

[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 examples, 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 or operably linked, 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 examples, the multispecific polypeptide construct, in an inactive state, is composed of a first component and a second component, wherein the first and second components are operably linked, wherein each of the first and second components comprises an antigen binding domain that binds a tumor associated antigen (TAA), wherein the first component comprises an Fc region, wherein the second component comprises a CD3-binding region, and wherein the first and second components are coupled by a cleavable linker. In some examples, the CD3-binding region binds CD3 (CD3ε).

[0007a] In one aspect, the present disclosure refers to a multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising a heterodimeric 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 amino-terminally relative to the CD3-binding region;

the CD3-binding region is an anti-CD3 antibody or antigen binding fragment that is a Fv antibody fragment comprising a variable heavy chain region (VH) and a variable light chain region (VL) and the VH and VL are linked to opposite polypeptides of the heterodimeric Fc; and

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

[0007b] In another aspect, the present disclosure refers to 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 linker, wherein the Fc region is positioned amino-terminally relative to the CD3-binding region; and

the multispecific polypeptide construct further comprises at least one antigen binding domain that binds a tumor associated antigen (TAA) and is positioned carboxy-terminally relative to the CD3-binding region.

[0007c] In another aspect, the present disclosure refers to 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 linker, wherein the Fc region is positioned amino-terminally relative to the CD3-binding region; and

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

[0007d] In another aspect, the present disclosure refers to 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 cleavable linker, wherein the Fc region is positioned amino-terminally relative to the CD3-binding region; and

the multispecific polypeptide construct further comprises at least one antigen binding domain that binds a tumor associated antigen (TAA).

[0008] In some examples, 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 examples, 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 examples, 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.

[0009] 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; 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; 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; and a CD3 binding region that binds CD3 (CD3ε).

[0010] Among examples of the present disclosure are multispecific polypeptide constructs that bind at least CD3 and a second antigen, such as a tumor associated antigen (TAA). The multispecific polypeptide constructs provided herein include at least a first component that includes one or more copies of an antigen-binding domain that bind an antigen 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 CD3 binding region, which are used interchangeably herein), and a linker, such as a cleavable linker, that joins the first component and the second component.

[0011] 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 examples, in the uncleaved/inactive state, 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) are bound to their cognate antigen. This is advantageous as it prevents systemic binding of the CD3 binding region to T-cells and focuses it to site of antigen expression. This is beneficial as it eliminates a major binding sink of peripheral T-cells, allowing more favorable distribution and localization at site of antigen expression, e.g., tumor cells or the tumor microenvironment. In some cases, binding and/or engagement of CD3 is amplified or increased by inclusion of a cleavable linker joining component #1 and component # 2, in which upon cleavage of the cleavable linker, such as by proteolysis, increased binding by the CD3 binding region is enabled.

[0012] In the inactive, *i.e.*, uncleaved state, component #1 and component #2 of the multispecific polypeptide constructs are operably linked and do not bind or otherwise engage CD3 unless the antigen binding domain(s) is bound to their cognate antigen. In some examples, the uncleaved 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

examples, the uncleaved multispecific polypeptide constructs are capable of interacting with complement proteins, namely C1q, and mediating complement dependent cytotoxicity.

[0013] The multispecific polypeptide constructs of the disclosure generally have more than one antigen-binding domain(s). In provided examples in which the multispecific polypeptide constructs contain a cleavable linker, once the linker joining the first and second component is cleaved, such as by protease, each component maintains at least one antigen binding domain. The first component (i.e., component #1) contains at least an Fc region and antigen binding domain. The second component (i.e., component #2) contains at least an anti-CD3 binding domain and an antigen binding domain.

[0014] Cleavage, such as by proteolysis, within the cleavable linker physically separates component #1 and component #2, each of which has therapeutic utility albeit rely on different effector cells. Component #1 contains at least one antigen binding domain and an Fc region. In some examples, component #1 is capable of eliciting innate immune effector functions, for example ADCC, cytokine release, degranulation and/or phagocytosis. Component #2 contains at least domain CD3 binding region and an antigen binding domain, the former of which is capable of binding CD3 (when separated from component #1). Component #2 is capable of forming an immune synapse between an antigen expression cell and a T-cell. This co-engagement mediates antigen dependent T-cell activation, cytotoxicity, cytokine release, degranulation and proliferation. In the cleaved/activated state component #2 is not operably linked to the Fc-region of component #1 and thereby component #2 does not interact with FcRn and has enhanced serum clearance if localized to a site without an antigen expressing cell. This is advantageous as it limits the systemic exposure of the activated anti-CD3 binding domain and focuses directly into antigen expressing tissues, e.g., tumor cells or the tumor microenvironment.

[0015] In some examples, the multispecific polypeptide is in an inactive state, *i.e.*, uncleaved state, and binding of the CD3-binding region to CD3 is inhibited or substantially reduced when the multispecific polypeptide construct is in an uncleaved state compared to a cleaved state. In some examples, the multispecific polypeptide is in an activated state, and the first and second components are not operably linked. In some examples, the multispecific polypeptide is in an activated state, *i.e.*, cleaved state, and the second component binds the epsilon chain of CD3 (CD3 ϵ) and a tumor associated antigen (TAA).

