

cytokines, in addition to the fact that some bispecific compositions have a very short half-life, necessitating continuous infusions of four to eight weeks in order to maintain circulating concentrations within the therapeutic window for sufficient time to achieve a therapeutic effect, or have a variable effect. Thus, there is an unmet need in the field for the development of effective bispecific antibodies for use in cancer treatment.

SUMMARY OF THE INVENTION

[0004] The present invention relates to anti-cluster of differentiation 3 (CD3) antigen binding fragments incorporated into chimeric fusion proteins and methods of using the same.

[0004a] In one aspect, disclosed herein is a polypeptide comprising an antigen binding fragment, wherein the antigen binding fragment, comprises light chain complementarity-determining regions (CDR-L) and heavy chain complementarity-determining regions (CDR-H), light chain framework regions (FR-L), and heavy chain framework regions (FR-H), and wherein the antigen binding fragment: specifically binds to cluster of differentiation 3 T cell receptor (CD3); comprises a variable heavy (VH) amino acid sequence having at least 90% sequence identity to an amino acid sequence of SEQ ID NO: 28; and comprises a variable light (VL) amino acid sequence having: (i) at least 99% sequence identity to an amino acid sequence of SEQ ID NO: 27; or (ii) at least 90% identity to an amino acid sequence of SEQ ID NO: 27, wherein the VL comprises a CDR-L1 sequence comprising an amino acid sequence of SEQ ID NO: 1; a CDR-L2 sequence comprising an amino acid sequence of SEQ ID NO: 4; and a CDR-L3 sequence comprising an amino acid sequence of SEQ ID NO: 6.

[0005] In one aspect, disclosed herein is a polypeptide comprising an antigen binding fragment, wherein the antigen binding fragment, comprises light chain complementarity-determining regions (CDR-L) and heavy chain complementarity-determining regions (CDR-H), and wherein the antigen binding fragment, a. specifically binds to cluster of differentiation 3 T cell receptor (CD3); and b. comprises CDR-H1, CDR-H2, and CDR-H3, having amino acid sequences of SEQ ID NOs: 8, 9, and 10, respectively.

[0006] In another aspect, disclosed herein is a polypeptide comprising an anti-CD3 antigen binding fragment, wherein the antigen binding fragment comprises light chain complementarity-determining regions (CDR-L) and heavy chain complementarity-determining regions (CDR-H), and wherein the antigen binding fragment a. specifically binds to CD3; b. comprises CDR-H1, CDR-H2, and CDR-H3, wherein CDR-H3 comprises an amino acid sequence of SEQ ID NO:10; and c. exhibits a higher thermal stability, as evidenced by in an in vitro assay, (i) a higher melting temperature (T_m) relative to that of an antigen binding fragment consisting of a sequence shown in SEQ ID NO:41, or (ii) upon incorporating said anti-CD3 antigen binding fragment into an anti-CD3 bispecific antibody, the bispecific antibody exhibits a higher T_m relative to a control bispecific antibody, wherein said anti-CD3 bispecific antibody comprises said anti-CD3 binding fragment and a reference antigen binding fragment that binds to an antigen other than CD3, and wherein said control bispecific antigen binding fragment consists of SEQ ID NO:41 and said reference antigen binding fragment.

[0007] In some embodiments, the T_m of the antigen binding fragment is at least 2 °C greater, or at least 3°C greater, or at least 4°C greater, or at least 5°C greater than the T_m of an antigen binding fragment consisting of a sequence of SEQ ID NO:41.

[0008] In yet another aspect, disclosed herein is a polypeptide comprising an antigen binding fragment, wherein the antigen binding fragment comprises light chain complementarity-determining regions (CDR-L) and heavy chain complementarity-determining regions (CDR-H), wherein the antigen binding fragment a. specifically binds to CD3; b. comprises CDR-H1, CDR-

[0043] In another aspect, disclosed herein is use of the polypeptide disclosed herein in the preparation of a medicament for the treatment of a disease in a subject. In some embodiments, the disease is selected from the group consisting of carcinomas, Hodgkin's lymphoma, non-Hodgkin's lymphoma, B cell lymphoma, diffuse large B cell lymphoma, T-cell lymphoma, follicular lymphoma, mantle cell lymphoma, blastoma, breast cancer, colon cancer, prostate cancer, head and neck cancer, any form of skin cancer, melanoma, genito-urinary tract cancer, ovarian cancer, ovarian cancer with malignant ascites, vaginal cancer, vulvar cancer, Ewing sarcoma, peritoneal carcinomatosis, uterine serous carcinoma, parathyroid cancer, endometrial cancer, cervical cancer, colorectal cancer, an epithelia intraperitoneal malignancy with malignant ascites, uterine cancer, mesothelioma in the peritoneum kidney cancers, lung cancer, laryngeal cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, stomach cancer, small intestine cancer, liver cancer, hepatocarcinoma, retinoblastoma, hepatoblastoma, liposarcoma, pancreatic cancer, gall bladder cancer, testicular cancer, cancers of the bile duct, cancers of the bone, salivary gland carcinoma, thyroid cancer, craniopharyngioma, carcinoid tumor, epithelial cancer, arrhenoblastoma, adenocarcinoma, sarcomas of any origin, primary hematologic malignancies including acute or chronic lymphocytic leukemias, acute or chronic myelogenous leukemias, B-cell derived chronic lymphatic leukemia, hairy cell leukemia, myeloproliferative neoplastic disorders, or myelodysplastic disorders, myasthenia gravis, Morbus Basedow, Kaposi sarcoma, neuroblastoma, Hashimoto thyroiditis, Wilms tumor, or Goodpasture syndrome.

