

CHIMERIC POLYPEPTIDE ASSEMBLY AND METHODS OF MAKING AND USING THE SAME

BACKGROUND OF THE INVENTION

[0001] Many approved cancer therapeutics are cytotoxic drugs that kill normal cells as well as tumor cells. The therapeutic benefit of these cytotoxic drugs depends on tumor cells being more sensitive than normal cells, thereby allowing clinical responses to be achieved using doses that do not result in unacceptable side effects. However, essentially all of these non-specific drugs result in some if not severe damage to normal tissues, which often limits treatment suitability.

[0002] Bispecific antibodies offer a different approach to cytotoxic drugs in that they direct immune effector cells to kill cancer cells. Bispecific antibodies combine the benefits of different binding specificities derived from two monoclonal antibodies into a single composition, enabling approaches or combinations of coverages that are not possible with monospecific antibodies. This approach relies on binding of one arm of the bispecific antibody to a tumor-associated antigen or marker, while the other arm, upon binding the CD3 molecule on T cells, triggers their cytotoxic activity by the release of effector molecules such as such as TNF- α , IFN- γ , interleukins 2, 4 and 10, perforin, and granzymes. Advances in antibody engineering have led to the development of a number of bispecific antibody formats and compositions for redirecting effector cells to tumor targets, including Bi-specific T-cell Engagers (BiTEs®) such as blinatumomab. BiTEs function by recruiting and activating polyclonal populations of T-cells at tumor sites, and do so without the need for co-stimulation or conventional MHC recognition. There remains, however, the dual problems of certain patients experiencing serious side effects referred to as “cytokine storm” or “cytokine release syndrome” (Lee DW et al. Current concepts in the diagnosis and management of cytokine release syndrome. Blood. 2014 124(2):188-195) mediated by the release of TNF- α and IFN- γ , amongst other cytokines, in addition to the fact that BiTE compositions have a very short half-life, necessitating continuous infusions of four to eight weeks in order to maintain BiTE within the therapeutic window for sufficient time to achieve a therapeutic effect.

SUMMARY

[0003] There remains a considerable need for alternative therapeutics that offer the pharmacologic advantages of such bispecific antibody formats but with increased safety,

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 disclosure 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 example, 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 examples 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 example, 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 example, 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 example, the chimeric polypeptide assembly is a fusion protein. In another example, the second and third portions are a fusion protein and the first portion is conjugated to the second portion. In one example 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 maleimide or other cross-linking agents known in the art. In another example, 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.

[0008] The first and the second binding domains are generally antibody fragments derived from monoclonal antibodies. In one example, the first and the second binding domains of the first portion of the chimeric polypeptide assembly compositions are scFv or configured as a diabody. In other examples, the first and the second binding domains of the first portion of the chimeric polypeptide assembly compositions are selected from the group consisting of Fv, Fab, Fab', Fab'-SH, F(ab')₂, linear antibodies, a single domain antibody, a non-antibody scaffold, and a single domain camelid antibody. In other examples, the first and the second binding domains of the first portion of the chimeric polypeptide assembly compositions are selected from the group of peptides, non-antibody scaffolds such as anticalins, adnectins, fynomers, affilins, affibodies, centyrins, DARPins. In other examples the binding domain for the tumor cell target is a variable domain of a T cell receptor that has been engineered to bind MHC that is loaded with a peptide fragment of a protein that is overexpressed by tumor cells.

[0009] In one example of the chimeric polypeptide assembly, the first binding domain of the first portion has binding affinity to a target cell marker. Target cells include any cell types of eukaryotes such as those of ectoderm, mesoderm or endoderm origin. Of particular interest are tumor cells and markers expressed by the tumor cells. Tumor cell can arise from a cell selected from the group consisting of stromal cell, fibroblasts, myofibroblasts, glial cells, epithelial cells, fat cells, lymphocytic cells, vascular cells, smooth muscle cells, mesenchymal cells, breast tissue cells, prostate cells, kidney cells, brain cells, colon cells, ovarian cells, uterine cells, bladder cells, skin cells, stomach cells, genito-urinary tract cells, cervix cells, uterine cells, small intestine cells, liver cells, pancreatic cells, gall bladder cells, bile duct cells, esophageal cells, salivary gland cells, lung cells, and thyroid cells. In some cases, the tumor specific marker include 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, VEGFR2, DLK1, SP17, ROR1, and EphA2. In another example of the chimeric polypeptide assembly, the first binding domain of the first portion has binding affinity to a target cell marker that is an inflammatory marker.

[0010] In one example, the first binding domain of the first portion of the chimeric polypeptide assembly compositions comprises VH and VL regions with specific binding affinity to a tumor-specific marker or an antigen of a target cell. In one example of the foregoing, the first binding domain VH and VL are derived from a monoclonal antibody VH and VL selected from the group of paired sequences set forth in Table 2. The VH and VL regions of the first and second binding domains can be configured in different orders, with

respect to the N-terminus to C-terminus order. In one example, the first binding domain VH and VL regions are arranged in the order VH—VL. In another example, the first binding domain VH and VL regions are arranged in the order VL—VH. In other cases, 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-H3 region, wherein each of said regions is derived from monoclonal antibody sequences selected from the group of sequences set forth in Table 2. The various configurations of VH and VL regions as well as the CDRs contained therein typically retain the desired binding specificity to an intended target cell marker.

