

reduced side effects, increased selectivity, and/or enhanced pharmacokinetic properties, such as requiring less frequent dosing or merely dosing by a single injection.

[0004] The present invention discloses a chimeric polypeptide assembly useful in the treatment or prevention of diseases, including but not limited to cancers, autoimmune, and inflammatory disorders. In a first aspect, the present disclosure provides a cleaveable chimeric polypeptide assembly. The cleavable chimeric polypeptide assembly compositions address an unmet need and are superior in one or more aspects including enhanced terminal half-life, targeted delivery, and reduced toxicity to healthy tissues compared to conventional bispecific antibody preparations in use.

[0005] A subject polypeptide assembly typically comprises a first portion, a second portion, and a third portion, wherein: said first portion comprises (i) a first binding domain with binding specificity to a target cell marker; and (ii) a second binding domain with binding specificity to an effector cell antigen; said second portion comprises a peptidyl release segment (RS) capable of being cleaved by one or more mammalian proteases; and said third portion comprises a bulking moiety; wherein said bulking moiety is capable of being released from said first portion by action of said mammalian protease on said second portion.

[0005a] In one aspect, the present invention provides a chimeric polypeptide assembly comprising a first portion, a second portion, and a third portion wherein:

- a. said first portion is a polypeptide comprising
 - i. a first binding domain with binding specificity to a tumor-specific marker; and
 - ii. a second binding domain with binding specificity to a T-cell antigen;
- b. said second portion comprises a peptidyl release segment (RS) capable of being cleaved by one or more mammalian proteases; and
- c. said third portion comprises a bulking moiety, wherein the bulking moiety is an extended recombinant polypeptide (XTEN);

wherein said bulking moiety is capable of being released from said first portion by action of said mammalian protease on said second portion,

wherein the binding affinity of the first binding domain to the tumor cell, as measured by K_d constant in the in vitro assay, is at least one order of magnitude greater compared to the binding affinity of the second binding domain to the T-cell antigen.

[0006] The various components in a subject chimeric polypeptide assembly can be configured in a variety of different orders. In one embodiment, the chimeric polypeptide assembly is configured, from N-terminus to C-terminus, wherein the first portion is linked to

the second portion, which in turn is linked to the third portion. In another embodiment, the chimeric polypeptide assembly is configured, from N-terminus to C-terminus, wherein the third portion is linked to the second portion, which in turn is linked to the first portion. In one embodiment, the chimeric polypeptide assembly is a fusion protein. In another embodiment, the second and third portions are a fusion protein and the first portion is conjugated to the second portion. In one embodiment of the chemically conjugated polypeptide assembly composition, the C-terminus of the first portion polypeptide can be conjugated to the N-terminus of the second portion polypeptide via a cysteine or other suitable amino acids amenable for cross-linking by agents such as maleamide or other cross-linking agents known in the art. In another embodiment, the first portion and the second portion is a monomeric fusion protein and the third portion is chemically conjugated to the second portion.

[0007] Optionally, the chimeric polypeptide assembly compositions may comprise an additional bulking moiety linked to the composition by a second release segment linked to the opposite end of the composition, thereby enclosing the first and second portions.

polypeptide assembly in the same subject. Pharmacokinetic parameters can be measured using plasma samples from a subject being administered with the subject chimeric polypeptide assembly using methods described herein or conventional methods known in the art. In another embodiment, upon or following administration to a subject, the intact chimeric polypeptide assembly exhibits reduced extravasation from the blood circulatory system in the subject compared to the chimeric polypeptide assembly in which the RS is cleaved, releasing the first portion and the third portion. In the foregoing embodiments of this paragraph, the subject can be mouse, rat, monkey, dog, and human.

