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(71) Applicant: MOLECULAR TEMPLATES, INC. [US/US]; 9301 Amberglen Boulevard; Suite 100, Austin, TX 78729 (US).

(72) Inventors: POMA, Eric; 165 Christopher Street, New York, NY 10014 (US). WILLERT, Erin; 2230 Mockingbird Drive, Round Rock, TX 78681 (US).

(74) Agent: ROGERS, Eric; MTEM, Harborside 5, 185 Hudson St, STE 1510, Jersey City, NJ 7311 (US).

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Schematic Drawing of Exemplary HER2-Targeting Molecules of the Present Invention

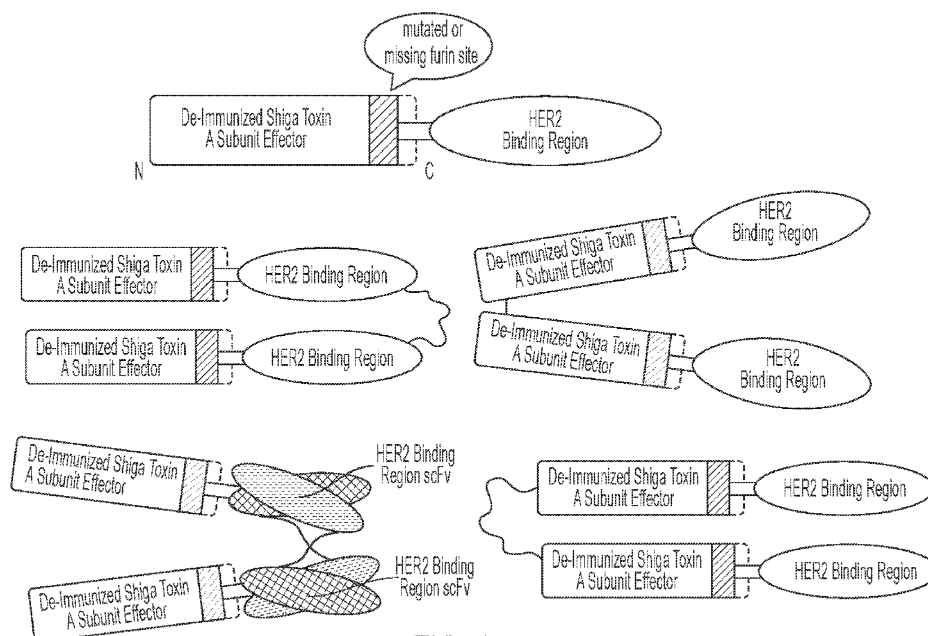

FIG. 1

(57) Abstract: Provided herein are HER2-targeting molecules comprising Shiga toxin A Subunit derived polypeptides having 1) de-immunization and 2) reduced, protease-cleavage sensitivity while retaining Shiga toxin function(s), such as, *e.g.*, potent cytotoxicity via ribosome inhibition. Certain HER2-targeting molecules of the present invention exhibit reduced immunogenic potential in mammals and are well-tolerated by mammals while retaining aforementioned features. The HER2-targeting molecules of the present invention have uses for selectively killing specific cells (*e.g.*, HER positive tumor cells); for selectively delivering cargos to specific cells (*e.g.*, HER positive tumor cells), and as therapeutic and/or diagnostic molecules for treating and diagnosing a variety of conditions, including cancers and tumors involving the expression or over-expression of cell-surface HER2.

[Continued on next page]

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HER2-TARGETING MOLECULES COMPRISING DE-IMMUNIZED, SHIGA TOXIN A SUBUNIT SCAFFOLDS

TECHNICAL FIELD

[1] The present invention relates to HER2-targeing molecules comprising Shiga toxin effector polypeptides, derived from the A Subunits of naturally occurring Shiga toxins, that comprise a combination of mutations providing (1) de-immunization, (2) a reduction in protease sensitivity, and/or (3) an embedded, T-cell epitope(s); wherein the Shiga toxin effector polypeptides retain one or more Shiga toxin functions, such as, *e.g.*, potent cytotoxicity. The HER2-targeting molecules of the present invention are useful for administration to multicellular organisms, such as, *e.g.*, when it is desirable to (1) eliminate or reduce non-specific toxicities and/or (2) eliminate or reduce certain immune responses. The HER2-targeting molecules of the present invention are useful (1) for selectively killing specific HER2-positive cell type(s) amongst other cells and (2) as therapeutic molecules for treating a variety of diseases, disorders, and conditions involving HER2-expressing cells, including cancers and tumors.

BACKGROUND

[2] HER2 is a particularly attractive molecular target for therapeutics because of its overexpression on the surfaces of tumor and/or cancer cells, its correlation with poor prognoses, and its functional roles in tumorigenesis and cancer development, such as invasiveness and metastasis, and anti-neoplastic drug resistance (Nielsen D et al., *Breast* 22: 1-12 (2013); Ocaña A, Pandiella A, *Curr Pharm Des* 19: 808-17 (2013)).

[3] HER2 (human epidermal growth factor receptor 2) is a type I transmembrane tyrosine kinase receptor of the ErbB family (Yamamoto T et al., *Nature* 319: 230-4 (1986); Slamon D et al., *Science* 235: 177-82 (1987)). Members of the ErbB family are integral glycoproteins which regulate cell growth, differentiation, and survival by binding to growth factor ligands as dimers (Chantry A, *J Biol Chem* 270: 3068-73 (1995)).

[4] HER2 is prominently associated with the pathogenesis, progression, and prognosis of certain breast cancers, among other cancers (Citri A, Yarden Y, *Nat Rev Mol Cell Biol* 7: 505-16 (2006)). The proto-oncogene *HER2*, which encodes HER2, was found to be amplified and overexpressed in breast cancer cells (King et al., *Science* 229: 974-6 (1985); Slamon et al. *Science* 235: 177-82 (1987)). Amplification and/or over-expression of HER2 occurs in approximately 15-30% of breast cancers, and the presence of HER2 in breast cancer is strongly associated with aggressive malignancy, increased disease recurrence, and poor prognosis (Slamon D et al., *Science* 244: 707-12 (1989)); Bernstein H, *N Engl J Med* 353: 1652-4 (2005); Pritchard I et al., *N Engl J Med* 354: 2103-11 (2006); Tan M, Yu D, *Adv Exp Med Biol* 608: 119-29 (2007); Mitri Z et al., *Chemother Res Pract* 2012: 743193 (2012)).

[5] HER2 is overexpressed in many other diverse cancers and may functionally contribute to tumorigenesis generally. HER2 overexpression has been observed in breast, colorectal, endometrial, esophageal, gastric, head and neck, lung, ovarian, prostate, pancreatic, and testicular germ cell tumor cells (Kern J et al., *Cancer Res* 50: 5184-7 (1990); Natali P et al., *Int J Cancer* 45: 457-61 (1990); Jaehne

J et al., *J Cancer Res Clin Oncol* 118: 474-9 (1992); Signoretti S et al., *J Natl Cancer Inst* 92: 1918-25 (2000); Di Lorenzo G et al., *Clin Cancer Res* 8: 3438-44 (2002); Owens M et al., *Clin Breast Cancer* 5: 63-9 (2004); Roskoski R, *Biochem Biophys Res Commun* 319: 1-11 (2004); Cohen G et al., *Cancer Res* 66: 5656-64 (2006); Santin A et al., *Int J Gynaecol Obstet* 102: 128–31 (2008); Vermeij J et al., *BMC Cancer* 8: 3 (2008); Chen P et al., *J Clin Pathol* 66: 113-9 (2013); Chou et al., *Genome Med* 5: 78 (2013); Cros J et al., *Ann Oncol* 24: 2624-9 (2013); König A et al., *Anticancer Res* 33: 4975-82 (2013); Sugishita Y et al., *Int J Oncol* 42: 1589-96 (2013)). In addition, overexpression of HER2 in a tumor cell can confer drug resistance to anti-neoplastic agents (Koutras A et al., *Crit Rev Oncol Hematol* 74: 73-8 (2010)).