[0011] In other examples of the chimeric polypeptide assembly, the second binding domain of the first portion has binding affinity to an effector cell. Where desired, the effector cell can be immune cells, including but not limited to plasma cell, T cell, B cell, cytokine induced killer cell (CIK cell), master cell, dendritic cell, regulatory T cell (RegT cell), helper T cell, myeloid cell, and NK cell. The second binding domain typically exhibits binding specificity to an antigen expressed by an effector cell. In some examples, the antigen is expressed on the cell surface of an effector cell. In another example, the second binding domain has binding specificity to an effector cell antigen expressed on a T cell. Non-limiting exemplary effector cell antigens include CD3, CD4, CD8, $\alpha\beta$ TCR, CD25, CD45RO, CD69, CD127, and CD196 (CCR6). Of particular interest is a second binding domain adopting a scFv configuration having VH and VL regions derived from a monoclonal antibody that binds specifically to human CD3. In one example, the second binding domain VH and VL are derived from a monoclonal antibody VH and VL selected from the group of sequences set forth in Table 1. In another example, the second binding domain comprises VH and VL regions derived from a monoclonal antibody capable of binding human CD3 ϵ .

[0012] The VH and VL of the scFv of the binding domains can be arranged in different configurations without affecting the utility of the resulting composition. In one example, the second binding domain scFv comprises VH and VL regions arranged in the order VH—VL or VL—VH in the N-terminal to C-terminal direction. The binding domains can also be created from CDR regions. In one example, 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-H3 region, wherein each is derived from a monoclonal antibody of Table 1. In the foregoing examples of the paragraph, the VH and VL regions and the first binding domain and the second binding domain are linked by flexible polypeptide linkers selected from the group of sequences set forth in Table 8 and Table 9. In another example, the first portion of the chimeric polypeptide assembly compositions has a sequence with at least about 90%, or

91%, or 92%, or 93%, or 94%, or 95%, or 96%, or 97%, or 98%, or 99% identity to a sequence selected from the group consisting of the sequences of Table 13.

[0013] One advantage of the subject chimeric polypeptide assembly is that it is assembled in form of a prodrug, wherein the intact composition can be activated in proximity to a target tissue or a certain cellular environment in which mammalian proteases are present, releasing the first portion binding domains at the site where its activity is most desirable. For example, the first portion binding domain, when present in the intact assembly, has lower binding affinity due to the shielding effect of the bulking moiety. Upon release via cleavage of the RS by a mammalian protease preferentially expressed in a target issue, for example, a tumor tissue, the first portion binding domain becomes “activated” without being shielded by the bulking moiety. In another example, the disclosure provides a chimeric polypeptide assembly, wherein the mammalian protease capable of cleaving the RS is preferentially expressed in an inflammatory tissue. In one example, the chimeric polypeptide assembly comprises an RS, wherein the RS comprises an amino acid sequence selected from the group consisting of the sequences set forth in Table 4. Where desired, the RS comprises an amino acid sequence selected from the group consisting of the sequences LSGRSDNHSPLGLAGS, SPLGLAGSLSGRSDNH, SPLGLSGRSDNH, LAGRSDNHSPLGLAGS, LSGRSDNHVPLSLKMG, SPLGLAGS, GPLALARG, LSGRSDNH, VPLSLTMG, VPLSLKMG, VPLSLSMG, EPLELVAG, EPLELRAG, EPAALMAG, EPASLMAG, RIGSLRTA, RIQFLRTA, EPFHLMAG, VPLSLFMG, EPLELPAG, and EPLELAAG. Where desired, the release segment of the chimeric polypeptide assembly composition comprises the amino acid sequence the sequence LSGRSDNHSPLGLAGS. In the RS examples, 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.

[0014] In another example, the third portion of the chimeric polypeptide assembly compositions comprise a bulking moiety. Exemplary bulking moieties include but are not limited to: extended recombinant polypeptides (XTEN), albumin binding domain, albumin, IgG binding domain, polypeptides consisting of proline, serine, and alanine; fatty acid, ELP biopolymer, Fc domain, polyethylene glycol (PEG), PLGA, and hydroxylethyl starch. In one example, the bulking moiety is an XTEN sequence. Where desired, the XTEN of the third portion 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.

[0015] In another example, the subject chimeric polypeptide assembly exhibits the ability to bind and link effector cells and target cells, thereby forming an immunological synapse such that the effector cell can mediate its biological effect in a target cell specific manner. For example, a subject chimeric polypeptide assembly possesses the ability to (1) bind specifically to a target cell marker such as a tumor-specific marker, and to (2) bind specifically to an antigen expressed on an effector cell (e.g., an antigen expressed by a T-cell). The concurrent binding of the T cell and the tumor cell mediates killing, damage, and/or lysis of the tumor cell. In one example, upon cleavage of the second portion by the one or more mammalian proteases and release of the first portion, 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 in an in vitro assay comprising both the T cells and the tumor cells. In an exemplary design characteristic of the inventive compositions, upon cleavage of the second portion RS to release the first portion and the third portion from said chimeric polypeptide assembly, the released first portion has a molecular weight that is at least 2-fold, 3-fold, 4-fold, or 5-fold less than the third portion and has a molecular weight that is at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60% less than the intact chimeric polypeptide assembly. In an example, upon cleavage of the second portion RS, the released said first portion from said chimeric polypeptide assembly has increased binding affinity to the effector T cell bearing the CD3 antigen and/or the tumor cell marker compared to the chimeric binding assembly wherein the second portion has not been cleaved. The increased binding affinity of the released first portion to the T cell bearing the human CD3 antigen and/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 human CD3 antigen or the tumor cell marker wherein the RS has not been cleaved. In another example, upon cleavage of the second portion RS and the release of said first portion from said chimeric polypeptide assembly, the concurrent binding of the first portion to the T cell and the tumor cell yields cytotoxic activity against the tumor cell in an in vitro assay comprising a population of T cells and tumor cells. In another example, 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. For example, 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. In one example, the