[0016] In some examples, 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 examples, 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.

[0017] In some examples, 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.

[0018] In some examples, the first component includes one or more copies of an antigen-binding domain. In some examples, the first component contains at least two antigen binding domains, such as two antigen binding domains. In some examples, 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 binds to a different epitope of the same TAA. In some instances, the at least two antigen binding domains of the first component binds to a different epitope of the same TAA. In some examples, the at least two antigen binding domain of the first component bind to a different TAA.

[0019] In some examples, the antigen-binding domain of the first component, which, in some cases is a first antigen binding domain, includes one or more copies of an antibody or an antigen-binding fragment thereof. In some examples, the antigen binding domain of the first component, such as the first antigen-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 examples, the first antigen-binding domain 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 camelid heavy chain only antibodies. V_{NARS} 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.

[0020] In some examples, the antigen binding domain of the first component, such as the first antigen binding domain, binds an antigen, such as a tumor associated antigen (TAA). In some examples, 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, 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.

[0021] In some examples, the Fc region is a homodimeric Fc region. In some examples, the Fc region is a heterodimeric Fc region.

[0022] In some examples, 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 examples, 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 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. In some of any such examples, 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. In some examples, 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.

[0023] In some examples, 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 examples, 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.

[0024] In some examples, 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. In some examples, 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 examples, 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.

[0025] In some examples, the Fc is a heterodimeric Fc. 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 examples, each of the Fc polypeptides of the heterodimeric Fc independently comprise at least one amino acid modification. In some cases, 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. In some examples, the amino acid modification is a knob-into-hole modification.

[0026] In some examples, 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.

[0027] In some examples, the amino acid modification is a charge mutation to increase electrostatic complementarity of the polypeptides. In some examples, 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 examples, 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 examples, at least the first or second Fc polypeptides each comprise a modification in a complementary position, wherein the modification is replacement with an amino acid having an opposite charge to the complementary amino acid of the other polypeptide. In some examples, 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.

[0028] In some examples, 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 examples, 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. In some examples, the Fc region comprises a polypeptide that lacks Lys447.

[0029] In some examples, 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 examples, 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.

[0030] In some examples, 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 examples, 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.

[0031] In some examples, 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.

[0032] In some examples, 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 examples, 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.

[0033] In some examples, the Fc region comprises a polypeptide comprising at least one modification to enhance FcγR binding. In some cases, the modification is modification at Ser239 or Ile332. In some examples, the glycosylation of the Fc region is modified to enhance FcγR binding as compared to an unmodified Fc region. In some examples, the Fc region lacks or has reduced fucose content.

[0034] In some examples, the CD3 binding region is an anti-CD3 antibody or antigen-binding fragment. In some examples, 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 examples, the CD3 binding region is monovalent.

[0035] In some examples, 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 examples, 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. In some examples, 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. In some examples, 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.

[0036] In some examples, the multispecific polypeptide construct contains a linker that is a polypeptide linker. In some examples, 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 examples, 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. In some cases, the linker is a cleavable linker.

[0037] In some examples, the first antigen binding domain and the immunoglobulin Fc polypeptide are operably linked via amino acid linkers. In some examples, these intra-component linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present disclosure can be of various lengths, for example 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length.

[0038] In some examples, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, *i.e.*, (GGG)₂ (SEQ ID NO: 10); GGSGGGSGGS, *i.e.*, (GGG)₃ (SEQ ID NO: 11); GGSGGGSGGGSGGS, *i.e.*, (GGG)₄ (SEQ ID NO: 12); and GGSGGGSGGGSGGGSGGS, *i.e.*, (GGG)₅ (SEQ ID NO: 13).

[0039] In some examples, the second component also includes one or more copies of an anti-CD3 binding domain. In some examples, the anti-CD3 binding domain includes

one or more copies of an antibody or an antigen-binding fragment thereof. In some examples, 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. In some examples, the anti-CD3 binding domain includes an Fv antibody fragment that binds CD3ε (referred to herein as an anti-CD3ε Fv fragment). In some examples, the anti-CD3ε Fv antibody fragment includes an amino acid sequence selected from the group of SEQ ID NO: 32-81. In some examples, the anti-CD3ε 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 examples, the anti-CD3ε 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 examples, the anti-CD3ε 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.

[0040] In some examples, the anti-CD3ε Fv antibody fragment is a disulfide stabilized anti-CD3 binding Fv fragment (dsFv).

[0041] In some examples, the second component also includes one or more copies of an antigen-binding domain. In certain examples, the second component contain at least two antigen binding domains, such as two antigen binding domains. In some examples, 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 binds to a different epitope of the same TAA. In some instances, the at least two antigen binding domains of the second component binds to a different epitope of the same TAA. In some examples, the at least two antigen binding domain of the second component bind to a different TAA.

[0042] In some examples, 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 examples, the second antigen-binding domain of the second component binds the same antigen as the first antigen-binding domain of the first component. In some examples, the second antigen-binding domain of the second component binds a different epitope on the same antigen as the first antigen-binding domain of the first component. In some examples, the second antigen-binding domain of the second component binds the epitope on the same antigen as the first antigen-binding domain of the first component.