[0043a] In one aspect, disclosed herein is a use of the polypeptide as described herein in the preparation of a medicament for the treatment of cancer in a subject.

[0044] In yet another aspect, disclosed herein is a method of treating a disease in a subject, comprising administering to the subject in need thereof one or more therapeutically effective doses of the pharmaceutical composition disclosed herein. In certain embodiments, the subject is selected from the group consisting of mouse, rat, monkey, and human. In some embodiments, the disease is selected from the group consisting of carcinomas, Hodgkin's lymphoma, non-Hodgkin's lymphoma, B cell lymphoma, T-cell lymphoma, follicular lymphoma, mantle cell lymphoma, blastoma, breast cancer, colon cancer, prostate cancer, head and neck cancer, any form of skin cancer, melanoma, genito-urinary tract cancer, ovarian cancer, ovarian cancer with malignant ascites, peritoneal carcinomatosis, uterine serous carcinoma, endometrial cancer, cervical cancer, colorectal cancer, an epithelia intraperitoneal malignancy with malignant ascites, uterine cancer, mesothelioma in the peritoneum kidney cancers, lung cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, stomach cancer, small intestine cancer, liver cancer, hepatocarcinoma, hepatoblastoma, liposarcoma, pancreatic cancer, gall bladder cancer, cancers of the bile duct, salivary gland carcinoma, thyroid cancer, epithelial cancer, adenocarcinoma, sarcomas of any origin, primary hematologic malignancies including acute or

chronic lymphocytic leukemias, acute or chronic myelogenous leukemias, myeloproliferative neoplastic disorders, or myelodysplastic disorders, myasthenia gravis, Morbus Basedow, Hashimoto thyroiditis, or Goodpasture syndrome.

[0045] In other embodiments, the pharmaceutical composition is administered to the subject as one or more therapeutically effective doses administered twice weekly, once a week, every two weeks, every three weeks, every four weeks, or monthly. In certain embodiments, the pharmaceutical composition is administered to the subject as one or more therapeutically effective doses over a period of at least two weeks, or at least one month, or at least two months, or at least three months, or at least four months, or at least five months, or at least six months. In some embodiments, the dose is administered intradermally, subcutaneously, intravenously, intra-arterially, intra-abdominally, intraperitoneally, intrathecally, or intramuscularly.

[0046] In one aspect, disclosed herein is an isolated nucleic acid, the nucleic acid comprising (a) a polynucleotide encoding a polypeptide disclosed herein; or (b) the complement of the polynucleotide of (a).

[0047] In a related aspect, disclosed herein is an expression vector comprising the polynucleotide sequence disclosed herein and a recombinant regulatory sequence operably linked to the polynucleotide sequence.

[0048] In another aspect, disclosed herein is an isolated host cell, comprising the expression vector disclosed herein. In some embodiments, the host cell is a prokaryote. In one embodiment, the host cell is *E. coli*.

[0048a] In another aspect, disclosed herein is the polypeptide as described herein for use in therapy.

INCORPORATION BY REFERENCE

[0049] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0050] Various features of this disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0051] FIGURE 1(FIG. 1) depicts the individual components of a bispecific antigen binding fragment composition. FIG. 1A depicts an antigen binding fragment having affinity to a target cell marker. FIG. 1B depicts an antigen binding fragment having affinity to an effector cell. FIGS. 1C and 1D depict XTEN polypeptides of different length. FIG. 1E depicts a cleavable release segment.

CLAIMS

WHAT IS CLAIMED IS:

1. A polypeptide comprising an antigen binding fragment, wherein the antigen binding fragment, comprises light chain complementarity-determining regions (CDR-L) and heavy chain complementarity-determining regions (CDR-H), light chain framework regions (FR-L), and heavy chain framework regions (FR-H), and wherein the antigen binding fragment:
 - specifically binds to cluster of differentiation 3 T cell receptor (CD3);
 - comprises a variable heavy (VH) amino acid sequence having at least 90% sequence identity to an amino acid sequence of SEQ ID NO: 28; and
 - comprises a variable light (VL) amino acid sequence having:
 - (i) at least 99% sequence identity to an amino acid sequence of SEQ ID NO: 27; or
 - (ii) at least 90% identity to an amino acid sequence of SEQ ID NO: 27, wherein the VL comprises a CDR-L1 sequence comprising an amino acid sequence of SEQ ID NO: 1; a CDR-L2 sequence comprising an amino acid sequence of SEQ ID NO: 4; and a CDR-L3 sequence comprising an amino acid sequence of SEQ ID NO: 6.
2. The polypeptide of claim 1, wherein the antigen binding fragment comprises CDR-H1, CDR-H2, and CDR-H3, having amino acid sequences of SEQ ID NOs: 8, 9, and 10, respectively.
3. The polypeptide of claim 1, wherein the T_m of the antigen binding fragment is at least 2 °C greater, or at least 3°C greater, or at least 4°C greater, or at least 5°C greater than the T_m of an antigen binding fragment consisting of a sequence of SEQ ID NO:41.
4. The polypeptide of claim 1, wherein the antigen binding fragment comprises: CDR-H1 having an amino acid sequence of SEQ ID NO: 8; CDR-H2 having an amino acid sequence of SEQ ID NO: 9; CDR-H3 having an amino acid sequence of SEQ ID NO:

10; CDR-L1 having an amino acid sequence of SEQ ID NO: 1; CDR-L2 having an amino acid sequence of SEQ ID NO: 4; and CDR-L3 having an amino acid sequence of SEQ ID NO: 6.

5. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment comprises:
 - a. a FR-L1 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:12;
 - b. a FR-L2 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:13;
 - c. a FR-L3 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:14;
 - d. a FR-L4 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:19;
 - e. a FR-H1 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:20;
 - f. a FR-H2 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:23;
 - g. a FR-H3 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:24; and
 - h. a FR-H4 consisting of an amino acid sequence that is at least 90% identical to amino acid sequence of SEQ ID NO:26.
6. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment comprises a variable heavy (VH) amino acid sequence having at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity or is identical to an amino acid sequence of SEQ ID NO:28.
7. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment comprises a variable light (VL) amino acid sequence having at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity or is identical to an amino acid sequence of SEQ ID NO: 27.

8. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment comprises an amino acid sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity or is identical to an amino acid sequence of any one of SEQ ID NOs:36-40.
9. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment specifically binds human or cynomolgus monkey (cyno) CD3.
10. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment specifically binds human and cynomolgus monkey (cyno) CD3.
11. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment binds a CD3 complex subunit selected from CD3 epsilon, CD3 delta, CD3 gamma, CD3 zeta, CD3 alpha and CD3 beta epsilon unit of CD3.
12. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment binds a CD3 epsilon fragment of CD3.
13. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment exhibits an isoelectric point (pI) that is less than or equal to 6.6.
14. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment exhibits a pI that is between 6.0 and 6.6, inclusive.
15. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment exhibits a pI that is at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 pH units lower than the pI of a reference antigen binding fragment consisting of a sequence shown in SEQ ID NO: 41.
16. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment specifically binds human or cyno CD3 with a dissociation constant (K_d) constant between about 10 nM and about 400 nM, as determined in an in vitro antigen-binding assay comprising a human or cyno CD3 antigen.
17. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment specifically binds human or cyno CD3 with a dissociation constant (K_d) of less than about 10 nM, or less than about 50 nM, or less than about 100 nM, or less than about 150 nM, or less than about 200 nM, or less than about 250 nM, or less than about 300 nM, or less than about 350 nM, or less than about 400 nM as determined in an in vitro antigen-binding assay.
18. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment exhibits a binding affinity to CD3 that is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or at least 10-fold weaker relative to that of an

antigen binding fragment consisting of an amino acid sequence of SEQ ID NO:41, as determined by the respective dissociation constants (K_d) in an in vitro antigen-binding assay.

19. The polypeptide of any one of the preceding claims, further comprising a first release segment peptide (RS1), wherein the RS1 is a substrate for cleavage by a mammalian protease.
20. The polypeptide of claim 19, wherein the RS1 is a substrate for a protease selected from the group consisting of legumain, MMP-2, MMP-7, MMP-9, MMP-11, MMP-14, uPA, and matriptase.
21. The polypeptide of claim 19 or 20, wherein the RS1 comprises an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence selected from SEQ ID NOs:42-660.
22. The polypeptide of any one of claims 19-21, wherein the RS1 comprises an amino acid sequence selected from the sequences of RSR-2089, RSR-2295, RSR-2298, RSR-2488, RSR-2599, RSR-2485, RSR-2486, RSR-2728, RSN-2089, RSN-2295, RSN-2298, RSN-2488, RSN-2599, RSN-2485, RSN-2486, RSN-2728, RSC-2089, RSC-2295, RSC-2298, RSC-2488, RSC-2599, RSC-2485, RSC-2486, and RSC-2728, each of which being forth in Table 5.
23. The polypeptide of any one of the preceding claims, further comprising a first extended recombinant polypeptide, wherein the first extended recombinant polypeptide is characterized in that
 - a. it has at least about 100 amino acids;
 - b. at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the amino acid residues of the XTEN1 sequence are selected from glycine (G), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P); and
 - c. it has at least 4-6 different amino acids selected from G, A, S, T, E and P.
24. The polypeptide of claim 23, wherein the first extended recombinant polypeptide have at least about 100 to about 1000 amino acids.
25. The polypeptide of claim 23 or 24, wherein the first extended recombinant polypeptide comprises an amino acid sequence selected from at least three of SEQ ID NOs:661-664.