cytotoxic activity and/or cell lysis of the tumor cell is mediated by target specific activation of the T cell, 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. As the RS of the chimeric binding assembly may be subject to partial cleavage of the RS during an *in vitro* cytotoxic assay, for purposes of determining the maximum comparative difference in cytotoxicity, the RS of the assembly can be substituted with a non-cleavable peptide and assayed in comparison to a sample of the released first portion. Where desired, the *in vitro* assay can be assays selected from 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, or by the methods described herein in the Examples, below.

[0016] In another example, the disclosure provides chimeric polypeptide assembly compositions comprising a first portion wherein said first portion comprises i) a second binding domain with binding specificity to an effector cell antigen; and ii) a first binding domain with binding specificity to a tumor-specific marker or an antigen of a target cell; a second portion wherein said second portion comprises a first release segment (RS) capable of being cleaved by a mammalian protease, a third portion comprising a first 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, a fourth portion comprising a release segment (RS) that may be the same or may be different from the second portion RS, and a fifth portion comprising a second bulking moiety that may be the same or may be different from the third portion wherein said bulking moiety is capable of being released from said first portion by action of said mammalian protease on said fourth portion. In one example of the foregoing, the second release segment of the chimeric polypeptide assembly composition comprises an amino acid sequence selected from the group consisting of the sequences set forth in Table 4. In another example of the foregoing, the second release segment of the chimeric polypeptide assembly composition comprises an amino acid sequence selected from

the group consisting of the sequences LSGRSDNHSPLGLAGS, SPLGLAGSLSGRSDNH, SPLGLSGRSDNH, LAGRSDNHSPLGLAGS, LSGRSDNHVPLSLKMG, SPLGLAGS, GPLALARG, LSGRSDNH, VPLSLTMG, VPLSLKMG, VPLSLSMG, EPLELVAG, EPLELRAG, EPAALMAG, EPASLMAG, RIGSLRTA, RIQFLRTA, EPFHLMAG, VPLSLFMG, EPLELPAG, and EPLELAAG. In another example of the foregoing, the second release segment of the chimeric polypeptide assembly composition comprises an amino acid sequence capable of being cleaved by a protease selected from the group of proteases set forth in Table 3. In another example of the foregoing, the bulking moiety of the fifth portion of the composition is selected from the group consisting of: XTEN; albumin binding domain; albumin; IgG binding domain; a polypeptide of at least 350 amino acid residues consisting of proline, serine, and alanine; fatty acid; and Fc domain. In another example of the foregoing, the bulking moiety of the fifth portion of the composition is XTEN. In another example of the foregoing, the bulking moiety of the composition is an XTEN comprising an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, when optimally aligned, to a sequence selected from the group of sequences set forth in Table 5. In one example, the disclosure provides a chimeric polypeptide assembly composition, comprising an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence without the signal peptide, as set forth in Table 10, when optimally aligned. In another example, the disclosure provides a chimeric polypeptide assembly composition, comprising an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence, as set forth in Table 12, when optimally aligned. In another example, the disclosure provides a chimeric polypeptide assembly composition, comprising an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence, as set forth in FIG. 36 or FIG. 37. In another example, the disclosure provides chimeric polypeptide assembly consisting of an amino acid sequence having a polypeptide sequence selected from the group consisting of the sequences set forth in FIG. 36 or FIG. 37.

[0017] In an exemplary characteristic of the chimeric polypeptide assembly compositions, the ability to effect cytolysis of the target cells after release of the first portion binding domains (compared to the intact composition) is proportionally greater than the increased binding affinity to the target cell marker of released first portion as compared to that of the intact composition. In one example of this characteristic, the relative cytotoxicity expressed as EC50 integer, compared to the binding affinity expressed as the log of the Kd in an *in vitro*

assay is at least about 2:1, or at least 10:1, or at least 50:1, or at least 100:1, or at least 300:1, or at least 500:1, or at least 1000:1. In another example, the ratio of cytotoxicity (e.g., expressed as the EC₅₀ integer), to the binding affinity (e.g., expressed as the log of the K_d) of the released first portion of the chimeric polypeptide assembly in an *in vitro* assay is at least about 2-fold, at least about 3-fold, at least about 5-fold, at least about 10-fold, at least about 30-fold, at least about 50-fold, or at least about 100-fold greater.