[0043] In some examples, the antigen binding domain of the second component, such as the second antigen-binding domain, includes one or more copies of an antibody or an antigen-binding fragment thereof. In some examples, the second antigen-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 examples, the second antigen-binding domain 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 camelid heavy chain only antibodies. V_{NARS} 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.

[0044] In some examples, the antigen binding domain of the second component, such as the second antigen-binding domain, binds an antigen, such as a tumor associated antigen (TAA). In some examples, 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.

[0045] In some examples, the antigen binding domain of the second component, such as the second antigen binding domain, and the anti-CD3-binding domain are operably linked via amino acid linkers. In some examples, these intra-component linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present disclosure 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.

[0046] In some examples, 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).

[0047] 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 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).

[0047a] In another aspect, the present disclosure refers to 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 cleavable linker, wherein the heterodimeric Fc region is positioned amino-terminally relative to the anti-CD3 antibody; and

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

[0048] In some examples, binding of the CD3-binding region to CD3 is substantially reduced when the multispecific polypeptide construct is in an uncleaved state compared to a cleaved state. In some examples, in a cleaved state, the first and second components are not linked.

[0049] In some examples, the cleavable linker is a polypeptide. In some examples, the cleavable linker is a polypeptide that is a substrate for a protease. In some examples, the protease is produced by an immune effector cell, by a tumor, or by cells present in the tumor microenvironment. In some examples, the protease is produced by a tumor that is in proximity to cells that express CD3 ϵ and/or is produced by a tumor that is co-localized with cells that express CD3 ϵ in a tissue, and wherein the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease. In some examples, the protease is produced by a tumor that is in proximity to cells that express one or more tumor associated antigens (TAA) and/or is produced by a tumor that is co-localized with cells that express the target TAA(s) in a tissue, and wherein the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease. In some examples, the protease is produced by an immune effector cell. In some examples, the protease is produced by an immune effector cell that is in proximity to cells that express the TAA. In some examples, 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 examples, the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease. In some examples, the protease is produced an immune effector cell that is in proximity to cells that express the TAA and wherein the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease.

[0050] In some examples, the cleavable linker is a polypeptide of up to 50 amino acids in length. In some examples, the cleavable linker is a polypeptide of up to 25 amino acids in length. In some examples, the cleavable linker is a polypeptide of up to 15 amino acids in length.

[0051] In some examples, the cleavable linker is a substrate for a protease selected from the proteases described herein. In some examples, the cleavable linker is a substrate for a protease selected from the group consisting of uPA, legumain, matriptase (also referred to herein as MT-SP1 or MTSP1), ADAM17, BMP-1, TMPRSS3, TMPRSS4, MMP-9, MMP-12, MMP-13, MMP-14, and any combination thereof. In some examples, the cleavable linker is a substrate for a protease selected from the group consisting of uPA, legumain, and matriptase. In some examples, the protease is selected from among matriptase, a matrix metalloprotease (MMP), granzyme B, and combinations thereof.

[0052] In some examples, the protease is granzyme B. In some examples, the cleavable linker comprises an amino acid sequence of the general formula $P_4 P_3 P_2 P_1 \downarrow P_1'$ (SEQ ID NO: 150), wherein P_4 is amino acid I, L, Y, M, F, V, or A; P_3 is amino acid A, G, S, V, E, D, Q, N, or Y; P_2 is amino acid H, P, A, V, G, S, or T; P_1 is amino acid D or E; and P_1' is amino acid I, L, Y, M, F, V, T, S, G or A. In some examples, the cleavable linker comprises an amino acid sequence of the general formula $P_4 P_3 P_2 P_1 \downarrow P_1'$ (SEQ ID NO: 151), wherein P_4 is amino acid I or L; P_3 is amino acid E; P_2 is amino acid P or A; P_1 is amino acid D; and P_1' is amino acid I, V, T, S, or G. In some examples, the cleavable linker comprises the amino acid sequence IEPDI (SEQ ID NO:136), LEPDG (SEQ ID NO:152), 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) or LEADG (SEQ ID NO:153). In some cases, the cleavable linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:22, 105-112, 136-141, 148,150-153.

[0053] In some examples, the protease is matriptase. In some cases, the cleavable linker comprises the sequence $P_1QAR\downarrow(A/V)$ (SEQ ID NO: 154), wherein P_1 is any amino acid; or the cleavable linker comprises the sequence $RQAR(A/V)$ (SEQ ID NO: 155). In some examples, the cleavable linker comprises the sequence $RQARV$ (SEQ ID NO: 156). In some cases, the cleavable linker comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 23, 154-156.

[0054] In some examples, the protease is an MMP. In some examples, the MMP is MMP-2. In some examples, the cleavable linker comprises the general formula $P_3 P_2 P_1 \downarrow P_1'$ (SEQ ID NO: 157), wherein P_3 is P, V or A; P_2 is Q or D; P_1 is A or N; and P_1' is L, I or M. In some cases, the cleavable linker comprises the general formula $P_3 P_2 P_1 \downarrow P_1'$ (SEQ ID NO: 158), wherein P_3 is P; P_2 is Q or D; P_1 is A or N; and P_1' is L or I. In some examples, the cleavable linker comprises the sequence PAGL (SEQ ID NO:24). In some examples, the cleavable linker is a substrate for a matrix metalloprotease (MMP).