[6] There is an urgent need for new therapeutics to supplement present day therapies for HER2-bearing neoplasms. Thus, it would be desirable to have cytotoxic cell-targeting molecules which target HER2 for use as therapeutic molecules to treat a variety of diseases, such as, *e.g.*, cancers and tumors, that can be treated by selective killing of, or selective delivery of a beneficial agent into, a HER2 positive cell. In particular, it would be desirable to have HER2-binding, cytotoxic, cell-targeting molecules exhibiting low antigenicity and/or immunogenicity, low off-target toxicity, and potent cytotoxicity. Furthermore, it would be desirable to have HER2-targeting therapeutic and/or diagnostic molecules exhibiting low antigenicity and/or immunogenicity, low off-target toxicity, high stability, and/or the ability to deliver peptide-epitope cargos for presentation by the MHC class I system of a target cell. For example, it would be desirable to have cytotoxic HER2-targeting molecules comprising Shiga toxin A Subunit derived components which maintain potent cytotoxicity to target cells while 1) reducing the potential for unwanted antigenicities and/or immunogenicities, 2) reducing the potential for non-specific toxicities, 3) allowing for drug tolerability over a wide range of dosages, 4) allowing for drug tolerance after repeated administration, and 5) retaining effectiveness in the presence of one or more additional HER2-targeted therapies.

SUMMARY OF THE INVENTION

[7] The Shiga toxin A Subunit derived components of the HER2-targeting molecules of the present invention each comprise a combination of features (*e.g.*, de-immunized sub-region(s) and a protease-cleavage resistant sub-region). Certain combination Shiga toxin effector polypeptides of the present invention are more useful because they provide several Shiga toxin effector functions in a single polypeptide, such as, *e.g.*, promoting cellular internalization, directing sub-cellular routing to the cytosol, ribosome inactivation, and/or delivering cargos to subcellular compartments. Certain HER2-targeting molecules of the present invention are more useful because they provide a combination of several properties in a single molecule, such as, *e.g.*, efficient cellular internalization, potent cell-targeted cytotoxicity, selective cytotoxicity, de-immunization, low non-specific toxicity at high dosages, high stability, CD8+ T-cell hyper-immunization, and/or 5) retention of effectiveness in the presence of one or more additional HER2-targeted therapies. Different embodiments of the HER2-targeting molecules of the present invention are described below with reference to sets of embodiments numbered #1–3.

Embodiment Set #1 – HER2-Targeting Molecule Comprising a De-Immunized Shiga Toxin Effector Polypeptide Comprising an Embedded or Inserted, Heterologous, T-Cell Epitope and a Non-Overlapping De-Immunized Sub-Region

[8] The present invention provides cell-targeting molecules, each comprising (i) a binding region capable of specifically binding an extracellular target biomolecule (HER2/neu/ErbB2) and (ii) a de-immunized, Shiga toxin A Subunit effector polypeptide. For example, certain embodiments of Set #1 is the cell-targeting molecule comprising (i) a binding region capable of specifically binding an extracellular target biomolecule and (ii) a de-immunized, Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) at least one inserted or embedded, heterologous epitope; and (b) at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region which does not overlap with the embedded or inserted, heterologous, T-cell epitope. For certain further embodiments, the Shiga toxin effector polypeptide is capable of exhibiting at least one Shiga toxin effector function, such as, *e.g.*, directing intracellular routing to the endoplasmic reticulum and/or cytosol of a cell in which the polypeptide is present, inhibiting a ribosome function, enzymatically inactivating a ribosome, causing cytostasis, and/or causing cytotoxicity. The Shiga toxin effector polypeptide of Embodiment Set #1 may be truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A Subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions. The Shiga toxin effector polypeptide of Embodiment Set #1 may comprise a disrupted furin-cleavage motif at the carboxy-terminus of the A1 fragment region. In certain embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin effector polypeptide as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit. For example, the present invention provides a Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, epitope; (b) a disruption of at least one, endogenous, B-cell and/or CD4+ T-cell epitope region; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function. In a further example, the present invention provides a Shiga toxin A subunit effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region; (b) a disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; and wherein the Shiga toxin A subunit effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function.

Accordingly, the present invention provides a HER2-targeting molecule that comprises: (i) an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, 5 a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor; and ii) a Shiga toxin A subunit effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell 10 epitope region; (b) a disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; and wherein the Shiga toxin A subunit effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or 15 CD4+ T-cell epitope regions; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function. For certain further embodiments, the cell-targeting molecule is capable when introduced to cells of exhibiting a cytotoxicity comparable or better than a reference molecule, such as, *e.g.*, a second cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide components comprise a wild-type Shiga toxin furin- 20 cleavage site at the carboxy terminus of its A1 fragment region.

[9] For certain embodiments of Embodiment Set #1, the cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A Subunit or a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type 25 Shiga toxin A1 fragment.

[10] In certain embodiments of Embodiment Set #1, the binding region and Shiga toxin effector polypeptide are linked together, either directly or indirectly.

[11] In certain embodiments of Embodiment Set #1, the binding region comprises a polypeptide comprising an immunoglobulin or immunoglobulin-type binding region. In certain further embodiments, 30 the binding region comprising a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid antibody (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), 35 complementary determining region 3 fragment (CDR3), constrained FR3-CDR3-FR4 polypeptide (FR3-CDR3-FR4), Fd fragment, small modular immunopharmaceutical (SMIP) domain, antigen-binding fragment (Fab), Armadillo repeat polypeptide (ArmRP), fibronectin-derived 10th fibronectin type III domain (10Fn3), tenascin type III domain (TNfn3), ankyrin repeat motif domain, low-density-

lipoprotein-receptor-derived A-domain (LDLR-A), lipocalin (anticalin), Kunitz domain, Protein-A-derived Z domain, gamma-B crystallin-derived domain, ubiquitin-derived domain, Sac7d-derived polypeptide (affitin), Fyn-derived SH2 domain, miniprotein, C-type lectin-like domain scaffold, engineered antibody mimic, and any genetically manipulated counterparts of any of the foregoing which retain binding functionality. In certain embodiments, the binding region comprises a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid antibody (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), Fd fragment, and antigen-binding fragment (Fab). In certain embodiments, the cell-targeting molecule of the present invention comprises an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor. In certain embodiments, the binding region comprises, consists essentially of, or consists of a single-chain variable fragment (scFv). In certain embodiments, the binding region comprises a single-chain variable fragment (scFv). In certain embodiments, the binding region comprises, consists essentially of, or consists of a V_HH fragment derived from a camelid antibody.

[12] In certain embodiments of Embodiment Set #1, the binding region and the Shiga toxin effector polypeptide are fused, either directly or indirectly, forming a continuous polypeptide such that the binding region is associated, either directly or indirectly, with the carboxy-terminus of the Shiga toxin effector polypeptide.

[13] For certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention is capable of exhibiting (i) a catalytic activity level comparable to a wild-type Shiga toxin A1 fragment or wild-type Shiga toxin effector polypeptide, (ii) a ribosome inhibition activity with a half-maximal inhibitory concentration (IC_{50}) value of 10,000 picomolar or less, and/or (iii) a significant level of Shiga toxin catalytic activity.

[14] For certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention and/or its Shiga toxin effector polypeptide is (i) capable of exhibiting subcellular routing efficiency comparable to a reference cell-targeting molecule, such as, *e.g.*, a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment, and/or (ii) capable of exhibiting a significant level of intracellular routing activity to the endoplasmic reticulum and/or cytosol from an endosomal starting location of a cell.

[15] For certain embodiments of Embodiment Set #1, whereby administration of the cell-targeting molecule of the present invention to a cell physically coupled with the extracellular target biomolecule of

the cell-targeting molecule's binding region, the cell-targeting molecule is capable of causing death of the cell. For certain further embodiments, administration of the cell-targeting molecule of the invention to two different populations of cell types which differ with respect to the presence or level of the extracellular target biomolecule, the cell-targeting molecule is capable of causing cell death to the cell-types physically coupled with an extracellular target biomolecule of the cytotoxic cell-targeting molecule's binding region at a CD_{50} at least three times or less than the CD_{50} to cell types which are not physically coupled with an extracellular target biomolecule of the cell-targeting molecule's binding region. For certain embodiments, whereby administration of the cell-targeting molecule of the present invention to a first populations of cells whose members are physically coupled to extracellular target biomolecules of the cell-targeting molecule's binding region, and a second population of cells whose members are not physically coupled to any extracellular target biomolecule of the binding region, the cytotoxic effect of the cell-targeting molecule to members of said first population of cells relative to members of said second population of cells is at least 3-fold greater. For certain embodiments, whereby administration of the cell-targeting molecule of the present invention to a first populations of cells whose members are physically coupled to a significant amount of the extracellular target biomolecule of the cell-targeting molecule's binding region, and a second population of cells whose members are not physically coupled to a significant amount of any extracellular target biomolecule of the binding region, the cytotoxic effect of the cell-targeting molecule to members of said first population of cells relative to members of said second population of cells is at least 3-fold greater. For certain embodiments, whereby administration of the cell-targeting molecule of the present invention to a first population of target biomolecule positive cells, and a second population of cells whose members do not express a significant amount of a target biomolecule of the cell-targeting molecule's binding region at a cellular surface, the cytotoxic effect of the cell-targeting molecule to members of the first population of cells relative to members of the second population of cells is at least 3-fold greater.