26. The polypeptide of any one of claims 23-25, wherein the first extended recombinant polypeptide comprises an amino acid sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence selected from SEQ ID NOs: 665-718 and 922-926.
27. The polypeptide of any one of claims 23-26, wherein the first extended recombinant polypeptide comprises an amino acid sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence selected from the sequences of AE144_1A, AE144_2A, AE144_2B, AE144_3A, AE144_3B, AE144_4A, AE144_4B, AE144_5A, AE144_6B, AE144_7A, AE284, AE288_1, AE288_2, AE288_3, AE292, AE293, AE300, AE576, AE584, AE864, AE864_2, AE865, AE866, AE867, and AE868, each of which being set forth in Table 7.
28. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment is a chimeric or a humanized antigen binding fragment.
29. The polypeptide of any one of the preceding claims, wherein the antigen binding fragment is selected from the group consisting of Fv, Fab, Fab', Fab'-SH, linear antibody, single domain antibody (sdAb), and single-chain variable fragment (scFv).
30. The polypeptide of any one of the preceding claims expressed as a fusion protein, wherein the fusion protein, in an uncleaved state, has a structural arrangement from N-terminus to C-terminus of AF1-RS1- first extended recombinant polypeptide or first extended recombinant polypeptide -RS1-AF1, wherein AF1 is a first antigen binding fragment that is the antigen binding fragment of claim 1.
31. The polypeptide of any one of the preceding claims, further comprising a second antigen binding fragment (AF2) that specifically binds to a target cell marker other than CD3.
32. The polypeptide of claim 31, wherein the AF2 is fused to the AF1 by a flexible peptide linker.
33. The polypeptide of claim 32, wherein the flexible linker comprises 2 or 3 types of amino acids selected from the group consisting of glycine, serine, and proline.
34. The polypeptide of any one of claims 31-33, wherein (1) the AF2 fragment is selected from the group consisting of Fv, Fab, Fab', Fab'-SH, linear antibody, a single domain antibody, and single-chain variable fragment (scFv), or (2) the AF1 and AF2 are configured as an (Fab')₂ or a single chain diabody.

35. The polypeptide of any one of claims 31-34, wherein the target cell marker is a tumor antigen.
36. The polypeptide of claim 35, wherein the target cell marker is selected from 1-40- β -amyloid, 4-1BB, 5AC, 5T4, 707-AP, A kinase anchor protein 4 (AKAP-4), activin receptor type-2B (ACVR2B), activin receptor-like kinase 1 (ALK1), adenocarcinoma antigen, adipophilin, adrenoceptor β 3 (ADRB3), AGS-22M6, α folate receptor, α -fetoprotein (AFP), AIM-2, anaplastic lymphoma kinase (ALK), androgen receptor, angiopoietin 2, angiopoietin 3, angiopoietin-binding cell surface receptor 2 (Tie 2), anthrax toxin, AOC3 (VAP-1), B cell maturation antigen (BCMA), B7-H3 (CD276), Bacillus anthracis anthrax, B-cell activating factor (BAFF), B-lymphoma cell, bone marrow stromal cell antigen 2 (BST2), Brother of the Regulator of Imprinted Sites (BORIS), C242 antigen, C5, CA-125, cancer antigen 125 (CA-125 or MUC16), Cancer/testis antigen 1 (NY-ESO-1), Cancer/testis antigen 2 (LAGE-1a), carbonic anhydrase 9 (CA-IX), Carcinoembryonic antigen (CEA), cardiac myosin, CCCTC-Binding Factor (CTCF), CCL11 (eotaxin-1), CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD11, CD123, CD125, CD140a, CD147 (basigin), CD15, CD152, CD154 (CD40L), CD171, CD179a, CD18, CD19, CD2, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD24, CD25 (α chain of IL-2 receptor), CD27, CD274, CD28, CD3, CD3 ϵ , CD30, CD300 molecule-like family member f (CD300LF), CD319 (SLAMF7), CD33, CD37, CD38, CD4, CD40, CD40 ligand, CD41, CD44 v7, CD44 v8, CD44 v6, CD5, CD51, CD52, CD56, CD6, CD70, CD72, CD74, CD79A, CD79B, CD80, CD97, CEA-related antigen, CFD, ch4D5, chromosome X open reading frame 61 (CXORF61), claudin 18.2 (CLDN18.2), claudin 6 (CLDN6), Clostridium difficile, clumping factor A, CLCA2, colony stimulating factor 1 receptor (CSF1R), CSF2, CTLA-4, C-type lectin domain family 12 member A (CLEC12A), C-type lectin-like molecule-1 (CLL-1 or CLECL1), C-X-C chemokine receptor type 4, cyclin B1, cytochrome P4501B1 (CYP1B1), cyp-B, cytomegalovirus, cytomegalovirus glycoprotein B, dabigatran, DLL4, DPP4, DR5, E. coli shiga toxin type-1, E. coli shiga toxin type-2, ecto-ADP- ribosyltransferase 4 (ART4), EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2), EGF-like-domain multiple 7 (EGFL7), elongation factor 2 mutated (ELF2M), endotoxin, Ephrin A2, Ephrin B2, ephrin type-A receptor 2, epidermal growth factor receptor (EGFR), epidermal growth factor receptor variant III (EGFRvIII), episialin, epithelial cell adhesion molecule (EpCAM), epithelial glycoprotein 2 (EGP-2), epithelial glycoprotein 40 (EGP-40), ERBB2, ERBB3, ERBB4, ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene), Escherichia coli, ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML), F protein of respiratory syncytial virus, FAP, Fc fragment of IgA receptor (FCAR or CD89), Fc receptor-like 5 (FCRL5), fetal acetylcholine receptor, fibrin II β chain, fibroblast activation protein α (FAP), fibronectin extra domain-B, FGF-5, Fms-Like Tyrosine Kinase 3 (FLT3), folate binding protein (FBP), folate hydrolase, folate receptor 1, folate receptor α , folate receptor β , Fos-related antigen 1, Frizzled receptor, Fucosyl GM1, G250, G protein-coupled receptor 20 (GPR20), G protein-coupled receptor class C group 5, member D (GPRC5D), ganglioside G2 (GD2), GD3 ganglioside, glycoprotein 100 (gp100), glypican-3 (GPC3), GMCSF receptor α -chain, GPNMB, GnT-V, growth differentiation factor 8, GUCY2C, heat shock protein 70-2 mutated (mut hsp70-2), hemagglutinin, Hepatitis A virus cellular receptor 1 (HAVCR1), hepatitis B surface antigen, hepatitis B virus, HER1, HER2/neu, HER3, hexasaccharide portion of globoH glycosphingolipid (GloboH), HGF, HHGFR, high molecular weight-melanoma-