[0055] In some examples, 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.

[0056] In some of any of the provided examples, the antigen binding domain(s) results in monovalent, bivalent, trivalent, or tetravalent binding to the TAA. In some examples, the one or more antigen binding domains that bind TAA independently are selected from an sdAb, an scFv or a Fab. In some examples, the one or more antigen binding domains that binds a TAA is 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 examples, 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.

[0057] In some examples, 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.

[0058] In some examples, the at least one of the antigen binding domain(s) is a Fab. In some examples, 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 examples, 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 at the carboxy-terminally relative to the CD3 binding region of one of the first or second polypeptide of the multispecific polypeptide construct. In some examples, 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.

[0059] 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, 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, 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.

[0060] In some examples, 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 binds a different epitope of the same TAA. In some instances, the first antigen binding domain and the second antigen binding domain binds the same epitope of the same TAA. In some examples, 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 a different TAA.

[0061] In some examples, 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 examples, 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 examples, 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.

[0062] In some examples, the multispecific polypeptide construct in the uncleaved state 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 (such as a cleavable linker) – anti-CD3 binding domain – LP2 – second antigen binding domain. In some examples, the multispecific polypeptide construct in the uncleaved state has the structural arrangement from N-terminus to C-terminus as follows: second antigen binding domain – LP2 – anti-CD3 binding domain (CD3 binding region) – linker (such as a cleavable linker) – immunoglobulin Fc polypeptide linker region – LP1 – first antigen binding domain. In some examples, the linker is a cleavable linker. In some examples, the two linking peptides 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.

[0063] In some examples, the multispecific construct is a construct having any of the structural arrangements shown in FIG. 1. In some examples, the multispecific construct is a bispecific construct having the structural arrangement shown in FIG. 2. In some examples, the bispecific construct 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 examples, the central immunoglobulin Fc polypeptide region is heterodimeric. The immunoglobulin Fc polypeptide region is coupled to a cleavable linker that contains one or more proteolytic cleavage sites located at a position C-terminal to the end of the immunoglobulin Fc polypeptide region. In some examples, the one or more proteolytic cleavage sites is a substrate for matriptase, a matrix metalloprotease (MMP), or granzyme B. The cleavable linker is attached to an anti-CD3 binding sequence located C-terminal from the Fc region and in some cases, at the distal end of the second component.

[0064] In some examples, the anti-CD3 antibody or antigen binding fragment is an Fv antibody fragment. In some examples, the Fv antibody fragment comprises a disulfide stabilized anti-CD3 binding Fv fragment (dsFv). In some examples, the anti-CD3 binding sequence is an Fv antibody fragment that has an 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 examples, the VH and VL domains that comprise the anti-CD3 Fv are operably linked to opposite members of a heterodimeric Fc region. In these examples, the anti-CD3 Fv binds CD3 in a monovalent fashion. The anti-CD3 dsFv does not engage CD3 when the cleavable linkers are intact, *i.e.*, in an uncleaved or inactive state. The C-terminus of the bispecific construct includes a second antigen binding domain that binds a TAA. In some examples, the second antigen binding domain binds the same TAA as the first antigen binding domain located in within the first component. In some examples, 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 example, the second antigen binding domain binds a distinct TAA from that of the first antigen binding domain.

[0065] In some examples, 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 examples, 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 examples, 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 examples, 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_{NARS} 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.

[0066] In some examples, the antibody or antigen-binding fragment is an sdAb. In some cases, the sdAb is a human or humanized sdAb. In some examples, the sdAb is VHH, VNAR, an engineered VH domain or an engineered VK 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.

[0067] In some of any of the provided examples, the anti-CD3 antibody or antigen-binding fragment comprises a VH CDR1 comprising the amino acid sequence TYAMN (SEQ ID NO: 16); a VH CD2 comprising the amino acid sequence RIRSKYNNYATYYADSVKD (SEQ ID NO: 17); 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).

[0068] In some examples, the anti-CD3 dsFv comprises: a VH having the amino acid sequence of any of SEQ ID NOS: 14, 44, 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, 44, and 32-62; and a VL having the amino acid sequence of any of SEQ ID NOS: 15, 72, 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, 44, and 32-62. 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 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.

[0069] In some examples, 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 immunoglobulin Fc region is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1-6. In some examples, 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.

[0070] In some examples, 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. In some examples, 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 examples, 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. In some examples, modifications within the Fc region reduce binding to Fc-receptor-gamma receptors while have minimal impact on binding to the neonatal Fc receptor (FcRn). In some examples, 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.

[0071] In some examples, the first antigen binding domain and the immunoglobulin Fc polypeptide are operably linked via amino acid linkers. In some examples, these intra-component linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present disclosure 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.