[0023] In another aspect, the invention relates to pharmaceutical compositions of the chimeric polypeptide assembly. In one embodiment, the invention provides pharmaceutical compositions comprising the chimeric polypeptide assembly of any of the chimeric polypeptide assembly disclosed herein, and one or more pharmaceutically suitable excipients and, optionally, one or more carriers or stabilizers. In another embodiment, the pharmaceutical composition is formulated for intradermal, subcutaneous, intravenous, intra-arterial, intraabdominal, intraperitoneal, intrathecal, or intramuscular administration. In another embodiment, the pharmaceutical composition is in a liquid form. In a related embodiment, the pharmaceutical composition in a liquid form is supplied in a pre-filled syringe for a single injection. In other embodiments, the pharmaceutical composition is formulated as a lyophilized powder to be reconstituted prior to administration.

[0023a] In another aspect, the invention relates to a pharmaceutical composition comprising the chimeric polypeptide assembly as described herein and one or more pharmaceutically suitable excipients,

[0024] In another aspect, the invention relates to methods and uses of the chimeric polypeptide assembly or a pharmaceutical composition comprising the chimeric polypeptide assembly. In one embodiment, the invention provides a chimeric polypeptide assembly or a pharmaceutical composition comprising the chimeric polypeptide assembly for the preparation of a medicament for the treatment of a disease in a subject. In a related embodiment, the medicament is used in a disease, wherein the disease is selected from the group consisting of carcinoma, Hodgkin's lymphoma, and non-Hodgkin's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, mantle cell lymphoma, blastoma, breast cancer, ER/PR+ breast cancer, Her2+ breast cancer, triple-negative breast cancer, colon cancer, colon cancer with malignant ascites, mucinous tumors, prostate cancer, head and neck cancer, skin cancer, melanoma, genito-urinary tract cancer, ovarian cancer, ovarian cancer with malignant ascites, peritoneal carcinomatosis, uterine serous carcinoma, endometrial cancer, cervix cancer, colorectal, uterine cancer, mesothelioma in the peritoneum, kidney cancer,

Wilm's tumor, lung cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, stomach cancer, small intestine cancer, liver cancer, hepatocarcinoma, hepatoblastoma,

least 50-fold, or at least 60-fold, or at least 70-fold, or at least 80-fold, or at least 90-fold, or at least 100-fold lower potential to extravasate from the circulation when administered to a subject compared to the first portion not linked to the chimeric polypeptide assembly when administered at a comparable dose to a subject.

[0030] In another aspect, the invention relates to nucleic acids encoding the subject compositions. In one embodiment, the invention provides an isolated nucleic acid comprising a polynucleotide sequence selected from (a) a polynucleotide encoding the chimeric polypeptide assembly of any one of the embodiments disclosed herein, or (b) the complement of the polynucleotide of (a). In another embodiment, the invention provides an isolated nucleic acid comprising a polynucleotide sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a polynucleotide sequence set forth in Table 10 or Table 14. In another embodiment, the invention provides an expression vector comprising a polynucleotide sequence of the foregoing embodiments and a recombinant regulatory sequence operably linked to the polynucleotide sequence. In another embodiment, the invention provides an isolated host cell, comprising the foregoing expression vector. In one embodiment, the host cell is *E. coli*.

[0030a] In another aspect, the invention provides an isolated host cell, comprising the expression vector as described herein, wherein the host cell is *E. coli*.

[0031] In another aspect, the invention relates to T cell binding compositions and nucleic acids that encode them. In one embodiment, the invention provides a monomeric fusion protein comprising an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of the amino acid sequences set forth in Table 7, wherein the monomeric fusion protein exhibits binding affinity to a CD3 antigen of a T cell. In another embodiment, the invention provides an isolated nucleic acid comprising a polynucleotide sequence selected from (a) a polynucleotide encoding the fusion protein of the foregoing T cell binding composition, (b) a polynucleotide sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a polynucleotide sequence selected from the group consisting of the polynucleotide sequences set forth in Table 7; or (c) the complement of the polynucleotide of (a) or (b).