associated antigen (HMW-MAA), histone complex, HIV-1, HLA-DR, HNGF, Hsp90, HST-2 (FGF6), human papilloma virus E6 (HPV E6), human papilloma virus E7 (HPV E7), human scatter factor receptor kinase, human Telomerase reverse transcriptase (hTERT), human TNF, ICAM-1 (CD54), iCE, IFN- α , IFN- β , IFN- γ , IgE, IgE Fc region, IGF-1, IGF-1 receptor, IGHE, IL-12, IL-13, IL-17, IL-17A, IL-17F, IL-1 β , IL-20, IL-22, IL-23, IL-31, IL-31RA, IL-4, IL-5, IL-6, IL-6 receptor, IL-9, immunoglobulin lambda-like polypeptide 1 (IGLL1), influenza A hemagglutinin, insulin-like growth factor 1 receptor (IGF-I receptor), insulin-like growth factor 2 (ILGF2), integrin α 4 β 7, integrin β 2, integrin α 2, integrin α 4, integrin α 5 β 1, integrin α 7 β 7, integrin α Ib β 3, integrin α v β 3, interferon α / β receptor, interferon γ -induced protein, Interleukin 11 receptor α (IL-11R α), Interleukin-13 receptor subunit α -2 (IL-13R α 2 or CD213A2), intestinal carboxyl esterase, kinase domain region (KDR), KIR2D, KIT (CD117), L1-cell adhesion molecule (L1-CAM), legumain, leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2), leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), lymphocyte antigen 6 (Ly-6), Lewis-Y antigen, LFA-1 (CD11a), LINGO-1, lipoteichoic acid, LOXL2, L-selectin (CD62L), lymphocyte antigen 6 complex, locus K 9 (LY6K), lymphocyte antigen 75 (LY75), lymphocyte-specific protein tyrosine kinase (LCK), lymphotoxin- α (LT- α) or Tumor necrosis factor- β (TNF- β), Lysosomal Associated Membrane Protein 1 (LAMP1), macrophage migration inhibitory factor (MIF or MMIF), M-CSF, mammary gland differentiation antigen (NY-BR-1), MCP-1, melanoma cancer testis antigen-1 (MAD-CT-1), melanoma cancer testis antigen-2 (MAD-CT-2), melanoma inhibitor of apoptosis (ML-IAP), melanoma-associated antigen 1 (MAGE-A1), mesothelin, mucin 1, cell surface associated (MUC1), MUC-2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, MUC16, mucin CanAg, myelin-associated glycoprotein, myostatin, N-Acetyl glucosaminyl-transferase V (NA17), NCA-90 (granulocyte antigen), Nectin-4, nerve growth factor (NGF), neural apoptosis-regulated proteinase 1, neural cell adhesion molecule (NCAM), neurite outgrowth inhibitor (e.g., NOGO-A, NOGO-B, NOGO-C), neuropilin-1 (NRP1), N-glycolylneuraminic acid, NKG2D, Notch receptor, o-acetyl-GD2 ganglioside (OAcGD2), olfactory receptor 51E2 (OR51E2), oncofetal antigen (h5T4), oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl), Oryctolagus cuniculus, OX-40, oxLDL, p53 mutant, paired box protein Pax-3 (PAX3), paired box protein Pax-5 (PAX5), pannexin 3 (PANX3), P-cadherin, phosphate-sodium co-transporter, phosphatidylserine, placenta-specific 1 (PLAC1), platelet-derived growth factor receptor α (PDGF-R α), platelet-derived growth factor receptor β (PDGFR- β), polysialic acid, proacrosin binding protein sp32 (OY-TES1), programmed cell death protein 1 (PD-1), Programmed death-ligand 1 (PD-L1), proprotein convertase subtilisin/kexin type 9 (PCSK9), prostase, prostate carcinoma tumor antigen-1 (PCTA-1 or Galectin 8), melanoma antigen recognized by T cells 1 (MelanA or MART1), P15, P53, PRAME, prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), prostatic acid phosphatase (PAP), prostatic carcinoma cells, prostein, Protease Serine 21 (Testisin or PRSS21), Proteasome (Prosome, Macropain) Subunit, β Type, 9 (LMP2), Pseudomonas aeruginosa, rabies virus glycoprotein, RAGE, Ras Homolog Family Member C (RhoC), receptor activator of nuclear factor kappa-B ligand (RANKL), Receptor for Advanced Glycation Endproducts (RAGE-1), receptor tyrosine kinase-like orphan receptor 1 (ROR1), renal ubiquitous 1 (RU1), renal ubiquitous 2 (RU2), respiratory syncytial virus, Rh blood group D antigen, Rhesus factor, sarcoma translocation breakpoints, sclerostin

