the TAA antigen binding domains were composed as a Fab (designated MAB formats). In this example, an anti-B7H3 Fab was used composed of a heavy chain Fd set forth in SEQ ID NO:127 and a light chain set forth in SEQ ID NO:128. Polynucleotides were generated to encode polypeptide chains of exemplary multispecific polypeptide constructs containing non-cleavable linkers. These included cx5067, cx6083, and cx6084, as depicted in **FIG. 3C**. Constructs were engineered without a disulfide stabilized anti-CD3 antibody Fv or were engineered with a disulfide linkage stabilizing the VH and VL domains of the anti-CD3 antibody (anti-CD3 VH with the mutation G44C and VL with the mutation G100C). A list of exemplary Fab constructs is provided below in **Table E1.2**.

Table E1.2: Exemplary B7H3-targeted constrained CD3 engaging constructs

Construct ID	Chain	N-term Domain	Fc	Linker	CD3 Binding Domain	C-term Domain	Disulfide Stabilized
cx5067	1	B7H3-Fab (SEQ ID NOs: 127, 128)	Fc-Het-1 (SEQ ID NO:194)	GGGGSGGGGSGGGG S (SEQ ID NO:170)	VH32 (SEQ ID NO: 196)	None	no
	2	B7H3-Fab (SEQ ID NOs: 127, 128)	Fc-Het-2 (SEQ ID NO:195)	GGGGSGGGGSGGGG S (SEQ ID NO:170)	VL20 (SEQ ID NO: 199)	None	
cx6083	1	B7H3-Fab (SEQ ID NOs: 127, 128)	Fc-Het-1 (SEQ ID NO:194)	GGGGSGGGGSGGGG S (SEQ ID NO:170)	VH33 (SEQ ID NO: 197)	None	yes
	2	B7H3-Fab (SEQ ID NOs: 127, 128)	Fc-Het-2 (SEQ ID NO:195)	GGGGSGGGGSGGGG S (SEQ ID NO:170)	VL21 (SEQ ID NO: 200)	None	
cx6084	1	B7H3-Fab (SEQ ID NOs: 127, 128)	Fc-Het-1 (SEQ ID NO:194)	GGGGSGGGGSGGGG S (SEQ ID NO:170)	VH13 (SEQ ID NO: 44)	None	yes

	2	B7H3-Fab (SEQ ID NOs: 127, 128)	Fc-Het-2 (SEQ ID NO:195)	GGGSGGGSGGGG S (SEQ ID NO:170)	VL10 (SEQ ID NO: 72)	None
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B. Expression and purification of generated constructs

[0384] Separate plasmids encoding each chain of the heterodimeric constrained CD3 binding protein were transiently transfected at an equimolar ratio into mammalian cells (either HEK293 or CHO) using polyethylenimine. Recombinant protein secreted into the supernatant was collected after 3-14 days, and purified by protein A chromatography, followed by either preparative size exclusion chromatography (SEC) or flow-through hydrophobic interaction chromatography (HIC). In some cases, heterodimeric protein was enriched for during purification due to a mutation designed into one chain of the heterodimeric Fc at position I253R or H435R (e.g. in the hole-Fc) such that it did not bind protein A, and thus homodimers of I253R or H435R were not purified. The second chromatography step by SEC (AKTA with Superdex-200 resin) or FT-HIC (AKTA with butyl/phenyl sepharose) was used to remove undesired cross-paired species containing two heterodimeric Fcs that were more hydrophobic and twice the expected molecular weight.

[0385] The method favored production of heterodimeric multispecific polypeptide constructs, containing properly paired species of heterodimeric Fc and the anti-CD3 Fv (e.g. disulfide stabilized anti-CD3 Fv). Purified heterodimeric constrained CD3 binding protein was stable and did not accumulate cross-paired species upon prolonged incubation at 4°C or increased protein concentration.

EXAMPLE 2: Assessment of Binding of Constrained CD3 Binding Constructs to Cancer Cells and Primary T cells by Flow Cytometry

[0386] This Example describes studies assessing binding of exemplary constructs to T cells or to cancer cells. These studies were carried out in single cultures containing either only the T cells or only the cancer cells in isolation from each other.

A. FR-alpha Binding

[0387] Binding of exemplary multispecific polypeptide constructs of the disclosure containing an antigen-binding domain directed against Folate Receptor Alpha (FR α) to CD3 on the surface of primary T cells and to FR α expressing cells (Ovar-5) was assessed by flow cytometry. The tumor antigen binding domains of the tested constructs bind the Folate Receptor Alpha (FR α), which is not expressed on the primary T cells. The tested constructs

included cx1356 and cx681, containing a non-cleavable linker of 3 amino acids in cx1356 or a non-cleavable linker of 18 amino acids in cx681 (see FIG. 2B and Table E1.1).

[0388] For the studies described in FIG. 5A and 5B, 100nM of each construct, cx1356 or cx681 was used and assessed for binding to either the Ovar-5 cells or primary T-cells. An anti-CD3 antibody was included as a control. A titration of the constrained CD3 engaging constructs on either FR α expressing cells (Ovar-5; **FIG. 5C**) or isolated primary T-cells (FR α negative; **FIG. 5D**), was conducted to assess binding. Bound constructs were detected using fluorophore-conjugated anti-human IgG Fc secondary antibody.

[0389] The tested FR α -targeting constructs with various linkers between the Fc and the component of the CD3 binding domains were found to bind FR α expressing cells (Ovar-5) (**FIG. 5A** and **5C**), but lacked the capacity to bind T-cells (**FIG. 5B** and **5D**). These results are consistent with a finding that binding to CD3 on T cells in isolation is constrained in the provided formats.

B. B7H3 Binding

[0390] Binding of exemplary multispecific constructs containing an antigen-binding domain directed against B7H3 were assessed for binding to B7H3 positive A375 tumor cells or primary T-cells. The constructs were generated containing antigen-binding domain(s) that were either sdAbs or a FABs, and that were positioned either only N-terminal to the Fc or both N-terminal to the Fc and C-terminal to the anti-CD3 binding domain (see **FIGS. 3A** and **3C** and Table E1.1). Among the various formats of constructs tested included: sdAb-Fc-dsFV-sdAb (cx3072, cx5952) sdAb-Fc-FV (cx6079), sdAb-Fc-dsFV (cx6080, cx6081), MAB-FV (cx5067) and MAB-dsFV (cx6083, cx6084), where the FV represents the anti-CD3 binding domain composed of VH and VL domain pairs and “ds” notes disulfide stabilized via an engineered interdomain disulfide bond.

[0391] **FIGS. 6A-F** demonstrate that these constructs are capable of binding to B7H3 but not T-cells in isolation. Binding was assessed as described above via flow cytometry using a fluorophore-conjugated anti-human Fc secondary antibody. cx3072 bound to A375 cells with high affinity (**FIG. 6A**) but not to isolated T-cells (**FIG. 6B**). The tested sdAb-Fc-dsFV-sdAb (cx5952) displayed higher binding affinity compared to a FAB containing MAB-FV (cx5067) and MAB-dsFV constructs (cx6083, cx6084) (**FIG. 6C**). The B7H3 -targeting sdAb containing constructs (cx5952, cx6079, cx6080, and cx6081) bound to B7H3 positive cells with similar affinities (depicted in **FIG. 6E**), with cx5952 displaying higher maximal binding. cx6079, cx6080 and cx6081 contain two identical B7H3-targeting sdAbs, whereas cx5952, and cx3072 contain two distinct B7H3-targeting sdAb that bind different epitopes.

The MAB-FV, cx5067, contains two identical B7H3-targeting FAB domains. Notably none of the exemplary B7H3-targeted constrained CD3 engaging constructs bound isolated primary human T-cells, as depicted in **FIGS. 6B, 6D, and 6F**. These results further support that binding to CD3 on T cells in isolation is constrained in the provided formats.

EXAMPLE 3: Assessment of CD3 Signaling Activity in Co-cultures with Antigen-expressing Target Cells and Impact of Linker Length on Activity

[0392] The effect of various length linkers between the Fc and the component domains (VH and VL) that comprise the CD3 binding region on T-cell activating capacity was tested using a Jurkat reporter assay. The CD3 reporter cells were developed from Jurkat cells that naturally express CD3 and were engineered to express NFAT-driven green fluorescence protein (GFP). Agonism of CD3 results in NFAT signaling and production of green fluorescence.

[0393] Antigen targeting constrained CD3 engaging constructs were titrated onto co-cultures of target cells and Jurkat CD3 reporter cells. In this assay, target cells included either IGROV1 (FR α positive) or NCI-460 (FR α negative). For reporter assays utilizing adherent antigen expressing target cells, target cells were seeded, allowed to settle at room temperature for uniform distribution, and incubated for several hours at 37°C to permit adherence prior to addition of reporter cells and antigen targeting constrained CD3 engaging constructs. Assay plates were serially imaged using an IncuCyte ZOOM system and CD3 reporter cell activation was determined by measuring GFP expression as the total integrated green object in the well.

[0394] FR α -targeting constrained CD3 engaging constructs, generated as described in Example 1 containing GlySer-based linkers of varying lengths as listed in **Table E3** were used in these assays.

Table E3: Tested Linker Lengths	
SEQ ID NO	Linker
-	gs3: GGS
10	gs6: GGSGGS
11	gs9: GGSGGSGGS
12	gs12: GGSGGSGGSGGS
13	gs15: GGSGGSGGSGGSGGS
119	gs18: GGGGGSGGGGGSGGGGGS
147	gs18: GGSGGGGSGGGGGSGGGGGS

[0395] As shown in **FIGS. 7A-7F**, the T-cell activating activity as determined by fluorescent intensity was dependent on co-culture with FR α -antigen expressing target cells. The length of the linker and T-cell activating capacity were positively correlated. T-cell activating capacity was shown to directly relate to linker length, indicating shorter linkers restrict CD3 binding to a greater extent (see **FIGS. 7A, 7C and 7E**). Importantly, T-cell engagement of the constructs is dependent on TAA-binding, as these constructs did not demonstrate a T-cell binding capacity in isolation (e.g. solution form when unbound to target TAA) as shown above in Example 2. Further, no observable fluorescence was measured in co-cultures with FR α negative cells (**FIGS. 7B, 7D and 7E**). Together, these constructs displayed restricted or substantially reduced binding to CD3, yet were capable of activating T-cells in a target dependent manner.

EXAMPLE 4: Assessment of T cell activating activity of B7H3-targeted Constrained CD3 Binding Constructs

[0396] Constructs containing either a B7H3-targeted sdAb or a Fab as the tumor-associated antigen-binding domain were assessed for T-cell activating activity in a T cell reporter assay and in a T cell cytotoxicity assay. Activity of B7H3-targeted constrained CD3 engaging constructs that were formatted with an anti-B7H3 sdAb (e.g. cx5823, cx6079, cx6080, cx6081, cx3072 and cx5952) or anti-B7H3 MAB constructs formatted with a Fab (e.g. cx5067, cx6083 or cx6084) as the antigen-binding domain(s) were assessed (see **FIGS. 3A-3C** and Table E1.1). All tested constructs, except cx5067 and cx6079, contained a disulfide-stabilized anti-CD3 Fv (dsFv) containing an interchain disulfide bond created by the modification of anti-CD3 VH G44C paired with VL G100C. The anti-CD3 Fv of cx5067, designated MAB-Fv, was not disulfide-stabilized.

A. T cell Reporter Activity

[0397] The NFAT-GFP CD3 Jurkat reporter described in Example 3 was used to compare the CD3 agonistic properties of B7H3-targeted constrained CD3 engaging constructs when co-cultured in the presence of B7H3-positive cells (A375) or non-target CCRF-CEM cells that naturally lack B7H3 expression. In this assay, anti-B7H3 sdAb constructs, cx5823, cx6079, cx6080 and cx6081, or anti-B7H3 Fabs constructs, cx5067, cx6083 and cx6084, were used as the B7H3-targeting domains. As shown in **FIG. 8A**, the constructs containing B7H3-targeted sdAb displayed similar potencies of antigen-dependent CD3 activation. As shown in **FIG. 8C** the exemplary cx5823 construct containing B7H3-targeted sdAbs was found to be superior at mediating antigen-dependent CD3 activation

compared to the constructs containing B7H3-targeted Fabs. Although cx5823 is formatted with a binding domain for a costimulatory receptor, it is unlikely that this component contributed to the difference in results, since Jurkat T cells do not express the costimulatory receptor. None of the constructs demonstrated activity against the B7H3-negative CCRF-CEM cells (**FIGS. 8B** and **8D**).

B. Cytotoxicity

[0398] To further assess activity of the molecules, exemplary B7H3-targeted constructs cx3072 and cx5952 (each formatted as sdAb-dsFv), cx6083 and cx6084 (MAB-dsFv), cx5067 (MAB-Fv), cx6079 (sdAb-Fv), and cx6080 and cx 6081 (sdAb-dsFv) were tested in a T-cell-mediated cytotoxicity assay. Target cells included the B7H3 positive cell line, A375, and either modified A375 cells, wherein B7H3 gene was disrupted by CRISPR (A375:B7H3 KD), or CCRF-CEM cells that naturally lacked B7H3 expression. Target cells were seeded at 1.0×10^4 cells per well, allowed to settle at room temperature for uniform distribution, and incubated for several hours at 37°C. Primary T cells were negatively enriched from PBMCs isolated from healthy human donor leukopaks and added at a 10:1 T cell-to-target cell ratio. Green caspase-3/7 reagent was added, which fluorescently labels nuclear DNA of cells undergoing apoptosis was added. Multispecific constructs with constrained CD3 engaging activity were titrated onto the co-culture and assay plates were serially imaged using an IncuCyte ZOOM system. Target cell death was determined by measuring total red/green overlap object area.

[0399] As shown in **FIGS. 9A** and **9B**, exemplary constructs cx3072 and cx5952 containing sdAb B7H3-targeted antigen-binding domains induced potent T-cell-mediated cytotoxicity of B7H3 positive (A375) but not B7H3 negative cell lines.

[0400] When compared to exemplary B7H3-targeting constrained CD3 engagers with Fab B7H3-targeting domains (cx5067, cx6083 and cx6084), the exemplary cx5952 sdAb B7H3-targeting constrained CD3 engager mediated enhanced target-dependent T-cell cytotoxicity (**FIG. 10A**). No measurable T cell cytotoxicity was observed against the B7H3 negative cell line CCRF-CEM for any of the tested constructs, consistent with the capacity to potently induce antigen-dependent T-cell activation (**FIG. 10B**). Of the constructs tested, representative MAB-dsFV constructs cx6084 and cx6083 contained the engineered disulfide, whereas the representative MAB-FV construct cx5067 lacked this stabilizing modification. Notably cx6083 and cx5067 are identical with the exception of the presence (cx6083) or absence (cx5067) of the engineered disulfide within the anti-CD3 FV domain (depicted in **FIG. 3C**). The engineered disulfide was created by the modification of G44C within VH and

G100C within VL. As shown in **FIG. 10A**, cx6083 displayed superior potency in mediating target-dependent T-cell cytotoxicity compared to cx5067, suggesting that the incorporation of the inter-domain disulfide is beneficial in T-cell mediated cytotoxicity, likely by enhancing proper association of the VH and VL domains that comprise the anti-CD3 FV.

[0401] When compared to other exemplary B7H3-targeting constrained CD3 engagers with sdAb B7H3-targeting domains (cx6079, cx6080 and cx6081), the exemplary cx5952 sdAb B7H3-targeting constrained CD3 engager mediated enhanced target-dependent T-cell cytotoxicity (**FIG. 10C**). No measurable T cell cytotoxicity was observed against the B7H3 negative cell line CCRF-CEM for any of the tested constructs, consistent with the capacity to potentially induce antigen-dependent T-cell activation (**FIG. 10D**). Of the constructs tested, sdAb-dsFV constructs cx5952, cx6080 and cx6081 contained the engineered disulfide linkage, whereas the sdAb-FV construct cx6079 lacked this stabilizing modification. The engineered disulfide was created by the modification of G44C within VH and G100C within VL. Notably, cx5952 was engineered to have two distinct B7H3 targeting domains, one located at the amino terminal and one located at the carboxy terminal. cx6079, cx6080, and cx6081 were engineered to have two identical B7H3 targeting domains, both located at the amino terminal (see **FIG. 3A**).

C. T Cell Modulation

[0402] To further assess T cell modulation, exemplary multispecific CD3 constrained binding constructs was assessed by monitoring the ability of the constructs to modulate T cell activation markers. To assess T cell activation, suspension cells from T cell cytotoxicity assays above, involving culture of T cells with B7H3 positive (A375) or B7H3 negative cell lines (CCRF-CEM) in the presence of an exemplary B7H3-targeted constrained CD3 engaging construct, cx5952, were collected. Cells were stained with a live/dead stain and fluorophore-conjugated anti-CD4, anti-CD8, anti-CD25, anti-CD69, and/or anti-CD71 antibodies. Cells were analyzed using a SONY SA3800 spectral analyzer and CD4+ or CD8+ T cell activation was determined by measuring expression levels of CD25, CD69 or CD71 or percent CD25-, CD69- or CD71-positive.

[0403] Results are shown for CD25 expression (**FIG. 11A**), CD69 expression (**FIG. 11B**) and CD71 expression (**FIG. 11C**) on CD4+ and CD8+ T cells following the co-culture with B7H3 positive (A375) or B7H3 negative cell lines (CCRF-CEM) in the presence of cx5952. The results showed that cx5952 mediated a dose-dependent B7H3-dependent T-cell activation via CD3 binding, as evidenced by increased expression of CD25, CD69 and CD71 in CD4+ and CD8+ T cells.

[0404] When compared to the other exemplary B7H3-targeting constrained CD3 engagers with sdAb B7H3-targeting domains (cx6079, cx6080 and cx6081), the exemplary cx5952 sdAb B7H3-targeting constrained CD3 engager mediated increased T cell activation as evidenced by increased expression of CD25 in CD4+ T cells (**FIG. 11D**) and in CD8+ T cells (**FIG. 11H**) and increased expression of CD71 in CD4+ T cells (**FIG. 11F**) and in CD8+ T cells (**FIG. 11J**). Increased expression of the surface markers on T cells was not observed in the presence of the B7H3-targeting constrained CD3 engager constructs in cultures with B7H3 negative cell lines (**FIGS. 11E and 11G** for CD4+ T cells and **FIGS. 11I and 11K** for CD8+ T cells).

D. T Cell Cytokine Production

[0405] Supernatants from T cell cytotoxicity tumor cell co-culture assays, involving co-culture of T cells with B7H3 positive, A375 or negative, CCRF-CEM cells in the presence of cx5952, cx6083, cx6084 or cx5067, were analyzed for IFN γ content by sandwich ELISA. A standard curve was generated from which cytokine concentration values of supernatant samples were interpolated. Samples that had absorbance values below the lower limit of detection were assigned a cytokine concentration equal to half that of the lowest standard concentration. As shown in **FIG. 12A**, the representative sdAb-Fc-dsFV-sdAb construct, cx5952, was superior to the tested B7H3-targeted FAB containing constructs, cx6083, cx6084 and cx5067 at eliciting target-dependent cytokine release from activated T-cells. Importantly, the MAB-dsFV constructs, cx6083 and cx6084 were superior to the MAB-FV construct, cx5067, demonstrating the importance of interdomain disulfide stabilizing modification for enhancing T-cell function.

[0406] When compared to the other exemplary B7H3-targeting constrained CD3 engagers with sdAb B7H3-targeting domains (cx6079, cx6080 and cx6081), the exemplary cx5952 sdAb B7H3-targeting constrained CD3 engager mediated substantially increased production of IFN γ in the presence of B7H3-target cells T cells but not in cultures with B7H3 negative cell lines (**FIG. 12B**)

E. Summary

[0407] These observations further support that the antigen-targeted constrained CD3 format provided herein lack or exhibit reduced T-cell binding in isolation while maintaining potent B7H3-dependent T-cell cytotoxicity inducing capacities. Without wishing to be bound by theory, together these results show that utilization of antigen targeted sdAbs instead of a Fabs may reduce the immune synapse distance between the TAA expressing tumor cell and the CD3 expressing T-cells and enhance T cell activity and cytotoxicity. Notably, it was

found that the inclusion of an interchain disulfide bond created by the modification of anti-CD3 VH G44C paired with VL G100C greatly enhanced the activity of constrained CD3 engaging constructs. Further, the more potent B7H3-dependent T cell activity by cx5952 compared to other sdAb B7H3-targeting domain constructs suggests that the positioning of the B7H3-targeting sdAb C-terminal to the anti-CD3 binding domain or the fact that cx5952 binds two distinct epitopes on B7H3 whereas the other constructs tested bind to a single epitope in a bivalent manner, contributed to this enhanced activity.

EXAMPLE 5: Assessment of CD3-constrained Multispecific Constructs Containing Single or Multiple B7H3-Binding Targeting Domains

[0408] Activity of constructs containing a monovalent sdAb antigen-binding domain (positioned at either the N or C-terminus) was compared to activity of dual binding (bivalent) constructs that contained antigen-targeting sdAbs positioned at both the N and C-termini. Binding was assessed substantially as described in Example 2 and T cell activity was assessed in the Jurkat reporter assay and T cell cytotoxicity assays substantially as described in Examples 3 and 4.

A. Binding

[0409] As shown in **FIG. 13A** the bivalent B7H3-targeting constrained CD3 engaging constructs, cx5187 and cx5823, displayed higher affinity binding to B7H3 positive A375 cells, compared to the monovalent versions, cx5873 and cx5965. None of these constructs displayed any detectable binding to B7H3 negative CCRF-CEM cells or isolated T-cells (**FIG. 13B**).

B. T Cell Reporter Activity

[0410] B7H3 Antigen-dependent CD3 agonistic capacities of antigen-targeted constrained CD3 engaging constructs that engage the antigen in a monovalent or bivalent manner were assessed using CD3-NFAT Jurkat reporter cells, in an assay substantially as described above. As shown in **FIG. 13C**, substantially increased fluorescence reporter activity was observed in the presence of the exemplary bivalent B7H3-targeted construct cx5187 compared to reporter activity for the exemplary monovalent constructs cs5873 and cx5965. No reporter activity was observed when constructs were incubated with Jurkat reporter cells co-cultured with B7H3-negative CCRF target cells (**FIG. 13D**).

C. Cytotoxic Activity

[0411] Cytotoxicity of B7H3-targeted CD3 constrained binding constructs was assessed against a melanoma cell line, A375, and a T-cell acute lymphoblastic leukemia cell line,

CCRF-CEM, which were used as B7H3 positive and negative cell lines, respectively. Cytotoxicity was assessed substantially as described in Example 4. As shown in **FIG. 14A** an exemplary bivalent B7H3-targeted constrained CD3 engaging construct, cx5187, displayed enhanced target-dependent T-cell mediated cytotoxicity compared to the monovalent versions of the constructs, cx5873 and cx5965. In these assays, no cytotoxicity was observed in the absence of B7H3 expression of the target cells, as shown in **FIG. 14B** wherein the CCRF-CEM cells were used as target cells.

D. T Cell Modulation

[0412] T cell modulation was assessed by monitoring expression of CD25, substantially as described in Example 4, in suspension cells from T cell cytotoxicity assays above, involving culture of T cells with B7H3 positive (A375) or B7H3 negative cell lines (CCRF-CEM) in the presence of cx5187, cx5873 or cx5965. As shown in **FIG. 15A** and **15B**, an exemplary bivalent B7H3-targeted constrained CD3 engaging construct, cx5187, displayed enhanced target-dependent T-cell mediated activation compared to the monovalent versions of the constructs, cx5873 and cx5965, as evidenced by enhanced potency of CD25 upregulation on CD4 and CD8 T-cells. In these assays, no T-cell activation was observed in the absence of B7H3 expression of the target cells, as shown in **FIG. 15C** and **15D**, wherein the CCRF-CEM cells were used as target cells. These results demonstrated that the B7H3-targeting constrained CD3 engaging constructs induced potent antigen-dependent activation of both CD4 and CD8 T-cells.

D. Summary

[0413] Together, these results demonstrate that bivalent antigen-targeted constrained CD3 engaging constructs displayed superior antigen-dependent CD3 binding and activity than the monovalent antigen-targeted constrained CD3 engaging constructs. These results are consistent with a finding that constructs containing dual antigen-binding domains positioned at both the N and C-termini have superior binding and T cell activity than monovalent constructs containing only a single monovalent antigen-binding domain. Furthermore, without wishing to be bound by theory, positioning one of the sdAbs C-terminal to the CD3 binding domain may form a more optimal immune synapse compared to constructs wherein the sdAbs are only positioned N-terminal to the Fc as the latter may increase the immune synapse distance.

EXAMPLE 6: Assessment of a CD3-constrained Multispecific Constructs Containing B7H3-Targeting sdAb and Fab Domains

[0414] Constructs containing either B7H3-targeted sdAb(s) or a Fab as the tumor-associated antigen-binding domain were assessed for T-cell activating activity. Activity of B7H3-targeted constrained CD3 engaging constructs that were formatted with anti-B7H3 sdAbs (e.g. cx5952 and cx6079) or anti-B7H3 MAB constructs formatted with a Fab (e.g. cx5067, cx6083 or cx6084) as the antigen-binding domain(s) were assessed (see **FIGS. 3A** and **3C** and Table E1.1 and E1.2). All tested constructs, except cx6079 and cx5067, contained a disulfide-stabilized anti-CD3 Fv (dsFv) containing an interchain disulfide bond created by the modification of anti-CD3 VH G44C paired with VL G100C. The anti-CD3 Fv of cx5067, designated MAB-Fv, and the anti-CD3 Fv of cx6079, designated sdAb-Fc-Fv, were not disulfide-stabilized. Additionally, cx5952 was engineered to contain two distinct B7H3-targeting sdAb domains, with one located N-terminal to the Fc domain and one located C-terminal to the CD3-binding domain. By contrast, cx6079 was engineered to contain two identical B7H3-targeting sdAb domains, both located N-terminal to the Fc domain. The Fvs of all three Fab constructs were engineered to be N-terminal to the Fc domain.