[0032] In another embodiment, the invention provides a method of use of the nucleic acid encoding the fusion protein of the foregoing T cell binding composition in a method of making a polynucleotide sequence encoding the chimeric polypeptide assembly of any one of the chimeric polypeptide assembly embodiments disclosed herein, the method comprising

operably linking a polynucleotide sequence encoding a scFv with binding affinity to a target cell marker disclosed herein or selected from the group of targets set forth in Table 2, in

CLAIMS

WHAT IS CLAIMED IS:

1. A chimeric polypeptide assembly comprising a first portion, a second portion, and a third portion wherein:
 - a. said first portion is a polypeptide comprising
 - i. a first binding domain with binding specificity to a tumor-specific marker; and
 - ii. a second binding domain with binding specificity to a T-cell antigen;
 - b. said second portion comprises a peptidyl release segment (RS) capable of being cleaved by one or more mammalian proteases; and
 - c. said third portion comprises a bulking moiety, wherein the bulking moiety is an extended recombinant polypeptide (XTEN);wherein said bulking moiety is capable of being released from said first portion by action of said mammalian protease on said second portion, wherein the binding affinity of the first binding domain to the tumor cell, as measured by K_d constant in the in vitro assay, is at least one order of magnitude greater compared to the binding affinity of the second binding domain to the T-cell antigen.
2. The chimeric polypeptide assembly of claim 1, wherein from N-terminus to C-terminus, the first portion is linked to the second portion, which in turn is linked to the third portion.
3. The chimeric polypeptide assembly of claim 1, wherein from N-terminus to C-terminus, the third portion is linked to the second portion, which in turn is linked to the first portion.
4. The chimeric polypeptide assembly of any one of claims 1-3, wherein the mammalian protease is expressed in a tumor tissue.
5. The chimeric polypeptide assembly of any one of the preceding claims, wherein the first binding domain and the second binding domain are each an scFv, a single chain diabody, a single domain antibody, a single domain camelid antibody, or a non-antibody scaffold.
6. The chimeric polypeptide assembly of any one of the preceding claims, wherein second binding domain has binding specificity to the T-cell antigen expressed on a T cell selected from the group consisting of T cell, regulatory T cell (RegT cell), and helper T cell.
7. The chimeric polypeptide assembly of claim 6 wherein the T-cell antigen is CD3 or CD3 ϵ .
8. The chimeric polypeptide assembly of claim 7, wherein the second binding domain comprises a VL and/or VH region derived from a monoclonal antibody capable of binding human CD3 or CD3 ϵ .
9. The chimeric polypeptide assembly of claim 7, wherein the second binding domain VL and/or VH are selected from the sequences set forth in Table 1.
10. The chimeric polypeptide assembly of claim 8 or claim 9, wherein the second binding domain is an scFv wherein the VH and VL regions are arranged in the order VH—VL or VL—VH in the N-terminal to C-terminal direction.