[0072] In some examples, the GS-linker comprises an amino acid sequence selected from the group consisting of GGS GGS, *i.e.*, (GGS)₂ (SEQ ID NO: 10); GGS GGS GGS, *i.e.*, (GGS)₃ (SEQ ID NO: 11); GGS GGS GGS GGS, *i.e.*, (GGS)₄ (SEQ ID NO: 12); and GGS GGS GGS GGS GGS, *i.e.*, (GGS)₅ (SEQ ID NO: 13).

[0073] In some examples, the anti-CD3ε dsFv antibody fragment includes an amino acid sequence selected from the group consisting of SEQ ID NO: 32-81. In some examples, the anti-CD3ε dsFv 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 examples, the anti-CD3ε 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 examples, the anti-CD3ε 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.

[0074] In some examples, the second antigen binding domain and the anti-CD3-binding domain are operably linked via amino acid linkers. In some examples, these intra-component linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present disclosure 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.

[0075] In some examples, the GS-linker comprises an amino acid sequence selected from the group consisting of GGS₂, *i.e.*, (GGS)₂ (SEQ ID NO: 10); GGS₃, *i.e.*, (GGS)₃ (SEQ ID NO: 11); GGS₄, *i.e.*, (GGS)₄ (SEQ ID NO: 12); and GGS₅, *i.e.*, (GGS)₅ (SEQ ID NO: 13).

[0076] In some examples, the cleavable linker is a polypeptide. In some examples, the cleavable linker is a polypeptide that is a substrate for a protease. In some examples, the protease is produced by a tumor that is in proximity to cells that express CD3ε and/or is produced by a tumor that is co-localized with cells that express CD3ε in a tissue, and wherein the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease. In some examples, the protease is produced by a tumor that is in proximity to cells that express one or more tumor associated antigens (TAA) and/or is produced by a tumor that is co-localized with cells that express the target TAA(s) in a tissue, and wherein the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease.

[0077] In some examples, the cleavable linker is a polypeptide of up to 50 amino acids in length. In some examples, the cleavable linker is a polypeptide of up to 25 amino acids in length. In some examples, the cleavable linker is a polypeptide of up to 15 amino acids in length. In some examples, the cleavable linker is a substrate for a protease selected from the proteases described herein. In some examples, the cleavable linker is a substrate for a protease selected from the group consisting of uPA, legumain, matriptase (also referred to herein as MT-SP1 or MTSP1), ADAM17, BMP-1, TMPRSS3, TMPRSS4, MMP-9, MMP-12, MMP-13, MMP-14, and any combination thereof. In some examples, the cleavable linker is a substrate for a protease selected from the group consisting of uPA, legumain, and matriptase. In some examples, the cleavable linker is a substrate for a matrix metalloprotease (MMP).

[0078] In some examples, the multispecific construct also includes an agent conjugated to the multispecific construct. In some examples, the agent is a therapeutic agent. In some examples, the agent is a detectable moiety. In some examples, the detectable moiety is a diagnostic agent. In some examples, the agent is conjugated to the multispecific construct via a linker. In some examples, the linker is a cleavable linker. In some examples, the linker is a non-cleavable linker.

[0079] In some examples, the anti 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.

[0080] In some examples, 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 examples, 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.

[0081] In some examples, the multispecific construct naturally contains one or more disulfide bonds. In some examples, the multispecific construct can be engineered to include one or more disulfide bonds.

[0082] 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.

[0083] Among the provided examples is a polynucleotide(s) encoding any of the provided multispecific polypeptide constructs. In another aspect, the present disclosure refers to a polynucleotide(s) encoding the multispecific polypeptide construct as disclosed herein. In another aspect, the present disclosure refers to a polynucleotide encoding a polypeptide chain of any of the multispecific polypeptide constructs as disclosed herein. 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 examples, 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 examples, 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).

[0084] Provided herein is a vector comprising any of the provided polynucleotides. In some examples, 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.

[0084a] In another aspect, the present disclosure refers to a vector, comprising a polynucleotide or polynucleotides as disclosed herein.

[0085] 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.

[0085a] In another aspect, the present disclosure refers to a cell, comprising a polynucleotide or polynucleotides as disclosed herein, or the vector as disclosed herein.

[0086] 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 examples, the cell comprises such a vector.

[0087] 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 examples, the method further includes isolating or purifying the multispecific polypeptide construct from the cell. In some cases, the multispecific polypeptide construct is a heterodimer.

[0087a] In another aspect, the present disclosure refers to a method of producing a multispecific polypeptide construct, the method comprising introducing into a cell a polynucleotide or polynucleotides as disclosed herein, or the vector as disclosed herein, and culturing the cell under conditions to produce the multispecific polypeptide construct.

[0088] Provided herein is a multispecific polypeptide construct produced by any of the provided methods.

[0088a] In another aspect, the present disclosure refers to a multispecific polypeptide construct produced by the method as disclosed herein.

[0089] 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 examples, the target cell is a tumor cell expressing the tumor associated antigen (TAA).

[0089a] In another aspect, the present disclosure refers to 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 as disclosed herein or the pharmaceutical composition as disclosed herein, said target cell expressing a tumor associated antigen recognized by the multispecific polypeptide construct.