[0018] In some examples, wherein in a comparison of a) the relative cytotoxicity, which is measured as a ratio between the cytotoxicity of (i) the released first portion to the target tumor cell in an *in vitro* assay comprising both the T cells 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) the relative binding affinity to the effector cell antigen, which is measured as a ratio between the binding affinity of (i) the released first portion to the effector cell antigen and (ii) the binding affinity to the effector cell antigen 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, where the ratio between the relative cytotoxicity and the relative binding affinity is greater than at least 3:1, 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. In one example of the foregoing, the non-cleavable peptide has the sequence glycine-serine, serine-glycine, or multiple units of either dipeptide and the effector cell antigen is CD3. In one example, wherein in a comparison of a) the relative cytotoxicity, which is measured as a ratio between the cytotoxicity of (i) the released first portion to the target tumor cell in an *in vitro* assay comprising both the T cells 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) the relative binding affinity to the target cell marker, which is measured as a ratio between the binding affinity of (i) the released first portion to the target cell marker and (ii) the binding affinity to the target cell marker 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, where the ratio between the relative cytotoxicity and the relative

binding affinity is greater than at least 3:1, 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. In one example of the foregoing, the non-cleavable peptide has the sequence glycine-serine, serine-glycine, or multiple units of either dipeptide and the effector cell antigen is CD3. In another example, wherein in a comparison of a) the relative cytotoxicity, which is measured as a ratio between the cytotoxicity of (i) the released first portion to the target tumor cell in an in vitro assay comprising both the T cells 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; b) the relative effector cell antigen binding affinity, which is measured as a ratio between the binding affinity of (i) the released first portion to the effector cell antigen and (ii) the binding affinity 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 c) the relative binding affinity to the target cell marker, which is measured as a ratio between the binding affinity of (i) the released first portion to the target cell marker and (ii) the binding affinity to the target cell marker 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, where the ratio between the relative cytotoxicity and the relative effector cell antigen binding affinity multiplied with the relative binding affinity to the target cell marker is greater than at least 3:1, 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. In one example of the foregoing, the non-cleavable peptide has the sequence glycine-serine, serine-glycine, or multiple units of either dipeptide and the effector cell antigen is CD3.

[0019] In one example, the disclosure provides chimeric polypeptide assembly compositions in which the EC₅₀ value of the released first portion of the chimeric polypeptide assembly composition in an in vitro cytotoxicity assay comprising both the T cells and tumor cells bearing the target cell marker is ≤ 5000 pg/ml, even more preferably ≤ 1000 pg/ml, even more preferably ≤ 500 pg/ml, even more preferably ≤ 350 pg/ml, even more preferably ≤ 250 pg/ml, even more preferably < 100 pg/ml, even more preferably ≤ 50 pg/ml, even more preferably < 10 pg/ml, and most preferably ≤ 5 pg/ml. In one example, the EC₅₀ value of the released first

portion of the chimeric polypeptide assembly composition in the in vitro assay is at least 10-fold, or at least 20-fold, or at least 30-fold, or at least 40-fold, or at 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, or at least 120-fold less than the EC50 value of the intact chimeric polypeptide assembly composition.

[0020] In some cases, 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. In one example, the binding affinity of the first binding domain of the released first portion to the target 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 CD3 antigen. In other examples, the binding affinity of the first binding domain of the released first portion to the target cell, as measured by K_d constant in an in vitro binding assay 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. The binding affinity can be determined by standard cell-based assays, ELISA, assays described in the Examples, herein, or in other in vitro assays known in the art.

[0021] In another example, the disclosure relates to the enhanced properties of the chimeric polypeptide assembly when administered to a subject. It is specifically contemplated that the intact chimeric polypeptide assembly compositions comprising release segments exhibit less cytotoxicity and/or reduced capacity to elicit the production of proinflammatory cytokines compared to the released first portion component. In one example, the disclosure provides chimeric polypeptide assembly compositions wherein upon or following administration of a composition comprising the chimeric polypeptide assembly to a subject, the second portion of the assembly is cleaved in proximity to a tumor expressing a protease capable of cleaving the RS. Upon cleavage of the second portion by said mammalian protease and release of the first portion from the 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 in the subject. In one example, the concurrent binding of the released first portion to a T cell bearing the CD3 antigen and the tumor cell bearing the tumor cell marker results in the release of T cell-derived effector molecules. In the foregoing, 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, or other T cell effector molecules known in the art. As a consequence of the concurrent binding of the effector cell and the target cell, an

immunological synapse is created, which effects lysis of the target cell by the T cell and the effector molecules.

[0022] In another example, the disclosure relates to chimeric polypeptide assembly compositions with increased terminal half-life and other properties imparted by the bulking moiety; e.g., XTEN. In one example, the disclosure provides chimeric polypeptide assembly compositions wherein the intact composition exhibits a half-life upon or 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 upon or following administration to a subject at a comparable dose. In another example, upon or 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. In a related example, the plasma C_{max} concentration of the released first portion upon or following a single administration of the chimeric polypeptide composition 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. In another related example, the plasma C_{max} concentration of the released first portion upon or following a single administration of the intact chimeric polypeptide composition 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 plasma C_{max} concentration of the intact chimeric 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 example, 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 examples of this paragraph, the subject can be mouse, rat, monkey, dog, and human.