[16] For certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting a cytotoxicity with a half-maximal inhibitory concentration (CD_{50}) value of 300 nM or less and/or capable of exhibiting a significant level of Shiga toxin cytotoxicity. For certain further embodiments, the cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A Subunit or a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment.

[17] In certain embodiments of Embodiment Set #1, the heterologous, T-cell epitope is a CD8+ T-cell epitope, such as, *e.g.*, with regard to a human immune system. For certain further embodiments, the heterologous, T-cell epitope is capable of being presented by a MHC class I molecule of a cell. In certain further embodiments, the cell-targeting molecule of the present invention is capable of one or more the following: entering a cell, inhibiting a ribosome function, causing cytostasis, causing cell death, and/or delivering the embedded or inserted, heterologous, T-cell epitope to a MHC class I molecule for

presentation on a cellular surface. For certain further embodiments, the cell-targeting molecule is capable when introduced to cells of exhibiting a cytotoxicity comparable or better than a reference molecule, such as, *e.g.*, a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1
5 fragment.

[18] For certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention is capable of delivering an embedded or inserted, heterologous, CD8+ T-cell epitope to a MHC class I presentation pathway of a cell for cell-surface presentation of the epitope bound by a MHC class I molecule.

10 [19] In certain embodiments of Embodiment Set #1, the cell-targeting molecule comprises a molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region.

[20] For certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention is capable when introduced to a chordate of exhibiting improved *in vivo* tolerability and/or
15 stability compared to a reference molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region. In certain further embodiments, the Shiga toxin effector polypeptide is not cytotoxic and the molecular moiety is cytotoxic.

20 [21] In certain embodiments of Embodiment Set #1, the cell-targeting molecule comprises a molecular moiety associated with the carboxy-terminus of the Shiga toxin effector polypeptide. In certain embodiments, the molecular moiety comprises or consists of the binding region. In certain embodiments, the molecular moiety comprises at least one amino acid and the Shiga toxin effector polypeptide is linked to at least one amino acid residue of the molecular moiety. In certain further
25 embodiments, the molecular moiety and the Shiga toxin effector polypeptide are fused forming a continuous polypeptide.

[22] In certain embodiments of Embodiment Set #1, the cell-targeting molecule further comprises a cytotoxic molecular moiety associated with the carboxy-terminus of the Shiga toxin effector polypeptide. For certain embodiments, the cytotoxic molecular moiety is a cytotoxic agent, such as, *e.g.*, a small
30 molecule chemotherapeutic agent, anti-neoplastic agent, cytotoxic antibiotic, alkylating agent, antimetabolite, topoisomerase inhibitor, and/or tubulin inhibitor known to the skilled worker and/or described herein. For certain further embodiments, the cytotoxic molecular moiety is cytotoxic at concentrations of less than 10,000, 5,000, 1,000, 500, or 200 pM.

[23] In certain embodiments of Embodiment Set #1, the binding region is linked, either directly or
35 indirectly, to the Shiga toxin effector polypeptide by at least one covalent bond which is not a disulfide bond. In certain further embodiments, the binding region is fused, either directly or indirectly, to the carboxy-terminus of the Shiga toxin effector polypeptide to form a single, continuous polypeptide. In

certain further embodiments, the binding region is an immunoglobulin or immunoglobulin-type binding region.

[24] In certain embodiments of Embodiment Set #1, the disrupted furin-cleavage motif comprises one or more mutations in the minimal, furin-cleavage site relative to a wild-type Shiga toxin A Subunit. In certain embodiments, the disrupted furin-cleavage motif is not an amino-terminal truncation of sequences that overlap with part or all of at least one amino acid residue of the minimal furin-cleavage site. In certain embodiments, the mutation in the minimal, furin-cleavage site is an amino acid deletion, insertion, and/or substitution of at least one amino acid residue in the R/Y-x-x-R furin cleavage motif. In certain further embodiments, the disrupted furin-cleavage motif comprises at least one mutation relative to a wild-type Shiga toxin A Subunit, the mutation altering at least one amino acid residue in the region natively positioned (1) at 248–251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 variant sequence (*e.g.* SEQ ID NOs: 4–6); or (2) at 247–250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant sequence (*e.g.* SEQ ID NOs: 7–18), or the equivalent amino acid sequence position in any Shiga toxin A Subunit. In certain further embodiments, the mutation is an amino acid residue substitution of an arginine residue with a non-positively charged, amino acid residue.

[25] In certain embodiments of Embodiment Set #1, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6 and 37; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18 and 75–89; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6, 37, and 75–89; or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3; wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and at least one (two, three, four or more) disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region(s) which does not overlap with the embedded or inserted, heterologous, T-cell epitope.

[26] In certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity comparable to a cytotoxicity of a reference molecule, such as, *e.g.*, a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment.

[27] In certain embodiments of Embodiment Set #1, the binding region may comprise at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, SEQ ID NO:52, and SEQ ID NO:53, respectively; and at least one light-chain variable domain polypeptide comprising: (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:54, SEQ ID NO:55, and SEQ ID NO:56, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 57, SEQ ID NO:58, and SEQ ID NO:59, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:60, SEQ ID NO:61, and SEQ

ID NO:62, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 63, SEQ ID NO:64, and SEQ ID NO:65, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:68, respectively. The binding region having these CDRs may be an immunoglobulin binding region comprising a single-chain variable fragment.

[28] In certain embodiments of Embodiment Set #1, the binding region may comprises the binding region comprises a polypeptide selected from the group consisting of: a) a heavy chain only variable (V_HH) domain comprising (i) a HCDR1 comprising or consisting essentially of the amino acid sequences as shown in SEQ ID NO:69 or SEQ ID NO:72; (ii) a HCDR2 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:70 or SEQ ID NO:73; and (iii) a HCDR3 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:71 or SEQ ID NO:74. The binding region having these CDRs may be an immunoglobulin binding region comprising a heavy chain only variable (V_HH) domain derived from a camelid antibody.

[29] In certain embodiments of Embodiment Set #1, the binding region may comprise: (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 387 of SEQ ID NOs: 26, 29, 30, or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; amino acids 401 to 522 of SEQ ID NO:36, or amino acids 401 to 520 of SEQ ID NO:28; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 375 of SEQ ID NO: 24, 27, or 28; amino acids 393 to 499 of SEQ ID NO:26; amino acids 403 to 513 of SEQ ID NO:25; amino acids 408 to 514 of SEQ ID NO:36; and amino acids 413 to 519 of SEQ ID NO: 29 or 30. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:29; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 413 to 519 of SEQ ID NO:29. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:36; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 408 to 514 of SEQ ID NO:36

[30] In certain embodiments of Embodiment Set #1, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any one of the following polypeptide sequences: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; or amino acids 269 to 514 of SEQ ID NO:36. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 518 of SEQ ID

NO:102. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 386 of SEQ ID NO:31. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 370 of SEQ ID NO:34. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 367 of SEQ ID NO:35. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 514 of SEQ ID NO:36.

[31] In certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention comprises, consists essentially of, or conists of the polypeptide shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 29, 31, 34, 35, 36, 102, 104, and 106–108. In certain further embodiments, the cell-targeting molecule of the present invention further comprises an amino terminal methionine residue. In certain further embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO: 29 or 102.

[32] In certain embodiments of Embodiment Set #1, the binding region sterically covers the carboxy-terminus of the A1 fragment region.

[33] In certain embodiments of Embodiment Set #1, the molecular moiety sterically covers the carboxy-terminus of the A1 fragment region. In certain further embodiments, the molecular moiety comprises the binding region.

[34] In certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention comprises a binding region and/or molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region. In certain further embodiments, the mass of the binding region and/or molecular moiety is at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater.

[35] In certain embodiments of Embodiment Set #1, the cell-targeting molecule comprises a binding region with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein (*e.g.*, cytotoxicity and/or intracellular routing).