[0090] In some examples, the multispecific polypeptide construct comprises a cleavable linker that functions as a substrate for a protease and the inducing or stimulating the immune response is increased in the presence of the protease. In some cases, the protease is produced by an immune effector cell, by a tumor, or by cells present in the tumor microenvironment.

[0091] In some examples, 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 instances, the immune effector cell is in proximity to cells that express the antigen. In some examples, the protease is produced by a tumor that is in proximity to cells that express the TAA in a tissue and/or produced by a tumor that is co-localized with TAA in a tissue, and wherein the protease cleaves the cleavable linker in the multispecific polypeptide construct when the multispecific polypeptide construct is exposed to the protease. In some examples, the protease is selected from among matrilysin, a matrix metalloprotease (MMP), granzyme B, and combinations thereof. In some instances, the protease is granzyme B.

[0092] In some examples, the contacting is carried out ex vivo or in vitro. In some examples, the contacting is carried out in vivo in a subject.

[0093] 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 examples, the method increases T-cell activity. In some examples, the method increases cytolytic T-cell (CTL) activity. In some examples, the immune response is increased against a tumor or cancer. In some examples, the method treats a disease or condition in the subject.

[0093a] In another aspect, the present disclosure refers to a composition comprising a therapeutically effective amount of the multispecific conjugate as disclosed herein or the pharmaceutical composition as disclosed herein for use in stimulating or inducing an immune response in a subject.

[0093b] In another aspect, the present disclosure refers to use of a composition comprising the multispecific polypeptide construct as disclosed herein or the pharmaceutical composition as disclosed herein in the manufacture of a medicament for stimulating or inducing an immune response in a subject.

[0094] 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 examples, the disease or condition is a tumor or a cancer.

[0094a] In another aspect, the present disclosure refers to a composition comprising a therapeutically effective amount of the multispecific polypeptide construct as disclosed herein or the pharmaceutical composition as disclosed herein for use in treating a disease or condition in a subject.

[0094b] In another aspect, the present disclosure refers to use of a composition comprising the multispecific polypeptide construct as disclosed herein or the pharmaceutical composition as disclosed herein in the manufacture of a medicament for the treatment of a disease or condition in a subject.

[0095] In some examples of any of the provided method, the subject, such as the subject to be treated is, *e.g.*, human or other mammal. In some examples of any of the provided method, the subject is a human. In some examples, 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 examples, the subject is a rodent.

[0096] A multispecific polypeptide construct of the disclosure used in any of the examples 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.

[0097] A multispecific polypeptide construct of the disclosure used in any of the examples of these methods and uses can be used in a treatment regimen comprising neoadjuvant therapy.

[0098] A multispecific polypeptide construct of the disclosure used in any of the examples 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 examples, 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 examples, the additional agent is a taxane, such as paclitaxel (*e.g.*, Abraxane®). In some examples, the additional agent is an anti-metabolite, such as gemcitabine. In some examples, the additional agent is an alkylating agent, such as platinum-based chemotherapy, such as carboplatin or cisplatin. In some examples, the additional agent is a targeted agent, such as a kinase inhibitor, *e.g.*, sorafenib or erlotinib. In some examples, 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 examples, the additional agent is a proteasome inhibitor, such as bortezomib or

carfilzomib. In some examples, the additional agent is an immune modulating agent, such as lenolidomide or IL-2. In some examples, the additional agent is radiation. In some examples, the additional agent is an agent considered standard of care by those skilled in the art. In some examples, the additional agent is a chemotherapeutic agent well known to those skilled in the art. In some examples, the multispecific polypeptide construct and the additional agent(s) are formulated in a single composition. In some examples, the multispecific polypeptide construct and the additional agent(s) are administered as two or more separate compositions. In some examples, the multispecific polypeptide construct and the additional agent(s) are administered simultaneously. In some examples, the multispecific polypeptide construct and the additional agent(s) are administered sequentially.

[0099] In some examples, 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.

[00100] In some examples, 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 examples, 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 examples, 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 examples, 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)).

[00101] In some examples, 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 examples, the kinase inhibitor is selected from the group consisting of B-RaFi, MEKi, and Btk inhibitors, such as ibrutinib. In some examples, the kinase inhibitor is crizotinib. In some examples, 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 examples, the agonist is selected from the group consisting of OX40, GITR, CD137, CD28, ICOS, CD27, and HVEM. In some examples, the checkpoint inhibitor is an antibody that binds a target selected from CTLA-4, PD-1, and/or PD-L1. In some examples, 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 examples, the checkpoint inhibitor is an anti-CTLA4 antibody such as, *e.g.*, Yervoy™. In some examples, the checkpoint inhibitor is an anti-PD-1 antibody such as, *e.g.*, Opdivo™ and/or Keytruda™.