(SOST), selectin P, sialyl Lewis adhesion molecule (sLe), sperm protein 17 (SPA17), sphingosine-1-phosphate, squamous cell carcinoma antigen recognized by T Cells 1, 2, and 3 (SART1, SART2, and SART3), stage-specific embryonic antigen-4 (SSEA-4), Staphylococcus aureus, STEAP1, syndecan 1 (SDC1)+A314, SOX10, survivin, survivin-2B, synovial sarcoma, X breakpoint 2 (SSX2), T-cell receptor, TCR Γ Alternate Reading Frame Protein (TARP), telomerase, TEM1, tenascin C, TGF- β (e.g., TGF- β 1, TGF- β 2, TGF- β 3), thyroid stimulating hormone receptor (TSHR), tissue factor pathway inhibitor (TFPI), Tn antigen ((Tn Ag) or (GalNAc α -Ser/Thr)), TNF receptor family member B cell maturation (BCMA), TNF- α , TRAIL-R1, TRAIL-R2, TRG, transglutaminase 5 (TGS5), tumor antigen CTAA16.88, tumor endothelial marker 1 (TEM1/CD248), tumor endothelial marker 7-related (TEM7R), tumor protein p53 (p53), tumor specific glycosylation of MUC1, tumor-associated calcium signal transducer 2 (TROP-2), tumor-associated glycoprotein 72 (TAG72), tumor-associated glycoprotein 72 (TAG-72)+A327, TWEAK receptor, tyrosinase, tyrosinase-related protein 1 (TYRP1 or glycoprotein 75), tyrosinase-related protein 2 (TYRP2), uroplakin 2 (UPK2), vascular endothelial growth factor (e.g., VEGF-A, VEGF-B, VEGF-C, VEGF-D, PIGF), vascular endothelial growth factor receptor 1 (VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2), vimentin, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), von Willebrand factor (VWF), Wilms tumor protein (WT1), X Antigen Family, Member 1A (XAGE1), β -amyloid, κ -light chain, Fibroblast Growth Factor Receptor 2 (FGFR2), LIV-1 Protein, estrogen regulated (LIV1, aka SLC39A6), Neurotrophic Receptor Tyrosine Kinase 1 (NTRK1, aka TRK), Ret Proto-Oncogene (RET), B Cell Maturation Antigen (BCMA, aka TNFRSF17), Transferrin Receptor (TFRC, aka CD71), Activated Leukocyte Cell Adhesion Molecule (ALCAM, aka CD166), Somatostatin Receptor 2 (SSTR2), KIT Proto-Oncogene Receptor Tyrosine Kinase (cKIT), V-Set Immunoregulatory Receptor (VSIR, aka VISTA), Glycoprotein Nmb (GPNMB), Delta Like Canonical Notch Ligand 3 (DLL3), Interleukin 3 Receptor Subunit Alpha (IL3RA, aka CD123), Lysosomal Associated Membrane Protein 1 (LAMP1), Cadherin 3, Type 1, P-Cadherin (CDH3), Ephrin A4 (EFNA4), Protein Tyrosine Kinase 7 (PTK7), Solute Carrier Family 34 Member 2 (SLC34A2, aka NaPi-2b), GCC, PLAUR Domain Containing 3 (LYPD3, aka LY6 or C4.4a), Mucin 17, Cell Surface Associated (MUC17), Fms Related Receptor Tyrosine Kinase 3 (FLT3), NKG2D ligands (e.g. ULBP1, ULBP2, ULBP3, H60, Rae-1 α , Rae-1 β , Rae-1 δ , Rae-1 γ , MICA, MICB, hHLA-A), SLAM Family Member 7 (SLAMF7), Interleukin 13 Receptor Subunit Alpha 2 (IL13RA2), C-Type Lectin Domain Family 12 Member A (CLEC12A aka CLL-1), CEA Cell Adhesion Molecule 5 (CEACAM aka CD66e), Interleukin 3 Receptor Subunit Alpha (IL3RA), CD5 Molecule (CD5), UL16 Binding Protein 1 (ILBP1), V-Set Domain Containing T Cell Activation Inhibitor 1 (VTCN1 aka B7-H4), Chondroitin Sulfate Proteoglycan 4 (CSPG4), Syndecan 1 (SDC1 aka CD138), Interleukin 1 Receptor Accessory Protein (IL1RAP), Baculoviral IAP Repeat Containing 5 (BIRC5 aka Survivin), CD74 Molecule (CD74), Hepatitis A Virus Cellular Receptor 1 (HAVCR1 aka TIM1), SLIT and NTRK Like Family Member 6 (SILTRK6), CD37 Molecule (CD37), Coagulation Factor III, Tissue Factor (CD142 aka F3), AXL Receptor Tyrosine Kinase (AXL), Endothelin Receptor Type B (EDNRB aka ETBR), Cadherin 6 (CDH6), Fibroblast Growth Factor Receptor 3 (FGFR3), Carbonic Anhydrase 6 (CA6), CanAg glycoform of MUC1, Integrin Subunit Alpha V (ITGAV), Teratocarcinoma-Derived Growth Factor 1 (TDGF1, aka Crypto 1), SLAM Family Member 6 (SLAMF6 aka CD352), and Notch Receptor 3 (NOTCH3).