[0023] In another example, the disclosure relates to pharmaceutical compositions of the chimeric polypeptide assembly. In one example, the disclosure 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 example, the pharmaceutical composition is formulated for intradermal,

subcutaneous, intravenous, intra-arterial, intraabdominal, intraperitoneal, intrathecal, or intramuscular administration. In another example, the pharmaceutical composition is in a liquid form. In a related example, the pharmaceutical composition in a liquid form is supplied in a pre-filled syringe for a single injection. In other examples, 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 example, the disclosure relates to methods and uses of the chimeric polypeptide assembly or a pharmaceutical composition comprising the chimeric polypeptide assembly. In one example, the disclosure 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 example, 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,

liposarcoma, pancreatic cancer, gall bladder cancer, cancers of the bile duct, esophageal cancer, salivary gland carcinoma, thyroid cancer, epithelial cancer, arrhenoblastoma, adenocarcinoma, sarcoma, and B-cell derived chronic lymphatic leukemia.

[0025] In another example, the disclosure relates to chimeric polypeptide assembly or a pharmaceutical composition comprising the chimeric polypeptide assembly for use in a method of treating a disease in a subject, wherein the method comprises administering the chimeric polypeptide assembly or the pharmaceutical composition to a subject with the disease, including but not limited to cancer. Where desired, the method comprises administering to the subject in need thereof a therapeutically effective dose of a pharmaceutical composition comprising the chimeric polypeptide assembly and one or more excipients. In one example of the method of treatment, 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. In another example of the method of treatment, 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, or monthly. In another example of the method of treatment, the pharmaceutical composition is administered to the subject as one or more 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 another example of the method of treatment, the dose is administered intradermally, subcutaneously, intravenously, intra-arterially, intra-abdominally, intraperitoneally, intrathecally, or

intramuscularly. In another example of the method of treatment, the dose is administered as a bolus dose or by infusion of 5 minutes to 96 hours as tolerated for maximal safety and efficacy. In another example of the method of treatment, the dose is selected from the group consisting of at least about 0.005 mg/kg, at least about 0.01 mg/kg, at least about 0.02 mg/kg, at least about 0.04 mg/kg, at least about 0.08 mg/kg, at least about 0.1 mg/kg, at least about 0.12 mg/kg, at least about 0.14 mg/kg, at least about 0.16 mg/kg, at least about 0.18 mg/kg, at least about 0.20 mg/kg, at least about 0.22 mg/kg, at least about 0.24 mg/kg, at least about 0.26 mg/kg, at least about 0.27 mg/kg, at least about 0.28 mg/kg, at least 0.3 mg/kg, at least 0.4 mg/kg, at least about 0.5 mg/kg, at least about 0.6 mg/kg, at least about 0.7 mg/kg, at least about 0.8 mg/kg, at least about 0.9 mg/kg, at least about 1.0 mg/kg, at least about 1.5 mg/kg, or at least about 2.0 mg/kg. In another example of the method of treatment, the initial dose is selected from the group consisting of at least about 0.005 mg/kg, at least about 0.01 mg/kg, at least about 0.02 mg/kg, at least about 0.04 mg/kg, at least about 0.08 mg/kg, at least about 0.1 mg/kg, and a subsequent dose is selected from the group consisting of at least about 0.1 mg/kg, at least about 0.12 mg/kg, at least about 0.14 mg/kg, at least about 0.16 mg/kg, at least about 0.18 mg/kg, at least about 0.20 mg/kg, at least about 0.22 mg/kg, at least about 0.24 mg/kg, at least about 0.26 mg/kg, at least about 0.27 mg/kg, at least about 0.28 mg/kg, at least 0.3 mg/kg, at least 0.4 mg/kg, at least about 0.5 mg/kg, at least about 0.6 mg/kg, at least about 0.7 mg/kg, at least about 0.8 mg/kg, at least about 0.9 mg/kg, at least about 1.0 mg/kg, at least about 1.5 mg/kg, or at least about 2.0 mg/kg. In another example of the method of treatment, the administration of the therapeutically effective dose of the pharmaceutical composition to the subject results in a plasma concentration of the chimeric polypeptide assembly of at least about 0.1 ng/mL to at least about 2 ng/mL or more in the subject for at least about 3 days, at least about 7 days, at least about 10 days, at least about 14 days, or at least about 21 days.

[0026] In another examples, the disclosure provides a pharmaceutical composition for use in a method for the treatment of a disease, the method comprising administering the pharmaceutical composition to a subject with the disease according to a treatment regimen comprising one or more consecutive doses using a therapeutically effective dose. In one example of the pharmaceutical composition for the use in the method for the treatment of a disease, 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. In another example of the pharmaceutical composition for the use in the treatment of a disease, the treatment regimen is part of a specified treatment cycle, wherein the specified treatment cycle comprises administration of the pharmaceutical composition twice a week, every week, every 10 days, every two weeks, every three weeks, or every month per each treatment cycle. In one example, the treatment regimen results in the improvement of a clinical parameter or endpoint associated with the disease in the subject, wherein the clinical parameter or endpoint is selected from one or any combination of the group consisting of tumor shrinkage as a complete, partial or incomplete response; time-to-progression, time to treatment failure, biomarker response; progression-free survival; disease free-survival; time to recurrence; time to metastasis; time of overall survival; improvement of quality of life; and improvement of symptoms. In the foregoing examples of the method, the subject is selected from the group consisting of mouse, rat, monkey, and human.