[00102] In some examples, the inhibitor is a CTLA-4 inhibitor. In some examples, the inhibitor is a LAG-3 inhibitor. In some examples, the inhibitor is a PD-1 inhibitor. In some examples, the inhibitor is a PDL1 inhibitor. In some examples, the inhibitor is a TIGIT inhibitor. In some examples, the inhibitor is a TIM-3 inhibitor. In some examples, the inhibitor is a B7H3 inhibitor. In some examples, the inhibitor is a B7H4 inhibitor. In some examples, the inhibitor is a Vista inhibitor. In some examples, the inhibitor is a B-RAFi inhibitor. In some examples, the inhibitor is a MEKi inhibitor. In some examples, the inhibitor is a Btk inhibitor. In some examples, the inhibitor is ibrutinib. In some examples, the inhibitor is crizotinib. In some examples, the inhibitor is an IDO inhibitor. In some examples, the inhibitor is an α -CSF1R inhibitor. In some examples, the inhibitor is an α -CCR4 inhibitor. In some examples, the inhibitor is a TGF-beta. In some examples, the inhibitor is a myeloid-derived suppressor cell. In some examples, the inhibitor is a T-regulatory cell.

[00103] In some examples, the agonist is OX40. In some examples, the agonist is GITR. In some examples, the agonist is CD137. In some examples, the agonist is CD28. In some examples, the agonist is ICOS. In some examples, the agonist is CD27. In some examples, the agonist is HVEM.

[00104] In some examples, 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 examples, 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.

[00105] In some examples, 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 examples, 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.

[00106] 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.

[00107] 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.

[00108] 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 examples, the agent is a dolastatin. In some examples, the agent is an auristatin or derivative thereof. In some examples, the agent is a maytansinoid or maytansinoid derivative. In some examples, the agent is DM1 or DM4. In some examples, the agent is a duocarmycin or derivative thereof. In some examples, the agent is a calicheamicin or derivative thereof. In some examples, the agent is a pyrrolobenzodiazepine.

[00109] In some examples, the linker between the multispecific polypeptide construct and the cytotoxic agent is cleavable. In some examples, the linker is non-cleavable. In some examples, 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.

[00110] 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 examples, the cancer is a squamous cell cancer. In some examples, the cancer is a skin squamous cell carcinoma. In some examples, the cancer is an esophageal squamous cell carcinoma. In some examples, the cancer is a head and neck squamous cell carcinoma. In some examples, the cancer is a lung squamous cell carcinoma.

[00111] 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.

[00111a] In another aspect, the present disclosure refers to a pharmaceutical composition comprising the multispecific polypeptide construct as disclosed herein and a pharmaceutically acceptable carrier.

[00112] 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.

CLAIMS

What is claimed is:

1. A multispecific polypeptide construct, the multispecific polypeptide construct comprising a first component comprising a heterodimeric 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 amino-terminally relative to the CD3-binding region;

the CD3-binding region is an anti-CD3 antibody or antigen binding fragment that is a Fv antibody fragment comprising a variable heavy chain region (VH) and a variable light chain region (VL) and the VH and VL are linked to opposite polypeptides of the heterodimeric Fc; and

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

2. 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 linker, wherein the Fc region is positioned amino-terminally relative to the CD3-binding region; and

the multispecific polypeptide construct further comprises at least one antigen binding domain that binds a tumor associated antigen (TAA) and is positioned carboxy-terminally relative to the CD3-binding region.

3. 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 linker, wherein the Fc region is positioned amino-terminally relative to the CD3-binding region; and

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

4. The multispecific polypeptide construct of any of claims 1-3, wherein the linker is a polypeptide linker.

5. The multispecific polypeptide construct of any of claims 1-4, wherein the linker is a cleavable linker.

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 first and second components are coupled by a cleavable linker, wherein the Fc region is positioned amino-terminally relative to the CD3-binding region; and

the multispecific polypeptide construct further comprises at least one antigen binding domain that binds a tumor associated antigen (TAA).

7. 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 cleavable linker, wherein the heterodimeric Fc region is positioned amino-terminally relative to the anti-CD3 antibody; and

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

8. The multispecific polypeptide construct of any of claims 1, 2 and 4-7, wherein the at least one antigen binding domain is positioned amino-terminally relative to the Fc region and/or positioned carboxy-terminally relative to the CD3-binding region.

9. The multispecific polypeptide construct of any of claims 1, 2, and 4-8, 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 binds a tumor associated antigen (TAA).

10. The multispecific polypeptide construct of any of claims 1-9, wherein the CD3-binding region binds CD3 (CD3ε).

11. The multispecific polypeptide construct of any of claims 3, 9, and 10, wherein the first antigen binding domain is positioned amino-terminally relative to the Fc region and the second antigen binding domain is positioned carboxy-terminally relative to the CD3-binding region.

12. The multispecific polypeptide construct of any of claims 1-11, 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.

13. The multispecific polypeptide construct of any of claims 2-6 and 8-12, wherein the Fc region is a heterodimeric Fc region.

14. The multispecific polypeptide construct of any of claims 1, 7, and 13, wherein one or both Fc polypeptide of the heterodimeric Fc region comprises at least one amino acid modification to promote 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 any of claims 1, 7, 13, and 14, wherein each of the Fc polypeptides of the heterodimeric Fc comprises a knob-into-hole modification or comprises a charge mutation to increase electrostatic complementarity of the polypeptides.

16. The multispecific polypeptide construct of any of claims 1-15, wherein the Fc region comprises a polypeptide comprising at least one amino acid modification to enhance FcRn binding.

17. The multispecific polypeptide construct of any of claims 1-16, wherein the Fc region comprises a polypeptide comprising at least one amino acid modification that reduces effector function and/or reduces binding to Fc gamma receptor or C1q.