37. The polypeptide of any one of claims 31-36, wherein the AF2 specifically binds the target cell marker with a K_d between about 0.1 nM and about 100 nM, as determined in an in vitro antigen-binding assay comprising the target cell marker.
38. The polypeptide of any one of claims 31-37, wherein the binding affinity of the AF2 to the target cell marker is at least 10-fold greater, or at least 100-fold greater, or at least 1000-fold greater than the binding affinity of the AF1 to CD3, as measured in an in vitro antigen-binding assay.
39. The polypeptide of any one of claims 31-38, wherein the AF2 comprises a CDR of a monoclonal antibody having binding affinity to the target cell marker.
40. The polypeptide of claim 38, wherein the CDR of the AF2 is selected from the sequences of SEQ ID NOs:719-918.
41. The polypeptide of any one of claims 31-40, wherein the AF2 comprises VL and VH of a monoclonal antibody having binding affinity to the target cell marker.
42. The polypeptide of claim 41, wherein the VL is selected from the sequences of SEQ ID NOs:819-918, and the VH of the AF2 is selected from the sequences of SEQ ID NOs:719-818.
43. The polypeptide of any one of claims 19-42, further comprising a second release segment (RS2), wherein the RS2 is a substrate for cleavage by a mammalian protease.
44. The polypeptide of claim 43, wherein the RS2 is a substrate for a protease selected from legumain, MMP-2, MMP-7, MMP-9, MMP-11, MMP-14, uPA, and matriptase.
45. The polypeptide of claim 43 or claim 44, wherein the RS2 comprises an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to a sequence selected from SEQ ID NOs:42-660.
46. The polypeptide of any one of claims 43-45, wherein the sequences of RS1 and RS2 are identical.
47. The polypeptide of any one of claims 43-46, wherein the sequences of RS1 and RS2 are not identical.
48. The polypeptide of any one of claims 43-47, wherein the RS1 and RS2 are each a substrate for cleavage by multiple proteases at one, two, or three cleavage sites within each release segment sequence.
49. The polypeptide of any one of claims 43-48, further comprising a second extended recombinant polypeptide, wherein the second extended recombinant polypeptide is characterized in that
 - a. it has at least about 100 amino acids;