A. Cytotoxicity

[0415] Cytotoxicity of B7H3-targeted CD3 constrained binding constructs was assessed substantially as described in Example 4. Cytotoxicity was assessed against a melanoma cell line, A375, and a T-cell acute lymphoblastic leukemia cell line, CCRF-CEM, which were used as B7H3 positive and negative cell lines, respectively. As shown in **FIG. 16A** the exemplary constrained CD3 engaging constructs formatted with B7H3-targeting sdAbs, cx5952 and cx6079, were superior at eliciting antigen-dependent T-cell cytotoxicity compared to to the anti-B7H3 MAB constructs formatted with a Fab, cx5067, cx6083, and cx6084. Notably, cx5952 was more potent than cx6079, suggesting the positioning of the B7H3-targeting sdAb C-terminal to the anti-CD3 binding domain and/or the stabilization of the anti-CD3 FV via engineered disulfide contributed to this enhanced activity. In these assays, no cytotoxicity was observed in the presence of B7H3-negative CCRF-CEM cells target cells, as shown in **FIG. 16B**.

B. T Cell Modulation

[0416] To further assess T cell modulation, exemplary multispecific CD3 constrained binding constructs were assessed by monitoring the ability of the constructs to modulate T cell activation markers, substantially as described in Example 4. To assess T cell activation, suspension cells from T cell cytotoxicity assays above, involving culture of T cells with

B7H3 positive (A375) or B7H3 negative cell lines (CCRF-CEM) in the presence of an exemplary B7H3-targeted constrained CD3 engaging constructs, were collected. Tested constructs included anti-B7H3 constructs formatted with sdAbs (e.g. cx5952 and cx6079) and anti-B7H3 constructs formatted with a Fab (e.g. cx5067, cx6083 and cx6084)

[0417] Cells were stained with a live/dead stain and fluorophore-conjugated anti-CD4, anti-CD8, anti-CD25 and/or anti-CD71 antibodies. Cells were analyzed using a SONY SA3800 spectral analyzer and CD4+ or CD8+ T cell activation was determined by measuring expression levels of CD25 or CD71 or percent CD25- or CD71-positive.

[0418] Results are shown for CD25 expression (**FIG. 16C-F**) and CD71 expression (**FIG. 16G-J**) on CD4+ and CD8+ T cells following the co-culture with B7H3 positive (A375) or B7H3 negative cell lines (CCRF-CEM) in the presence of the described constructs. The results showed that cx5952 mediated a dose-dependent B7H3-dependent T-cell activation via CD3 binding, as evidenced by increased expression of CD25 and CD71 in CD4+ and CD8+ T cells. cx5952 was the most potent over other B7H3-targeted constrained CD3 engaging constructs and inducing T-dependent T-cell activation.

C. T Cell Cytokine Production

[0419] Supernatants from T cell cytotoxicity tumor cell co-culture assays, involving co-culture of T cells with B7H3 positive, A375 or negative, CCRF-CEM cells in the presence of cx5952, cx6079, cx6083, cx6084 or cx5067, were analyzed for IFN γ content by sandwich ELISA. A standard curve was generated from which cytokine concentration values of supernatant samples were interpolated. Samples that had absorbance values below the lower limit of detection were assigned a cytokine concentration equal to half that of the lowest standard concentration. As shown in **FIG. 16K**, the representative sdAb-Fc-dsFV-sdAb construct, cx5952, was superior to the tested B7H3-targeted FAB containing constructs, cx6083, cx6084 and cx5067 at eliciting target-dependent cytokine release from activated T-cells. This is consistent with the finding from the antigen dependent cytotoxicity and activation assays. Importantly, the MAB-dsFV constructs, cx6083 and cx6084 were superior to the MAB-FV construct, cx5067, demonstrating the importance of interdomain disulfide stabilizing modification for enhancing T-cell function.

D. Summary

[0420] Together, these results demonstrate that constrained anti-CD3 constructs formatted with anti-B7H3 sdAb binding domains were superior at eliciting antigen-dependent T-cell cytotoxicity compared to the anti-B7H3 MAB constructs formatted with a Fab B7H3 binding domain. Further, that cx5952 was more potent than cx6079 suggested that the

positioning of the B7H3-targeting sdAb C-terminal to the CD3 binding domain, the stabilization of the anti-CD3 Fv via engineered disulfide, or both, contribute to enhanced activity. Without wishing to be bound by theory, positioning one of the sdAbs C-terminal to the CD3 binding domain may form a more optimal immune synapse compared to constructs wherein the sdAbs are only positioned N-terminal to the Fc as the latter may increase the immune synapse distance.

EXAMPLE 7: Assessment of CD3-constrained Multispecific Constructs Containing Single or Multiple Antigen-Binding DLL3-Targeting Domains

[0421] This example describes the assessment and characterization of exemplary generated DLL3-targeted constrained CD3 engaging constructs in human primary T cell *in vitro* assays.

[0422] Binding and activity of DLL3-targeted constrained CD3 engaging constructs that were formatted with an anti-DLL3 sdAb (e.g. cx5352, cx5800, cx5801, and cx5499) as the antigen-binding domain(s) were assessed (see FIGS. 4A-4B and Table E1.1). All tested constructs contained a disulfide-stabilized anti-CD3 Fv (dsFv) containing an interchain disulfide bond created by the modification of anti-CD3 VH G44C paired with VL G100C. Further, the DLL-3-targeted constructs were engineered to contain a co-stimulatory receptor sdAb C-terminal to the CD3 dsFv, except for cx5499, which did not contain this co-stimulatory receptor sdAb domain.

A. Binding

[0423] Binding was assessed substantially as described in Example 2. As shown in FIG. 17A the bivalent DLL3-targeting constrained CD3 engaging constructs, cx5352 displayed higher affinity binding to DLL3 positive, SHP-77 cells compared to the monovalent versions, cx5800 and cx5801. None of the constructs tested displayed binding to DLL3-negative primary T cells, as depicted in FIG. 17B. These binding assays were conducted by flow cytometry, wherein bound constructs were detected using a fluorophore-conjugated anti-human IgG Fc secondary antibody.

A. T Cell Reporter Activity

[0424] T cell activity was assessed in a reporter assay substantially as described in Example 2, except that Jurkat cells expressing NFAT-driven Luciferase were used and luciferase activity was monitored. NFAT-driven Luciferase CD3 Jurkat reporter cells were co-cultured with SHP-77 (DLL3-positive) target cells in the presence of monovalent and bivalent constructs containing antigen-binding domains against the DLL3 antigen (see FIG.

4A). Specifically, as shown in **FIG. 17C**, the exemplary bivalent construct cx5352 induced substantially greater luciferase activity in this assay compared to the exemplary monovalent constructs cx5800 and cx5801. These results are consistent with results observed with B7H3-targeted constructs, thereby indicating that the activity of the constructs is not specific to a particular target antigen.

[0425]

C. Cytotoxicity

[0426] Cytotoxicity of cx5499, a DLL3-targeted CD3 constrained binding construct formatted with two distinct sdAb binding domains located at its amino and carboxy termini, was assessed against a DLL3 expressing cell line, SHP-77, using an assay substantially as described in Example 4. As shown in **FIG. 18A**, cx5499 induced potent T-cell mediated cytotoxicity directed toward the SHP-77 cell line.

D. T Cell Modulation

[0427] To further assess T cell modulation, exemplary multispecific CD3 constrained binding constructs were assessed by monitoring the ability of the constructs to modulate T cell activation markers. To assess T cell activation, suspension cells from T cell cytotoxicity assays above, involving culture of T cells with DLL3 positive SHP-77 cells in the presence of cx5499, in the presence of an exemplary DLL3-targeted constrained CD3 engaging constructs, were collected. Cells were stained with a live/dead stain and fluorophore-conjugated anti-CD4, anti-CD8, anti-CD25 and/or anti-CD69 antibodies. Cells were analyzed using a SONY SA3800 spectral analyzer and CD4+ or CD8+ T cell activation was determined by measuring expression levels of CD25 or CD69 or percent CD25- or CD69-positive.

[0428] **FIG. 18B** and **FIG. 18D** depict results for CD25 expression on CD4 T cells or CD8 T cells, respectively, upon culture of T cells with DLL3 positive, SHP-77 cells, in the presence of an exemplary DLL3-targeted constrained CD3 engaging construct, cx5499. **FIG. 18C** and **FIG. 18E** depict results for CD69 expression on CD4 cells or CD8 T cells, respectively, upon culture of T cells with DLL3 positive, SHP-77 cells, in the presence of an exemplary DLL3-targeted constrained CD3 engaging construct, cx5499. The results showed that cx5499 mediated a dose-dependent DLL3-dependent T-cell activation via CD3 binding, as evidenced by increased expression of CD25 and CD69 on CD4+ and CD8+ T cells.

E. Summary

[0429] Together, these results demonstrate that constrained anti-CD3 constructs formatted with anti-DLL3 sdAb binding domains are capable of binding to a DLL3-

expressing cell line, SHP-77, and eliciting antigen-dependent T-cell cytotoxicity and activation. This result is consistent with a finding that the constrained CD3 engaging constructs of the disclosure have broad applicability to specifically target numerous tumor antigens and elicit T-cell cytotoxicity and activation against target-expressing cells.

[0430] While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

SEQUENCE TABLE

SEQ ID NO	SEQUENCE	DESCRIPTION
1	PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSRDEL TKNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK	IgG1 Fc
2	PAPGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKT KPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVY TLP PSRDEL TKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPGK	xELL Fc
3	PAPPVAGPSV FLFPPKPKD TLMISRTPEV TCVVVDVSHED PEVQFNWYVD GVEVHNAKT KPREEQFNSTF RVVSVLTVVH QDWLNGKEYK CKVSNKGLPA PIEKTISKTK GQPREPQVY TLP SREEMTK NQVSLTCLVK GFYPSDISVE WESNGQPEN YKTTTPMLD DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKSLS LSPGK	IgG2 Fc
4	PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVQFKWYV DGVEVHNAKT KPREEQYNST FRVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKT KGQPREPQVY TLPPSREEMT KQVSLTCLV KGFYPSDIAV EWESSGQPEN NYNTTPMLD SDGSFFLYSK LTVDKSRWQQ GNIFSCSVMH EALHNRFTQK SLSLSPGK	IgG3 Fc
5	PAPEFLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT KQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK	IgG4 Fc
6	PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT KQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK	IgG4 Fc
7	EPKSSDKTHTCPPC	Hinge
8	DKTHTCPPC	Hinge
9	ESKYGPPCPPC	Hinge
10	GGSGGS	(GGG) ₂
11	GGSGGSGGS	(GGG) ₃
12	GGSGGSGGSGGS	(GGG) ₄
13	GGSGGSGGSGGSGGS	(GGG) ₅
14	EVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKDRFTISRDDSQSILYLQMNNLKTEDTAMYVCVR HGNFGNSYVSWFAYWGQGLTVTSA	anti-CD3 VH
15	QAVVTQESALTTSPTGETVTLTCSRSTGAVTTSNYANWVQEKPDHLFTGLIG GTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNLWVFGG GTKLTVL	anti-CD3 VL
16	TYAMN	anti-CD3 VH CDR1
17	RIRSKYNNYATYYADSVKD	anti-CD3 VH CDR2
18	HGNFGNSYVSWFAY	anti-CD3 VH CDR3
19	RSSTGAVTTSNYAN	anti-CD3 VL CDR1

20	GTNKRAP	anti-CD3 VL CDR2
21	ALWYSNLWV	anti-CD3 VL CDR3
22	LEAD	Granzyme B substrate
23	RQAR	Granzyme B substrate
24	PAGL	MMP substrate
25	TGLEADGSPAGLGRQARVG	Linker
26	TGLEADGSRQARVGPAGLG	Linker
27	TGSPAGLEADGSRQARVGS	Linker
28	TGPAGLGLEADGSRQARVG	Linker
29	TGRQARVGLEADGSPAGLG	Linker
30	TGSRQARVGPAGLEADGS	Linker
31	TGPAGLGSRQARVGLEADGS	Linker
32	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGR IRSKYNNYATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH1
33	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKDRFTISRDDSKSSLYLQMNNLKTEDTAMYYCVR HGNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH2
34	EVKLVESGGGLVKPGRSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVAR IRSKYNNYATYYADSVKDRFTISRDDSKSILYLQMNNLKTEDTAMYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH3
35	EVKLVESGGGLVKPGRSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVAR IRSKYNNYATYYADSVKDRFTISRDDSKSILYLQMNSLKTEDTAMYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH4
36	EVKLVESGGGLVKPGRSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVAR IRSKYNNYATYYADSVKDRFTISRDDSKSILYLQMNSLKTEDTAMYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH5
37	EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVSRI RSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH6
38	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMSWVRQAPGKGLEWVGR IRSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGDSYVSWFAYWGQGLTVTVSS	anti-CD3 VH7
39	EVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCV RHGNFGNSYISYWAYWGQGLTVTVS	anti-CD3 VH8
40	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVR HGNFGNSYVSWFAYWGQGTTVTVSS	anti-CD3 VH9
41	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVR HGNFGNSYVSYFAYWGQGTTVTVSS	anti-CD3 VH10
42	EVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKDRFTISRDDQSILYLQMNNLKTEDTAMYYCVR HGNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH11
43	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNYATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVR HGNFGNSYVSWFAYWGQGLTVTKP	anti-CD3 VH12
44	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNYATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTKP	anti-CD3 VH13

45	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVR HGNFGNSYVSWFAYWGQGLTVTKP	anti-CD3 VH14
46	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVAR IRSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH15
47	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKGLEWVSR IRSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH16
48	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVSR IRSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH17
49	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH18
50	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVSR IRSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH19
51	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKCLEWVSRI RSKYNNTATYYADSVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH20
52	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKCLEWVGR IRSKYNNTATYYADSVKDRFTISRDDSKNSLYLQMNSLKTEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH21
53	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKDRFTISRDDSKSSLYLQMNNLKTEDTAMYYCVR HGNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH22
54	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKDRFTISRDDSKSILYLQMNNLKTEDTAMYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH23
55	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKDRFTISRDDSKSILYLQMNSLKTEDTAMYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH24
56	EVKLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKDRFTISRDDSKSILYLQMNSLKTEDTAMYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH25
57	EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKCLEWVSRI RSKYNNTATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH26
58	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMSWVRQAPGKCLEWVGRI RSKYNNTATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGDSYVSWFAYWGQGLTVTVSS	anti-CD3 VH27
59	EVQLVESGGGLVQPGGSLRLSCAASGFTFNKYAMNWVRQAPGKCLEWVA RIRSKYNNTATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCV RHGNFGNSYISYWAYWGQGLTVTVS	anti-CD3 VH28
60	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH29
61	EVQLVESGGGLVQPGGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVAR IRSKYNNTATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGNSYVSFYFAYWGQGLTVTVSS	anti-CD3 VH30
62	EVQLVESGGGLVQPKGSLRLSCAASGFTFNTYAMNWVRQAPGKCLEWVA RIRSKYNNTATYYADSVKDRFTISRDDQSILYLQMNNLKTEDTAMYYCVR HGNFGNSYVSWFAYWGQGLTVTVSS	anti-CD3 VH31
63	QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIG GTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNLWVFGG GTKLTVL	anti-CD3 VL1

64	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGKSPRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNLWVFG CGTKLEIK	anti-CD3 VL2
65	QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIG GTNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFG GGTKLTVL	anti-CD3 VL3
66	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIG GTNKRAPGVPARFSGSLIGDKAALTITGAQADDESIYFCALWYSNLWVFGG GTKLTVL	anti-CD3 VL4
67	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIG GTNKRAPGVPARFSGSILGNKAALTITGAQADDESIYFCALWYSNLWVFGG GTKLTVL	anti-CD3 VL5
68	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIG GTNKRAPGVPARFSGSILGNKAALTITGAQADDESYYCALWYSNLWVFG GGTKLTVL	anti-CD3 VL6
69	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQEKPGQAFRGLIG GTNKRAPGTPARFSGSLLGGKAALTLSGAQPEDEAEYYCALWYSNLWVFG GGTKLTVL	anti-CD3 VL7
70	QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQAPRGLIG GTKFLAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFG GGTKLTVL	anti-CD3 VL8
71	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQAFRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNLWVFG GGTKLEIK	anti-CD3 VL9
72	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQAFRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNLWVFG CGTKLEIK	anti-CD3 VL10
73	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQCFRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNLWVFG EGTKLEIK	anti-CD3 VL11
74	QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIG GTNKRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNLWVFGC GTKLTVL	anti-CD3 VL12
75	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGKSPRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNLWVFG GGTKLEIK	anti-CD3 VL13
76	QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIG GTNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFG CGTKLTVL	anti-CD3 VL14
77	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIG GTNKRAPGVPARFSGSLIGDKAALTITGAQADDESIYFCALWYSNLWVFGG GTKLTVL	anti-CD3 VL15
78	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIG GTNKRAPGVPARFSGSILGNKAALTITGAQADDESIYFCALWYSNLWVFGC GTKLTVL	anti-CD3 VL16
79	QAVVTQEPSFSVSPGGTVTLTCRSSTGAVTTSNYANWVQQTPGQAFRGLIG GTNKRAPGVPARFSGSILGNKAALTITGAQADDESYYCALWYSNLWVFG CGTKLTVL	anti-CD3 VL17
80	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQEKPGQAFRGLIG GTNKRAPGTPARFSGSLLGGKAALTLSGAQPEDEAEYYCALWYSNLWVFG CGTKLTVL	anti-CD3 VL18
81	QTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGNYPNWVQQKPGQAPRGLIG GTKFLAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNRWVFG CGTKLTVL	anti-CD3 VL19
82	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFC SVMHEALHNHYTQKSLSLPT	Knob Fc

83	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMRSRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV KVSNAKALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSP	Hole Fc
84	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV NKAALPAIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSP	Knob Fc
85	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMRSRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV NKAALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSP	Hole Fc
86	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV VSNKALPAIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPG	Knob Fc
87	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMRSRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV KVSNAKALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPG	Hole Fc
88	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV NKAALPAIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS IAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPG	Knob Fc
89	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMRSRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV NKAALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPG	Hole Fc
90	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV VSNKALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV VMHEALHNRYTQKSLSLSP	Hole Fc
91	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV NKAALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNRYTQKSLSLSP	Hole Fc
92	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV VSNKALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV VMHEALHNRYTQKSLSLSPG	Hole Fc
93	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV NKAALPAIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNRYTQKSLSLSPG	Hole Fc
94	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV VSNKALPAIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPS SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSC SVVHEALHNHYTQKSLSLSP	Knob Fc

95	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDI AVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVV HEALHNHYTQKSLSLSP	Knob Fc
96	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVVHEALHNHYTQKSLSLSPG	Knob Fc
97	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDI AVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVV HEALHNHYTQKSLSLSPG	Knob Fc
98	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS DIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCS VVHEALHNRYTQKSLSLSP	Hole Fc
99	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIA VEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVVH EALHNRYTQKSLSLSP	Hole Fc
100	DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPS DIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCS VVHEALHNRYTQKSLSLSPG	Hole Fc
101	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIA VEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVVH EALHNRYTQKSLSLSPG	Hole Fc
102	PGGGG	Peptide Linker
103	GGGG	Peptide Linker
104	GPAGLGLPDGSRQARVG	Linker
105	GGSGGGGIEPDIGSGGS	Linker
106	GGSGGGGLEADTGGSGGS	Linker
107	GSIEPDIGS	Linker
108	GSLEADTGS	Linker
109	GGSGGGGIEPDGGSGGS	Linker
110	GGSGGGGIEPDVGGSGGS	Linker
111	GGSGGGGIEPDSGGSGGS	Linker
112	GGSGGGGIEPDTGGSGGS	Linker
113	GGGSLEPDGSGS	Linker
114	GPAGLGLEADGSRQARVG	Linker
115	GGEGGGGSGSGSGGS	Linker
116	GSSAGSEAGSGQAGVGS	Linker

117	GGSGGGGLEAEGSGGGGS	Linker
118	GGSGGGGIEPDPGSGGS	Linker
119	GGGGSGGGGGSGGGGS	Linker
120	QLQLQESGGGLVQPGGSLRLSCAASGFTLDNYAIGWFRQAPGKEREGVSCIS SSDGSTYYADSVKGRFTISRNNAKGTVYLLMNSLKPEDTAVYYCATELVPA CTYSNGRGLDGM DYWGKGTQVTVKP	FR alpha sdAb
121	EVQLLES GGGEVQPGGSLRLSCAASGSIFSIDATAWYRQAPGKQRELVAITS SGSTNYPE SVKGRFTISRDN AKNTVYLQMSSLRAEDTAVYYCNAITRYGGS TYDFWGQGT LVTVKP	FR alpha sdAb
122	EVQPGGSLRLSCAASETFGVVFTLGWYRQAPGKGREFVARVTGTDTVDYA ESVKGRFTISSDFARNTVYLQMNSLRAEDTAVYYCNTGAYWGQGT LVTVK P	FR alpha sdAb
123	EVQLVESGGGLVQPGGSLRLSCAASGFILDYYAIGWFRQAPGKEREGVLCID ASDDITYYADSVKGRFTISRDN AKNTVYLQMNSLKPEDTGVYYCATPIGLSS SCLLEYD YD YWGQGT LVTVKP	cMET sdAb
124	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAY ISSDSSAIYYADTVKGRFTISRDN AKNSLYLQMNSLRDEDTAVYYCGRGREN IYYGSRLDYWGQGT TTVTVSSSGGGSGGGSGGGSGDIQLTQSPSFLSASVG DRVTTITCKASQNVDTNVAWYQQKPGKAPKALIYSASYRYSVPSRFSGSGS GTDFTLTISSLQPEDFATYYCQQYNNYPFTFGGQGTKLEIK	B7H3 scFv
125	QVQLVQSGAEVKKPGSSVKVCKASGYAFSYSWINWVRQAPGQGLEWMG RIFPGDGD TDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARNV FDGYWL VYWGQGT LTVSGSGGGSGGGSGGGSGDIVMTQTPLSLPVT GEPASISCRSSKSLLSHNGITYLYWY LQKPGQSPQLLIYQMSNLVSGVPDRFS GSGSGTDFTLKISRVEAEDVG VYYCAQNLELPYTFGGG TKVEIK	CD20 scFv
126	QVQLQESGPGLVKPSSETLSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYVY YSGTTNYPNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCASI AVTGFY FDYWGQGT LTVTVSSSGGGSGGGSGGGSGSEIVLTQSPGTL SLSPGERVTLSC RASQRVNNNYLAWYQQRPQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLT ISRLEPEDFAVYYCQQYDRSPLTFGGG TKLEIK	DLL3 scFv
127	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAY ISSDSSAIYYADTVKGRFTISRDN AKNSLYLQMNSLRDEDTAVYYCGRGREN IYYGSRLDYWGQGT TTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNV NHKPSNTKVDKKVEPKSC	B7H3 Fd
128	DIQLTQSPSFLSASVGDRVTTITCKASQNVDTNVAWYQQKPGKAPKALIYSAS YRYSVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNNYPFTFGGQGTKLE IKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRKAKVQWKVDNALQSG NSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC	B7H3 LC
129	EVQLVESGGGLVQPKGSLKLSAASGFTFNTYAMNWVRQAPGKGLEWVA RIRSKSNYYATYYADSVKDRFTISRDDQSMLYLQMNNLKTEDTAMYYCV RQWDYDVRAMNYWGQGT SVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVEPKSC	5T4 Fd
130	DIVMTQSHIFMSTSVGDRVSITCKASQD VDTAVAWYQQKPGQSPKLLIYWA STRLTGVPDRFTGSGSGTDFTLTISNVQSEDLADYFCQQYSSYPYTFGGG TK LEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRKAKVQWKVDNALQ SGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTK SFNRGEC	5T4 LC
131	QVQLQESGPGLVKPSQTLSTCTVSGGSISSFNYYSWIRHHPGKGLEWIGY IYYSGSTYSNPSLKSRTISVDTSKNQFSLTLSSVTAADTAVYYCARGYNWN YFDYWGQGT LTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKP SNTKVDKKVEPKSC	gpNMB Fd

132	EIVMTQSPATLSVSPGERATLSCRASQSVDDNNLVWYQQKPGQAPRLLIYGAS TRATGIPARFSGSGSGTEFTLTISLSQSEDAVYYCQQYNNWPPWTFGQGTK VEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQ SGNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK SFNRGEC	gpNMB LC
133	QIQLVQSGPELKKPGETVKISCKASGYTFTNYGMNWVKQAPGKGLKWMMA WINTYTGEPTYADDFKGRFAFSLETSASTASLQIINLKNEDTATYFCARIGDS SPSDYWGGQTTTLTVSSSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVN HKPSNTKVDKKVEPKSC	DLL3 Fd
134	SIVMTQTPKFLLSAGDRVITITCKASQSVSNDVVWYQQKPGQSPKLLIYYAS NRYTGVPDRFAGSGYGTDFSTISTVQAEDLAVYFCQQDYTSPWTFGGGTK LEIRRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQ SGNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK SFNRGEC	DLL3 LC
135	GGGGGS	Peptide Linker
136	IEPDI	Linker
137	LEADT	Linker
138	IEPDG	Linker
139	IEPDV	Linker
140	IEPDS	Linker
141	IEPDT	Linker
142	LEPD	Linker
143	LEAE	Linker
144	IEPDP	Linker
145	QAVVTQEPSLTVSPGGTVTLTCRSSTGAVTTSNYANWVQQKPGQAPRGLIG GTNKRAPWTPARFSGSLLGGKAALTITGAQAEDEADYYCALWYSNLWVFG GGTKLTVLGGGGSGGGGEVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGM HWVRQAPGKGLEWVAYISSDSSAIYYADTVKGRFTISRDNKNSLYLQMNS LRDEDTAVYYCGRGRENIIYYSRLDYWGQGTTTVTVSSGGCGGGKVAALKE KVAALKEKVAALKEKVAALKE	Second Polypeptide Chain of B7-H3 x CD3 Bispecific DART-A Diabody
146	DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNAKALPAIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFY PSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFS CSVMHEALHNRYTQKSLSLSPGK	Third Polypeptide Chain of B7-H3 x CD3 Bispecific DART-A Diabody
147	GGSGGGSGGGSGGGGS	Linker
148	TGGSGGGGIEPDIGSGGS	Linker
149	GGGGS	Linker
150	X ₁ X ₂ X ₃ X ₄ X ₅ (P ₄ P ₃ P ₂ P ₁ ↓ P ₁ ') X ₁ = I, L, Y, M, F, V, or A; (P ₄ = I, L, Y, M, F, V, or A) X ₂ = A, G, S, V, E, D, Q, N, or Y; (P ₃ = A, G, S, V, E, D, Q, N, or Y) X ₃ = H, P, A, V, G, S, or T; (P ₂ = H, P, A, V, G, S, or T) X ₄ = D or E; (P ₁ = D or E) X ₅ = I, L, Y, M, F, V, T, S, G or A (P ₁ ' = I, L, Y, M, F, V, T, S, G or A)	Linker consensus
151	X ₁ E X ₃ D X ₅ (P ₄ P ₃ P ₂ P ₁ ↓ P ₁ ') X ₁ = I or L; (P ₄ = I or L) (P ₃ = E) X ₃ = P or A; (P ₂ = P or A) X ₅ = I, V, T, S, or G (P ₁ ' = I, V, T, S, or G)	Linker consensus