11. The chimeric polypeptide assembly of claim 7, wherein the second binding domain comprises a CDR-H1 region, a CDR-H2 region, a CDR-H3 region, a CDR-L1 region, a CDR-L2 region, and a CDR-L3 region, wherein each is derived from a monoclonal antibody of Table 1.
12. The chimeric polypeptide assembly of any one of claims 1-11, wherein the first binding domain comprises a VL and/or VH region derived from a monoclonal antibody capable of binding the tumor-specific marker, wherein the VH and VL regions are arranged in the order VH—VL or VL—VH in the N-terminal to C- terminal direction.
13. The chimeric polypeptide assembly of claim 12, wherein the first binding domain regions are derived from monoclonal antibody sequences set forth in Table 2.
14. The chimeric polypeptide assembly of claim 12, wherein the first binding domain is an scFv wherein the VH and VL regions are arranged in the order VH—VL or VL—VH in the N-terminal to C- terminal direction.
15. The chimeric polypeptide assembly of any one of claims 1-11, wherein the first binding domain comprises a CDR-H1 region, a CDR-H2 region, a CDR-H3 region, a CDR-L1 region, a CDR-L2 region, and a CDR-L3 region, wherein each of said regions is derived from monoclonal antibody sequences set forth in Table 2.
16. The chimeric polypeptide assembly of any one of claims 1-15, wherein the tumor specific marker is selected from the group consisting of alpha 4 integrin, Ang2, B7-H3, B7-H6, CEACAM5, cMET, CTLA4, FOLR1, EpCAM, CCR5, CD19, HER2, HER2 neu, HER3, HER4, HER1 (EGFR), PD-L1, PSMA, CEA, MUC1(mucin), MUC-2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, MUC16, β hCG, Lewis-Y, CD20, CD33, CD38, CD30, CD56 (NCAM), CD133, ganglioside GD3; 9-O- Acetyl-GD3, GM2, Globo H, fucosyl GM1, GD2, carbonicanhydrase IX, CD44v6, Sonic Hedgehog (Shh), Wue-1, plasma cell antigen 1, melanoma chondroitin sulfate proteoglycan (MCSP), CCR8, 6-transmembrane epithelial antigen of prostate (STEAP), mesothelin, A33 antigen, prostate stem cell antigen (PSCA), Ly-6, desmoglein 4, fetal acetylcholine receptor (fnAChR), CD25, cancer antigen 19-9 (CA19-9), cancer antigen 125 (CA-125), Muellierian inhibitory substance receptor type II (MISIIR), sialylated Tn antigen (s TN), fibroblast activation antigen (FAP), endosialin (CD248), epidermal growth factor receptor variant III (EGFRvIII), tumor-associated antigen L6 (TAL6), SAS, CD63, TAG72, Thomsen-Friedenreich antigen (TF-antigen), insulin-like growth factor I receptor (IGF-IR), Cora antigen, CD7, CD22, CD70, CD79a, CD79b, G250, MT-MMPs, F19 antigen, CA19-9, CA-125, alpha-fetoprotein (AFP), VEGFR1, VEGFR1, VEGFR2, DLK1, SP17, ROR1, and EphA2.
17. The chimeric polypeptide assembly of any one of claims 5-16, wherein the first binding domain and the second binding domain are linked by a flexible polypeptide linker selected from the group of sequences set forth in Table 8 and Table 9.
18. The chimeric polypeptide assembly of any one of the preceding claims, wherein the RS comprises an amino acid sequence capable of being cleaved by one or more proteases selected from the group consisting of the proteases set forth in Table 3.
19. The chimeric polypeptide assembly of claim 18, wherein the RS is a substrate for

legumain, MMP-2, MMP-7, MMP-9, MMP-14, uPA, and matriptase.

20. The chimeric polypeptide assembly of any one of the preceding claims, wherein the RS comprises an amino acid sequence selected from the group consisting of the sequences set forth in Table 4.

21. The chimeric polypeptide assembly of any one of the preceding claims, wherein the XTEN 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 group of sequences set forth in Table 5.

22. The chimeric polypeptide assembly of any one of the preceding claims, wherein the first portion of the chimeric polypeptide assembly 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 group of sequences set forth in Table 13.

23. The chimeric polypeptide assembly of any one of claims 16-22, wherein upon cleavage of the second portion by said mammalian protease and release of the first portion from the chimeric polypeptide assembly, the first portion is capable of concurrently binding to a T cell bearing the T-cell antigen and to a tumor cell bearing the tumor-specific marker in an *in vitro* assay comprising both the T cell and the tumor cell.

24. The chimeric polypeptide assembly of claim 23, wherein upon cleavage of the second portion to release said first portion and said third portion from said chimeric polypeptide assembly, said released first portion has a molecular weight that is at least 2-fold, 3-fold, 4-fold, or 5-fold less than said released third portion.

25. The chimeric polypeptide assembly of claim 23, wherein upon cleavage of the second portion, the released said first portion from said chimeric polypeptide assembly has increased binding affinity to the T cell bearing the effector cell antigen or the tumor cell marker compared to the chimeric binding assembly wherein the second portion has not been cleaved.