- b. at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the amino acid residues of the second extended recombinant polypeptide sequence are selected from glycine (G), alanine (A), serine (S), threonine (T), glutamate (E) and proline (P); and
 - c. it has at least 4-6 different amino acids selected from G, A, S, T, E and P.
- 50. The polypeptide of claim 49, wherein the second extended recombinant polypeptide comprises an amino acid sequence, wherein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the amino acid sequence comprises non-overlapping sequences selected from at least three of SEQ ID NOs:661-664.
- 51. The polypeptide of claim 49 or claim 50, wherein the second extended recombinant polypeptide comprises an amino acid sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence selected from SEQ ID NOs: 665-718 and 922-926.
- 52. The polypeptide of any one of claims 49-51, wherein the second extended recombinant polypeptide comprises an amino acid sequence having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence selected from the sequences of AE144_1A, AE144_2A, AE144_2B, AE144_3A, AE144_3B, AE144_4A, AE144_4B, AE144_5A, AE144_6B, AE144_7A, AE284, AE288_1, AE288_2, AE288_3, AE292, AE293, AE300, AE576, AE584, AE864, AE864_2, AE865, AE866, AE867, and AE868, each of which being set forth in Table 7.
- 53. The polypeptide of any one of claims 49-52, wherein the polypeptide has a structural arrangement from N-terminus to C-terminus as follows: first extended recombinant polypeptide-RS1-AF2-AF1-RS2- second extended recombinant polypeptide, first extended recombinant polypeptide-RS1-AF1-AF2-RS2- second extended recombinant polypeptide, second extended recombinant polypeptide -RS2-AF2-AF1-RS1-first extended recombinant polypeptide, second extended recombinant polypeptide -RS2-AF1-AF2-RS1-first extended recombinant polypeptide, second extended recombinant polypeptide -RS2-diabody-RS1-first extended recombinant polypeptide, or first extended recombinant polypeptide-RS1-diabody-RS2- second extended recombinant polypeptide, wherein the diabody comprises VL and VH of the AF1 and AF2, wherein the AF1 specifically binds CD3 and AF2 specifically binds a

- target cell marker, and wherein first extended recombinant polypeptide and second extended recombinant polypeptide are of different amino acid length or sequence..
54. A pharmaceutical composition comprising the polypeptide of any one of the preceding claims and one or more pharmaceutically suitable excipients.
55. The pharmaceutical composition of claim 54, wherein the pharmaceutical composition is formulated for intradermal, subcutaneous, intravenous, intra-arterial, intraabdominal, intraperitoneal, intrathecal, or intramuscular administration.
56. The pharmaceutical composition of claim 55, wherein the pharmaceutical composition is in a liquid form or a frozen form.
57. The pharmaceutical composition of any one of claims 54-56, wherein the pharmaceutical composition is in a pre-filled syringe for a single injection.
58. The pharmaceutical composition of claim 54, wherein the pharmaceutical composition is formulated as a lyophilized powder to be reconstituted prior to administration.
59. Use of the polypeptide of any one of claims 1-53 in the preparation of a medicament for the treatment of cancer in a subject.
60. The use of claim 59, wherein the cancer is selected from the group consisting of carcinomas, Hodgkin's lymphoma, non-Hodgkin's lymphoma, B cell lymphoma, diffuse large B cell lymphoma, T-cell lymphoma, follicular lymphoma, mantle cell lymphoma, blastoma, breast cancer, colon cancer, prostate cancer, head and neck cancer, any form of skin cancer, melanoma, genito-urinary tract cancer, ovarian cancer, ovarian cancer with malignant ascites, vaginal cancer, vulvar cancer, Ewing sarcoma, peritoneal carcinomatosis, uterine serous carcinoma, parathyroid cancer, endometrial cancer, cervical cancer, colorectal cancer, an epithelia intraperitoneal malignancy with malignant ascites, uterine cancer, mesothelioma in the peritoneum kidney cancers, lung cancer, laryngeal cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, stomach cancer, small intestine cancer, liver cancer, hepatocarcinoma, retinoblastoma, hepatoblastoma, liposarcoma, pancreatic cancer, gall bladder cancer, testicular cancer, cancers of the bile duct, cancers of the bone, salivary gland carcinoma, thyroid cancer, craniopharyngioma, carcinoid tumor, epithelial cancer, arrhenoblastoma, adenocarcinoma, sarcomas of any origin, primary hematologic malignancies including acute or chronic lymphocytic leukemias, acute or chronic myelogenous leukemias, B-cell derived chronic lymphatic leukemia, hairy cell leukemia, myeloproliferative neoplastic disorders, or

myelodysplastic disorders, myasthenia gravis, Morbus Basedow, Kaposi sarcoma, neuroblastoma, Hashimoto thyroiditis, Wilms tumor, or Goodpasture syndrome.

61. An isolated nucleic acid, the nucleic acid comprising (a) a polynucleotide encoding a polypeptide of any one of claims 1-53; or (b) the complement of the polynucleotide of (a).

62. An expression vector comprising the polynucleotide sequence of claim 61 and a recombinant regulatory sequence operably linked to the polynucleotide sequence.

63. An isolated host cell, comprising the expression vector of claim 62.

64. The host cell of claim 63, wherein the host cell is a prokaryote.

65. The host cell of claim 63 or claim 64, wherein the host cell is *E. coli*.

66. The polypeptide of any one of claims 1-53 for use in therapy.