

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 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 ;
 - 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 said bulking moiety is capable of being released from said first portion by action of said mammalian protease on said second portion.
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 target cell marker is a tumor specific marker or an inflammatory marker.
5. The chimeric polypeptide assembly of any one of claims 1-4, wherein the mammalian protease is expressed in a tumor tissue.
6. 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.
7. The chimeric polypeptide assembly of any one of the preceding claims, wherein second binding domain has binding specificity to an effector cell antigen expressed on an effector cell selected from the group consisting of plasma cell, T cell, B cell, cytokine induced killer cell (CIK cell), mast cell, dendritic cell, regulatory T cell (RegT cell), helper T cell, myeloid cell, and NK cell.
8. The chimeric polypeptide assembly of claim 7 wherein the effector cell antigen is CD3 or CD3ε.
9. The chimeric polypeptide assembly of claim 8, wherein the second binding domain comprises a VL and/or VH region derived from a monoclonal antibody capable of binding human CD3 or CD3ε.
10. The chimeric polypeptide assembly of claim 8, wherein the second binding domain VL and/or VH are selected from the sequences set forth in Table 1.
11. The chimeric polypeptide assembly of claim 9 or claim 10, 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.

12. The chimeric polypeptide assembly of claim 8, 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.
13. The chimeric polypeptide assembly of any one of claims 4-12, wherein the first binding domain comprises a VL and/or VH region derived from a monoclonal antibody capable of binding a 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.
14. The chimeric polypeptide assembly of claim 13, wherein the first binding domain regions are derived from monoclonal antibody sequences set forth in Table 2.
15. The chimeric polypeptide assembly of claim 13, 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.
16. The chimeric polypeptide assembly of any one of claims 4-12, wherein the first binding domain is capable of binding a tumor specific marker and 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.
17. The chimeric polypeptide assembly of any one of claims 4-16, 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), Muellerian 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.
18. The chimeric polypeptide assembly of any one claims 6-17, 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.
19. 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.

20. The chimeric polypeptide assembly of claim 19, wherein the RS is a substrate for legumain, MMP-2, MMP-7, MMP-9, MMP-14, uPA, and matriptase.
21. 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.
22. The chimeric polypeptide assembly of any one of the preceding claims, wherein the bulking moiety is an extended recombinant polypeptide (XTEN), 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..
23. 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.
24. The chimeric polypeptide assembly of any one of claims 17-23, 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 effector cell antigen and to a tumor cell bearing the tumor specific marker in an *in vitro* assay comprising both the T cells and the tumor cells.
25. The chimeric polypeptide assembly of claim 24, 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.
26. The chimeric polypeptide assembly of claim 24, 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.
27. The chimeric polypeptide assembly of claim 24, wherein the binding affinity of the released first portion to the T cell bearing the effector cell antigen or the tumor cell 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 bearing the effector cell antigen or the tumor cell marker wherein the second portion has not been cleaved.
28. The chimeric polypeptide assembly of claim 24, 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.
29. The chimeric polypeptide assembly of claim 24, 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.
30. The chimeric polypeptide assembly of claim 24, 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.

31. The chimeric polypeptide assembly of any one of claims 28-30, wherein the cytotoxic activity and/or cell lysis of the tumor cell is mediated by target specific activation of the T cell.

32. The chimeric polypeptide assembly of claim 31, 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.

33. The chimeric polypeptide assembly of any one of claims 28-32, 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 effector cells bearing the effector cell antigen and tumor cells bearing the target cell 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 effector cell antigen and the binding affinity of the composition of a(ii) to the effector 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.

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

35. The chimeric polypeptide assembly of any one of claims 24-34, 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.

36. The chimeric polypeptide assembly of claim 24, 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.

37. The chimeric polypeptide assembly of claim 24, wherein the binding affinity of the first binding domain to the target cell, as measured by K_d constant in the *in vitro* assay, is at least one

order of magnitude lower compared to the greater binding affinity of the second binding domain to the CD3 antigen.

38. The chimeric polypeptide assembly of claim 24, 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.

39. The chimeric polypeptide assembly of any one of claims 1-23, 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.

40. The chimeric polypeptide assembly of claim 39, 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 human CD3 antigen and to a tumor cell bearing the tumor specific marker.

41. The chimeric polypeptide assembly of claim 40, whereupon the concurrent binding to a T cell bearing the CD3 antigen and the tumor cell bearing the tumor cell marker by the first portion results in the release of T cell-derived effector molecules.

42. The chimeric polypeptide assembly of claim 41, 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.

43. The chimeric polypeptide assembly of claim 40, whereupon the concurrent binding of the first portion to a 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.

44. The chimeric polypeptide assembly of any one of claims 39-43, 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.

45. The chimeric polypeptide assembly of any one of claims 39-44, 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.

46. The chimeric polypeptide assembly of any one of claims 39-45, wherein the plasma Cmax 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.

47. The chimeric polypeptide assembly of any one of claims 39-45, wherein the plasma Cmax 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.

48. The chimeric polypeptide assembly of any of claims 39-47, 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.

49. The chimeric polypeptide assembly of any one of claims 39-48, wherein the subject is selected from the group consisting of mouse, rat, monkey, dog, and human.

50. A pharmaceutical composition comprising the chimeric polypeptide assembly of any one of the preceding claims and one or more pharmaceutically suitable excipients.

51. The pharmaceutical composition of claim 50, wherein the composition is in a liquid form.

52. The pharmaceutical composition of claim 51, wherein the composition is in a pre-filled syringe.

53. The pharmaceutical composition of claim 50, wherein the composition is formulated as a lyophilized powder to be reconstituted prior to administration.

54. An isolated nucleic acid comprising a polynucleotide sequence selected from (a) a polynucleotide encoding the chimeric polypeptide assembly of any one of claims 1-49, or (b) the complement of the polynucleotide of (a).

55. An expression vector comprising the polynucleotide sequence of claim 54 and a recombinant regulatory sequence operably linked to the polynucleotide sequence.

56. An isolated host cell, comprising the expression vector of claim 55, wherein the host cell is *E. coli*.