[0027] In another example, the disclosure relates to kits comprising the pharmaceutical composition. In one example, the disclosure provides a kit comprising the pharmaceutical composition of any one of examples disclosed herein, a container and a label or package insert on or associated with the container. In another example, the disclosure provides a kit comprising a pre-filled syringe containing the pharmaceutical composition of any one of examples disclosed herein, and a label or package insert on or associated with the syringe.

[0028] In another example, the disclosure relates to the differential characteristics and effects of the intact versus the cleaved chimeric polypeptide assembly compositions. In one example, the disclosure provides a chimeric polypeptide assembly of any of the examples disclosed herein, wherein the chimeric polypeptide assembly that is intact has at least a 10-fold, or at least 20-fold, or at least 30-fold, or at least 40-fold, or at 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, or at least 1000-fold lower potential to effect production of a Th1 cytokine from an effector cell compared to the corresponding first portion of the assembly that is not linked to the assembly, when said assembly is in contact with the effector cell and a target cell. In one example, the production of the Th1 cytokine is assayed in an in vitro assay comprising PBMC or CD3+ T cells and target cells having a tumor specific marker antigen 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, VEGFR2, DLK1, SP17, ROR1, and EphA2. In the foregoing example, the Th1 cytokine is selected from the group consisting of IL-2, TNF-alpha, and IFN-gamma. In another example, the production of the Th1 cytokine is assayed using blood or a fluid sample from a subject administered the chimeric polypeptide assembly compared to a subject administered the corresponding first portion not linked to the chimeric polypeptide assembly, with the result that the chimeric polypeptide assembly that is intact has at least a 10-fold, or at least 20-fold, or at least 30-fold, or at least 40-fold, or at 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, or at least 1000-fold lower potential to effect production of the Th1 cytokine. In the foregoing example, the subject is selected from the group consisting of mouse, rat, monkey, and human.

[0029] In other cases, the chimeric polypeptide assembly of any of the examples disclosed herein exhibits the characteristic that the chimeric polypeptide assembly that is intact has at least a 10-fold, or at least 20-fold, or at least 30-fold, or at least 40-fold, or at 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 example, the disclosure relates to nucleic acids encoding the subject compositions. In one aspect, 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 examples disclosed herein, or (b) the complement of the polynucleotide of (a). In another example, the disclosure 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 aspect, the invention provides an expression vector comprising a polynucleotide sequence of the foregoing examples and a recombinant regulatory sequence operably linked to the polynucleotide sequence. In another example, the disclosure provides an isolated host cell, comprising the foregoing expression vector. In one example, 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 example, the disclosure relates to T cell binding compositions and nucleic acids that encode them. In one example, the disclosure 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 example, the disclosure 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 example, the disclosure 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 examples 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

frame to a polynucleotide encoding the fusion protein of the foregoing disclosed T cell binding composition. In another example, the disclosure provides an expression vector comprising the foregoing polynucleotide sequence and a recombinant regulatory sequence operably linked to the polynucleotide sequence. The disclosure also provides an isolated host cell, comprising the expression vector, wherein the host cell is *E. coli*.

[0033] In yet another example, the disclosure relates to methods of producing the chimeric polypeptide assembly examples disclosed herein. In one example, the disclosure provides a method of producing a chimeric polypeptide assembly disclosed herein, the method comprising transforming a host cell with an expression vector encoding the chimeric polypeptide assembly, culturing the host cell under conditions permitting the chimeric polypeptide assembly to be expressed in the transformed host cell, and isolating the chimeric polypeptide assembly as a soluble fusion protein. In some examples, at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 97%, or at least 99% of the first and the second binding domains of the expressed fusion protein are correctly folded.

[0034] In other cases, the disclosure provides methods of inducing death of a target cell. The method typically comprises contacting said target cell with a chimeric polypeptide assembly of an example disclosed herein and an effector cell. In an example, the contact results in an effect in the target cell including but not limited to loss of membrane integrity, pyknosis, karyorrhexis, inducement of the intrinsic pathway of apoptosis, inducement of the extrinsic pathway of apoptosis, apoptosis, cell lysis, and cell death.

[0035] Cytotoxicity resulting in cell death (e.g., necrosis or apoptosis) can be determined by any suitable method, including, but not limited to, counting cells before and after treatment, or measuring the level of a marker associated with live or dead cells. Degree of cell death may be determined by any suitable method. In some examples, degree of cell death is determined with respect to a starting condition. For example, an individual may have a known starting amount of target cells, such as a starting cell mass or tumor of known size or circulating target cells at a known concentration. In another example, one can compare degree of cell death induced by one composition with respect to another (e.g. chimeric polypeptide assembly linked to a bulking moiety and a chimeric polypeptide assembly not linked to a bulking moiety). In such cases, degree of cell death may be expressed as a ratio of surviving cells after treatment to the starting cell population. In some examples, degree of cell death may be determined by a suitable cell death assay. In some example, degree of cell death may be determined by measurement of tumor size over time. A variety of cell death assays

are available, and may utilize a variety of detection methodologies. Examples of detection methodologies include, without limitation, the use of cell staining, microscopy, flow cytometry, cell sorting, and combinations of these. Further non-limiting examples of cell death assays are described in WO2011131472A1 and US20130052160, which is incorporated herein by reference.