152	LEPDG	Linker
153	LEADG	Linker
154	X ₁ QARX ₅ (P1QAR↓(A/V)) X ₁ = any amino acid; (P1 is any amino acid) X ₅ = A or V	Linker consensus
155	RQARX ₅ (RQAR(A/V)) X ₅ = A or V	Linker consensus
156	RQARV	Linker
157	X ₁ X ₂ X ₃ X ₄ (P ₃ P ₂ P ₁ ↓ P ₁ ') X ₁ = P, V or A; (P ₃ = P, V or A) X ₂ = Q or D; (P ₂ = Q or D) X ₃ = A or N; (P ₁ = A or N) X ₄ = L, I or M (P ₁ ' = L, I or M)	Linker consensus
158	PX ₂ X ₃ X ₄ (P ₃ P ₂ P ₁ ↓ P ₁ ') (P ₃ = P) X ₂ = Q or D; (P ₂ = Q or D) X ₃ = A or N; (P ₁ = A or N) X ₄ = L or I (P ₁ ' = L or I)	Linker consensus
159	GSGATNFSLLKQAGDVEENPGP	P2A
160	ATNFSLLKQAGDVEENPGP	P2A
161	QCTNYALLKLAGDVESNPGP	E2A
162	VKQTLNFDLLKLAGDVESNPGP	F2A
163	EGRGSLTTCGDVEENPGP	T2A
164	LEGGGEGRGSLTTCGDVEENPGPR	T2A
165	GGATCTGGAGCAACAACTTCTCACTACTCAAACAAGCAGGTGACGTGG AGGAGAATCCCGGACCC	P2A DNA
166	GSPAGLEADGSRQARVGS	Linker
167	EVQLVESGGGL VQPKGSLKLS CAASGFTFNT YAMNWVRQAP GKGLEWVARI RSKSNYYATY YADSVKDRFT ISRDDSQSM YLQMNLLKTE DTAMYXCVRQ WDYDVRAMNY WGQGTSTVTS S	anti-5T4 VH
168	DIVMTQSHIF MSTSVGDRVS ITCKASQD TAVAWYQQKP GQSPKLLIYW ASTRLTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCQQ YSSYPYTFGG GTKLEIK	anti-5T4 VL
169	DIQLTQSPSF LSASVGDRVT ITCKASQNVDTNVAWYQQKP GKAPKALIYS ASYRYSQVPS RFSGSGSGTD FTLTISLQPEDFATYYCQQ YNNYPFTFGQ GTKLEIKGGG SGGGGEVQLV ESGGGLVQPG GSLRLSCAAS GFTFSTYAMN WVRQAPGKGL EWVGRIRSKY NNYATYYADS VKDRFTISR DSKNSLYLQM NSLKTEDTAV YYCVRHGNFG NSYVSWFAYW GQGTLTVTSS GCGGGGEVAA LEKEVAALEK EVAALEKEVA ALEKGGGDKT HTPPCPAPE AAGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV KFNWYVDGVE VHNATKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP SREEMTKNQV SLWCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NHYTQKLSL SPGK	First Polypeptide Chain of B7-H3 x CD3 Bispecific DART-A Diabody
170	GGGGSGGGSGGGGS	Linker
171	GGG(GGS) _n wherein n is 0 to 10	Linker
172	(GGGGGS) _n wherein n is 1 to 4	Linker
173	(GGGGGS) _n wherein n is 1 to 10	Linker

174	Gly _x Xaa-Gly _y -Xaa-Gly _z Xaa is independently selected from A, V, L, I, M, F, W, P, G, S, T, C, Y, N, Q, K, R, H, D, or E x, y, and z are each integers in the range from 1-5	Linker
175	Gly-Gly-Gly-Xaa-Gly-Gly-Gly-Xaa-Gly-Gly-Gly Xaa is independently selected from A, V, L, I, M, F, W, P, G, S, T, C, Y, N, Q, K, R, H, D, or E	Linker
176	ATTGSSPGPT	Linker
177	GGGGG-C-GGGGG	Linker
178	(EAAAK) _n n=2-20	Linker
179	AS-(AP) _n -GT n=2-20	Linker
180	AS-(EAAAK) _n -GT n=2-20	Linker
181	(GGGGA) _n n=2-20	Linker
182	(PGGGS) _n n=2-20	Linker
183	(AGGGS) _n n=2-20	Linker
184	GG-(EGKSSGSGSESKST) _n -GG n=2-20	Linker
185	(SSSSG) _n n=1-9	Linker
186	SSSASASSA	Linker
187	GSPGSPG	Linker
188	QVQLQESGPG LVKPSSETLSL TCTVSGGSIS SYWWSWIRQP PGKGLEWIGY VYYSGTTNIN PSLKSRVTIS VDTSKNQFSL KLSSVTAADT AVYYCASIAY TGFYFDYWGQ GTLVTVSSGG GSGGGGSGG GGSEIVLTQS PGTLSLSPGE RVTLSRASQ RVNNNYLAWY QQRPGQAPRL LIYGASSRAT GIPDRFSGSG SGTDFTLTIS RLEPEDFAVY YCQQYDRSPL TFGGGTKLEI K	DLL3 scFv
189	QVQLVQSGAE VKKPGSSVKV SCKASGYAFS YSWINWVRQA PGQGLEWMGR IFPGDGDYDY NGKFKGRVTI TADKSTSTAY MELSSLRSED TAVYYCARNV FDGYWL VYWG QGTLTVSS	CD20 VH
190	DIVMTQTPLS LPVTPGEPAS ISCRSSKSL HSNIGITYLYW YLQKPGQSPQ LLIYQMSNLV SGVPDRFSGS GSGTDFTLKI SRVEAEDGVV YYCAQNLELP YTFGGGTKVE IKRTV	CD20 VL
191	QAVVTQEPSTVSPGGTVTLTCSSTGAVTTSNYANWVQKPGQAFRLIG GTNKRAPGVPARFSGSLGGKAALTISGAQPEDEADYYCALWYSNHWFVFG CGTKLTVL	anti-CD3 VL35 (CON)
192	GGGGG	linker
193	GGGGSGGGSGGGGS	linker
194	DKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVKHEDPEV KFNWYVDGVEVHNAKTKPREEEYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCDVSGFYPS DIAVEWESDGPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWEQGDVFSCS VMHEALHNHYTQKSLSLSPGK	Fc-Het-1
195	DKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVKHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSREQMTKNQVKLTCLVKGFPY SDIAVEWESNGQENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK	Fc-Het-2
196	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKGLEWVGR IRSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGDSYVSWFAYWGQGLTVTVSS	CD3-VH32

197	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKCLEWVGR IRSKYNNYATYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCVRH GNFGDSYVSWFAYWGQGLTVTVSS	CD3-VH33
198	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMNWVRQAPGKCLEWVAR IRSKYNNYATYYADTVKGRFTISRDDAKNTLYLQMSSLRAEDTAVYYCVR HGNFGDSYVSWFAYWGQGLTVTV	CD3-VH34
199	QAVVTQEPSTLVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGKSPRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNHWFVG GGTKLTVL	CD3-VL20
200	QAVVTQEPSTLVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGKSPRGLIG GTNKRAPGVPARFSGSLLGGKAALTISGAQPEDEADYYCALWYSNHWFVG CGTKLTVL	CD3-VL21
201	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSP	Knob Fc
202	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMRSRTPEVTCVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVSNNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSP	Hole Fc
203	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWKYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSP	Knob Fc
204	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMRSRTPEVTCVVDVSHEDPEVKF NWKYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSP	Hole Fc
205	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCS VMHEALHNRYTQKSLSLSP	Hole Fc
206	DKTHTCPPCPAPGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWKYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSV MHEALHNRYTQKSLSLSP	Hole Fc
207	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVVHEALHNHYTQKSLSLSP	Knob Fc
208	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVDVSHEDPEVKF WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSV HEALHNHYTQKSLSLSP	Knob Fc
209	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLYISRTPEVTCVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYP SDIAVEWESNGQPENNYKTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCS VVHEALHNRYTQKSLSLSP	Hole Fc

210	DKTHTCPPCPAPGGPSVFLFPPKPKDTLYISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKGFYPSDIA VEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSSVHH EALHNRYTQKSLSLSP	Hole Fc
211	GFTFNTYAMN	anti-CD3 VH CDR1
212	RIRSKYNNYATY	anti-CD3 VH CDR2
213	QVQLVQSGAE VKKPGSSVKV SCKASGYAFS YSWINWVRQA PGQGLEWMGR IFPGDGDY NGKFKGRVTI TADKSTSTAY MELSSLRSED TAVYYCARNV FDGYWL VYWG QGTLTVSSG GGGSGGGGSG GGGSDIVMTQ TPLSLPVTGP EPASISCRSS KSLLSHNGIT YLYWYLQKPG QSPQLLIYQM SNLVSGVPDR FSGSGSGTDF TLKISRVEAE DVGVIYCAQN LELPYTFGGG TKVEIK	CD20 scFv
214	EVQLVESGGGEVQPGGSLRLSCAASGFSFSSNVMMWVRQAPGKGLEWVSTIYSSG TGTFYAESVKGRFTISRDNANTLYLQMSSLRPEDTAVYYCATSGPVRGWGPRSQGT LVTVKP	B7H3 sdAb B7h3 hz1A5v51
215	EVQLVESGGGEVQPGGSLRLSCAASGTFSSYHMSWFRQAPGKQREPVATS HHGGTTNYAGSVKGRFTISRDNANTVYLQMNTLRAEDTAVYYCKADHG YQGRGYWGQGTLVTVKP	sdAb B7H3 hz58E05v27
216	EVQLVESGGGEVQPGGSLRLSCAASGTFSSYHMSWFRQAPGKQREL VATSHHGGT TNYAGSVKGRFTISRDNANTVYLQMNTLRAEDTAVYYCKADHG YQGRGYWGQGTL LVTVKP	sdAb B7H3 hz58E05v55
217	EVQLVESGGGEVQPGGSLRLSCAASGTFSSYHMSWFRQAPGKQREPVATS HHGGTTNYAGSVKGRFTISRDNANTVYLQMNTLRAEDTAVYYCKADHG YQGRGYWGQGTLVTVKP	sdAb B7H3 hz58E05v48
218	EVQLVESGGGEVQPGGSLRLSCAPSEITFTSTYTMGWFRQAPGKEREFVAVV NWGGGSKYYAESVKGRFTISRDNANTVYLQMSSLR AEDTAVYYCAAGG AYSGPYDTRQYTYWGQGTLVTVKPGG	sdAb B7H3 hz57B04v24
219	EVQLVESGGGEVQPGGSLRLSCAASGSIFSINAMGWYRQAPGKQREL VAGF TGDTNTIYAESVKGRFTISRDNANTVYLQMSSLR AEDTAVYYCAADVQLF SRDYEFYWGQGTLVTVKP	sdAb DLL3 hz10D9v7
220	EVQLVESGGGEVQPGGSLRLSCGPSEIITSDKSMGWVRQAPGKQRNLVAGIS NVGSTNYAQSVKGRFTISRDNANTVYLQMSSLR AEDTAVYYCYARDFEN EYWGQGTLVTVKP	sdAb DLL3 hz8E7v16
221	GFSFSINAMG	41BB CDR1
222	AIESGRNTV	41BB CDR2
223	LKGNRVVSPSVAY	41BB CDR3
224	HGNFGDSYVSWFAY	CD3-VH7, VH33 CDR3
225	ALWYSNHWV	CD3-VL2, VL21 CDR3
226	VLWYSNRWV	CD3-VL8 CDR3
227	GFTFSTYAMN	CD3 VH33 CDR1
228	RIRSKYNNYATY	CD3 VH33 CDR1
229	GSSTGAVTTSNYAN	CD3 VL21 CDR1
230	GTNKRAP	CD3 VL21 CDR2

CLAIMS

What is claimed is:

1. A multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein:

the CD3 binding region is an anti-CD3 antibody or antigen binding fragment that is an Fv antibody fragment comprising a variable heavy chain region (VH) and a variable light chain region (VL);

the Fc is a heterodimeric Fc comprising a first Fc polypeptide and a second Fc polypeptide and the VH and VL of the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc;

the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and

the first component comprises a first antigen binding domain and the second component comprises a second antigen binding domain, wherein each of the antigen binding domains bind a tumor associated antigen (TAA)..

2. The multispecific polypeptide construct of claim 1, wherein the CD3-binding region binds CD3 (CD3ε).

3. The multispecific polypeptide construct of claim 1 or claim 2, wherein the first antigen binding domain is positioned amino-terminally relative to the Fc region of the multispecific construct and the second antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific construct.

4. The multispecific polypeptide construct of any of claims 1-3, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

the first antigen binding domain that binds to a tumor-associated antigen (TAA);

the immunoglobulin Fc region;

the non-cleavable linker;

the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε); and

the second antigen binding domain that binds a tumor-associated antigen (TAA).

5. A multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein:

the CD3 binding region is an anti-CD3 antibody or antigen binding fragment that is a disulfide-stabilized Fv antibody fragment (dsFv) comprising a variable heavy chain (VH) and a variable light chain (VL);

the Fc is a heterodimeric Fc comprising a first Fc polypeptide and a second Fc polypeptide and the VH and VL of the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc;

the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and

one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA).

6. A multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising an immunoglobulin Fc region and a second component comprising a CD3-binding region, wherein:

the CD3 binding region is an anti-CD3 antibody or antigen binding fragment that is an Fv antibody fragment comprising a variable heavy chain (VH) and a variable light chain (VL);

the Fc is a heterodimeric Fc comprising a first Fc polypeptide and a second Fc polypeptide and the VH and VL of the anti-CD3 antibody or antigen binding fragment are linked to opposite polypeptides of the heterodimeric Fc;

the first and second components are coupled by a non-cleavable linker, wherein the Fc region is positioned N-terminal to the CD3-binding region; and

one or both of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA), wherein the antigen-binding domain is a single chain antibody fragment.

7. The multispecific polypeptide construct of claim 6, wherein the single chain antibody fragment is a single domain antibody or is a single chain variable fragment (scFv).

8. The multispecific polypeptide construct of any of claims 5-7, wherein the CD3-binding region binds CD3 (CD3ε).

9. The multispecific polypeptide construct of any of claims 5-8, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

the first antigen binding domain that binds to a tumor-associated antigen (TAA);

the immunoglobulin Fc region;

the non-cleavable linker;

the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε); and

the second antigen binding domain that binds a tumor-associated antigen (TAA).

10. The multispecific polypeptide construct of any of claims 5-8, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

the immunoglobulin Fc region;

the non-cleavable linker;

the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε); and

an antigen binding domain that binds a tumor-associated antigen (TAA).

11. The multispecific polypeptide construct of any of claims 5-8, wherein the multispecific construct comprises in order, from N-terminus to C-terminus:

the antigen binding domain that binds to a tumor-associated antigen (TAA);

the immunoglobulin Fc region;

the non-cleavable linker; and

the CD3 binding region, wherein the CD3 binding region binds CD3 (CD3ε).

12. The multispecific polypeptide construct of any of claims 1-11, wherein one or both of the first and second Fc polypeptides of the heterodimeric Fc region are a variant Fc polypeptide comprising at least one modification to induce heterodimerization compared to an Fc region of human IgG1, human IgG2 or human IgG4.

13. The multispecific polypeptide construct of any of claims 1-12, wherein one or both of the first and second Fc polypeptides of the heterodimeric Fc region are a variant Fc polypeptide comprising at least one modification to induce heterodimerization compared to

an Fc region of human IgG1, optionally compared to the Fc polypeptide set forth in SEQ ID NO:1 or an immunologically active fragment thereof .

14. The multispecific polypeptide construct of claim 12 or claim 13, wherein each of the Fc polypeptides of the heterodimeric Fc independently comprise at least one amino acid modification.

15. The multispecific polypeptide construct of any of claims 12-14, wherein the at least one modification is selected from a steric modification(s), a knob-into-hole modification(s), a charge mutation(s) to increase electrostatic complementarity of the polypeptides, a modification(s) to alter the isoelectric point (pI variant), or combinations thereof.

16. The multispecific polypeptide construct of any of claims 1-15, wherein the first Fc polypeptide of the heterodimeric Fc comprises the modification selected from among Thr366Ser, Leu368Ala, Tyr407Val, and combinations thereof and the second Fc polypeptide of the heterodimeric Fc comprises the modification T366W.

17. The multispecific polypeptide construct of claim 16, wherein the first and second Fc polypeptides further comprises a modification of a non-cysteine residue to a cysteine residue, wherein the modification of the first polypeptide is at one of the position Ser354 and Y349 and the modification of the second Fc polypeptide is at the other of the position Ser354 and Y349.

18. The multispecific polypeptide construct of any of claims 1-17, wherein the first Fc polypeptide comprises the modifications T366W/S354C and the second Fc polypeptide comprises the modifications T366S/L368A/Y407V/Y349C.

19. The multispecific polypeptide construct of any of claims 1-15, wherein the first Fc polypeptide comprises the modifications L368D/K370S and the second Fc polypeptide comprises the modifications S364K/E357Q.

20. The multispecific polypeptide construct of any of claims 1-15, wherein at least one of the first and second polypeptide comprises the modifications Q295E/N384D/Q418E/N421D.

21. The multispecific polypeptide construct of any of claims 1-20, wherein:
one of the first or second Fc polypeptide of the heterodimeric Fc further comprises a modification at residue Ile253, optionally wherein the modification is Ile253Arg; and/or one of the first or second Fc polypeptide of the heterodimeric Fc further comprises a modification at residue His435, optionally wherein the modification is His35Arg.

22. The multispecific polypeptide construct of any of claims 1-21, wherein one or both of the first and second Fc polypeptide comprises a polypeptide that lacks Lys447.

23. The multispecific polypeptide construct of any of claims 1-22, wherein the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:82, 86 or 201, and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:83, 87, 90, 92, 202 or 205.

24. The multispecific polypeptide construct of any of claims 1-23, wherein the first Fc polypeptide and the second Fc polypeptide comprises sequences selected from the group consisting of SEQ ID NOS: 82 and 83, respectively; SEQ ID NOS: 86 and 87, respectively; SEQ ID NOS: 201 and 202, respectively; SEQ ID NOS: 82 and 90, respectively; SEQ ID NOS: 86 and 92, respectively; and SEQ ID NOS: 201 and 205, respectively.

25. The multispecific polypeptide construct of any of claims 1-22, wherein the Fc region comprises a polypeptide comprising at least one modification to enhance FcRn binding.

26. The multispecific fusion polypeptide construct of claim 25, wherein the modification is at a position selected from the group consisting of Met252Y, Ser254T, Thr256E, Met428L, Met428V, Asn434S, and combinations thereof.

27. The multispecific fusion polypeptide construct of claim 25 or claim 26, wherein the modification is Met252Y and Met428L or is Met252Y and Met428V.

28. The multispecific polypeptide construct of any of claims 1-22 and 25-27, wherein the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 94, 96 or 207, and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 98, 100, or 209.

29. The multispecific polypeptide construct of any of claims 1-28, wherein the first Fc polypeptide and the second Fc polypeptide comprises sequences selected from the group consisting of SEQ ID NOS: 94 and 98, respectively; SEQ ID NOS: 96 and 100, respectively; and SEQ ID NOS: 207 and 209, respectively.

30. The multispecific polypeptide construct of any of claims 1-22 and 25-27, wherein the Fc region comprises a polypeptide comprising at least one amino acid modification that reduces effector function and/or reduces binding to an effector molecule selected from an Fc gamma receptor or C1q.

31. The multispecific polypeptide construct of claim 30, wherein the one or more amino acid modification is deletion of one or more of Glu233, Leu234 or Leu235.

32. The multispecific polypeptide construct of any of claims 1-22, 25-27, 30 and 31, wherein the first polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 84, 88, 95, 97, 203 or 208 and the second polypeptide of the heterodimeric Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS: 85, 89, 91, 93, 99, 101, 204, 206 or 210.

33. The multispecific polypeptide construct of any of claims 1-22, 25-27 and 30-32, wherein the first Fc polypeptide and the second Fc polypeptide comprises sequences selected from the group consisting of SEQ ID NOS: 84 and 85, respectively; SEQ ID NOS: 88 and 89, respectively; SEQ ID NOS: 203 and 204, respectively; SEQ ID NOS: 95 and 99, respectively; SEQ ID NOS: 97 and 101, respectively; SEQ ID NOS: 208 and 210,

respectively; SEQ ID NOS: 84 and 91, respectively; SEQ ID NOS: 88 and 93, respectively; and SEQ ID NOS: 203 and 206, respectively.

34. The multispecific polypeptide construct of any of claims 1-33, wherein the CD3 binding region is not able to, or is not substantially able to, bind or engage CD3 unless at least one of the antigen binding domain is bound to its TAA.

35. The multispecific polypeptide construct of any of claims 1-33, wherein the CD3 binding region is not able to, or is not substantially able, to bind or engage CD3 unless at least two of the antigen binding domain is bound to its TAA.

36. The multispecific polypeptide construct of any of claims 1-35, wherein the linker is a polypeptide linker.

37. The multispecific polypeptide construct of claim 36, wherein the linker is a polypeptide of up to 25 amino acids in length.

38. The multispecific polypeptide construct of claim 36 or claim 37, wherein the linker is a polypeptide of from or from about 2 to 24 amino acids, 2 to 20 amino acids, 2 to 18 amino acids, 2 to 14 amino acids, 2 to 12 amino acids, 2 to 10 amino acids, 2 to 8 amino acids, 2 to 6 amino acids, 6 to 24 amino acids, 6 to 20 amino acids, 6 to 18 amino acids, 6 to 14 amino acids, 6 to 12 amino acids, 6 to 10 amino acids, 6 to 8 amino acids, 8 to 24 amino acids, 8 to 20 amino acids, 8 to 18 amino acids, 8 to 14 amino acids, 8 to 12 amino acids, 8 to 10 amino acids, 10 to 24 amino acids, 10 to 20 amino acids, 10 to 18 amino acids, 10 to 14 amino acids, 10 to 12 amino acids, 12 to 24 amino acids, 12 to 20 amino acids, 12 to 18 amino acids, 12 to 14 amino acids, 14 to 24 amino acids, 14 to 20 amino acids, 14 to 18 amino acids, 18 to 24 amino acids, 18 to 20 amino acids or 20 to 24 amino acids.

39. The multispecific polypeptide construct of any of claims 36-38, wherein the linker is a polypeptide that is 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length.

40. The multispecific polypeptide construct of any of claims 36-39, wherein the linker is a polypeptide that is 3 to 18 amino acids in length.

41. The multispecific polypeptide construct of any of claims 36-40, wherein the linker is a polypeptide that is 12 to 18 amino acids in length.

42. The multispecific polypeptide construction of any of claims 36-40, wherein the linker is a polypeptide that is 15 to 18 amino acids in length.

43. The multispecific polypeptide construct of any of claims 1-42, wherein the non-cleavable linker comprises GS, GGS, GGGGS (SEQ ID NO:149), GGGGGS (SEQ ID NO:135) and combinations thereof.

44. The multispecific polypeptide construct of any of claims 1-43, wherein the non-cleavable linker comprises (GGS)_n, wherein n is 1 to 10.

45. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker comprises (GGGGS)_n (SEQ ID NO: 173), wherein n is 1 to 10.

46. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker comprises (GGGGGS)_n (SEQ ID NO:172), wherein n is 1 to 4.

47. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker is or comprises GGS.

48. The multispecific polypeptide construct of any of claims 1-43 and 45, wherein the non-cleavable linker is or comprises GGGGS (SEQ ID NO: 149).

49. The multispecific polypeptide construct of any of claims 1-43 and 46, wherein the non-cleavable linker is or comprises GGGGGS (SEQ ID NO: 135).

50. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker is or comprises (GGS)₂ (SEQ ID NO: 10).

51. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker is or comprises GGS GGSGSGGS (SEQ ID NO: 11).

52. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker is or comprises GSGSGSGSGSGS (SEQ ID NO: 12).

53. The multispecific polypeptide construct of any of claims 1-44, wherein the non-cleavable linker is or comprises GSGSGSGSGSGSGS (SEQ ID NO: 13).

54. The multispecific polypeptide construct of any of claims 1-43 and 46, wherein the non-cleavable linker is or comprises GGGGSGGGGSGGGGGS (SEQ ID NO: 119).

55. The multispecific polypeptide construct of any of claims 1-49, wherein the non-cleavable linker is or comprises GSGGGGSGGGGSGGGGS (SEQ ID NO: 147)

56. The multispecific polypeptide construct of any of claims 1-43 and 45, wherein the non-cleavable linker is or comprises and GGGGSGGGGSGGGGS (SEQ ID NO:170).

57. The multispecific polypeptide construct of any of claims 1-56, wherein the multispecific polypeptide construct comprises at least (i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment thereof; and (ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment thereof, wherein one or both of the first and second polypeptide comprise at least one antigen-binding domain that binds to a tumor associated antigen (TAA).