26. The chimeric polypeptide assembly of claim 23, wherein the binding affinity of the released first portion to the T-cell antigen or the tumor-specific marker is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold greater compared to the binding affinity of the chimeric polypeptide assembly to the T-cell antigen or the tumor-specific marker wherein the second portion has not been cleaved.

27. The chimeric polypeptide assembly of claim 23, wherein the concurrent binding of the first portion to the T cell and the tumor cell yields cytotoxic activity against the tumor cell in the *in vitro* assay.

28. The chimeric polypeptide assembly of claim 23, wherein the released first portion of the chimeric polypeptide assembly is capable of effecting a greater amount of cell lysis of the tumor cell compared to an intact chimeric binding assembly in the *in vitro* assay.

29. The chimeric polypeptide assembly of claim 23, wherein the amount of cell lysis effected by the released first portion of the chimeric polypeptide assembly is at least 10-fold greater, or at least 30-fold, or at least 100-fold, or at least 300-fold, or at least 1000-fold greater compared to the intact chimeric binding assembly in the *in vitro* assay.

30. The chimeric polypeptide assembly of any one of claims 27-29, wherein the cytotoxic activity and/or cell lysis of the tumor cell is mediated by target specific activation of the T cell.

31. The chimeric polypeptide assembly of claim 30, wherein the amount of activation of the T cell effected by the released first portion of the chimeric polypeptide assembly is at least 10-fold greater, or at least 30-fold, or at least 100-fold, or at least 300-fold, or at least 1000-fold greater compared to the intact chimeric binding assembly.

32. The chimeric polypeptide assembly of any one of claims 27-31, wherein in a comparison of:

- a) relative cytotoxicity, which is measured as a ratio between the cytotoxicity of (i) the released first portion to the tumor cell in an *in vitro* assay comprising both the T-cell antigen and the tumor-specific marker and (ii) the cytotoxicity of a composition comprising the corresponding first portion of the chimeric polypeptide assembly and the corresponding third portion of the chimeric polypeptide assembly linked by a non-cleavable peptide of 1 to about 10 amino acids; and
- b) relative binding affinity, which is measured as a ratio between the binding affinity of the second binding domain of the released first portion of a(i) to the T-cell antigen and the binding affinity of the composition of a(ii) to the T-cell antigen;

the ratio between the relative cytotoxicity and the relative binding affinity is greater than at least 10:1, or greater than at least 30:1, or greater than at least 50:1, or greater than at least 100:1, or greater than at least 300:1, or greater than at least 500:1, or greater than at least 1000:1.

33. The chimeric polypeptide assembly of claim 32, wherein the non-cleavable peptide has the sequence glycine-serine and the T-cell antigen is CD3 or CD3ε.

34. The chimeric polypeptide assembly of any one of claims 23-33, wherein the *in vitro* assay is selected from the group of assays consisting of cell membrane integrity assay, mixed cell culture assay, FACS based propidium Iodide assay, trypan Blue influx assay, photometric enzyme release assay, radiometric ⁵¹Cr release assay, fluorometric Europium release assay, CalceinAM release assay, photometric MTT assay, XTT assay, WST-1 assay, alamar blue assay, radiometric ³H-Thd incorporation assay, clonogenic assay measuring cell division activity, fluorometric rhodamine123 assay measuring mitochondrial transmembrane gradient, apoptosis assay monitored by FACS-based phosphatidylserine exposure, ELISA-based TUNEL test assay, sandwich ELISA, caspase activity assay, cell-based LDH release assay, and cell morphology assay, or any combination thereof.

35. The chimeric polypeptide assembly of claim 23, wherein the binding affinity of the first binding domain of the released first portion to the tumor-specific marker is greater compared to the binding affinity of the second binding domain of the released first portion to the CD3 antigen.

36. The chimeric polypeptide assembly of claim 1, wherein the K_d constant of the first binding domain of the chimeric polypeptide assembly is between 10^{-5} to 10^{-9} M and the K_d of the second binding domain is between 10^{-5} to 10^{-9} M.