[36] In certain embodiments of Embodiment Set #1, the binding region is comprised within a relatively large, molecular moiety comprising such as, *e.g.*, a molecular moiety with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein.

[37] In certain embodiments of Embodiment Set #1, the amino-terminus of the Shiga toxin effector polypeptide is at and/or proximal to an amino-terminus of a polypeptide component of the cell-targeting molecule. In certain further embodiments, the binding region is not located proximally to the amino-terminus of the cell-targeting molecule relative to the Shiga toxin effector polypeptide. In certain further

embodiments, the binding region and Shiga toxin effector polypeptide are physically arranged or oriented within the cell-targeting molecule such that the binding region is not located proximally to the amino-terminus of the Shiga toxin effector polypeptide. In certain further embodiments, the binding region is located within the cell-targeting molecule more proximal to the carboxy-terminus of the Shiga toxin effector polypeptide than to the amino-terminus of the Shiga toxin effector polypeptide. For certain further embodiments, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity that is greater than that of a third cell-targeting molecule having an amino-terminus and comprising the binding region and the Shiga toxin effector polypeptide which is not positioned at or proximal to the amino-terminus of the third cell-targeting molecule. For certain further embodiments, the cell-targeting molecule of the present invention exhibits cytotoxicity with better optimized, cytotoxic potency, such as, *e.g.*, 4-fold, 5-fold, 6-fold, 9-fold, or greater cytotoxicity as compared to the cytotoxicity of the third cell-targeting molecule. For certain further embodiments, the cytotoxicity of the cell-targeting molecule of the present invention to a population of target positive cells is 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or greater than the cytotoxicity of the third cell-targeting molecule to a second population of target positive cells as assayed by CD_{50} values. In certain further embodiments, the third cell-targeting molecule does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family (*e.g.* SEQ ID NO:109).

[38] In certain embodiments of Embodiment Set #1, the amino-terminus of the Shiga toxin effector polypeptide is at and/or proximal to an amino-terminus of a polypeptide component of the cell-targeting molecule. In certain further embodiments, the binding region is not located proximally to the amino-terminus of the cell-targeting molecule relative to the Shiga toxin effector polypeptide. In certain further embodiments, the binding region and Shiga toxin effector polypeptide are physically arranged or oriented within the cell-targeting molecule such that the binding region is not located proximally to the amino-terminus of the Shiga toxin effector polypeptide. In certain further embodiments, the binding region is located within the cell-targeting molecule more proximal to the carboxy-terminus of the Shiga toxin effector polypeptide than to the amino-terminus of the Shiga toxin effector polypeptide. For certain further embodiments, the cell-targeting molecule of the present invention is not cytotoxic and is capable when introduced to cells of exhibiting a greater subcellular routing efficiency from an extracellular space to a subcellular compartment of an endoplasmic reticulum and/or cytosol as compared to the cytotoxicity of a reference molecule, such as, *e.g.*, a fifth cell-targeting molecule having an amino-terminus and comprising the binding region and the Shiga toxin effector polypeptide which is not positioned at or proximal to the amino-terminus of the fifth cell-targeting molecule. In certain further embodiments, the fifth cell-targeting molecule does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family.

[39] In certain embodiments of Embodiment Set #1, the amino-terminus of the Shiga toxin effector polypeptide is at and/or proximal to an amino-terminus of a polypeptide component of the cell-targeting molecule. In certain further embodiments, the binding region is not located proximally to the amino-terminus of the cell-targeting molecule relative to the Shiga toxin effector polypeptide. In certain further

embodiments, the binding region and Shiga toxin effector polypeptide are physically arranged or oriented within the cell-targeting molecule such that the binding region is not located proximally to the amino-terminus of the Shiga toxin effector polypeptide. In certain further embodiments, the binding region is located within the cell-targeting molecule more proximal to the carboxy-terminus of the Shiga toxin effector polypeptide than to the amino-terminus of the Shiga toxin effector polypeptide. For certain further embodiments, the cell-targeting molecule of the present invention exhibits low cytotoxic potency (*i.e.* is not capable when introduced to certain positive target cell types of exhibiting a cytotoxicity greater than 1% cell death of a cell population at a cell-targeting molecule concentration of 1000 nM, 500nM, 100 nM, 75 nM, or 50 nM) and is capable when introduced to cells of exhibiting a greater subcellular routing efficiency from an extracellular space to a subcellular compartment of an endoplasmic reticulum and/or cytosol as compared to the cytotoxicity of a reference cell-targeting molecule, such as, *e.g.*, a fifth cell-targeting molecule having an amino-terminus and comprising the binding region and the Shiga toxin effector polypeptide which is not positioned at or proximal to the amino-terminus of the fifth cell-targeting molecule. In certain further embodiments, the fifth cell-targeting molecule does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family.

[40] In certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention, or a polypeptide component thereof, comprises a carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of a member of the KDEL family. For certain further embodiments, the carboxy-terminal endoplasmic reticulum retention/retrieval signal motif is selected from the group consisting of: KDEL (SEQ ID NO:109), HDEF (SEQ ID NO:110), HDEL (SEQ ID NO:111), RDEF (SEQ ID NO:112), RDEL (SEQ ID NO:113), WDEL (SEQ ID NO:114), YDEL (SEQ ID NO:115), HEEF (SEQ ID NO:116), HEEL (SEQ ID NO:117), KEEL (SEQ ID NO:118), REEL (SEQ ID NO:119), KAEL (SEQ ID NO:120), KCEL (SEQ ID NO:121), KFEL (SEQ ID NO:122), KGEL (SEQ ID NO:123), KHEL (SEQ ID NO:124), KLEL (SEQ ID NO:125), KNEL (SEQ ID NO:126), KQEL (SEQ ID NO:127), KREL (SEQ ID NO:128), KSEL (SEQ ID NO:129), KVEL (SEQ ID NO:130), KWEL (SEQ ID NO:131), KYEL (SEQ ID NO:132), KEDL (SEQ ID NO:133), KIEL (SEQ ID NO:134), DKEL (SEQ ID NO:135), FDEL (SEQ ID NO:136), KDEF (SEQ ID NO:137), KKEL (SEQ ID NO:138), HADL (SEQ ID NO:139), HAEL (SEQ ID NO:140), HIEL (SEQ ID NO:141), HNEL (SEQ ID NO:142), HTEL (SEQ ID NO:143), KTEL (SEQ ID NO:144), HVEL (SEQ ID NO:145), NDEL (SEQ ID NO:146), QDEL (SEQ ID NO:147), REDL (SEQ ID NO:148), RNEL (SEQ ID NO:149), RTDL (SEQ ID NO:150), RTEL (SEQ ID NO:151), SDEL (SEQ ID NO:152), TDEL (SEQ ID NO:153), SKEL (SEQ ID NO:154), STEL (SEQ ID NO:155), and EDEL (SEQ ID NO:156). In certain further embodiments, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity that is greater than that of a reference molecule, such as, *e.g.*, a sixth cell-targeting molecule consisting of the cell-targeting molecule except for it does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family. In certain further embodiments, the cell-targeting molecule of the present invention is capable of exhibiting a cytotoxicity

with better optimized, cytotoxic potency, such as, *e.g.*, 4-fold, 5-fold, 6-fold, 9-fold, or greater cytotoxicity as compared to the sixth cell-targeting molecule. In certain further embodiments, the cell-targeting molecule of the present invention is capable of exhibiting a cytotoxicity with better optimized, cytotoxic potency, such as, *e.g.*, 4-fold, 5-fold, 6-fold, 9-fold, or greater cytotoxicity as compared to the sixth cell-targeting molecule. In certain further embodiments, the cytotoxicity of the cell-targeting molecule of the present invention to a population of target positive cells is 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or greater than the cytotoxicity of the sixth cell-targeting molecule to a second population of target positive cells as assayed by CD_{50} values.