58. The multispecific polypeptide construct of any of claims 1-57, wherein the VH of the anti-CD3 antibody or antigen-binding fragment is on the same polypeptide as the at least one antigen-binding domain that binds to a tumor associated antigen (TAA).

59. The multispecific polypeptide construct of claim 58, wherein the polypeptide comprising the VL of the anti-CD3 antibody or antigen-binding fragment does not contain the at least one antigen-binding domain that binds to a tumor associated antigen (TAA).

60. The multispecific polypeptide construct of any of claims 1-4, 9 and 12-59, wherein the multispecific polypeptide construct exhibits bivalent, trivalent, or tetravalent binding to the TAA.

61. The multispecific polypeptide construct of any of claims 5-8 and 10-59, wherein the multispecific polypeptide construct exhibits monovalent, bivalent, trivalent, or tetravalent binding to the TAA.

62. The multispecific polypeptide construct of any of claims 1-61, wherein only one of the first and second polypeptide comprises the at least one antigen-binding domain that binds a TAA.

63. The multispecific polypeptide construct of any of claims 1-62, wherein the at least one antigen binding domain is positioned amino-terminally relative to the Fc region and/or is positioned carboxy-terminally relative to the CD3 binding region of one of the first or second polypeptide of the multispecific polypeptide construct.

64. The multispecific polypeptide construct of any of claims 1-62, wherein the at least one antigen binding domain is positioned amino-terminally relative to the Fc region of the multispecific construct and the second antigen binding domain is positioned carboxy-terminally relative to the CD3 binding region of the multispecific construct.

65. The multispecific polypeptide construct of any of claims 1-64, wherein the antigen binding domain, or independently each of the antigen binding domains, comprises an extracellular domain or binding fragment thereof of the native cognate binding partner of the TAA, or a variant thereof that exhibits binding activity to the TAA.

66. The multispecific polypeptide construct of any of claims 1-5 and 8-65, wherein the antigen binding domain, or independently each of the antigen binding domains, is an antibody or antigen-binding fragment thereof selected from the group consisting of a Fab fragment, a F(ab')₂ fragment, an Fv fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody.

67. The multispecific polypeptide construct of claim 66, wherein the antibody or antigen-binding fragment thereof is a Fv, a scFv, a Fab, or a single domain antibody (sdAb).

68. The multispecific polypeptide construct of any of claims 1-67, wherein the antibody or antigen-binding fragment thereof is an sdAb.

69. The multispecific polypeptide construct of claim 68, wherein the sdAb is a human or humanized sdAb.

70. The multispecific polypeptide construct of claim 68 or claim 69, wherein the sdAb is VHH, VNAR, an engineered VH domain or an engineered VK domain.

71. The multispecific polypeptide construct of any of claims 1-67, wherein the antibody or antigen-binding fragment thereof is an scFv.

72. The multispecific polypeptide construct of any of claims 1-5 and 8-67, wherein the antibody or antigen-binding fragment thereof is a Fab.

73. The multispecific polypeptide construct of claim 72, wherein the multispecific polypeptide construct comprises:

(i) a first polypeptide comprising the first Fc polypeptide of the heterodimeric Fc region, the linker and the VH domain of the anti-CD3 antibody or antigen binding fragment; (ii) a second polypeptide comprising the second Fc polypeptide of the heterodimeric Fc region, the linker and the VL domain of the anti-CD3 antibody or antigen binding fragment, and (iii) a third polypeptide comprising a VH-CH1 (Fd) or VL-CL of a Fab antibody fragment that binds to a tumor-associated antigen, wherein the first and/or second polypeptide further comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment.

74. The multispecific polypeptide construct of claim 73, wherein only one of the first and second polypeptide comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment.

75. The multispecific polypeptide construct of claim 73, wherein both the first and second polypeptide comprises the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment.

76. The multispecific polypeptide construct of claim 74 or claim 75, wherein the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment is positioned amino-terminally relative to the Fc region and/or at the carboxy-terminally relative to the CD3 binding region of one of the first and second polypeptide of the multispecific polypeptide construct.

77. The multispecific polypeptide construct of any of claims 74-76, wherein the other of the VH-CH1 (Fd) or VL-CL of the Fab antibody fragment is positioned amino-terminally relative to the Fc region of the first polypeptide or second polypeptide and carboxy-terminally relative to the CD3 binding region of the other of the first or second polypeptide.

78. The multispecific polypeptide construct of any of claims 1-77, wherein the antigen binding domain, or independently each of the antigen binding domains, binds to a tumor antigen selected from among 1-92-LFA-3, 5T4, Alpha-4 integrin, Alpha-V integrin, alpha4beta1 integrin, alpha4beta7 integrin, AGR2, Anti-Lewis-Y, Apelin J receptor, APRIL, B7-H3, B7-H4, BAFF, BTLA, C5 complement, C-242, CA9, CA19-9, (Lewis a), Carbonic anhydrase 9, CD2, CD3, CD6, CD9, CD11a, CD19, CD20, CD22, CD24, CD25, CD27, CD28, CD30, CD33, CD38, CD40, CD40L, CD41, CD44, CD44v6, CD47, CD51, CD52, CD56, CD64, CD70, CD71, CD74, CD80, CD81, CD86, CD95, CD117, CD123, CD125, CD132, (IL-2RG), CD133, CD137, CD138, CD166, CD172A, CD248, CDH6, CEACAM5 (CEA), CEACAM6 (NCA-90), CLAUDIN-3, CLAUDIN-4, cMet, Collagen, Cripto, CSFR, CSFR-1, CTLA-4, CTGF, CXCL10, CXCL13, CXCR1, CXCR2, CXCR4, CYR61, DL44, DLK1, DLL3, DLL4, DPP-4, DSG1, EDA, EDB, EGFR, EGFRviii, Endothelin B receptor (ETBR), ENPP3, EpCAM, EPHA2, EPHB2, ERBB3, F protein of RSV, FAP, FGF-2, FGF8, FGFR1, FGFR2, FGFR3, FGFR4, FLT-3, Folate receptor alpha (FR α), GAL3ST1, G-CSF, G-CSFR, GD2, GTR, GLUT1, GLUT4, GM-CSF, GM-CSFR, GP IIb/IIIa receptors, Gp130, GPIIB/IIIA, GPNMB, GRP78, HER2/neu, HER3, HER4, HGF, hGH, HVEM, Hyaluronidase, ICOS, IFN α , IFN β , IFN γ , IgE, IgE Receptor (FceRI), IGF,

IGF1R, IL1B, IL1R, IL2, IL11, IL12, IL12p40, IL-12R, IL-12Rbeta1, IL13, IL13R, IL15, IL17, IL18, IL21, IL23, IL23R, IL27/IL27R (wsx1), IL29, IL-31R, IL31/IL31R, IL2R, IL4, IL4R, IL6, IL6R, Insulin Receptor, Jagged Ligands, Jagged 1, Jagged 2, KISS1-R, LAG-3, LIF-R, Lewis X, LIGHT, LRP4, LRRC26, Ly6G6D, LyPD1, MCSP, Mesothelin, MRP4, MUC1, Mucin-16 (MUC16, CA-125), Na/K ATPase, NGF, Nicastrin, Notch Receptors, Notch 1, Notch 2, Notch 3, Notch 4, NOV, OSM-R, OX-40, PAR2, PDGF-AA, PDGF-BB, PDGFRalpha, PDGFRbeta, PD-1, PD-L1, PD-L2, Phosphatidyl-serine, P1GF, PSCA, PSMA, PSGR, RAAG12, RAGE, SLC44A4, Sphingosine 1 Phosphate, STEAP1, STEAP2, TAG-72, TAPA1, TEM-8, TGFbeta, TIGIT, TIM-3, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TMEM31, TNFalpha, TNFR, TNFRS12A, TRAIL-R1, TRAIL-R2, Transferrin, Transferrin receptor, TRK-A, TRK-B, uPAR, VAP1, VCAM-1, VEGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, VISTA, WISP-1, WISP-2, and WISP-3.

79. The multispecific polypeptide construct of any of claims 1-78, wherein the multispecific polypeptide construct comprises at least a first antigen binding domain and a second antigen binding domain, wherein the first antigen binding domain and second antigen binding domain bind to the same TAA.

80. The multispecific polypeptide construct of claim 79, wherein the first antigen binding domain and the second antigen binding domain binds different epitopes of the same TAA.

81. The multispecific polypeptide construct of claim 79, wherein the first antigen binding domain and the second antigen binding domain bind the same epitope of the same TAA.

82. The multispecific polypeptide construct of any of claims 1-78, wherein multispecific polypeptide construct comprises at least a first antigen binding domain and a second antigen binding domain wherein the first antigen binding domain and the second antigen binding domain bind a different TAA.

83. The multispecific polypeptide construct of any of claims 1-82, wherein:
the anti-CD3 antibody or antigen-binding fragment thereof comprises a VH CDR1 comprising the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CD2

comprising the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 comprising the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 comprising the amino acid sequence RSSTGAVTTSNYAN (SEQ ID NO: 19); a VL CDR2 comprising the amino acid sequence GTNKRAP (SEQ ID NO: 20); and a VL CDR3 comprising the amino acid sequence ALWYSNLWV (SEQ ID NO: 21); or

the anti-CD3 antibody or antigen-binding fragment comprises a VH CDR1 sequence that includes at least the amino acid sequence GFTFNTYAMN (SEQ ID NO: 211); a VH CDR2 sequence that includes at least the amino acid sequence RIRSKYNNYATY (SEQ ID NO: 212); a VH CDR3 sequence that includes at least the amino acid sequence HGNFGNSYVSWFAY (SEQ ID NO: 18), a VL CDR1 sequence that includes at least the amino acid sequence GSSTGAVTTSNYAN (SEQ ID NO: 229); a VL CDR2 sequence that includes at least the amino acid sequence GTNKRAP (SEQ ID NO: 230); and a VL CDR3 sequence that includes at least the amino acid sequence ALWYSNHWV (SEQ ID NO: 225).

84. The multispecific polypeptide construct of any of claims 1-4 and 6-83, wherein the anti-CD3 Fv comprises:

a VH having the amino acid sequence of any of SEQ ID NOS: 14, 32-43, 45-47, 48, 196 and 211 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 14, 32-43, 45-47, 48, 196 and 211; and

a VL having the amino acid sequence of any of SEQ ID NOS: 15, 63, 65-71, 73, 75, 77, and 199 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 15, 63, 65-71, 73, 75, 77, and 199.

85. The multispecific polypeptide construct of any of claims 1-4 and 6-84, wherein the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 14 and the amino acid sequence of SEQ ID NO: 15.

86. The multispecific polypeptide construction of any of claims 1-4 and 6-84, wherein the anti-CD3 Fv comprises the amino acid sequence of SEQ ID NO: 196 and the amino acid sequence of SEQ ID NO: 199.

87. The multispecific polypeptide construct of any of claims 1-4 and 6-83, wherein the Fv antibody fragment comprises a disulfide stabilized anti-CD3 binding Fv fragment (dsFv).

88. The multispecific polypeptide construct of claim 5 or claim 87, wherein the disulfide stabilized anti-CD3 binding Fv fragment (dsFv) comprises a VH chain with the mutation G44C and a VL chain with the mutation G100C, by Kabat numbering.

89. The multispecific polypeptide construct of any of claims 5, 87 and 88, wherein the anti-CD3 Fv comprises:

a VH having the amino acid sequence of any of SEQ ID NOS: 44 and 49-62, 197 and 198 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 44 and 49-62, 197 and 198; and

a VL having the amino acid sequence of any of SEQ ID NOS: 64, 72, 74, 76, 78-81, 191, 200 and 212 or a sequence that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 64, 72, 74, 76, 78-81, 191, 200 and 212.

90. The multispecific polypeptide construct of any of claims 5 and 87-89, wherein the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 44 and the amino acid sequence of SEQ ID NO: 72.

91. The multispecific polypeptide construct of any of claims 5 and 87-89, wherein the anti-CD3 dsFv comprises the amino acid sequence of SEQ ID NO: 198 and the amino acid sequence of SEQ ID NO: 200 or comprises the amino acid sequence of SEQ ID NO: 197 and the amino acid sequence of SEQ ID NO: 200.

92. The multispecific polypeptide construct of any of claims 1-91, wherein the multispecific polypeptide construct is conjugated to an agent.

93. The multispecific polypeptide construct of claim 92, wherein the agent is a therapeutic agent, an antineoplastic agent, a toxin or fragment thereof, a detectable moiety or a diagnostic agent.

94. The multispecific polypeptide construct of claim 93, wherein the agent is conjugated to the multispecific polypeptide construct via a linker.

95. A polynucleotide(s) encoding the mutispecific polypeptide constructs of any of claims 1-94.

96. A polynucleotide encoding a polypeptide chain of any of the mutispecific polypeptide constructs of any of claims 1-94.

97. A polynucleotide, comprising a first nucleic acid sequence encoding a first polypeptide of a mutispecific polypeptide construct of any of claims 1-94 and a second nucleic acid sequence encoding a second polypeptide of the mutispecific polypeptide construct, wherein the first and second nucleic acid sequence are separated by an internal ribosome entry site (IRES), or a nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping.

98. The polynucleotide of claim 97, wherein the first nucleic acid sequence and second nucleic acid sequence are operably linked to the same promoter.

99. The polynucleotide of claim 97 or claim 98, wherein the multispecific polypeptide construct comprises a third polypeptide chain, and the polynucleotide further comprises a third nucleic acid encoding the third polypeptide of the mutispecific polypeptide construct.

100. The polynucleotide of claim 99, wherein the third nucleic acid is separated from the first and/or second polypeptide by an internal ribosome entry site (IRES), or a nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping and/or the third nucleic acid sequence is operably linked to the same promoter as the first and/or second nucleic acid sequence.

101. The polynucleotide of any of claims 97-100, wherein the nucleic acid encoding a self-cleaving peptide or a peptide that causes ribosome skipping is selected from a T2A, a P2A, a E2A or a F2A.

102. A vector, comprising the polynucleotide of any of claims 95-101.
103. The vector of claim 102 that is an expression vector.
104. The vector of claim 102 or 103 that is a viral vector or a eukaryotic vector, optionally wherein the eukaryotic vector is a mammalian vector.
105. A cell, comprising polynucleotide or polynucleotides of any of claims 95-101, or a vector or vectors of any of claims 102-104.
106. The cell of claim 105, wherein the cell is recombinant or isolated.
107. The cell of claim 105 or claim 106, wherein the cell is a mammalian cell.
108. The cell of claim 107, wherein the cell is a HEK293 or CHO cell.
109. A method of producing a multispecific polypeptide construct, the method comprising introducing into a cell a polynucleotide or polynucleotides of any of claims 95-101 or a vector or vectors of any of claims 102-104 and culturing the cell under conditions to produce the multispecific polypeptide construct.
110. A method of producing a multispecific polypeptide construct, the method comprising culturing the cell of any of claims 105-108 under conditions in which the multispecific polypeptide is produced by the cell.
111. The method of claim 109 or claim 110, further comprising isolating or purifying the multispecific polypeptide construct from the cell.
112. The method of any of claims 109-111, wherein the multispecific polypeptide construct is a heterodimer.
113. A multispecific polypeptide construct produced by the method of any of claims 109-112.

114. A pharmaceutical composition comprising the multispecific polypeptide construct of any of claims 1-94 or claim 113 and a pharmaceutically acceptable carrier.

115. The pharmaceutical composition of claim 114 that is sterile.

116. A method of stimulating or inducing an immune response, the method comprising contacting a target cell and a T cell with the multispecific polypeptide construct of any of claims 1-94 or claim 113 or the pharmaceutical composition of claims 114 or claim 115, said target cell expressing a tumor associated antigen recognized by the multispecific polypeptide construct.

117. The method of claim 116, wherein the target cell is a tumor cell expressing the tumor associated antigen (TAA).

118. The method of claim 116 or claim 117, wherein the contacting is carried out ex vivo or in vitro.

119. The method of any of claims 116-118, wherein the contacting is carried out in vivo in a subject.

120. A method of stimulating or inducing an immune response in a subject, the method comprising administering, to a subject in need thereof, a therapeutically effective amount of the multispecific polypeptide construct of any of claims 1-94 or claim 113 or the pharmaceutical composition of claims 114 or claim 115.

121. The method of any of claims 116-120, which increases cell-mediated immunity.

122. The method of any of claims 116-121, which increases T-cell activity.

123. The method of any of claims 116-122, which increases cytolytic T-cell (CTL) activity.

124. The method of any of claims 116-123, wherein the immune response is increased against a tumor or cancer.

125. The method of any of claims 116-124, wherein the method treats a disease or condition in the subject.

126. A method of treating a disease or condition in a subject, the method comprising administering, to a subject in need thereof, a therapeutically effective amount of the multispecific conjugate of any of claims 1-94 or claim 113 or the pharmaceutical composition of claims 114 or claim 115.