37. The chimeric polypeptide assembly of any one of claims 1-22, wherein following administration of a composition comprising the chimeric polypeptide assembly to a subject, the

second portion of the chimeric polypeptide assembly is cleaved in proximity to a tumor expressing a protease capable of cleaving the RS, when said chimeric polypeptide assembly has been administered to a subject.

38. The chimeric polypeptide assembly of claim 37, wherein upon cleavage of the second portion by said mammalian protease and release of the first portion from the chimeric polypeptide assembly, the first portion is capable of concurrently binding to a T cell bearing a human CD3 antigen and to a tumor cell bearing the tumor-specific marker.

39. The chimeric polypeptide assembly of claim 38, whereupon the concurrent binding to the T cell bearing the CD3 antigen and the tumor cell bearing the tumor-specific marker by the first portion results in the release of T cell-derived effector molecules.

40. The chimeric polypeptide assembly of claim 39, wherein the effector molecule is selected from one or more effector molecules of the group consisting of TNF- α , IFN- γ , interleukin 2, perforin, and granzymes.

41. The chimeric polypeptide assembly of claim 38, whereupon the concurrent binding of the first portion to the T cell bearing the human CD3 antigen and to a tumor cell bearing the tumor-specific marker, lysis of the tumor cell is effected by the T cell.

42. The chimeric polypeptide assembly of any one of claims 38-41, wherein the assembly exhibits a half-life following administration to a subject that is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold greater compared to the half-life of the first portion not linked to said second and third portions after being administered to a subject at a comparable dose.

43. The chimeric polypeptide assembly of any one of claims 37-42, wherein following administration of the chimeric polypeptide assembly to a subject and cleavage of the second portion and release of said first portion and said third portion from said chimeric polypeptide assembly, said first portion has a half-life that is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold less compared to the intact chimeric polypeptide assembly in the subject.

44. The chimeric polypeptide assembly of any one of claims 37-43, wherein the plasma C_{max} concentration of the released first portion after a single administration of a composition comprising the chimeric polypeptide assembly to the subject does not exceed about 0.01 ng/ml, or about 0.1 ng/ml, or about 1 ng/ml, or about 10 ng/ml, or about 100 ng/ml.

45. The chimeric polypeptide assembly of any one of claims 37-43, wherein the plasma C_{max} concentration of the released first portion after a single administration of a composition comprising the chimeric polypeptide assembly to the subject is at least 3-fold lower, or at least 10-fold lower, or at least 30-fold lower, or at least 100-fold lower than the intact chimeric polypeptide assembly in the same subject.

46. The chimeric polypeptide assembly of any of claims 37-45, wherein the intact chimeric polypeptide assembly exhibits reduced extravasation from the blood circulatory system in a subject compared to the chimeric polypeptide assembly in which the RS is cleaved, releasing the first portion and the third portion.

47. The chimeric polypeptide assembly of any one of claims 37-46, wherein the subject is selected from the group consisting of mouse, rat, monkey, dog, and human.
48. A pharmaceutical composition comprising the chimeric polypeptide assembly of any one of the preceding claims and one or more pharmaceutically suitable excipients.
49. The pharmaceutical composition of claim 48, wherein the composition is in a liquid form.
50. The pharmaceutical composition of claim 49, wherein the composition is in a pre-filled syringe.
51. The pharmaceutical composition of claim 48, wherein the composition is formulated as a lyophilized powder to be reconstituted prior to administration.
52. An isolated nucleic acid comprising a polynucleotide sequence selected from (a) a polynucleotide encoding the chimeric polypeptide assembly of any one of claims 1-47, or (b) the complement of the polynucleotide of (a).
53. An expression vector comprising the polynucleotide sequence of claim 52 and a recombinant regulatory sequence operably linked to the polynucleotide sequence.
54. An isolated host cell, comprising the expression vector of claim 53, wherein the host cell is *E. coli*.