<u>Embodiment Set #2 – HER2-Targeting Molecule Comprising a Shiga Toxin Effector Polypeptide Comprising (i) an Embedded or Inserted, Heterologous, T-Cell Epitope and (ii) a Disrupted, Furin-Cleavage Motif</u>

[41] The present invention provides cell-targeting molecules, each comprising (i) a binding region capable of specifically binding an extracellular target biomolecule (HER2/neu/ErbB2) and (ii) a Shiga toxin A Subunit effector polypeptide comprising an inserted or embedded, heterologous, epitope; and (iii) a disrupted furin-cleavage motif. In certain embodiments, the cell-targeting molecule of the present invention comprises (i) a binding region capable of specifically binding an extracellular target biomolecule; (ii) a Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment derived region and a carboxy terminus, wherein the Shiga toxin effector polypeptide comprises: (a) an inserted or embedded, heterologous, epitope; and (b) a disrupted furin-cleavage motif at the carboxy-terminus of the A1 fragment region. For certain further embodiments, the Shiga toxin effector polypeptide is capable of exhibiting at least one Shiga toxin effector function, such as, *e.g.*, directing intracellular routing to the endoplasmic reticulum and/or cytosol of a cell in which the polypeptide is present, inhibiting a ribosome function, enzymatically inactivating a ribosome, causing cytostasis, and/or causing cytotoxicity. In certain embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin effector polypeptide as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit. The Shiga toxi effector polypeptide may be truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions. The Shiga toxin effector polypeptide of Embodiment Set #2 may further comprise at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region. In certain embodiments, the at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region does not overlap with the embedded or inserted, heterologous, epitope. For example, the present invention provides a Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, epitope; (b) a disruption of at least one, endogenous, B-cell and/or CD4+ T-cell epitope region; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function. In a further example, the present invention provides a Shiga toxin A subunit effector polypeptide

comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region; (b) a disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; wherein the Shiga toxin A subunit effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions; and wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function. In certain further embodiments, the heterologous, T-cell epitope is a CD8+ T-cell epitope, such as, *e.g.*, with regard to a human immune system. For certain further embodiments, the heterologous, T-cell epitope is capable of being presented by a MHC class I molecule of a cell. In certain further embodiments, the cell-targeting molecule of the present invention is capable of one or more the following: entering a cell, inhibiting a ribosome function, causing cytostasis, causing cell death, and/or delivering the embedded or inserted, heterologous, T-cell epitope to a MHC class I molecule for presentation on a cellular surface. For certain further embodiments, the cell-targeting molecule is capable when introduced to cells of exhibiting a cytotoxicity comparable or better than a reference molecule, such as, *e.g.*, a second cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide components comprise a wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region.

[42] In certain embodiments of Embodiment Set #2, the binding region and Shiga toxin effector polypeptide are linked together, either directly or indirectly.

[43] In certain embodiments of Embodiment Set #2, the binding region comprises a polypeptide comprising an immunoglobulin or immunoglobulin-type binding region. In certain further embodiments, the binding region comprising a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid antibody (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), complementary determining region 3 fragment (CDR3), constrained FR3-CDR3-FR4 polypeptide (FR3-CDR3-FR4), Fd fragment, small modular immunopharmaceutical (SMIP) domain, antigen-binding fragment (Fab), Armadillo repeat polypeptide (ArmRP), fibronectin-derived 10th fibronectin type III domain (10Fn3), tenascin type III domain (TNfn3), ankyrin repeat motif domain, low-density-lipoprotein-receptor-derived A-domain (LDLR-A), lipocalin (anticalin), Kunitz domain, Protein-A-derived Z domain, gamma-B crystallin-derived domain, ubiquitin-derived domain, Sac7d-derived polypeptide (affitin), Fyn-derived SH2 domain, miniprotein, C-type lectin-like domain scaffold, engineered antibody mimic, and any genetically manipulated counterparts of any of the foregoing which retain binding functionality. In certain embodiments, the binding region comprises a polypeptide

selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid antibody (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), Fd fragment, and antigen-binding fragment (Fab). In certain embodiments, the cell-targeting molecule of the present invention comprises an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor. In certain embodiments, the binding region comprises, consists essentially of, or consists of a single-chain variable fragment (scFv). In certain embodiments, the binding region comprises a single-chain variable fragment (scFv). In certain embodiments, the binding region comprises, consists essentially of, or consists of a V_HH fragment derived from a camelid antibody.

[44] In certain embodiments of Embodiment Set #2, the binding region and the Shiga toxin effector polypeptide are fused, either directly or indirectly, forming a continuous polypeptide such that the binding region is associated, either directly or indirectly, with the carboxy-terminus of the Shiga toxin effector polypeptide.

[45] In certain embodiments of Embodiment Set #2, the embedded or inserted, heterologous, T-cell epitope disrupts the endogenous, B-cell and/or CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: (i) 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; and 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, or the equivalent region in a Shiga toxin A Subunit or derivative thereof; (ii) 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; and 210–218 of SEQ ID NO:3; and (iii) 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent region in a Shiga toxin A Subunit or derivative thereof.

[46] In certain embodiments of Embodiment Set #2, the disrupted furin-cleavage motif comprises one or more mutations, relative to a wild-type Shiga toxin A Subunit, the mutation altering at least one amino acid residue in a region natively positioned at (1) at 248–251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 variant sequence (*e.g.* SEQ ID NOs: 4–6); or (2) at 247–250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant sequence (*e.g.* SEQ ID NOs: 7–18); or the equivalent region in a Shiga toxin A Subunit or derivative thereof. In certain further embodiments, the disrupted furin-cleavage motif comprises one or

more mutations, relative to a wild-type Shiga toxin A Subunit, in a minimal furin cleavage site of the furin-cleavage motif. In certain further embodiments the minimal furin cleavage site is represented by the consensus amino acid sequence R/Y-x-x-R and/or R-x-x-R.

[47] In certain embodiments of Embodiment Set #2, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6 and 37; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18 and 75–89; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6, 37, and 75–89; or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3; wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[48] In certain embodiments of Embodiment Set #2, the cell-targeting molecule comprises a molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region.

[49] In certain embodiments of Embodiment Set #2, the binding region sterically covers the carboxy-terminus of the A1 fragment region.

[50] In certain embodiments of Embodiment Set #2, the molecular moiety sterically covers the carboxy-terminus of the A1 fragment region. In certain further embodiments, the molecular moiety comprises the binding region.

[51] In certain embodiments of Embodiment Set #2, the cell-targeting molecule of the present invention comprises a binding region and/or molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region. In certain further embodiments, the mass of the binding region and/or molecular moiety is at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater.

[52] In certain embodiments of Embodiment Set #2, the cell-targeting molecule comprises a binding region with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein (*e.g.*, cytotoxicity and/or intracellular routing).

[53] In certain embodiments of Embodiment Set #2, the binding region is comprised within a relatively large, molecular moiety comprising such as, *e.g.*, a molecular moiety with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein.

[54] In certain embodiments of Embodiment Set #2, the binding region may comprise at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, SEQ ID NO:52, and SEQ ID NO:53, respectively; and at least one light-chain variable domain polypeptide comprising: (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:54, SEQ ID NO:55, and SEQ ID NO:56, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the

HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 57, SEQ ID NO:58, and SEQ ID NO:59, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively. For example, the binding region may comprises at least one heavy-chain
5 variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 63, SEQ ID NO:64, and SEQ ID NO:65, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:68, respectively. The binding region having these CDRs may be an immunoglobulin binding region comprising a single-chain variable
10 fragment.

[55] In certain embodiments of Embodiment Set #2, the binding region may comprises the binding region comprises a polypeptide selected from the group consisting of: a) a heavy chain only variable (V_HH) domain comprising (i) a HCDR1 comprising or consisting essentially of the amino acid sequences as shown in SEQ ID NO:69 or SEQ ID NO:72; (ii) a HCDR2 comprising or consisting essentially of the
15 amino acid sequence as shown in SEQ ID NO:70 or SEQ ID NO:73; and (iii) a HCDR3 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:71 or SEQ ID NO:74. The binding region having these CDRs may be an immunoglobulin binding region comprising a heavy chain only variable (V_HH) domain derived from a camelid antibody.

[56] In certain embodiments of Embodiment Set #2, the binding region may comprise: (a) at least one
20 heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 387 of SEQ ID NOs: 26, 29, 30, or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; amino acids 401 to 522 of SEQ ID NO:36, or amino acids 401 to 520 of SEQ ID NO:28; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 375 of SEQ ID NO: 24, 27, or 28; amino acids 393 to
25 499 of SEQ ID NO:26; amino acids 403 to 513 of SEQ ID NO:25; amino acids 408 to 514 of SEQ ID NO:36; and amino acids 413 to 519 of SEQ ID NO: 29 or 30. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:29; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 413 to 519 of SEQ ID NO:29.
30 For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:36; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 408 to 514 of SEQ ID NO:36

[57] In certain embodiments of Embodiment Set #2, the binding region comprises, consists essentially
35 of, or consists of the polypeptide represented by any one of the following polypeptide sequences: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; or amino acids 269 to 514 of SEQ ID

NO:36. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 518 of SEQ ID NO:102. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 386 of SEQ ID NO:31. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 370 of SEQ ID NO:34. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 367 of SEQ ID NO:35. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 514 of SEQ ID NO:36.