127. The method of claim 125 or claim 126, wherein the disease or condition is a tumor or a cancer.

128. The method of any of claims 119-127, wherein said subject is a human.

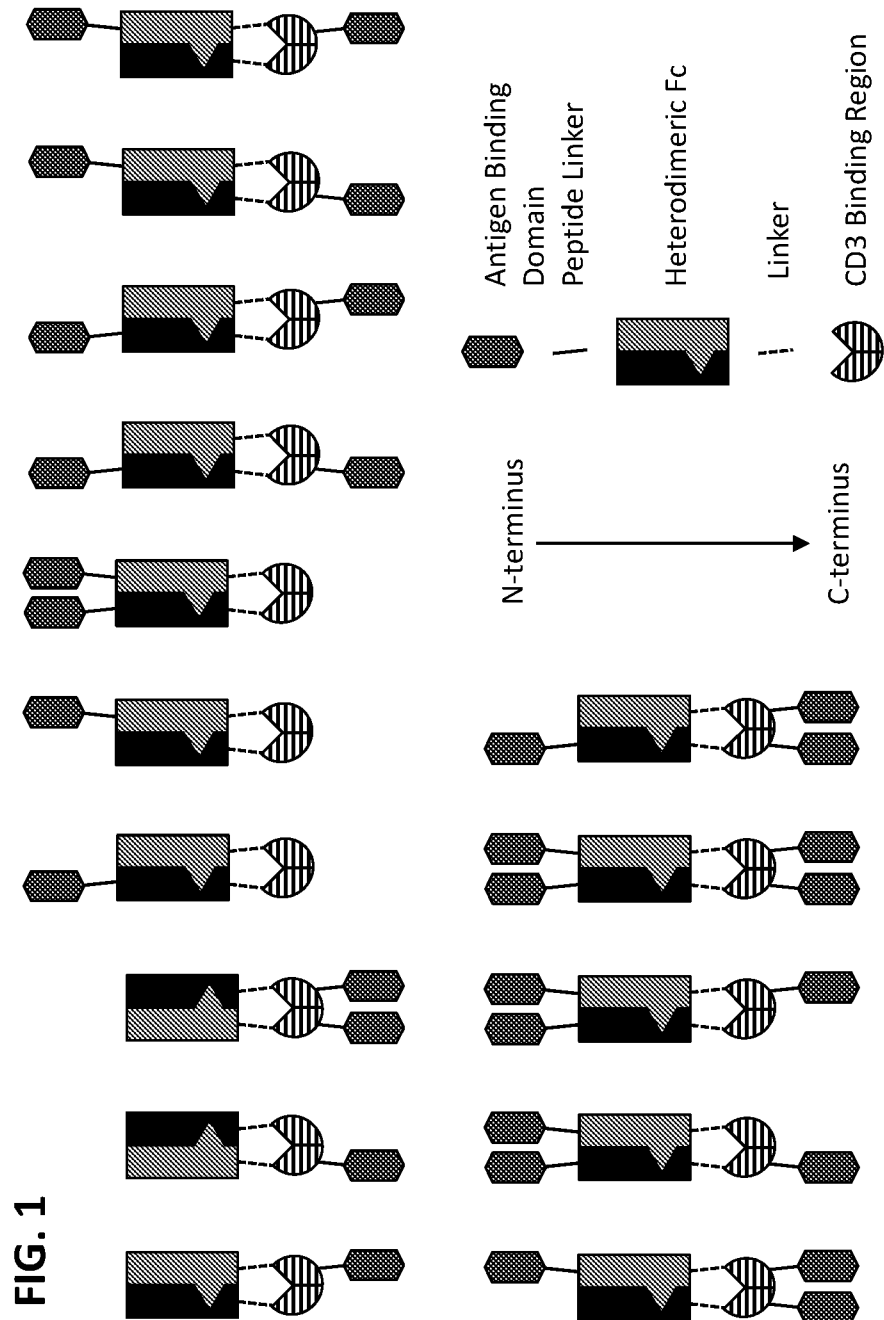


FIG. 2A

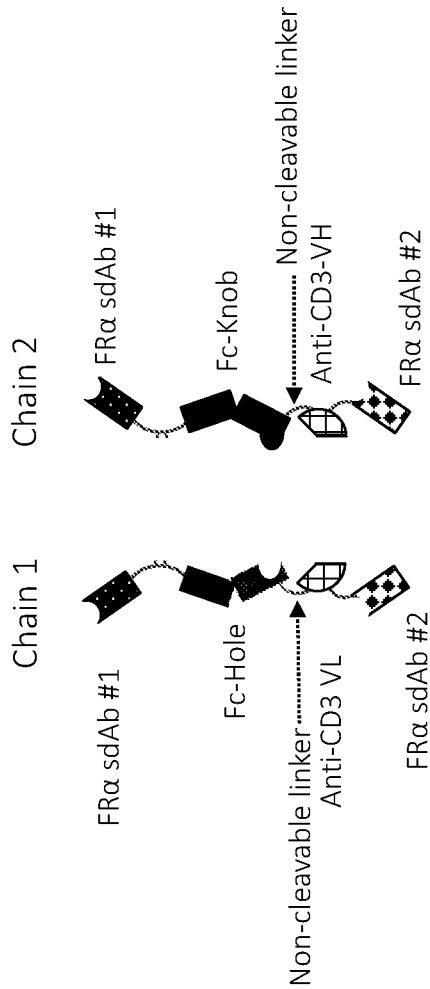


FIG. 2B

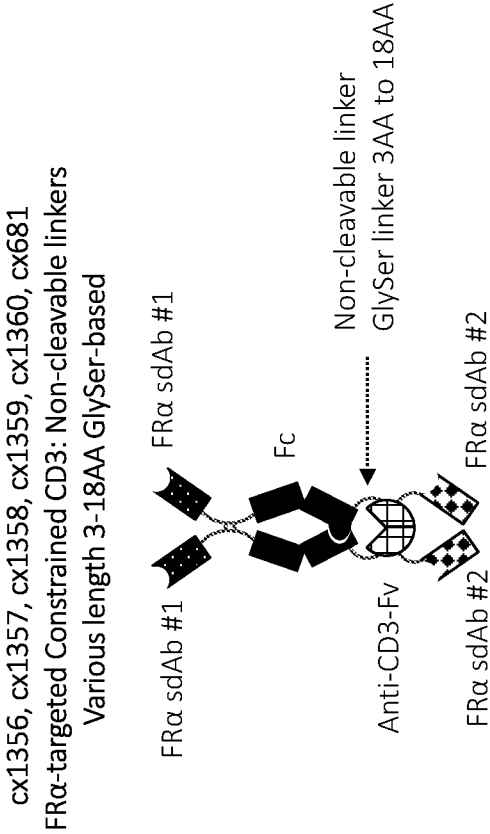


FIG. 3A

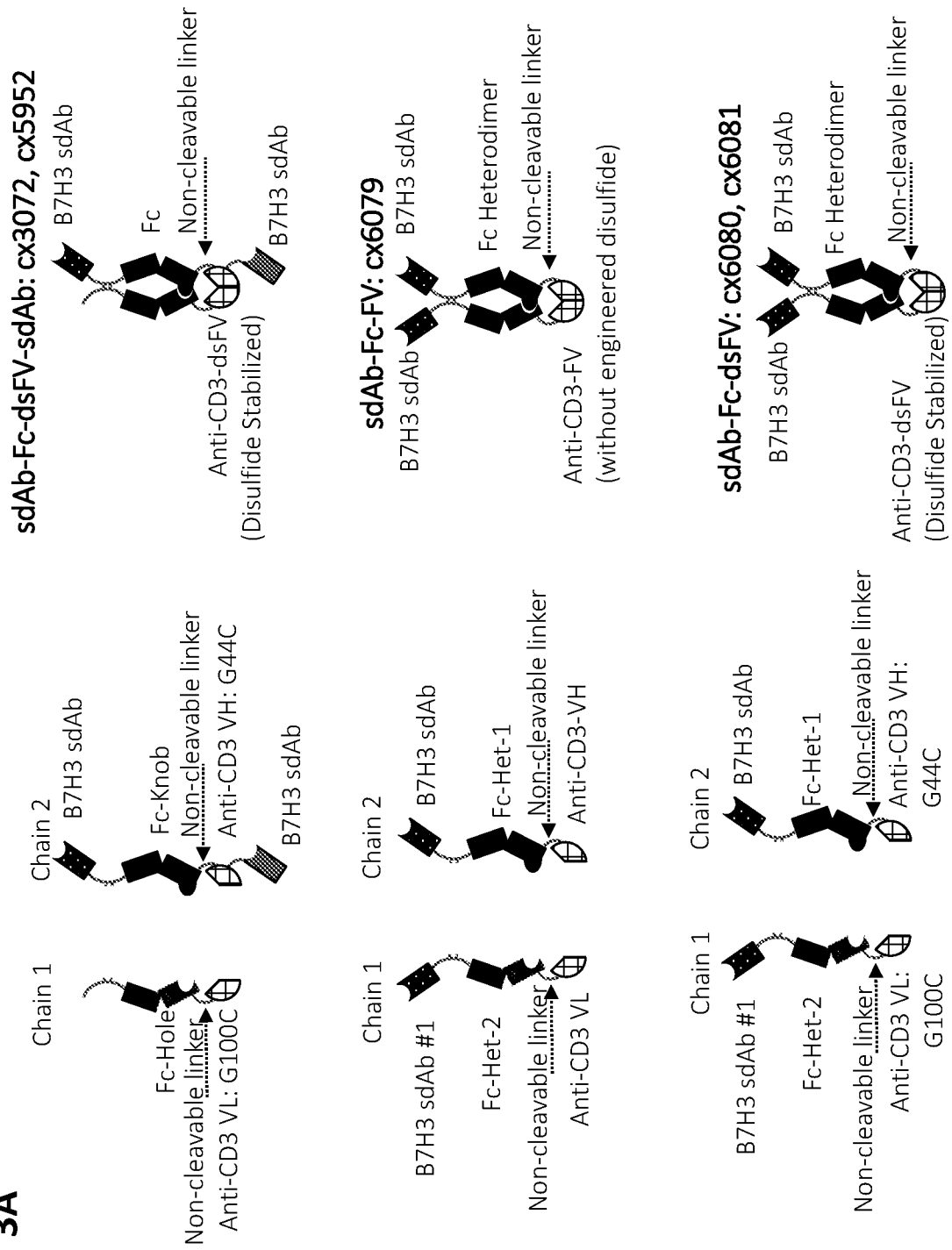
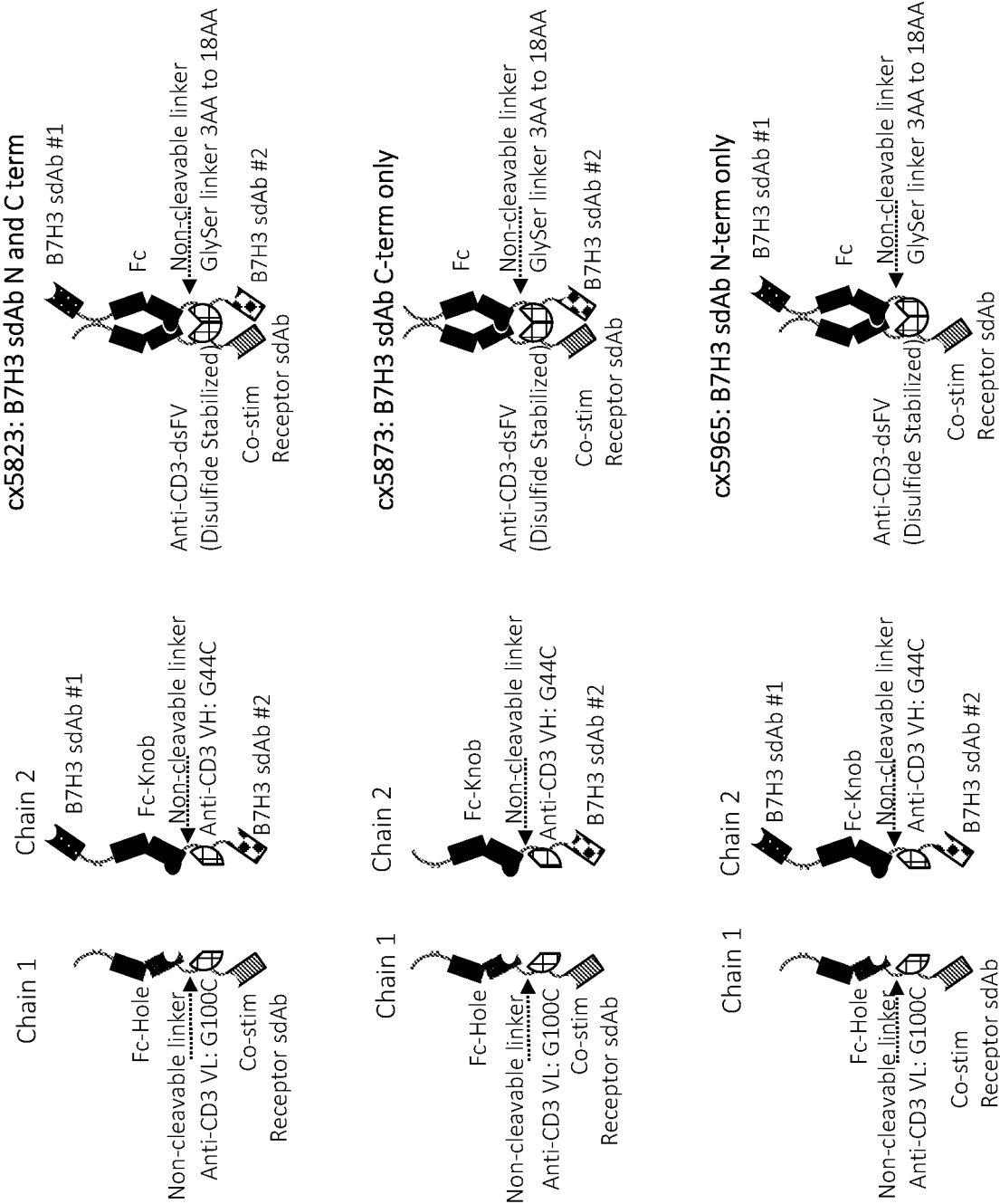


FIG. 3B



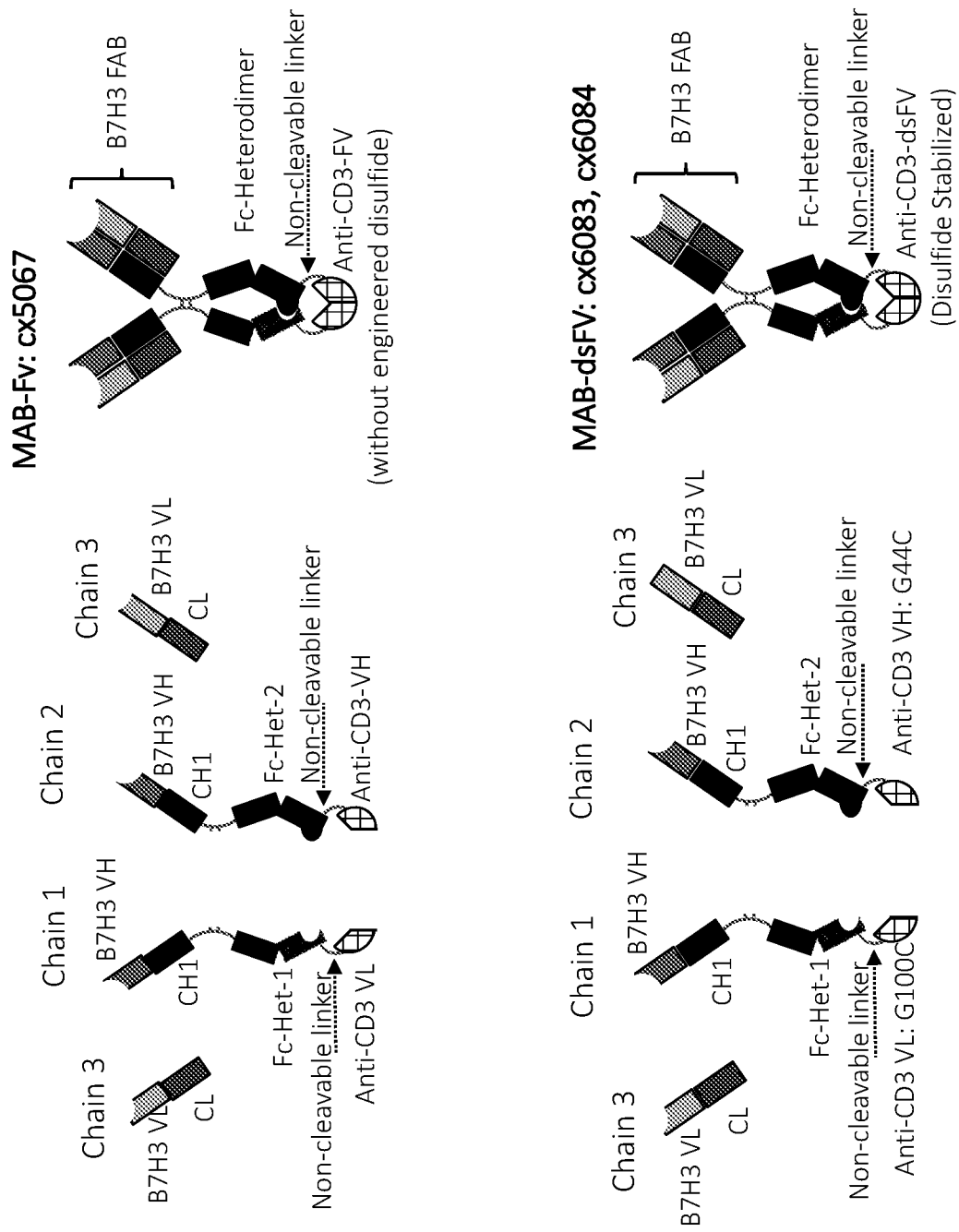


FIG. 4A

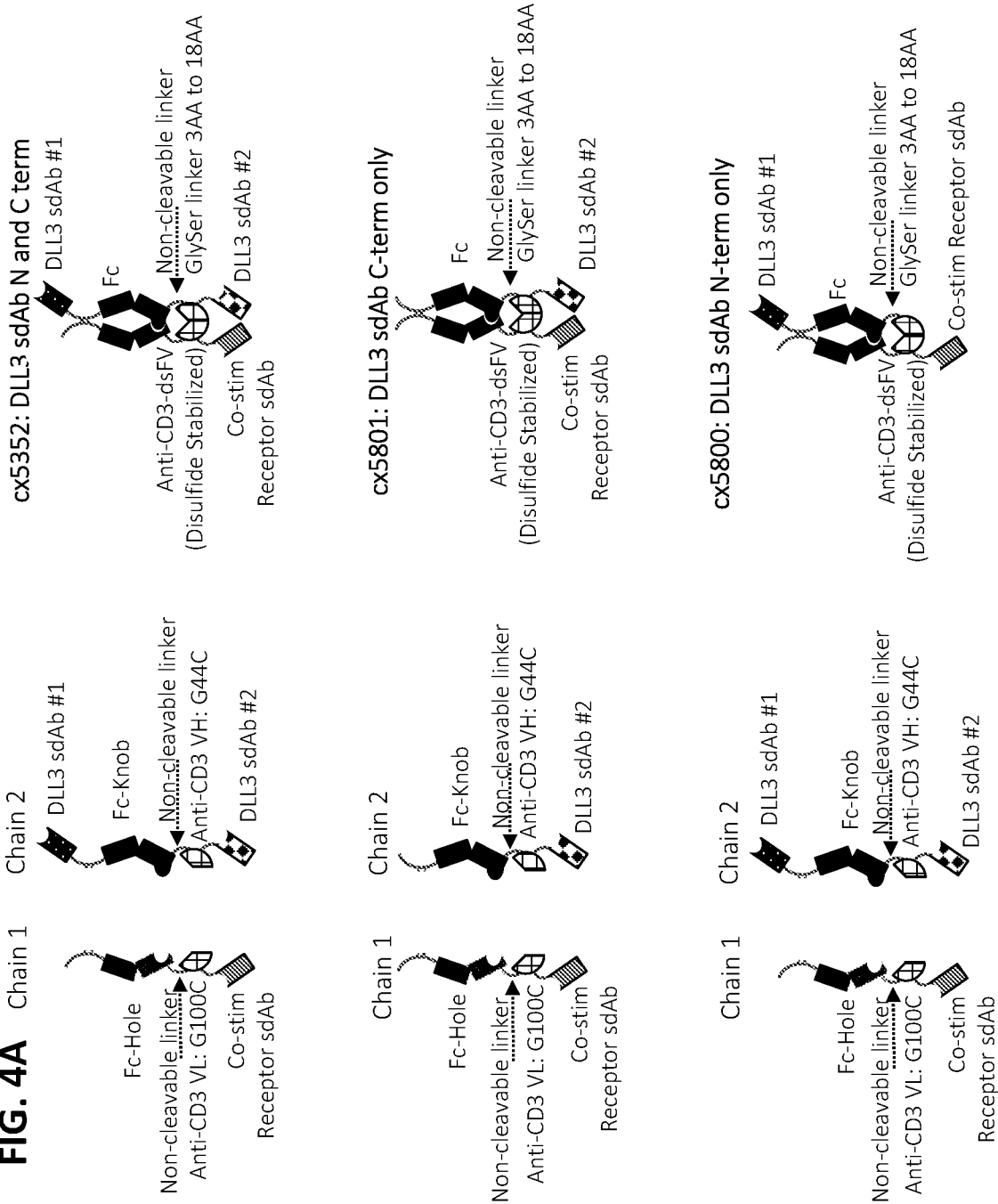


FIG. 4B

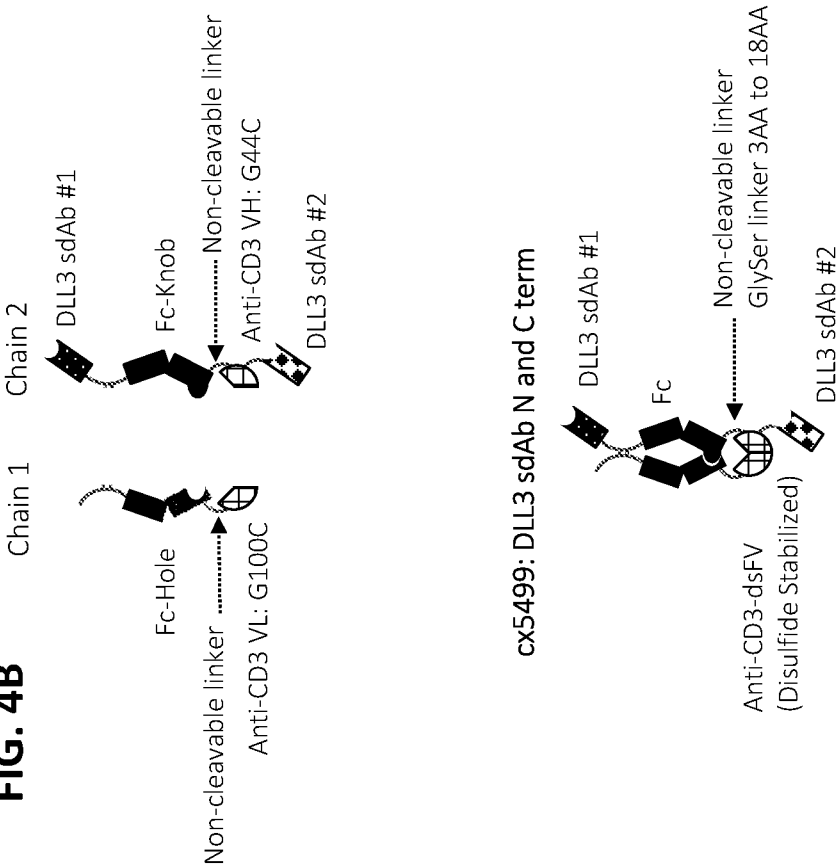


FIG. 5A

Binding to OVCAR5 cells

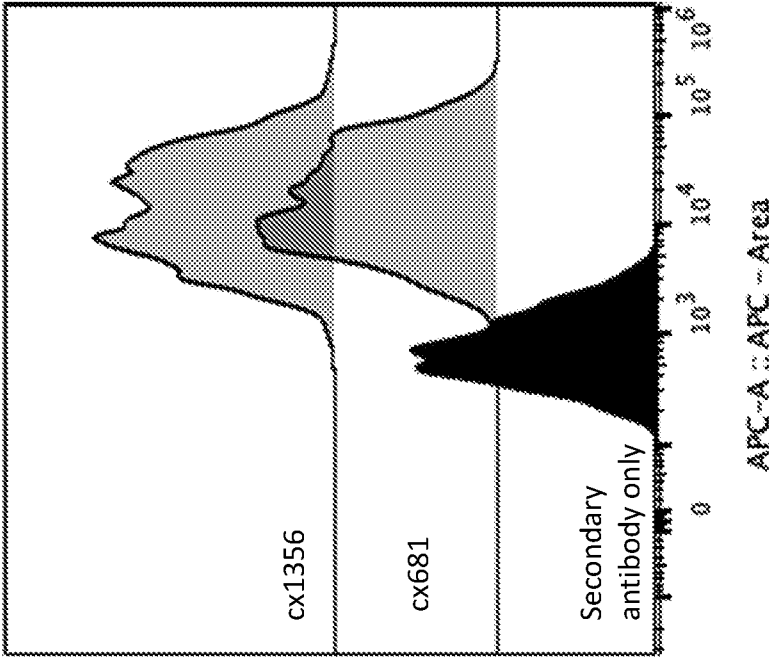
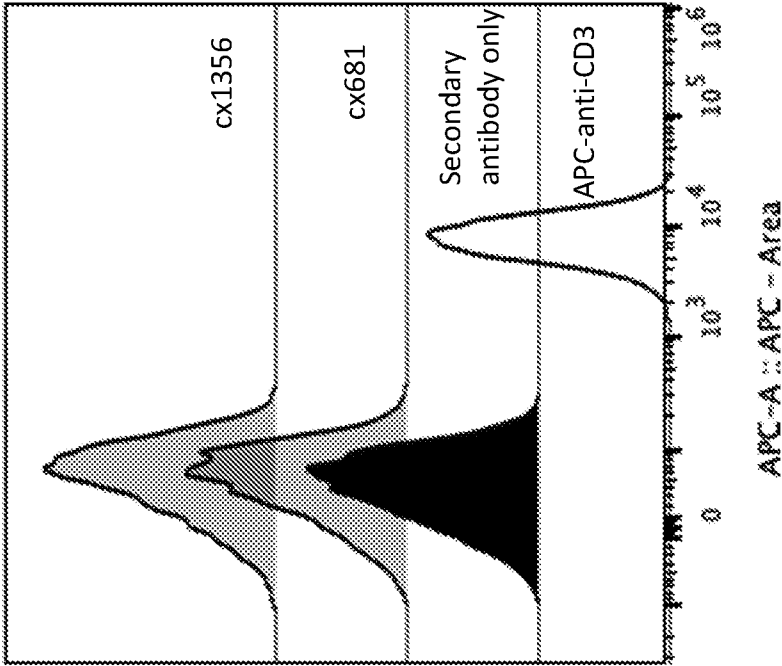


FIG. 5B

Binding to T cells



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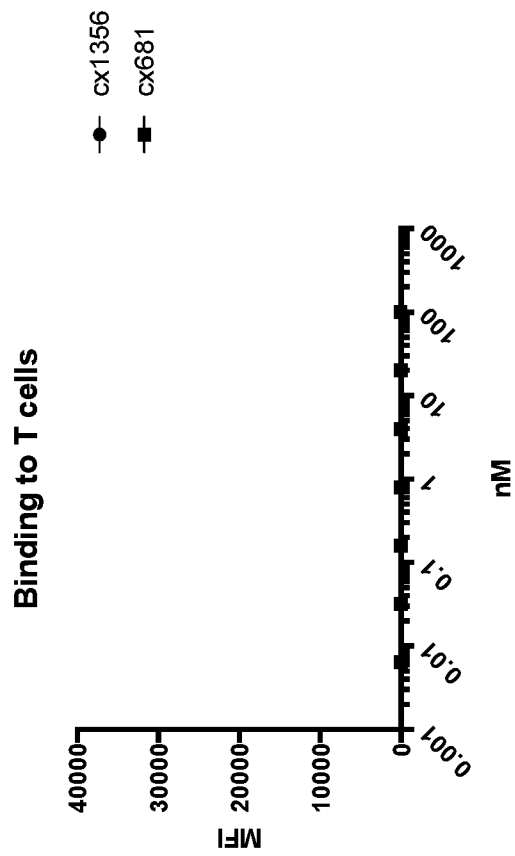
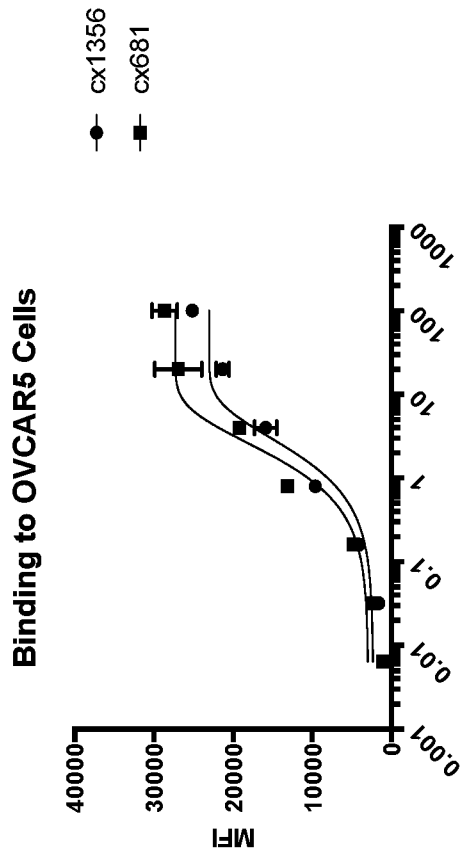


FIG. 6A

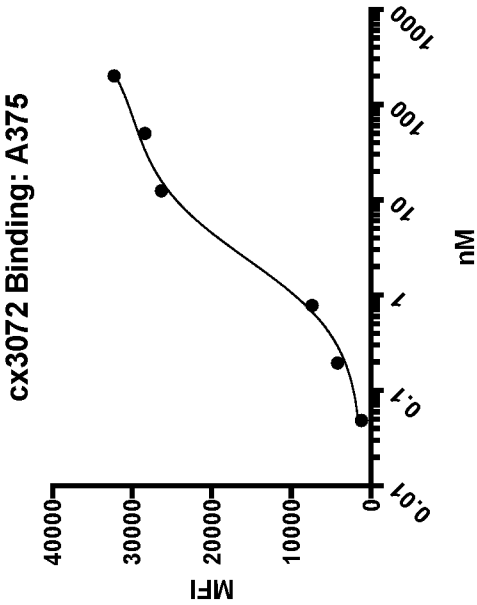
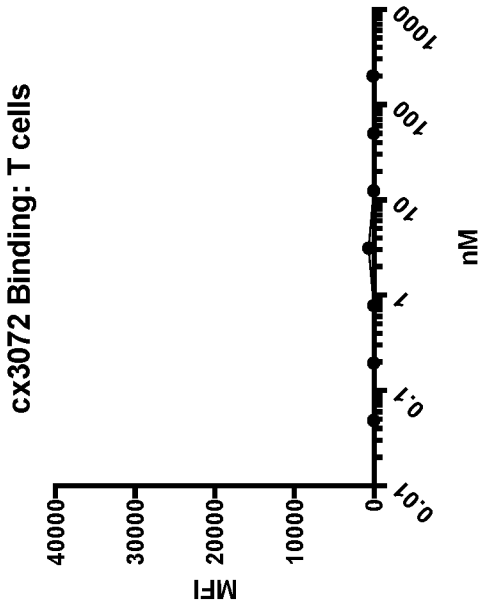
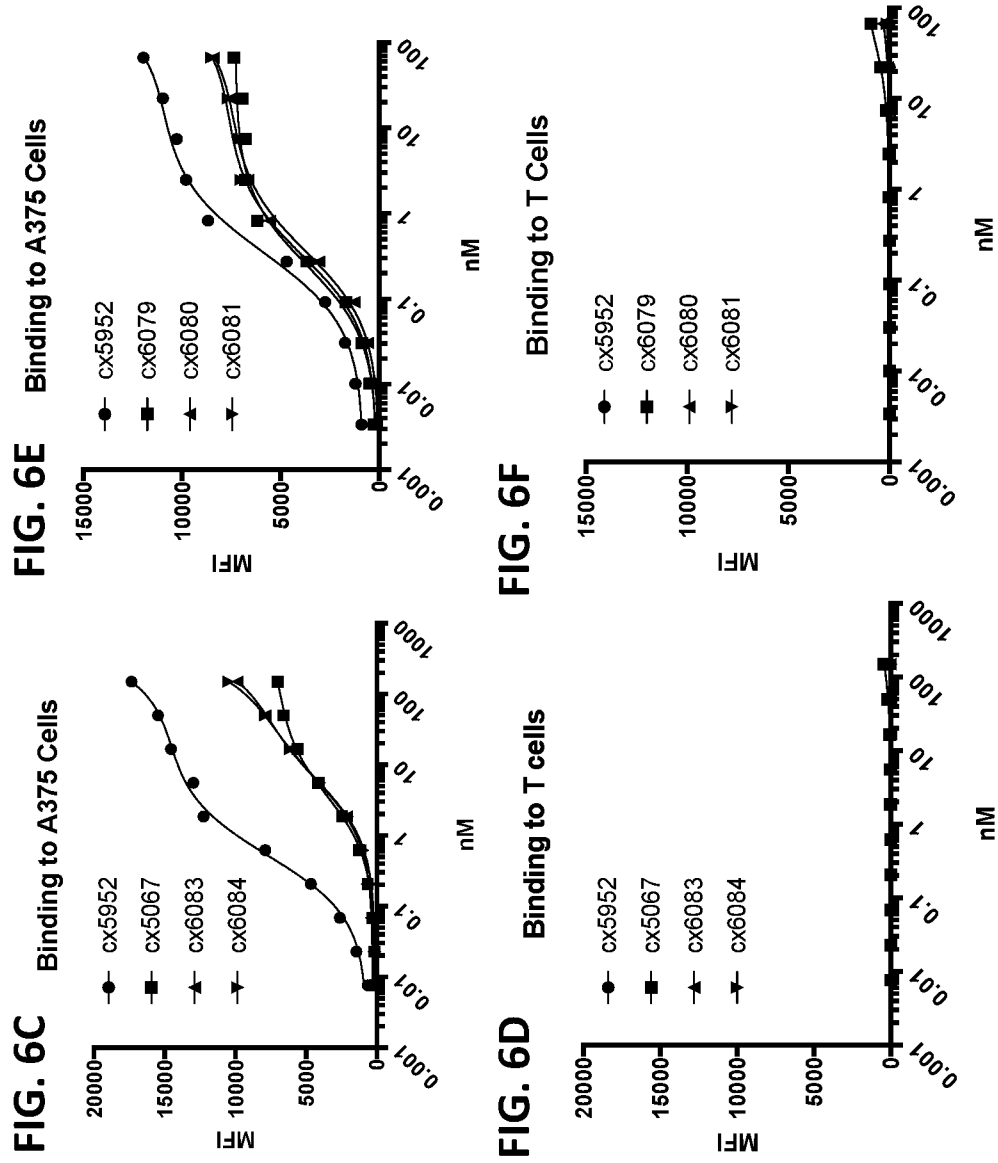