[0036] In one example of the foregoing, the method is employed in an in vitro cell-based assay comprising a mixed population of the target cells and the effector cells, and an effective amount of the chimeric polypeptide assembly having binding affinity for antigens of the target cell and the effector cell. In the assay, the target cell expresses a tumor specific marker antigen including but not limited to 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, VEGFR2, DLK1, SP17, ROR1, and EphA2 and the effector cell is a T cell wherein the effector cell antigen is CD3. In other examples, the method of inducing death of a target cell is employed in a subject having a cancer comprising a population of the target cell, wherein the method comprises administering a therapeutically effective amount of the chimeric polypeptide assembly to the subject. In one example of the foregoing, the method comprises administering the chimeric polypeptide assembly as one or more consecutively administered therapeutically effective doses of a pharmaceutical composition comprising the chimeric polypeptide assembly and one or more excipients. In another example of the foregoing, the method comprises a regimen of determining the amount of a pharmaceutical composition needed to achieve a therapeutic effect in the subject having the cancer and administering the

amount as one or more consecutively doses to the subject. In the methods of inducing death of a target cell in a subject, wherein the target cell is a cancer cell, where the cancer can be 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, liposarcoma, pancreatic cancer, gall bladder cancer, cancers of the bile duct, esophageal cancer, salivary gland carcinoma, thyroid cancer, epithelial cancer, arrhenoblastoma, adenocarcinoma, sarcoma, and B-cell derived chronic lymphatic leukemia. By use of the inventive method in the subject with a cancer, the method results in an improvement of a clinical parameter or endpoint wherein the clinical parameter or endpoint can be overall survival, symptom endpoints, disease-free survival, objective response rate, complete response, duration of response, progression-free survival, time to progression, time-to-treatment failure, tumor measurement, tumor size, tumor response rate, time to metastasis, and biomarker concentration. In other cases, use of the inventive method in the subject with a cancer results in a reduction in the frequency, duration, or severity in diagnostically associated side effects in the subject compared to administration of a comparable dose, in mmoles/kg, to a comparable subject of a composition comprising the first portion and an absence the second portion and third portion of the chimeric polypeptide assembly, wherein the side effects can be one or more of the following: increased plasma levels of IL-2, increased plasma levels of TNF-alpha, increased plasma levels of IFN-gamma, sepsis, febrile neutropenia, neurotoxicity, convulsions, encephalopathy, cytokine release syndrome, speech disturbance, equilibrium disturbance, fever, headache, confusion, hypotension, neutropenia, nausea, impaired consciousness, disorientation, and increased liver enzymes.

[0037] In still other cases, the disclosure provides methods of delivering a therapeutic agent to a tumor cell comprising a tumor specific marker, the method comprising administering to the target cell the chimeric polypeptide assembly of any one of the examples disclosed herein, wherein the therapeutic agent is delivered to the target cell via the first binding domain of the first portion specifically binding to the tumor specific marker. In the

foregoing, 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, VEGFR2, DLK1, SP17, ROR1, and EphA2. Where desired, the tumor specific marker is selected from the group consisting of Alpha 4 Integrin, Ang2, CEACAM5, CD19, CD20, CD33, CD38, cMET, CTLA4, EpCAM, EphA2, FOLR1, HER1(EGFR), HER2, HER3, HER1(EGFR)/HER3, HER2/3, Mesothelin, MUC1, PD-L1, PSMA, TAG-72, VEGFR1, VEGFR2. In one example of the method, the chimeric polypeptide assembly comprises an amino acid sequence having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least 100% sequence identity to a polypeptide sequence selected from the group consisting of the sequences of Table 12. In another example of the method, the chimeric polypeptide assembly comprises an amino acid sequence having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least 100% sequence identity to a polypeptide sequence selected from the group consisting of the sequences set forth in FIG. 36 or FIG. 37. In an example of the foregoing methods, the tumor cell resides in a tumor in a subject, wherein the subject can be mouse, rat, monkey, dog, and human.

[0038] In another example, the disclosure relates to physical characteristics of the chimeric polypeptide assembly compositions and resulting properties when administered to a subject. With respect to the chimeric polypeptide assembly examples disclosed herein, neither the second portion nor the third portion of the chimeric polypeptide assembly has specific binding affinity for the first portion. For each chimeric polypeptide assembly examples disclosed herein, the first portion accounts for less than 50% of the molecular weight of the

intact chimeric polypeptide assembly. In another example, the first portion of the chimeric polypeptide assembly examples disclosed herein accounts for less than 30%, or less than 40%, or less than 50% of the apparent molecular weight factor of the chimeric polypeptide assembly, when apparent molecular weight factor is assessed by size exclusion chromatography. Further, upon cleavage of the second portion and release of said first portion and said third portion from any of the chimeric polypeptide assembly examples disclosed herein, the hydrodynamic radius of the released first portion is less than about 30%, or less than about 40%, or less than about 50% of the hydrodynamic radius of the intact chimeric polypeptide assembly, when hydrodynamic radius is assessed by size exclusion chromatography. In one example, the disclosure provides a chimeric polypeptide assembly, wherein upon cleavage of the second portion and release of said first portion and said third portion from said chimeric polypeptide assembly, the hydrodynamic radius of the released first portion is less than about 5 nm, or less than about 4 nm, or less than about 3 nm when hydrodynamic radius is determined by size exclusion chromatography. Accordingly, the released first portion has greater ability to penetrate a tumor tissue compared to an intact chimeric polypeptide assembly. In another example, the hydrodynamic radius of an intact chimeric polypeptide assembly disclosed herein is greater than about 8 nm, or greater than about 9 nm, or greater than about 10 nm when hydrodynamic radius is determined by size exclusion chromatography. Accordingly, an intact chimeric polypeptide assembly administered to a subject with a tumor is less able to extravasate from vasculature of normal tissue of the subject compared to the ability to extravasate from vasculature of the tumor.