[58] In certain embodiments of Embodiment Set #2, the cell-targeting molecule of the present invention comprises, consists essentially of, or conists of the polypeptide shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments of Embodiment Set #2, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 29, 31, 34, 35, 36, 102, 104, and 106–108. In certain further embodiments, the cell-targeting molecule of the present invention further comprises an amino terminal methionine residue. In certain further embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO: 29 or 102.

[59] In certain embodiments of Embodiment Set #2, the disrupted furin-cleavage motif comprises an amino acid residue substitution in the furin-cleavage motif relative to a wild-type Shiga toxin A Subunit. In certain further embodiments, the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with a non-positively charged, amino acid residue selected from the group consisting of: alanine, glycine, proline, serine, threonine, aspartate, asparagine, glutamate, glutamine, cysteine, isoleucine, leucine, methionine, valine, phenylalanine, tryptophan, and tyrosine. In certain embodiments, the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with a histidine.

[60] In certain embodiments of Embodiment Set #2, the cell-targeting molecule is capable when introduced to cells of exhibiting cytotoxicity comparable to the cytotoxicity of a reference cell-targeting molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region. In certain further embodiments, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity that is in a range of from 0.1-fold, 0.5-fold, or 0.75-fold to 1.2-fold, 1.5-fold, 1.75-fold, 2-fold, 3-fold, 4-fold, or 5-fold of the cytotoxicity exhibited by the fourth cell-targeting molecule.

[61] In certain embodiments of Embodiment Set #2, the cell-targeting molecule is capable when introduced to a chordate of exhibiting improved, *in vivo* tolerability compared to *in vivo* tolerability of a reference molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting

molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region.

[62] In certain embodiments of Embodiment Set #2, the cell-targeting molecule is de-immunized due to the embedded or inserted, heterologous, epitope. In certain further embodiments, the cell-targeting molecule is capable of exhibiting less relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a seventh cell-targeting molecule consisting of the cell-targeting molecule except for it lacks one or more embedded or inserted epitopes present in the cell targeting molecule.

[63] In certain embodiments of Embodiment Set #2, the cell-targeting molecule is de-immunized due to the furin-cleavage motif disruption. In certain further embodiments, the cell-targeting molecule is capable of exhibiting less relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region.

[64] For certain embodiments of Embodiment Set #2, the cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A Subunit or a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment.

Embodiment Set #3 – HER2-Targeting Molecule Comprising a De-Immunized Shiga Toxin Effector Polypeptide Comprising a Disrupted, Furin-Cleavage Motif

[65] The present invention provides cell-targeting molecules, each comprising (i) a binding region capable of specifically binding an extracellular target biomolecule (HER2/neu/ErbB2) and (ii) a de-immunized, Shiga toxin A Subunit effector polypeptide comprising a disrupted furin-cleavage motif. In certain embodiments, the cell-targeting molecule of the present invention comprises (i) a binding region capable of specifically binding an extracellular target biomolecule and (ii) a de-immunized, Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment derived region and a carboxy terminus, wherein the Shiga toxin effector polypeptide comprises (a) a disrupted furin-cleavage motif at the carboxy-terminus of the A1 fragment region, and (b) at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope and/or epitope region. For certain further embodiments, the Shiga toxin effector polypeptide is capable of exhibiting at least one Shiga toxin effector function, such as, *e.g.*, directing intracellular routing to the endoplasmic reticulum and/or cytosol of a cell in which the polypeptide is present, inhibiting a ribosome function, enzymatically inactivating a ribosome, causing cytostasis, and/or causing cytotoxicity. In certain further embodiments, the cell-targeting molecule of the present invention is capable of one or more the following: entering a cell, inhibiting a ribosome function, causing

cytostasis, and/or causing cell death. In certain embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin effector polypeptide as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit. The Shiga toxin effector polypeptide may be truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions. The Shiga toxin effector polypeptide of Embodiment Set #3 may further comprise an inserted or embedded, heterologous, epitope; such as an embedded or inserted, heterologous, CD8+ T-cell epitope. The embedded or inserted, heterologous, CD8+ T-cell epitope may disrupt an endogenous, B-cell and/or CD4+ T-cell epitope region. For example, the present invention provides a Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises: a) an embedded or inserted, heterologous, epitope; (b) a disruption of at least one, endogenous, B-cell and/or CD4+ T-cell epitope region; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; and wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function. In a further example, the present invention provides a Shiga toxin A subunit effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region; (b) a disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; and wherein the Shiga toxin A subunit effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function.

[66] For certain embodiments of Embodiment Set #3, the cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A Subunit or a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment.

[67] In certain embodiments of Embodiment Set #3, the binding region and Shiga toxin effector polypeptide are linked together, either directly or indirectly.

[68] In certain embodiments of Embodiment Set #3, the binding region comprises a polypeptide comprising an immunoglobulin or immunoglobulin-type binding region. In certain further embodiments, the binding region comprising a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid antibody (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv),

complementary determining region 3 fragment (CDR3), constrained FR3-CDR3-FR4 polypeptide (FR3-CDR3-FR4), Fd fragment, small modular immunopharmaceutical (SMIP) domain, antigen-binding fragment (Fab), Armadillo repeat polypeptide (ArmRP), fibronectin-derived 10th fibronectin type III domain (10Fn3), tenascin type III domain (TNfn3), ankyrin repeat motif domain, low-density-lipoprotein-receptor-derived A-domain (LDLR-A), lipocalin (anticalin), Kunitz domain, Protein-A-derived Z domain, gamma-B crystallin-derived domain, ubiquitin-derived domain, Sac7d-derived polypeptide (affitin), Fyn-derived SH2 domain, miniprotein, C-type lectin-like domain scaffold, engineered antibody mimic, and any genetically manipulated counterparts of any of the foregoing which retain binding functionality. In certain embodiments, the binding region comprises a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid antibody (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), Fd fragment, and antigen-binding fragment (Fab). In certain embodiments, the cell-targeting molecule of the present invention comprises an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor. In certain embodiments, the binding region comprises, consists essentially of, or consists of a single-chain variable fragment (scFv). In certain embodiments, the binding region comprises a single-chain variable fragment (scFv). In certain embodiments, the binding region comprises, consists essentially of, or consists of a V_HH fragment derived from a camelid antibody.

[69] In certain embodiments of Embodiment Set #3, the binding region and the Shiga toxin effector polypeptide are fused, either directly or indirectly, forming a continuous polypeptide such that the binding region is associated, either directly or indirectly, with the carboxy-terminus of the Shiga toxin effector polypeptide.

[70] For certain embodiments of Embodiment Set #1, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting a cytotoxicity with a half-maximal inhibitory concentration (CD_{50}) value of 300 nM or less and/or capable of exhibiting a significant level of Shiga toxin cytotoxicity. For certain further embodiments, the cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A Subunit or a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment.

[71] For certain further embodiments, the cell-targeting molecule is capable when introduced to cells of exhibiting a cytotoxicity comparable or better than a reference molecule, such as, *e.g.*, a second cell-

targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide components comprise a wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region.

[72] In certain embodiments of Embodiment Set #3, the Shiga toxin effector polypeptide comprises a mutation, relative to a wild-type Shiga toxin A Subunit, in the B-cell and/or CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2, and 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; 285–293 of SEQ ID NO:1 or SEQ ID NO:2; 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; 236–258 of SEQ ID NO:1 or SEQ ID NO:2; and 274–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof. In certain further embodiments, there is no disruption which is a carboxy-terminal truncation of amino acid residues that overlap with part or all of at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope and/or epitope region (which may also disrupt an additional, different, endogenous, B-cell and/or CD4+ T-cell epitope region(s)).