FIG. 6B





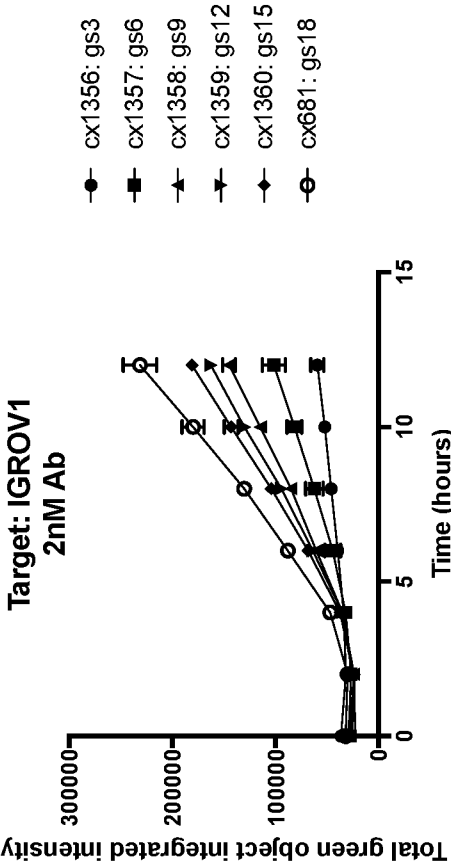


FIG. 7A

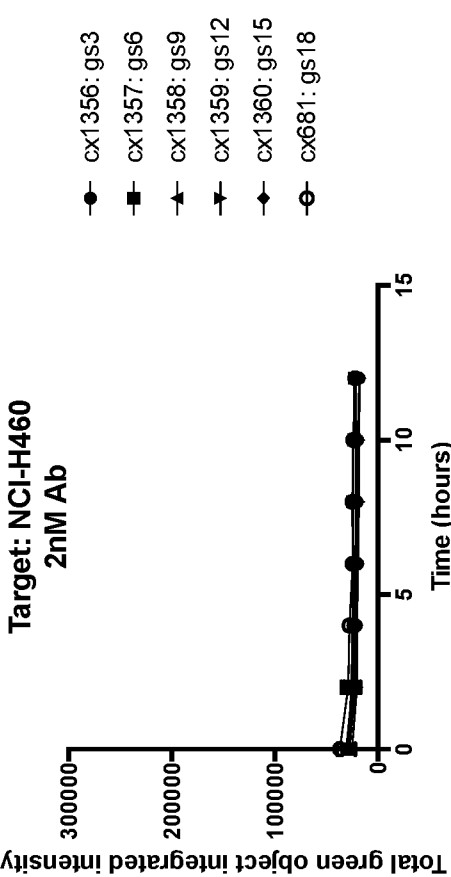


FIG. 7B

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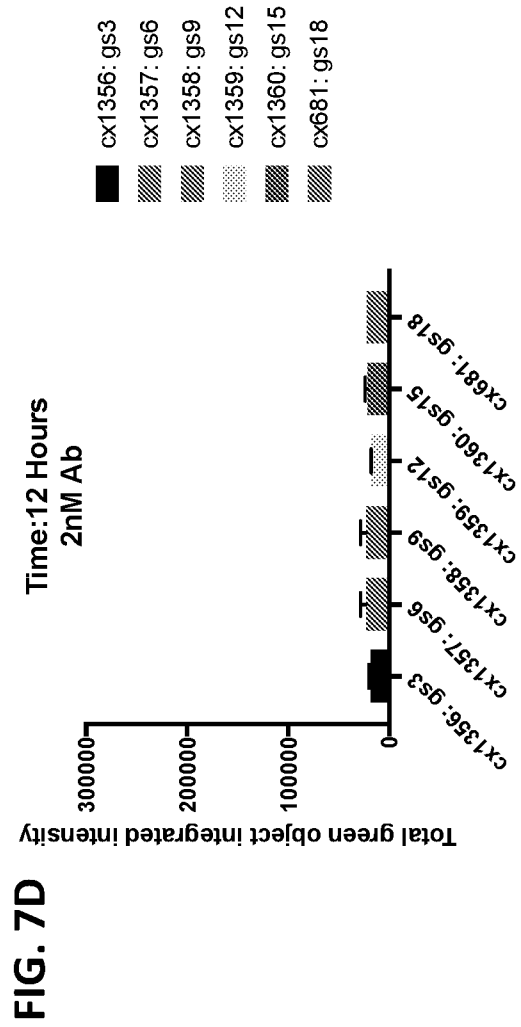
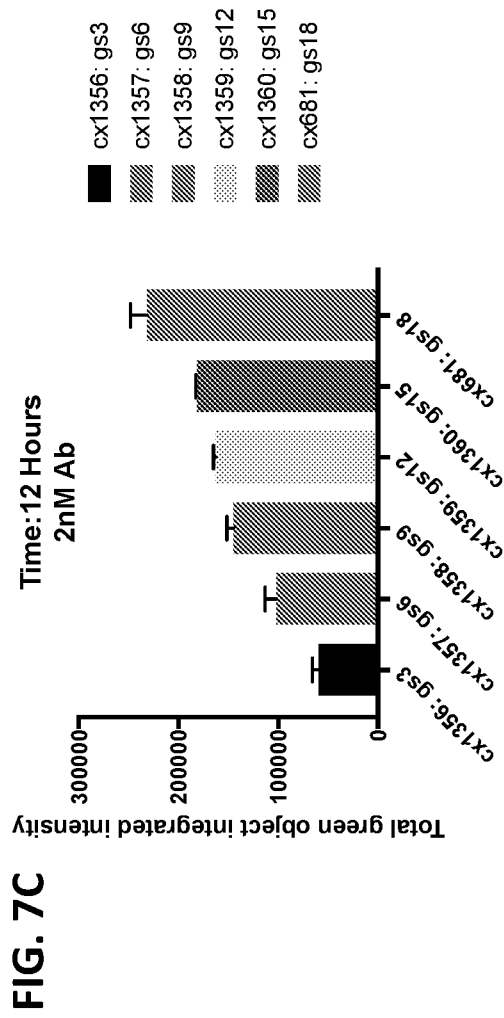


FIG. 7E

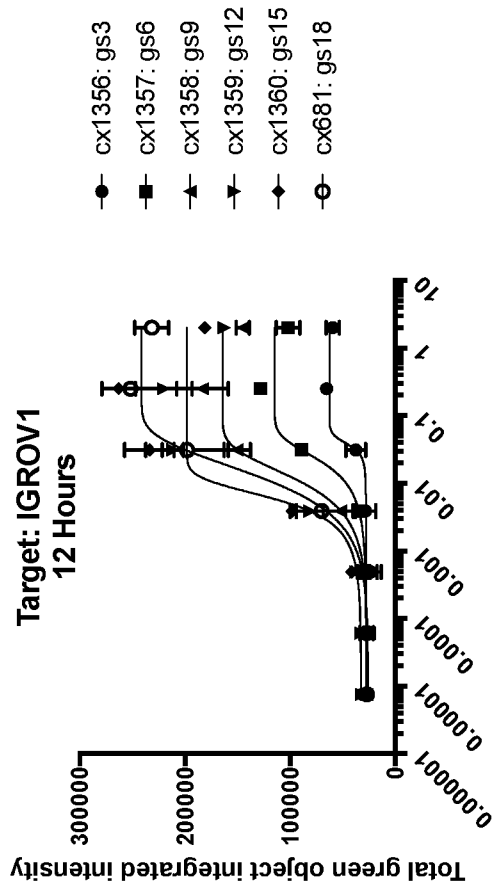
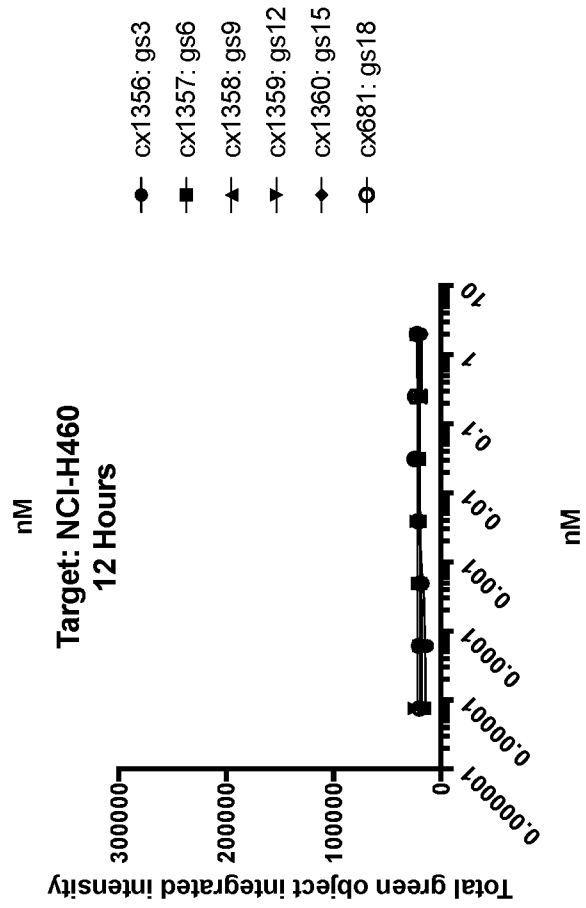
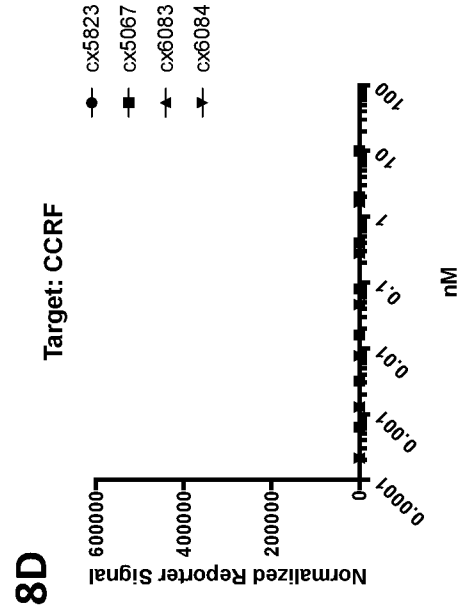
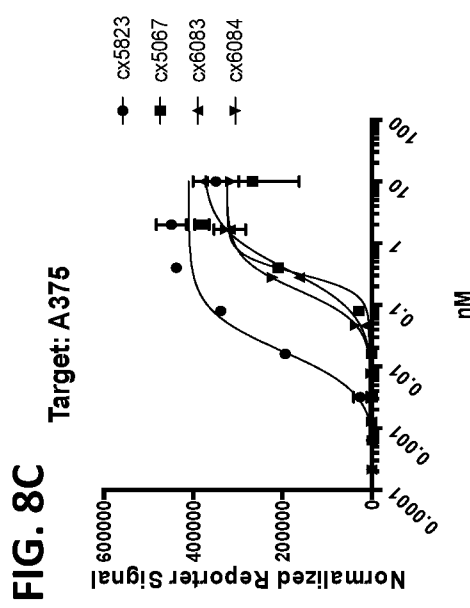
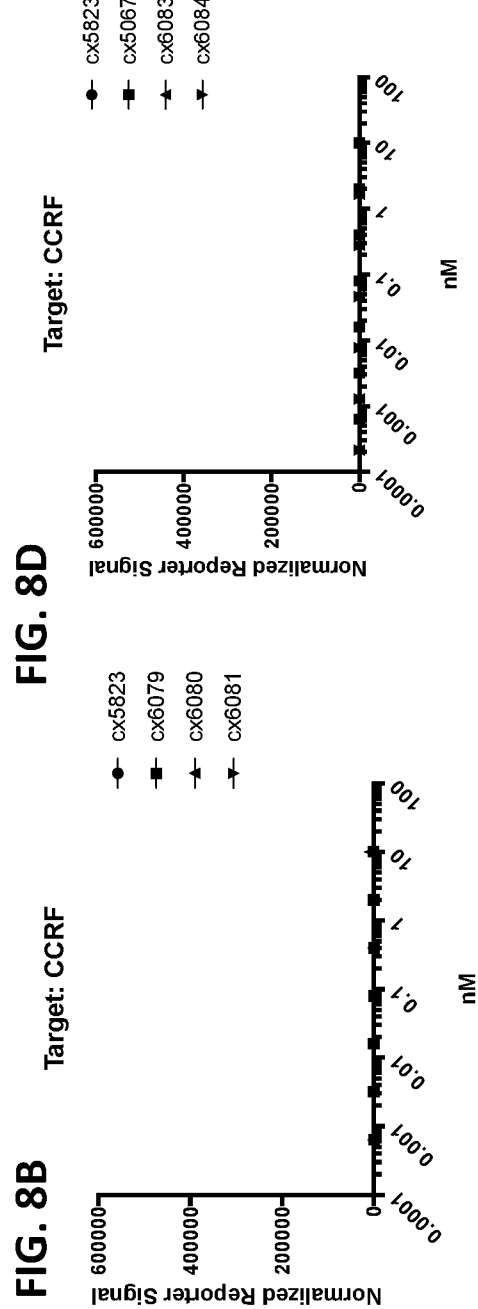
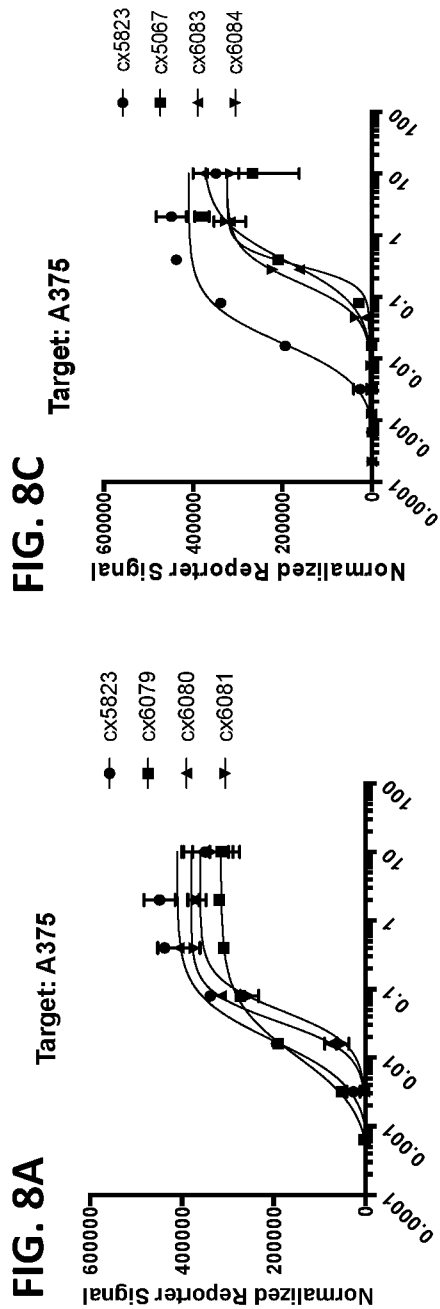
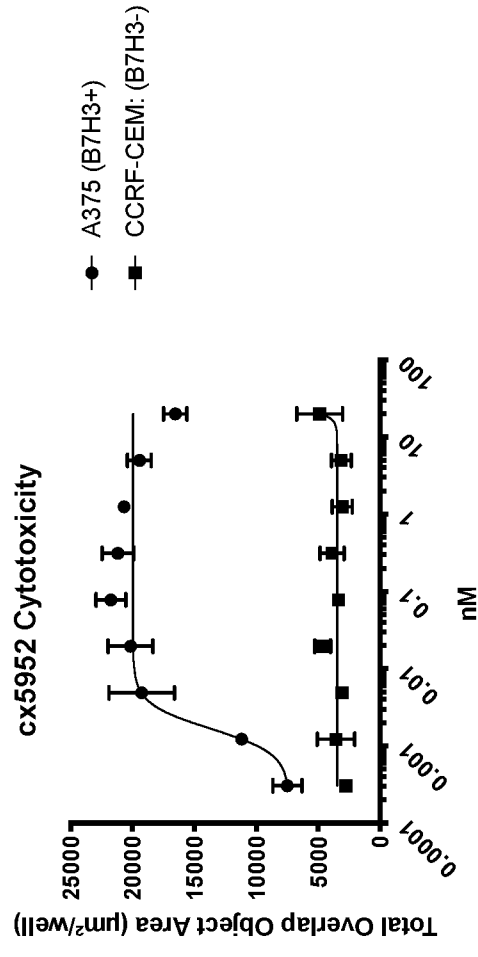
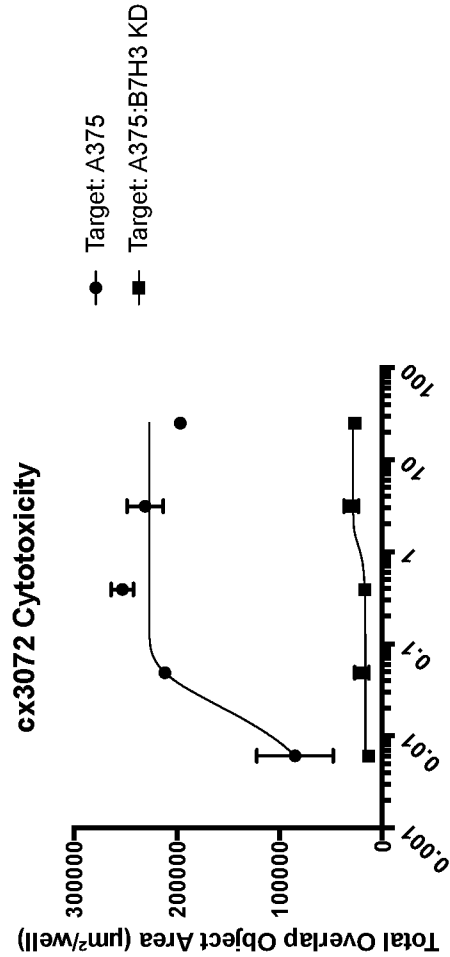


FIG. 7F







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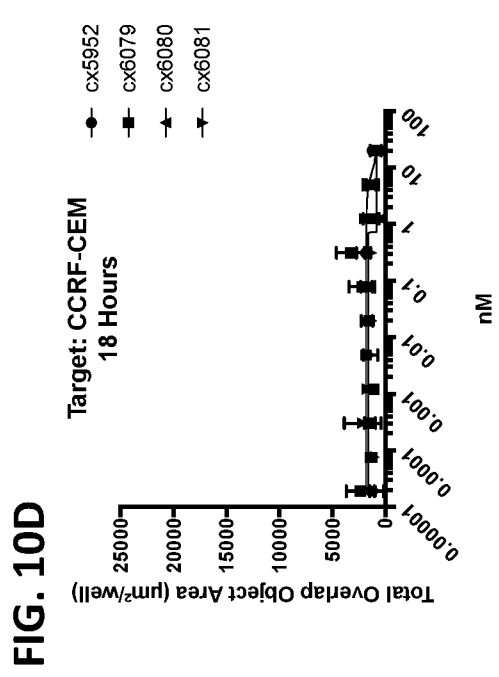
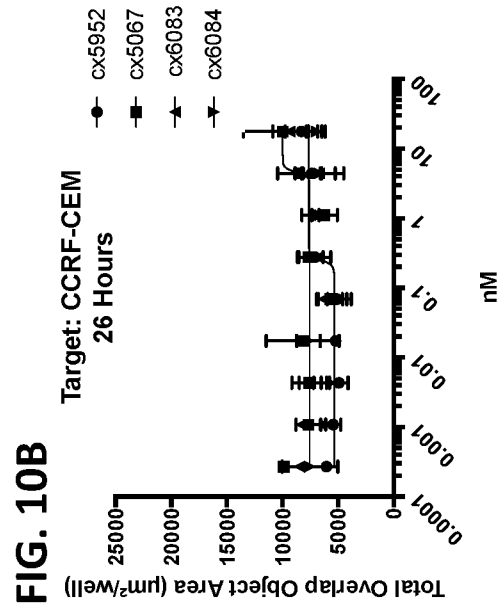
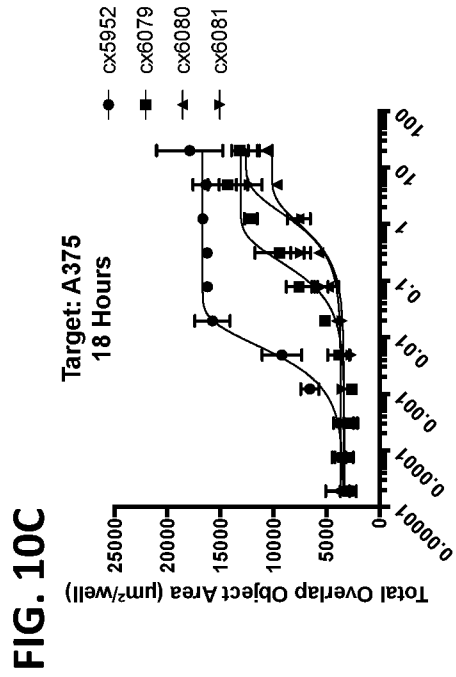
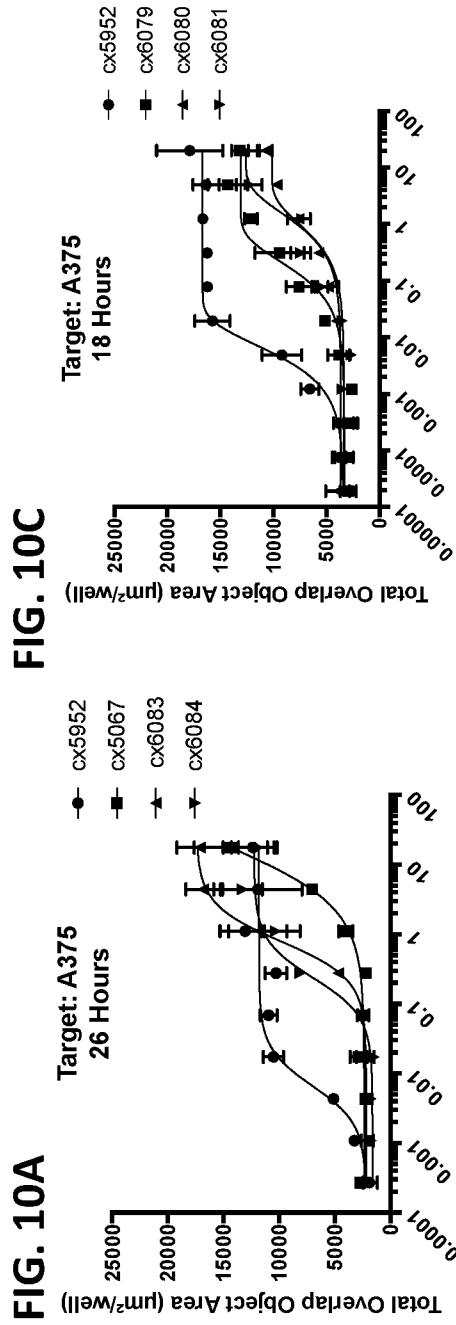