[0039] It is specifically contemplated that the chimeric polypeptide assembly composition examples can exhibit one or more or any combination of the properties disclosed herein. It is further specifically contemplated that the methods of treatment can exhibit one or more or any combination of the properties disclosed herein.

INCORPORATION BY REFERENCE

[0040] 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.

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 claims 1-21, further comprising a fourth portion, wherein said fourth portion comprises a peptidyl RS capable of being cleaved by one or more mammalian proteases.

23. The chimeric polypeptide assembly of claim 22, wherein the RS of said fourth portion comprises an amino acid sequence capable of being cleaved by said one or more proteases selected from the group consisting of the proteases set forth in Table 3.

24. The chimeric polypeptide assembly of claim 22 or claim 23, wherein the RS of said fourth portion is a substrate for legumain, MMP-2, MMP-7, MMP-9, MMP-14, uPA, and matriptase.

25. The chimeric polypeptide assembly of any one of claims 22-24, wherein the RS of said fourth portion comprises an amino acid sequence selected from the group consisting of the sequences set forth in Table 4.

26. The chimeric polypeptide assembly of any one of claims 1-25, further comprising a fifth portion, wherein said fifth portion comprises a bulking moiety, wherein said bulking moiety of said fifth portion is capable of being released from said first portion by action of said one or more mammalian proteases on said fourth portion.

27. The chimeric polypeptide assembly of claim 26, wherein the bulking moiety of the fifth portion is XTEN.

28. The chimeric polypeptide assembly of claim 26, wherein the bulking moiety of the fifth portion is XTEN comprising 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 the group of sequences set forth in Table 5.

29. The chimeric polypeptide assembly of any one of claims 26-28, wherein the chimeric polypeptide assembly has a configuration, from N-terminus to C-terminus, of (fifth portion)-(fourth portion)-(first portion)-(second portion)-(third portion).

30. 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.

31. The chimeric polypeptide assembly of any one of claims 16-30, 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.

32. The chimeric polypeptide assembly of claim 31, 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.

33. The chimeric polypeptide assembly of claim 31, 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.

34. The chimeric polypeptide assembly of claim 31, 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.

35. The chimeric polypeptide assembly of claim 31, 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.

36. The chimeric polypeptide assembly of claim 31, 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.

37. The chimeric polypeptide assembly of claim 31, 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.

38. The chimeric polypeptide assembly of any one of claims 35-37, wherein the cytotoxic activity and/or cell lysis of the tumor cell is mediated by target specific activation of the T cell.

39. The chimeric polypeptide assembly of claim 38, 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.

40. The chimeric polypeptide assembly of any one of claims 35-39, 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 the cytotoxicity of (II) 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.
41. The chimeric polypeptide assembly of claim 40, wherein the non-cleavable peptide has the sequence glycine-serine and the T-cell antigen is CD3 or CD3e.
42. The chimeric polypeptide assembly of any one of claims 31-41, 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.
43. The chimeric polypeptide assembly of claim 31, 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.
44. 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.
45. The chimeric polypeptide assembly of any one of claims 1-30, 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.
46. The chimeric polypeptide assembly of claim 45, 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.

47. The chimeric polypeptide assembly of claim 46, 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.
48. The chimeric polypeptide assembly of claim 47, 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.
49. The chimeric polypeptide assembly of claim 46, 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.
50. The chimeric polypeptide assembly of any one of claims 46-49, 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.
51. The chimeric polypeptide assembly of any one of claims 45-50, 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.
52. The chimeric polypeptide assembly of any one of claims 45-51, 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.
53. The chimeric polypeptide assembly of any one of claims 45-51, 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.
54. The chimeric polypeptide assembly of any of claims 45-53, 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.
55. The chimeric polypeptide assembly of any one of claims 45-54, wherein the subject is selected from the group consisting of mouse, rat, monkey, dog, and human.
56. A pharmaceutical composition comprising the chimeric polypeptide assembly of any one of the preceding claims and one or more pharmaceutically suitable excipients.
57. The pharmaceutical composition of claim 56, wherein the composition is in a liquid form.
58. The pharmaceutical composition of claim 57, wherein the composition is in a pre-filled syringe.

59. The pharmaceutical composition of claim 56, wherein the composition is formulated as a lyophilized powder to be reconstituted prior to administration.
60. An isolated nucleic acid comprising a polynucleotide sequence selected from (a) a polynucleotide encoding the chimeric polypeptide assembly of any one of claims 1-55, or (b) the complement of the polynucleotide of (a).
61. An expression vector comprising the polynucleotide sequence of claim 60 and a recombinant regulatory sequence operably linked to the polynucleotide sequence.
62. An isolated host cell, comprising the expression vector of claim 61, wherein the host cell is *E. coli*.