[73] In certain embodiments of Embodiment Set #3, the disrupted furin-cleavage motif comprises one or more mutations, relative to a wild-type Shiga toxin A Subunit, the mutation altering at least one amino acid residue in a region natively positioned at (1) at 248–251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 variant sequence (*e.g.* SEQ ID NOs: 4–6); or (2) at 247–250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant sequence (*e.g.* SEQ ID NOs: 7–18); or the equivalent region in a Shiga toxin A Subunit or derivative thereof. In certain further embodiments, the disrupted furin-cleavage motif comprises one or more mutations, relative to a wild-type Shiga toxin A Subunit, in a minimal furin cleavage site of the furin-cleavage motif. In certain further embodiments the minimal furin cleavage site is represented by the consensus amino acid sequence R/Y-x-x-R and/or R-x-x-R.

[74] In certain embodiments of Embodiment Set #3, the cell-targeting molecule comprises a molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region.

[75] In certain embodiments of Embodiment Set #3, the binding region sterically covers the carboxy-terminus of the A1 fragment region.

[76] In certain embodiments of Embodiment Set #3, the molecular moiety sterically covers the carboxy-terminus of the A1 fragment region. In certain further embodiments, the molecular moiety comprises the binding region.

[77] In certain embodiments of Embodiment Set #3, the cell-targeting molecule of the present invention comprises a binding region and/or molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region. In certain further embodiments, the mass of the binding region and/or molecular moiety is at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater.

[78] In certain embodiments of Embodiment Set #3, the cell-targeting molecule comprises a binding region with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein (*e.g.*, cytotoxicity and/or intracellular routing).

[79] In certain embodiments of Embodiment Set #3, the binding region is comprised within a relatively large, molecular moiety comprising such as, *e.g.*, a molecular moiety with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein.

[80] In certain embodiments of Embodiment Set #3, the disrupted furin-cleavage motif comprises an amino acid residue substitution in the furin-cleavage motif relative to a wild-type Shiga toxin A Subunit. In certain further embodiments, the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with a non-positively charged, amino acid residue selected from the group consisting of: alanine, glycine, proline, serine, threonine, aspartate, asparagine, glutamate, glutamine, cysteine, isoleucine, leucine, methionine, valine, phenylalanine, tryptophan, and tyrosine. In certain embodiments, the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with a histidine.

[81] In certain embodiments of Embodiment Set #3, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6 and 37; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18 and 75–89; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6, 37, and 75–89; or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3; wherein the Shiga toxin effector polypeptide comprises at least one at least one (two, three, four or more) disrupted, endogenous, B-cell and/or CD4+ T-cell epitope and/or epitope region(s) and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[82] In certain embodiments of Embodiment Set #3, the binding region may comprise at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, SEQ ID NO:52, and SEQ ID NO:53, respectively; and at least one light-chain variable domain polypeptide comprising: (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:54, SEQ ID NO:55, and SEQ ID NO:56, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the

HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 57, SEQ ID NO:58, and SEQ ID NO:59, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 63, SEQ ID NO:64, and SEQ ID NO:65, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:68, respectively. The binding region having these CDRs may be an immunoglobulin binding region comprising a single-chain variable fragment.

[83] In certain embodiments of Embodiment Set #3, the binding region may comprises the binding region comprises a polypeptide selected from the group consisting of: a) a heavy chain only variable (V_HH) domain comprising (i) a HCDR1 comprising or consisting essentially of the amino acid sequences as shown in SEQ ID NO:69 or SEQ ID NO:72; (ii) a HCDR2 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:70 or SEQ ID NO:73; and (iii) a HCDR3 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:71 or SEQ ID NO:74. The binding region having these CDRs may be an immunoglobulin binding region comprising a heavy chain only variable (V_HH) domain derived from a camelid antibody.

[84] In certain embodiments of Embodiment Set #3, the binding region may comprise: (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 387 of SEQ ID NOs: 26, 29, 30, or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; amino acids 401 to 522 of SEQ ID NO:36, or amino acids 401 to 520 of SEQ ID NO:28; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 375 of SEQ ID NO: 24, 27, or 28; amino acids 393 to 499 of SEQ ID NO:26; amino acids 403 to 513 of SEQ ID NO:25; amino acids 408 to 514 of SEQ ID NO:36; and amino acids 413 to 519 of SEQ ID NO: 29 or 30. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:29; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 413 to 519 of SEQ ID NO:29. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:36; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 408 to 514 of SEQ ID NO:36

[85] In certain embodiments of Embodiment Set #3, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any one of the following polypeptide sequences: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; or amino acids 269 to 514 of SEQ ID

NO:36. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 518 of SEQ ID NO:102. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 386 of SEQ ID NO:31. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 370 of SEQ ID NO:34. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 367 of SEQ ID NO:35. For example, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 514 of SEQ ID NO:36.

[86] In certain embodiments of Embodiment Set #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or conists of the polypeptide shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments of Embodiment Set #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 29, 31, 34, 35, 36, 102, 104, and 106–108. In certain further embodiments, the cell-targeting molecule of the present invention further comprises an amino terminal methionine residue. In certain further embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO: 29 or 102.

[87] In certain embodiments of Embodiment Set #3, the cell-targeting molecule is capable when introduced to cells of exhibiting cytotoxicity comparable to the cytotoxicity of a reference molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region. In certain further embodiments, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity that is in a range of from 0.1-fold, 0.5-fold, or 0.75-fold to 1.2-fold, 1.5-fold, 1.75-fold, 2-fold, 3-fold, 4-fold, or 5-fold of the cytotoxicity exhibited by the fourth cell-targeting molecule.

[88] In certain embodiments of Embodiment Set #3, the cell-targeting molecule is capable when introduced to a chordate of exhibiting improved, *in vivo* tolerability compared to *in vivo* tolerability of a reference molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region.

[89] In certain embodiments of Embodiment Set #3, the cell-targeting molecule is de-immunized due to the furin-cleavage motif disruption. In certain further embodiments, the cell-targeting molecule is capable of exhibiting less relative antigenicity and/or relative immunogenicity as compared to a reference cell-targeting molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type

Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the carboxy terminus of its A1 fragment region.

Further Embodiments of Embodiment Sets #1–#3

5 [90] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions.

[91] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide has a Shiga toxin A1 fragment derived region having a carboxy terminus and further comprises a disrupted 10 furin-cleavage motif at the carboxy-terminus of the A1 fragment region. In certain embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin effector polypeptide as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit. For example, the Shiga toxin effector polypeptide of the present invention may comprise a Shiga toxin A1 fragment derived region wherein the Shiga toxin A1 fragment region comprises a disrupted furin-cleavage motif at the carboxy-15 terminus of the Shiga toxin A1 fragment region, and wherein said furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin effector polypeptide as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit.

[92] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide further comprises at least one inserted or embedded, heterologous epitope. In certain embodiments of 20 Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises at least one embedded, heterologous epitope. In certain embodiments, the at least one inserted or embedded, heterologous epitope is a CD8+ T-cell epitope. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises at least one inserted or embedded, heterologous CD8+ T-cell epitope. In certain embodiments, the embedded or inserted, heterologous, CD8+ T-cell epitope disrupts an 25 endogenous, B-cell and/or CD4+ T-cell epitope region.

[93] In certain embodiments of Embodiment Sets #1 to #3, the amino-terminus of the Shiga toxin effector polypeptide is at and/or proximal to an amino-terminus of a polypeptide component of the cell-targeting molecule. In certain further embodiments, the binding region is not located proximally to the amino-terminus of the cell-targeting molecule relative to the Shiga toxin effector polypeptide. In certain 30 further embodiments, the binding region and Shiga toxin effector polypeptide are physically arranged or oriented within the cell-targeting molecule such that the binding region is not located proximally to the amino-terminus of the Shiga toxin effector polypeptide. In certain further embodiments, the binding region is located within the cell-targeting molecule more proximal to the carboxy-terminus of the Shiga toxin effector polypeptide than to the amino-terminus of the Shiga toxin effector polypeptide. For certain 35 further embodiments, the cell-targeting molecule of the present invention is not cytotoxic and is capable when introduced to cells of exhibiting a greater subcellular routing efficiency from an extracellular space to a subcellular compartment of an endoplasmic reticulum and/or cytosol as compared to the cytotoxicity of a reference molecule, such as, *e.g.*, an fifth cell-targeting molecule having an amino-terminus and

comprising the binding region and the Shiga toxin effector polypeptide which is not positioned at or proximal to the amino-terminus of the fifth cell-targeting molecule. For certain further embodiments, the cell-targeting molecule of the present invention exhibits cytotoxicity with better optimized, cytotoxic potency, such as, *e.g.*, 4-fold, 5-fold, 6-fold, 9-fold, or greater cytotoxicity as compared to the cytotoxicity of the fifth cell-targeting molecule. For certain further embodiments, the cytotoxicity of the cell-targeting molecule of the present invention to a population of target positive cells is 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or greater than the cytotoxicity of the fifth cell-targeting molecule to a second population of target positive cells as assayed by CD_{50} values. In certain further embodiments, the fifth cell-targeting molecule does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family.