FIG. 11A

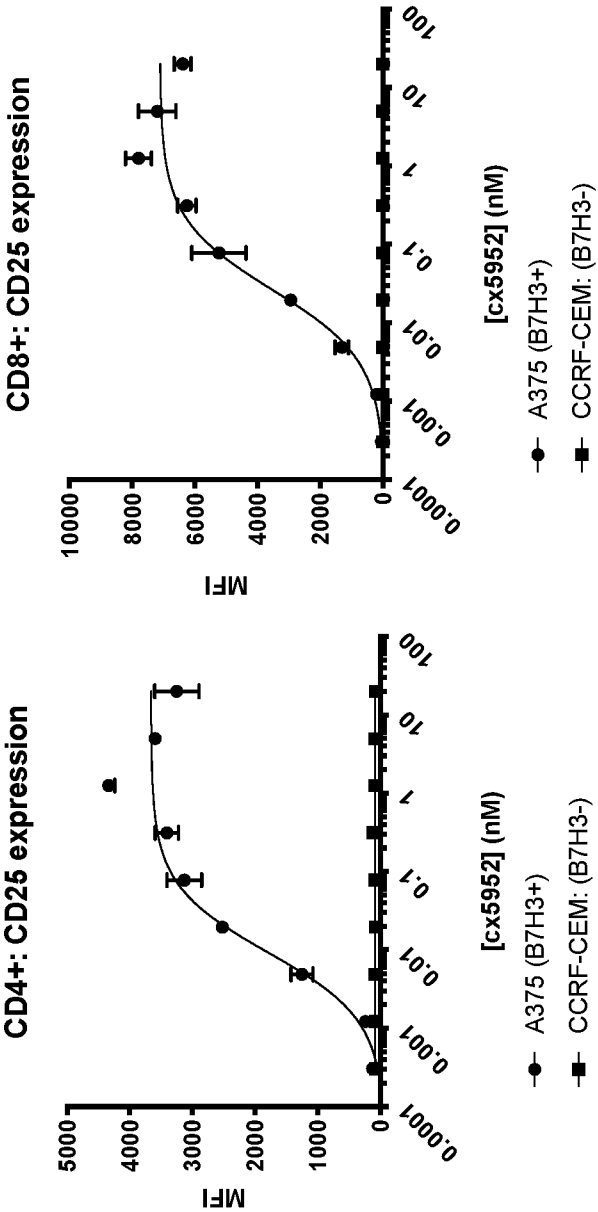


FIG. 11B

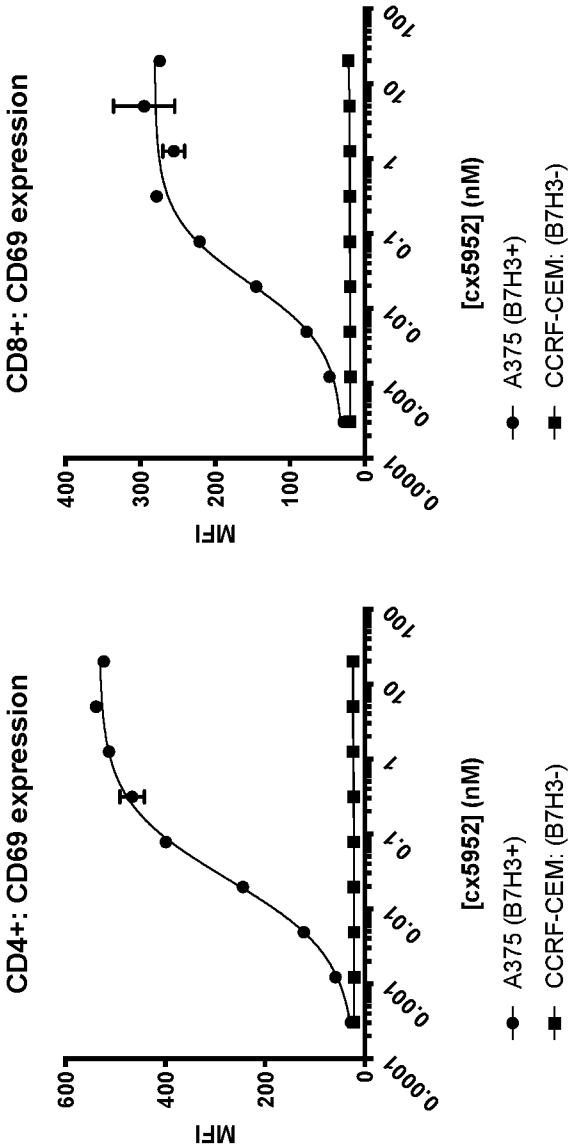
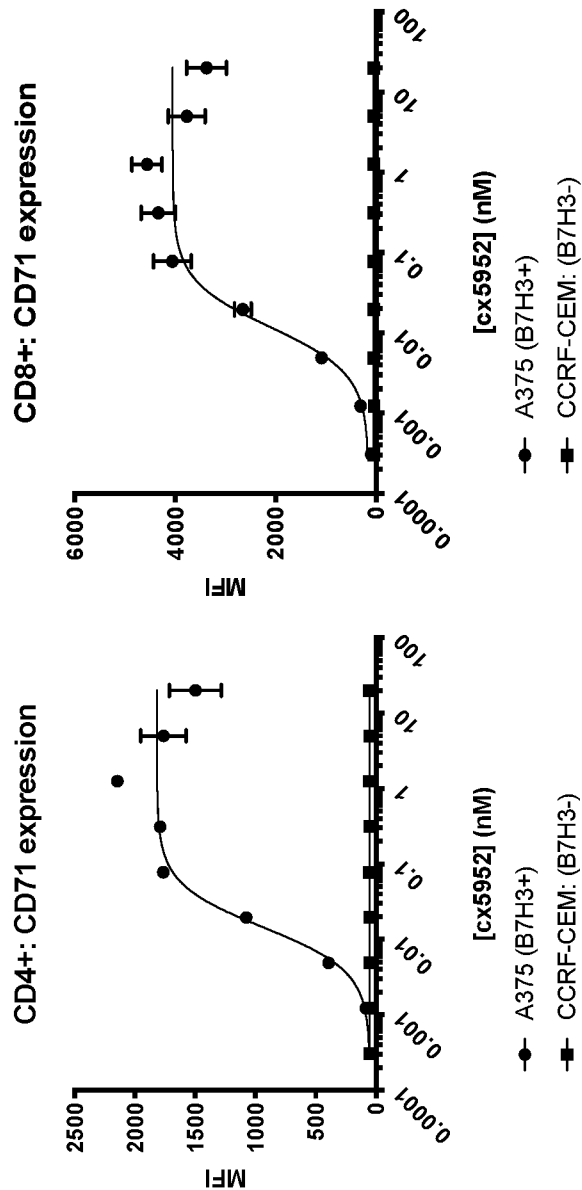
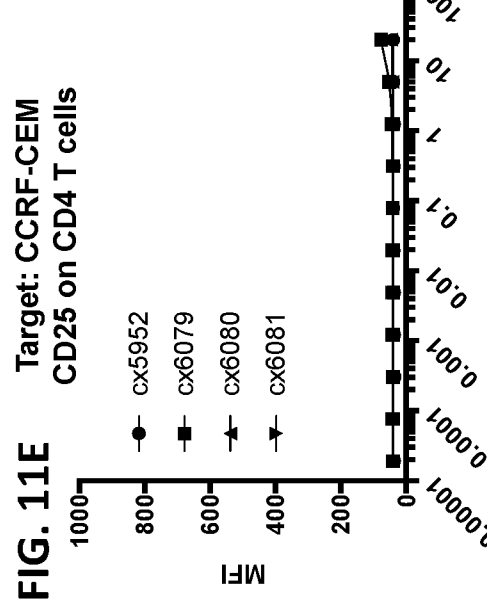
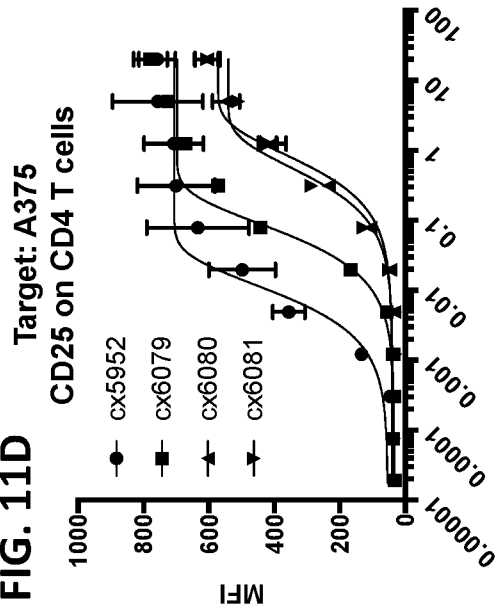
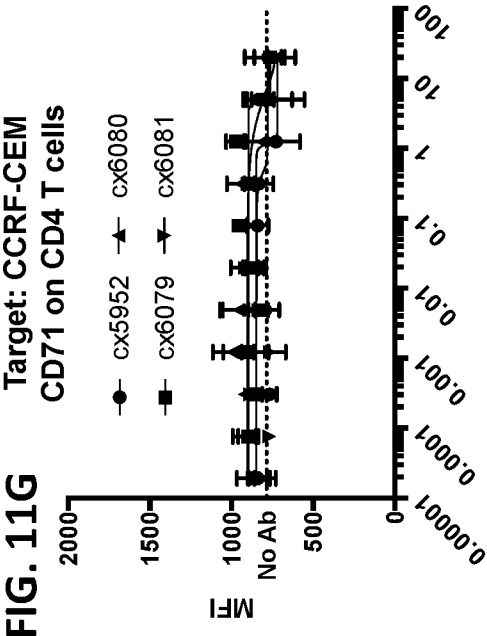
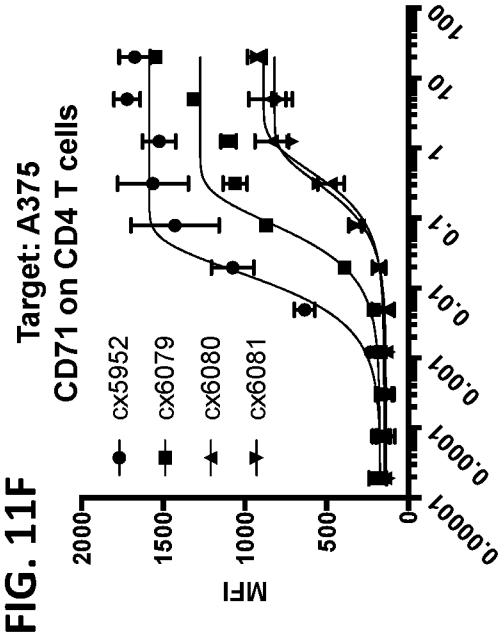
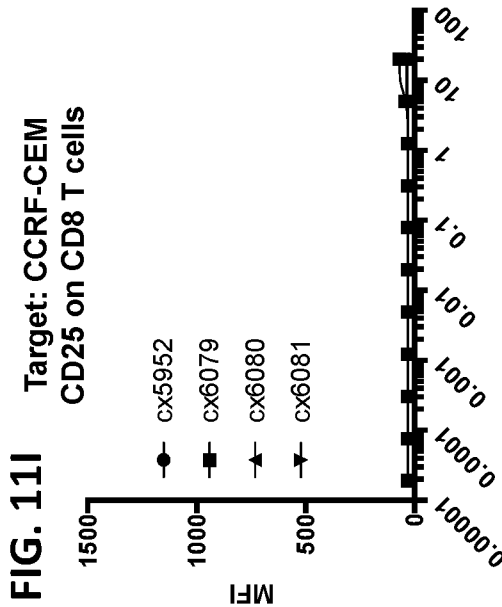
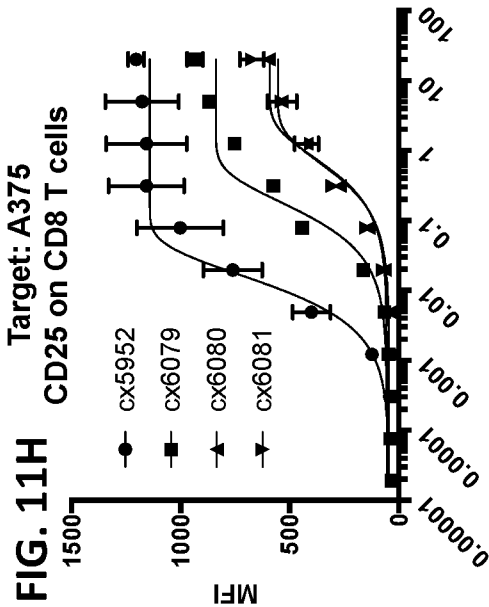
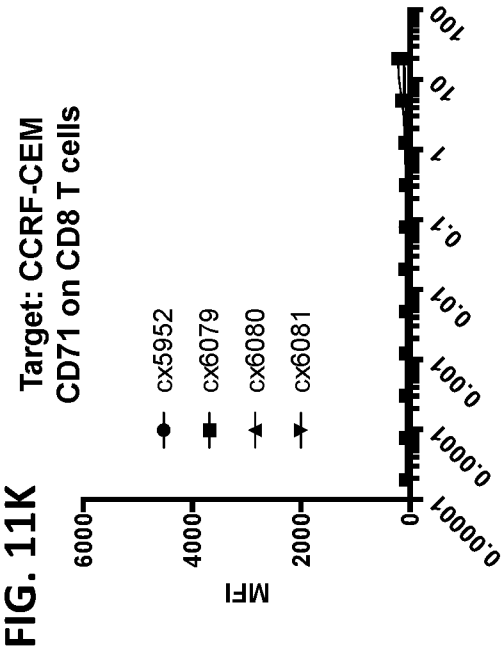
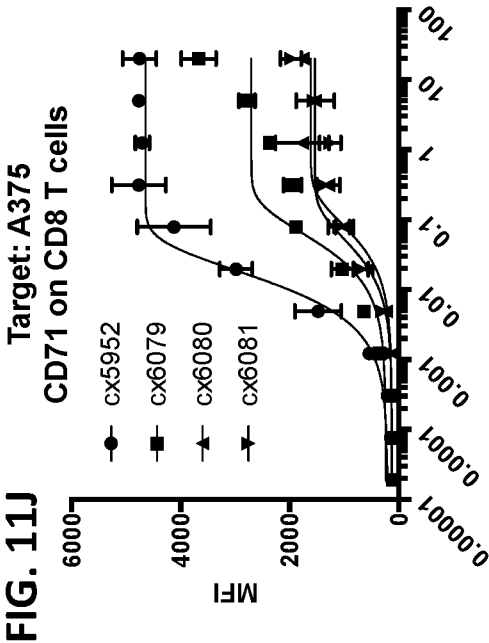
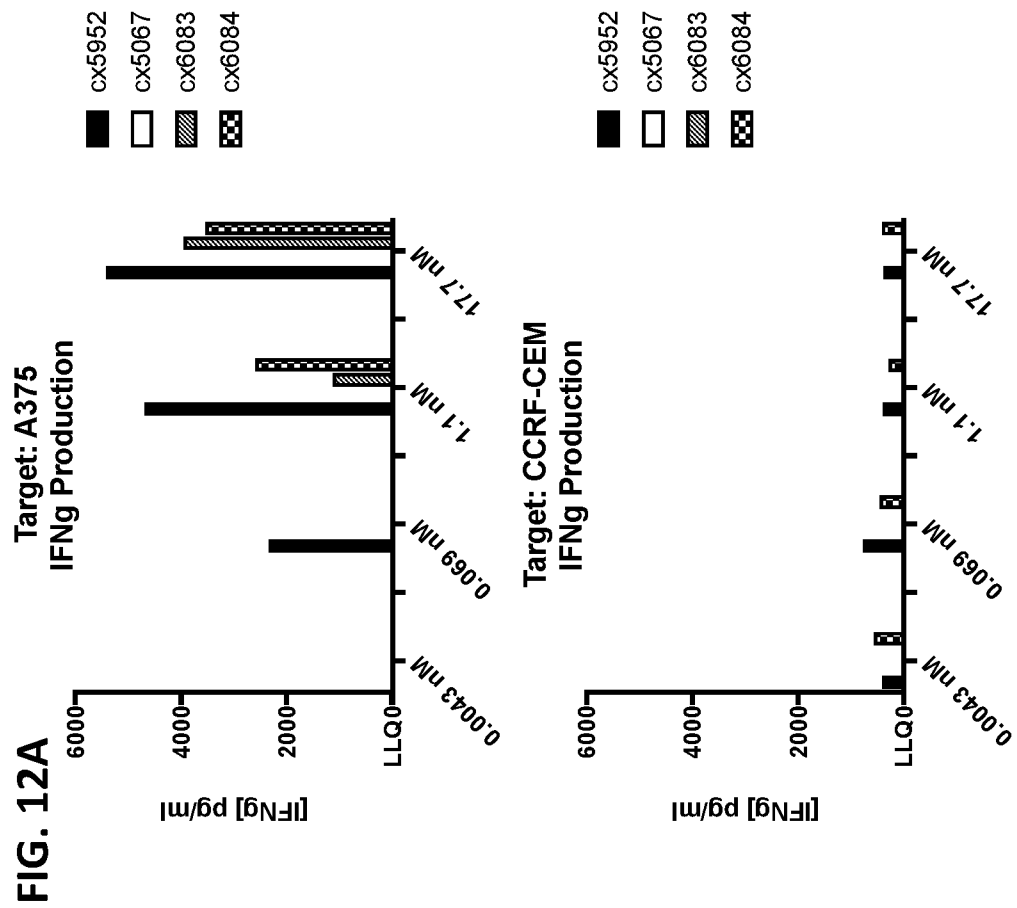


FIG. 11C









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FIG. 12B

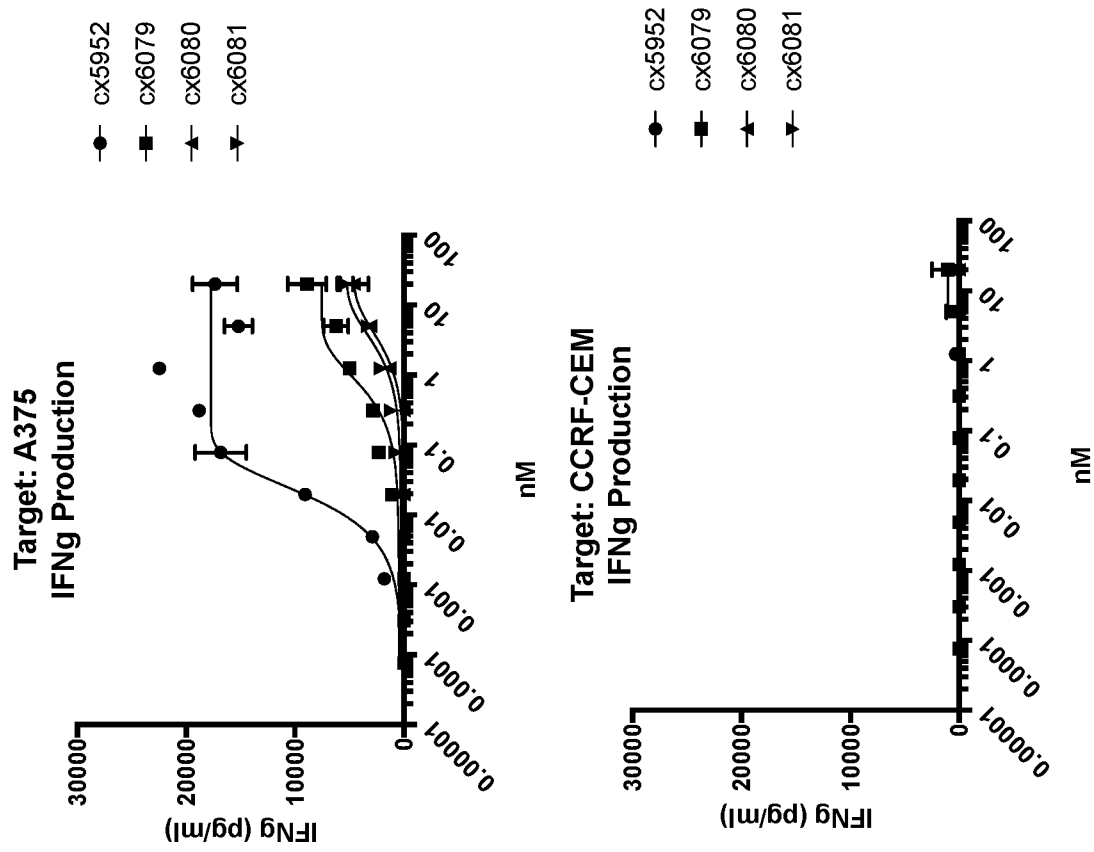


FIG. 13A

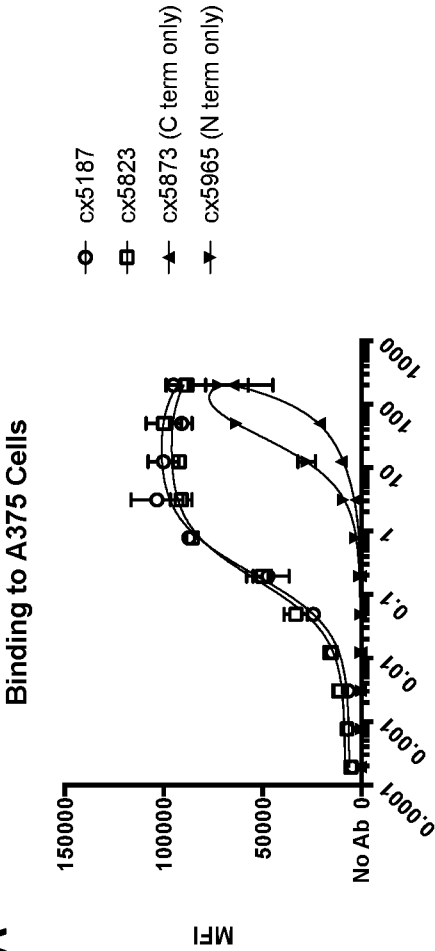
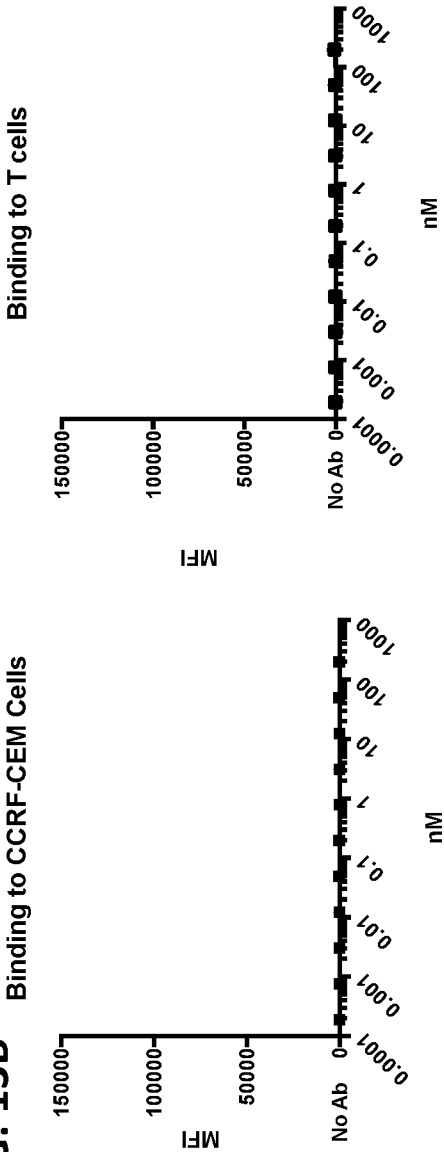


FIG. 13B



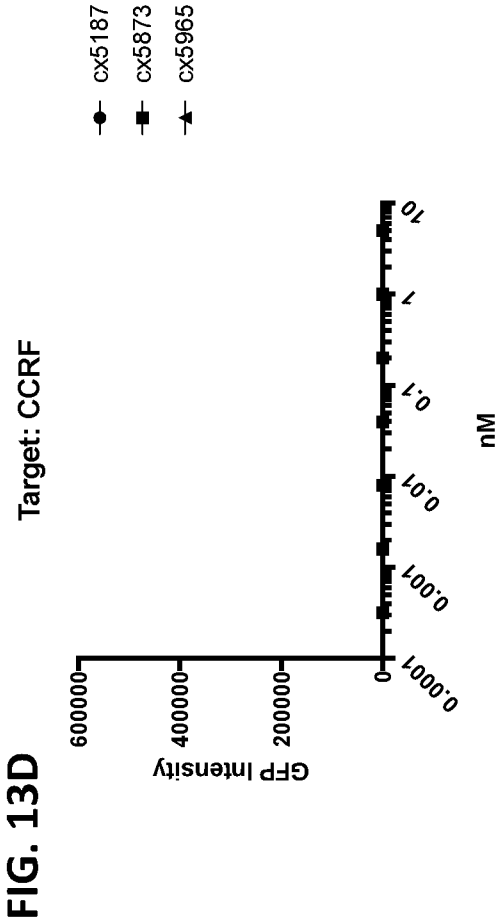
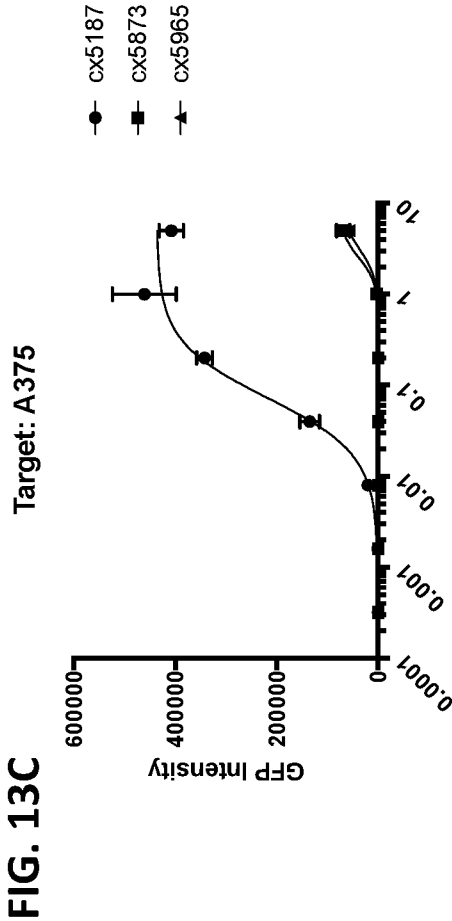