18. The multispecific polypeptide construct of any of claims 2-6 and 8-17, wherein the CD3-binding region is an anti-CD3 antibody or antigen-binding fragment.
19. The multispecific polypeptide construct of claim 18, wherein the anti-CD3 antibody or antigen binding fragment comprises a variable heavy chain region (VH) and a variable light chain region (VL).
20. The multispecific polypeptide construct of any of claims 1-19, wherein the CD3-binding region is monovalent.
21. The multispecific polypeptide construct of any of claims 1, 7, and 18-20, wherein the anti-CD3 antibody or antigen binding fragment is not a single-chain antibody, optionally is not a single-chain variable fragment (scFv).
22. The multispecific polypeptide construct of any of claims 19-21, 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.
23. The multispecific polypeptide construct of any of claims 1-22, wherein the CD3-binding region is not able to, or is not substantially able to, bind or engage CD3 unless at least one antigen binding domain is bound to its TAA, optionally wherein at least two antigen binding domains are bound to their TAA(s).
24. The multispecific polypeptide construct of any of claims 5-23, wherein binding of the CD3-binding region to CD3 is substantially reduced when the multispecific polypeptide construct is in an uncleaved state, compared to a cleaved state.
25. The multispecific polypeptide construct of any of claims 5-24, wherein the cleavable linker is a polypeptide that functions as a substrate for a protease.
26. The multispecific polypeptide construct of claim 25, wherein the protease is selected from the group consisting of: matriptase, a matrix metalloprotease (MMP), granzyme B, and combinations thereof.

27. The multispecific polypeptide construct of any of claims 1-26, 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.

28. The multispecific polypeptide construct of any of claims 1-27, wherein the antigen binding domain, or independently each of the antigen binding domains, is a single domain antibody (sdAb).

29. The multispecific polypeptide construct of any of claims 1-28, wherein the antigen binding domain, or independently each of the antigen binding domains, binds to a tumor antigen 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, IFNalpha, IFNbeta, IFNgamma, 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.

30. The multispecific polypeptide construct of any of claims 1-29, 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.

31. The multispecific polypeptide construct of any of claims 1-30, 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 the second antigen binding domain bind a different TAA.

32. The multispecific polypeptide construct of any of claims 7 and 18-31, wherein the anti-CD3 antibody or antigen binding fragment is an Fv antibody fragment.

33. The multispecific polypeptide construct of any of claims 1 and 32, wherein the Fv antibody fragment comprises a disulfide stabilized anti-CD3 binding Fv fragment (dsFv).

34. The multispecific polypeptide construct of any of claims 1-33, wherein the multispecific polypeptide construct is conjugated to an agent, optionally wherein the agent is a therapeutic agent, an antineoplastic agent, a toxin or fragment thereof, a detectable moiety, or a diagnostic agent.

35. A polynucleotide(s) encoding the multispecific polypeptide construct of any of claims 1-34.

36. A polynucleotide encoding a polypeptide chain of any of the multispecific polypeptide construct of any of claims 1-34.

37. A vector, comprising a polynucleotide or polynucleotides of claim 35 or claim 36.

38. A cell, comprising a polynucleotide or polynucleotides of claim 35 or claim 36, or the vector of claim 37.

39. A method of producing a multispecific polypeptide construct, the method comprising introducing into a cell a polynucleotide or polynucleotides of claim 35 or claim 36, or the vector of claim 37, and culturing the cell under conditions to produce the multispecific polypeptide construct.

40. A multispecific polypeptide construct produced by the method of claim 39.

41. A pharmaceutical composition comprising the multispecific polypeptide construct of any of claims 1-34 and 40 and a pharmaceutically acceptable carrier.

42. 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 1-34 and 40 or the pharmaceutical composition of claim 41, said target cell expressing a tumor associated antigen recognized by the multispecific polypeptide construct.

43. The method of claim 42, wherein the contacting is carried out ex vivo or in vitro.

44. A composition comprising a therapeutically effective amount of the multispecific conjugate of any of claims 1-34 and claim 40 or the pharmaceutical composition of claim 41 for use in stimulating or inducing an immune response in a subject.

45. Use of a composition comprising the multispecific polypeptide construct of any of claims 1-34 and 40 or the pharmaceutical composition of claim 41 in the manufacture of a medicament for stimulating or inducing an immune response in a subject.

46. The composition for use of claim 44 or the use of claim 45, wherein the immune response is increased against a tumor or cancer.

47. The composition for use of claim 44 or the use of claim 45, wherein the composition or the medicament treats a disease or condition in the subject.

48. A composition comprising a therapeutically effective amount of the multispecific polypeptide construct of any of claims 1-34 and 40 or the pharmaceutical composition of claim 41 for use in treating a disease or condition in a subject.

49. Use of a composition comprising the multispecific polypeptide construct of any of claims 1-34 and 40 or the pharmaceutical composition of claim 41 in the manufacture of a medicament for the treatment of a disease or condition in a subject.

50. The composition for use of claim 48 or the use of claim 49, wherein the disease or condition is a tumor or a cancer.