FIG. 14A

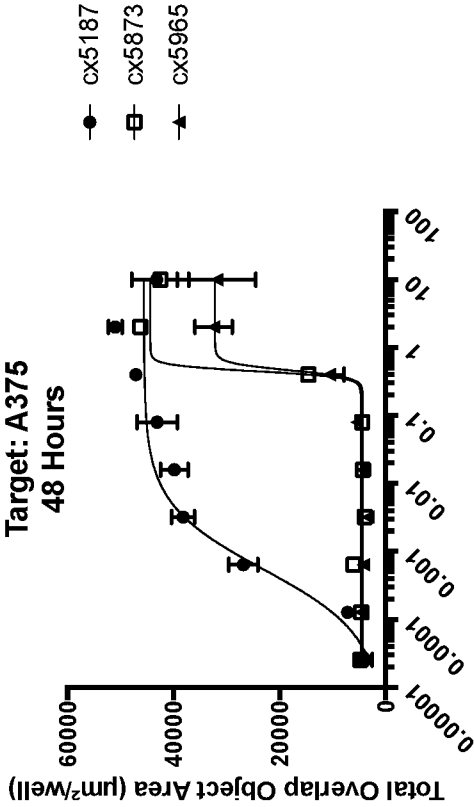
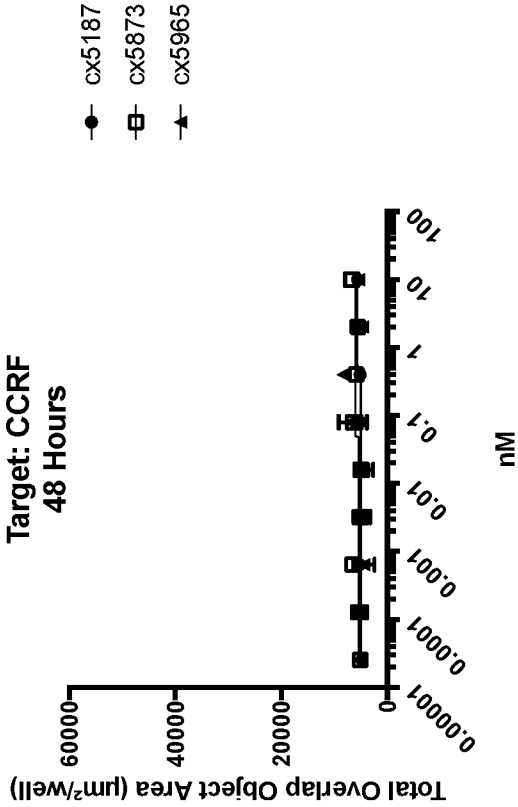
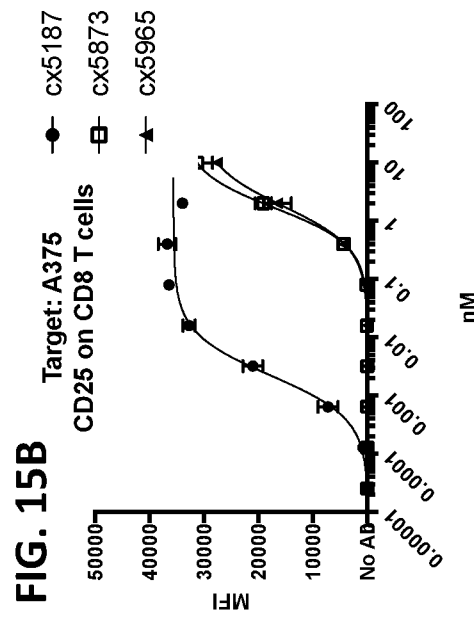
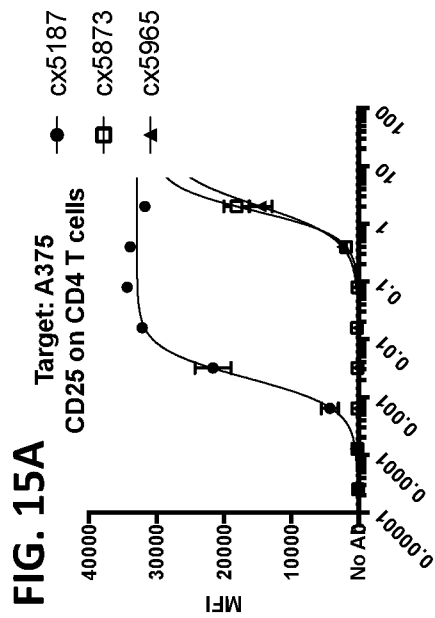
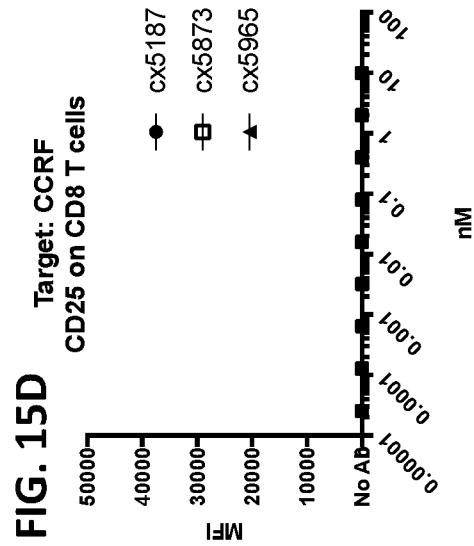
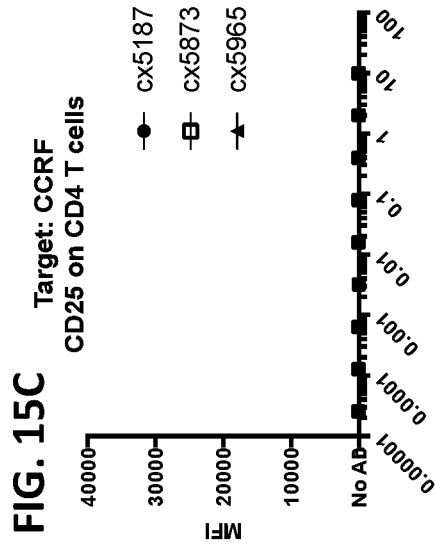
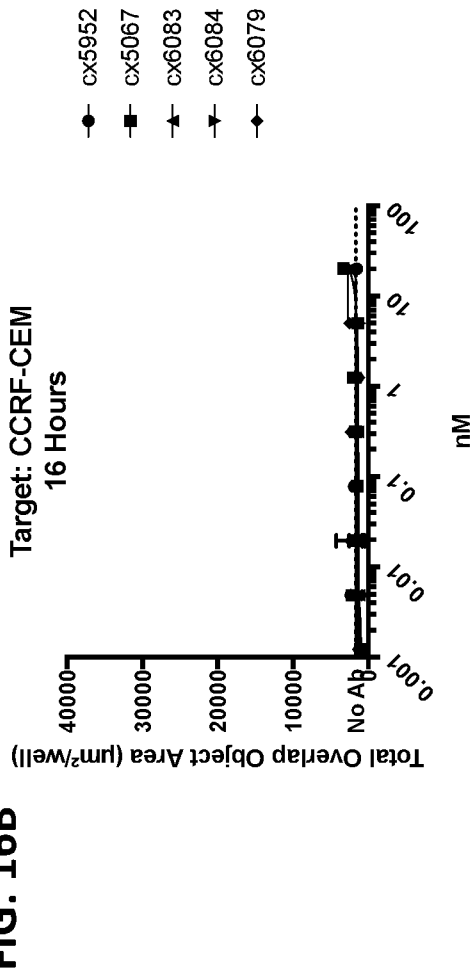
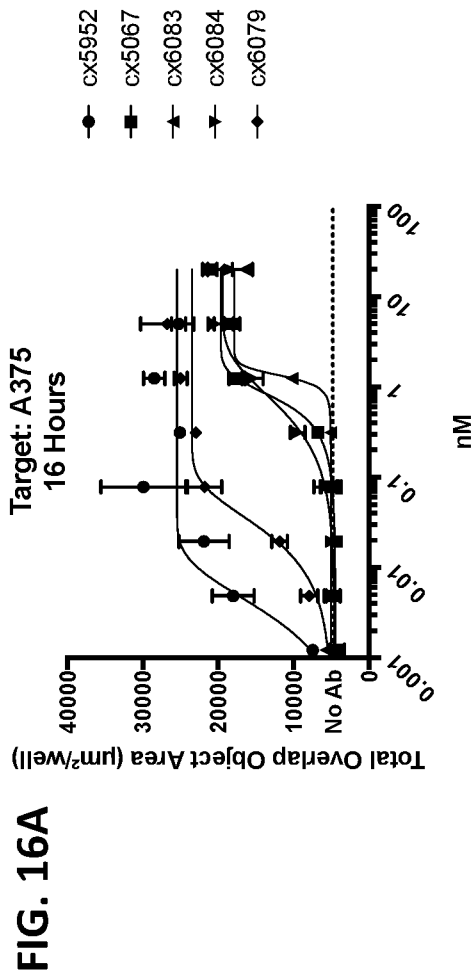


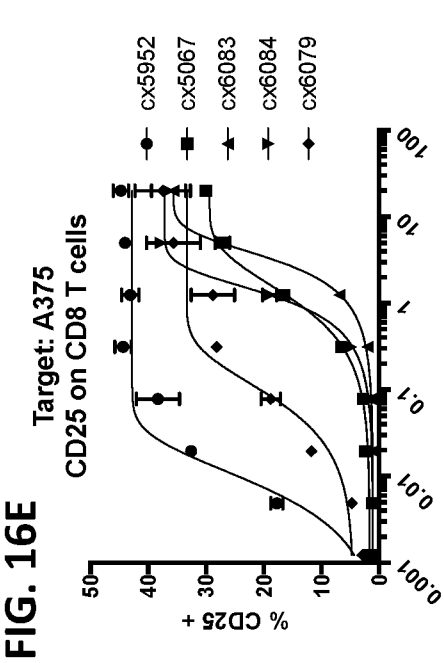
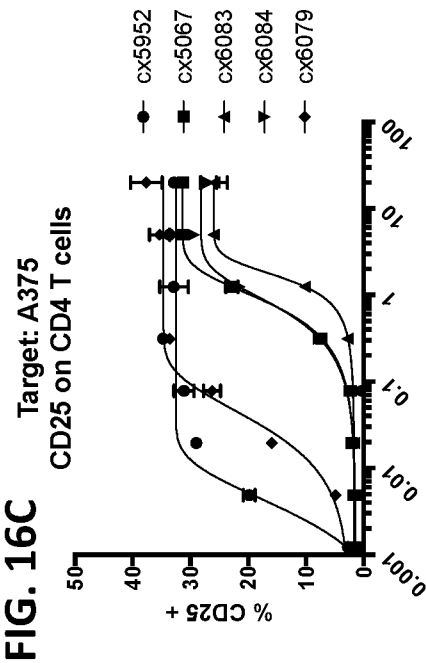
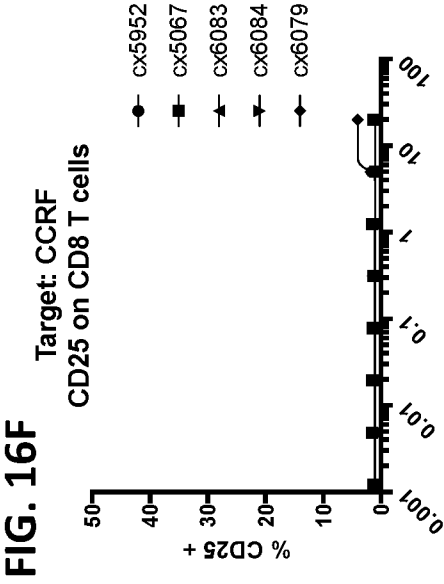
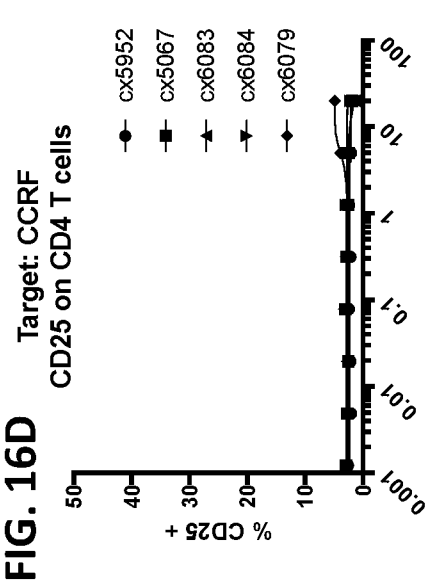
FIG. 14B

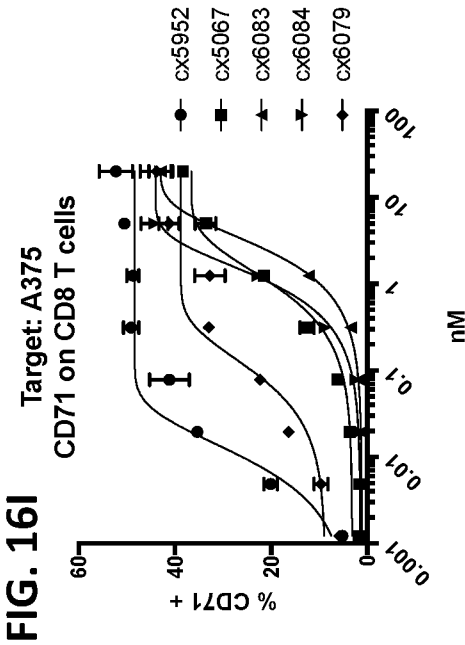
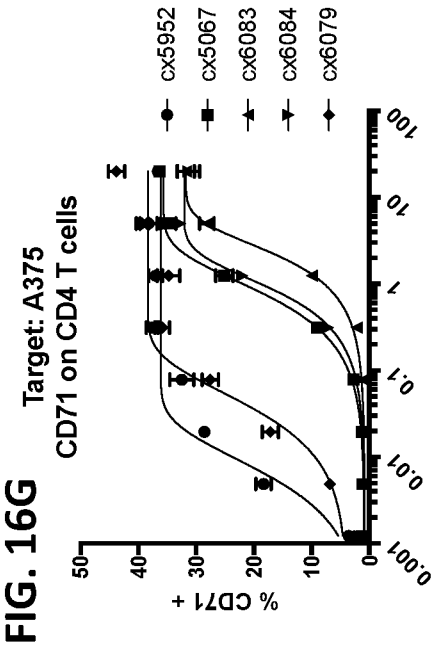
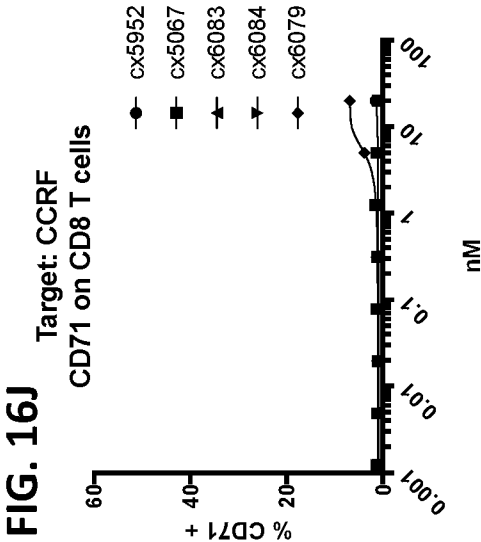
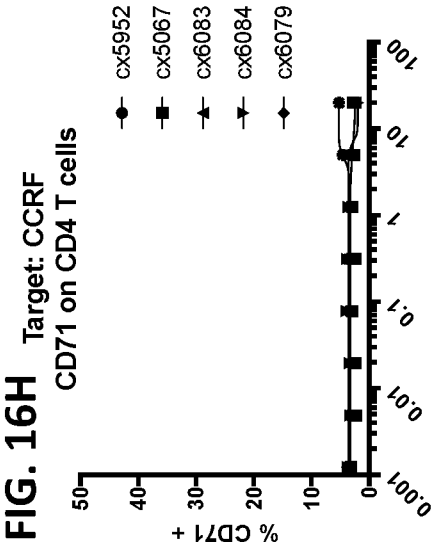


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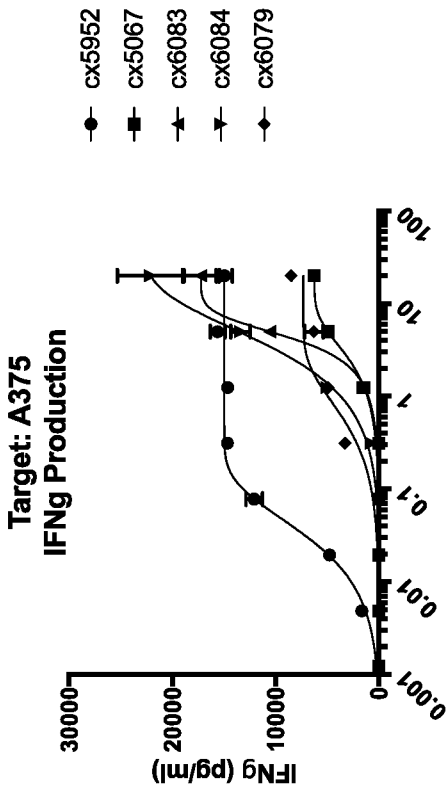


FIG. 16K

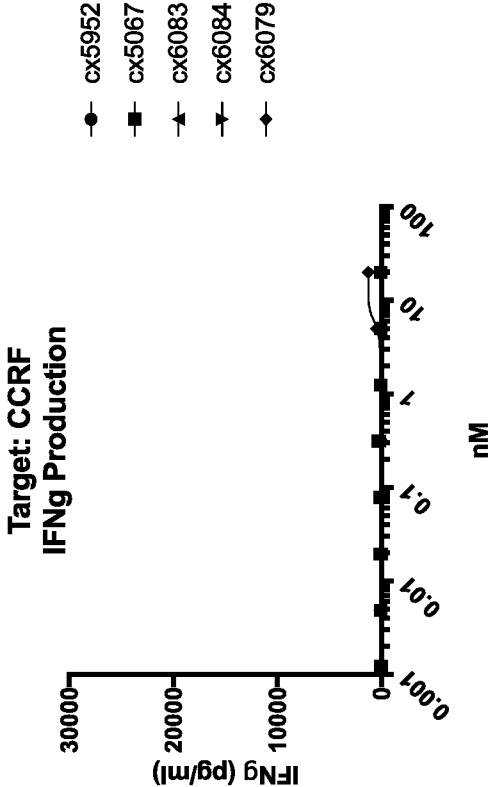
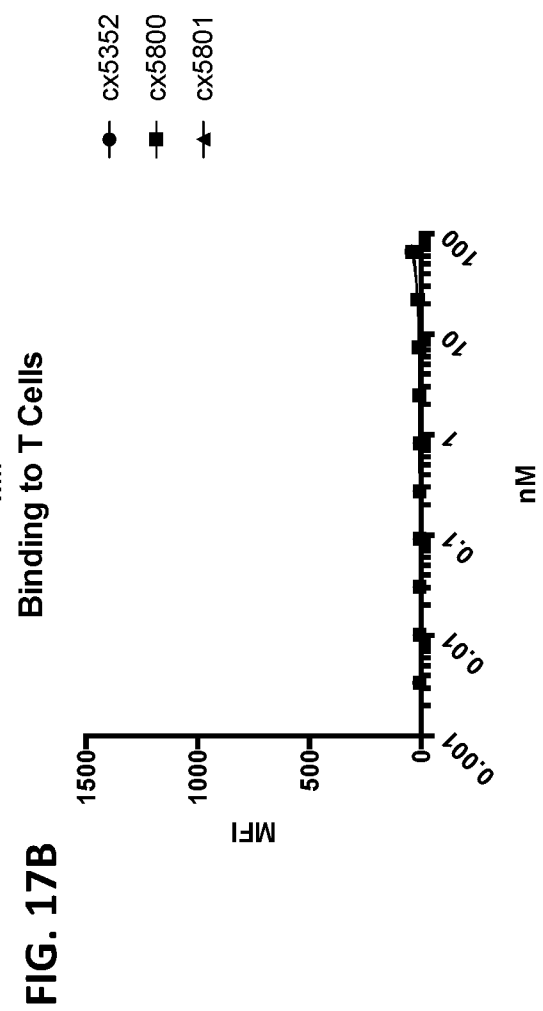
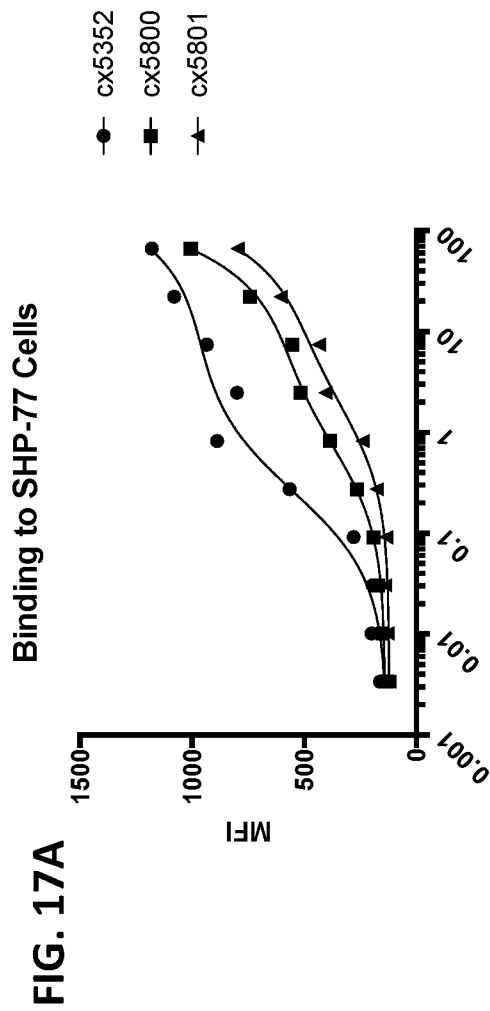


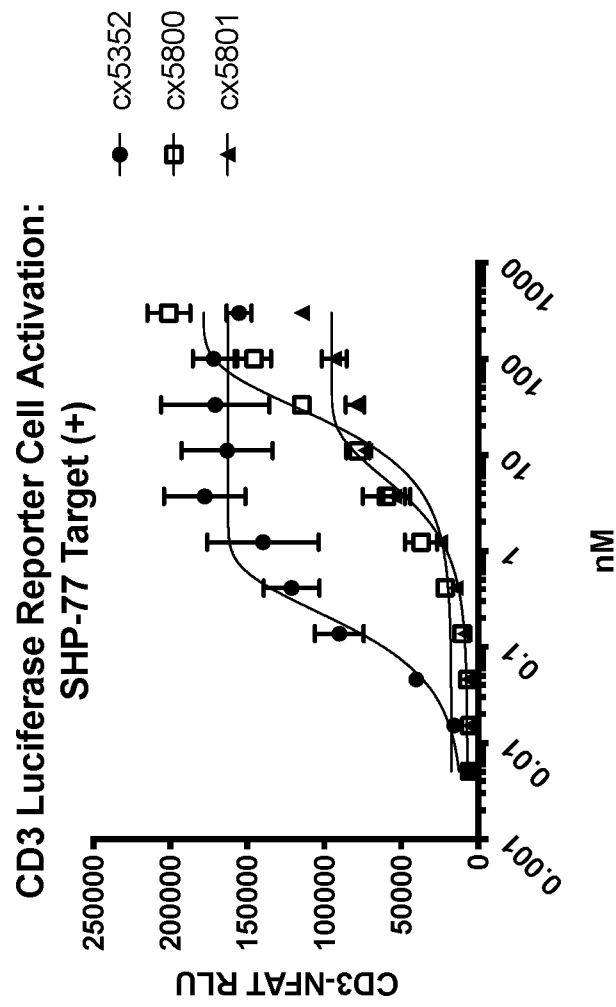
FIG. 16L

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FIG. 17C



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FIG. 18A

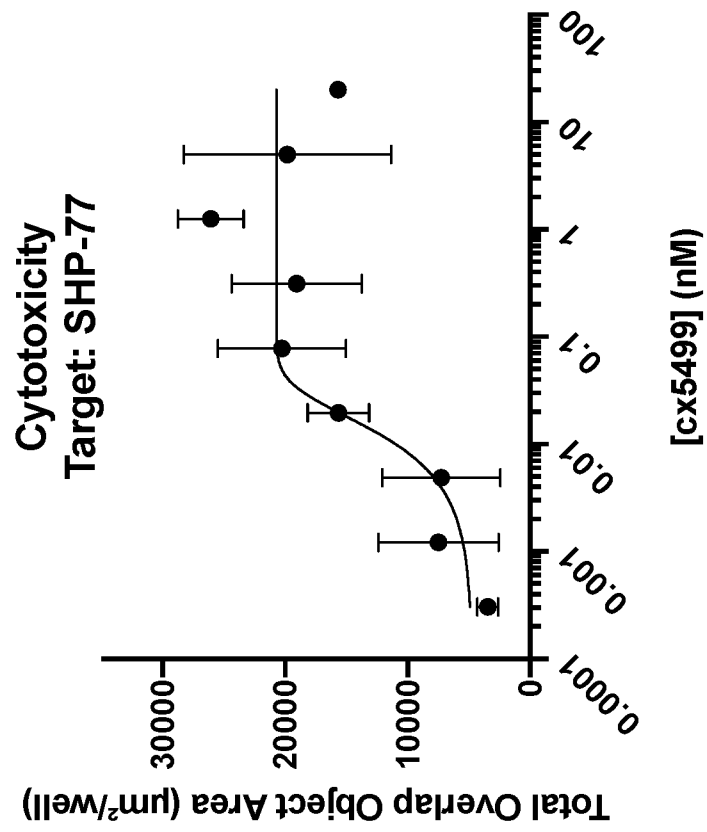


FIG. 18B CD25 Expression on CD4 T-cells

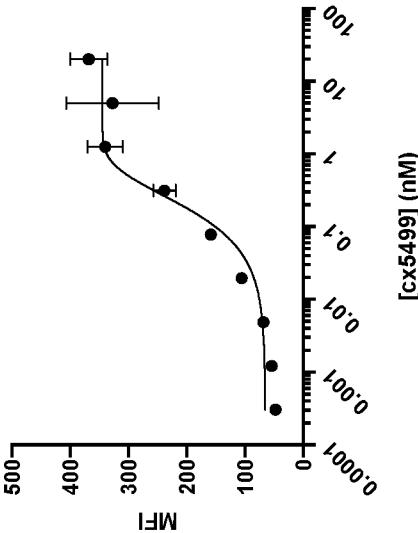


FIG. 18C CD69 Expression on CD4 T-cells

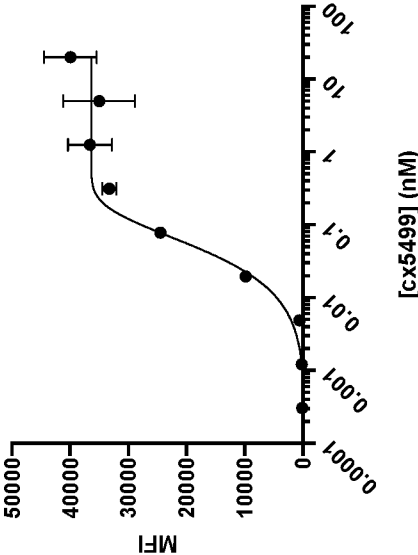


FIG. 18D CD25 Expression on CD8 T-cells

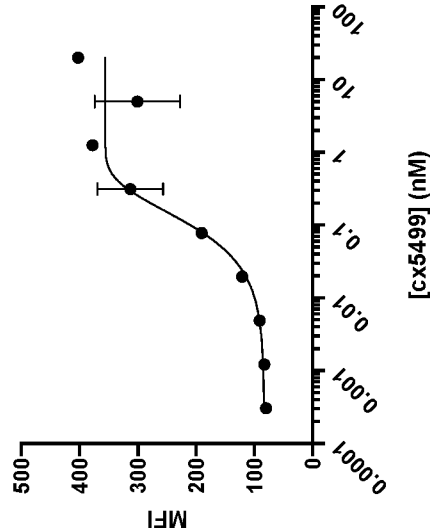


FIG. 18E CD69 Expression on CD8 T-cells

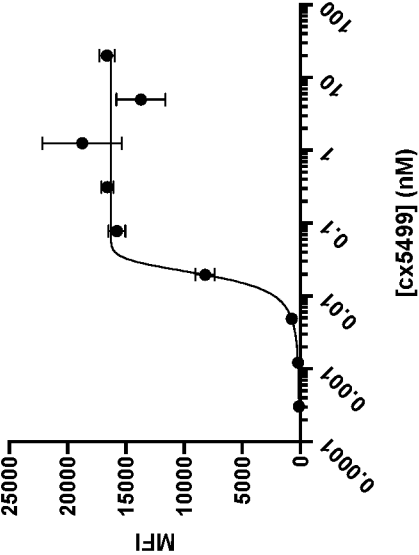


FIG. 1