[94] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule comprises a molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region.

[95] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention, or a polypeptide component thereof, comprises a carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of a member of the KDEL family. In certain further embodiments, the carboxy-terminal endoplasmic reticulum retention/retrieval signal motif is selected from the group consisting of: KDEL (SEQ ID NO:109), HDEF (SEQ ID NO:110), HDEL (SEQ ID NO:111), RDEF (SEQ ID NO:112), RDEL (SEQ ID NO:113), WDEL (SEQ ID NO:114), YDEL (SEQ ID NO:115), HEEF (SEQ ID NO:116), HEEL (SEQ ID NO:117), KEEL (SEQ ID NO:118), REEL (SEQ ID NO:119), KAEL (SEQ ID NO:120), KCEL (SEQ ID NO:121), KFEL (SEQ ID NO:122), KGEL (SEQ ID NO:123), KHEL (SEQ ID NO:124), KLEL (SEQ ID NO:125), KNEL (SEQ ID NO:126), KQEL (SEQ ID NO:127), KREL (SEQ ID NO:128), KSEL (SEQ ID NO:129), KVEL (SEQ ID NO:130), KWEL (SEQ ID NO:131), KYEL (SEQ ID NO:132), KEDL (SEQ ID NO:133), KIEL (SEQ ID NO:134), DKEL (SEQ ID NO:135), FDEL (SEQ ID NO:136), KDEF (SEQ ID NO:137), KKEL (SEQ ID NO:138), HADL (SEQ ID NO:139), HAEL (SEQ ID NO:140), HIEL (SEQ ID NO:141), HNEL (SEQ ID NO:142), HTEL (SEQ ID NO:143), KTEL (SEQ ID NO:144), HVEL (SEQ ID NO:145), NDEL (SEQ ID NO:146), QDEL (SEQ ID NO:147), REDL (SEQ ID NO:148), RNEL (SEQ ID NO:149), RTDL (SEQ ID NO:150), RTEL (SEQ ID NO:151), SDEL (SEQ ID NO:152), TDEL (SEQ ID NO:153), SKEL (SEQ ID NO:154), STEL (SEQ ID NO:155), and EDEL (SEQ ID NO:156). In certain further embodiments, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity that is greater than that of a reference molecule, such as, *e.g.*, a sixth cell-targeting molecule consisting of the cell-targeting molecule except for it does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family. In certain further embodiments, the cell-targeting molecule of the present invention is capable of exhibiting a cytotoxicity with better optimized, cytotoxic potency, such as, *e.g.*, 4-fold, 5-fold, 6-fold, 9-fold, or greater cytotoxicity as compared to a reference molecule, such as, *e.g.*, a sixth cell-targeting molecule consisting of the cell-targeting molecule except for it does not comprise any carboxy-terminal, endoplasmic

reticulum retention/retrieval signal motif of the KDEL family. In certain further embodiments, the cytotoxicity of the cell-targeting molecule of the present invention to a population of target positive cells is 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or greater than the cytotoxicity of the sixth cell-targeting molecule to a second population of target positive cells as assayed by CD_{50} values.

5 [96] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide further comprises at least one, two, three, four, five, six, seven, or eight disrupted, endogenous, B-cell and/or T-cell epitope regions. In certain further embodiments, the Shiga toxin effector polypeptide comprises a disruption of at least one, two, three, four, five, six, seven, or eight endogenous, B-cell and/or CD4+ T-cell epitopes and/or epitope regions described herein. In certain further embodiments, 10 the Shiga toxin effector polypeptide further comprises at least one (such as at least two, three, four, five, six, seven, or eight) disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region which does not overlap with at least one inserted or embedded, heterologous epitope, which may also disrupt an additional, different, endogenous, B-cell and/or CD4+ T-cell epitope region(s). In certain embodiments, the Shiga toxin effector polypeptide comprises a disruption of at least three, endogenous, B-cell and/or 15 CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope, which may also disrupt an additional, different, endogenous, B-cell and/or CD4+ T-cell epitope region(s).

[97] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide further comprises a disruption in the endogenous, B-cell and/or CD4+ T-cell epitope region selected from 20 the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179– 25 191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2, and 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; 285–293 of SEQ ID NO:1 or SEQ ID NO:2; 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID 30 NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; 236–258 of SEQ ID NO:1 or SEQ ID NO:2; and 274–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof. In certain further embodiments, there is no disruption which is a carboxy-terminal truncation of amino acid residues that overlap with part or all of at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope and/or epitope region.

35 [98] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide further comprises a disruption of at least one (such as at least two, three, four, five, six, seven, eight or more) endogenous, B-cell and/or CD4+ T-cell epitope region, wherein the B-cell region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or

SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18); and the CD4+ T-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; 236–258 of SEQ ID NO:1 or SEQ ID NO:2; and 274–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18). In certain embodiments, the B-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3; and 243–257 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18); and the CD4+ T-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18). For example, the B-cell epitope region may be selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; and 179–191 of SEQ ID NO:3; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18); and the CD4+ T-cell epitope region is 236–258 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or

derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18).

[99] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises a disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions, wherein the disruption comprises a mutation, relative to a wild-type Shiga toxin A Subunit, in the B-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3; and 243–257 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18); and/or the CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in the Shiga toxin effector polypeptides SEQ ID NOs: 4–18).

[100] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide further comprises a mutation, relative to a wild-type Shiga toxin A Subunit, in the B-cell immunogenic amino acid residue selected from the group of natively positioned Shiga toxin A Subunit amino acid residues: L49, D197, D198, R204, and R205.

[101] In certain embodiments of Embodiment Sets #1 to #3, the embedded or inserted, heterologous, T-cell epitope disrupts the endogenous, B-cell and/or CD4+ T-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: (i) 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; and 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18), wherein there is no disruption which is an amino-terminal truncation of sequences that overlap with part or all of at least one disrupted epitope region; (ii) 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; and 210–218 of SEQ ID NO:3; and (iii) 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1

or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

5 [102] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises a mutation, relative to a wild-type Shiga toxin A Subunit, in the B-cell and/or CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: (i) 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3;
10 and 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18), wherein there is no disruption which is an amino-terminal truncation of sequences that overlap with part or all of at least one disrupted epitope region; (ii)
15 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; and 210–218 of SEQ ID NO:3; and (iii) 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297
20 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18), wherein there is no disruption which is an amino-terminal truncation of sequences that overlap with part or all of at least one disrupted epitope region.

25 [103] In certain embodiments of Embodiment Sets #1 to #3, the embedded or inserted, heterologous, CD8+ T-cell epitope disrupts an endogenous, B-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; and 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID
30 NO:3, 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; and 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–
35 297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18); and/or an endogenous CD4+ T-cell epitope region

selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; 236–258 of SEQ ID NO:1 or SEQ ID NO:2; and 274–293 of SEQ ID NO:1 or SEQ ID NO:2 or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

[104] In certain embodiments of Embodiment Sets #1 to #3, the embedded or inserted, heterologous, CD8+ T-cell epitope disrupts an endogenous, B-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; and 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; and 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; and 243–257 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18); and/or an endogenous CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

[105] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises a disruption of at least one endogenous epitope region selected from the group of natively positioned Shiga toxin A Subunits consisting of: 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; or 210–218 of SEQ ID NO:3 or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and in any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

[106] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide does not comprise a heterologous, MHC class I-restricted, T-cell epitope. MHC class I-restricted, T-cell epitopes are known in the art or can be predicted by the skilled worker. The term heterologous refers to

MHC class I-restricted, T-cell epitopes which are not natively present in wild-type Shiga toxin A Subunits, such as, *e.g.*, the wild-type Shiga toxin A Subunit which is most closely related to the Shiga toxin effector polypeptide of interest.

[107] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises disruptions of at least two, three, four, five, six, seven, eight or more endogenous, B-cell and/or T-cell epitope regions. In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises disruptions of at least four endogenous, B-cell and/or T-cell epitope regions. In certain embodiments, the Shiga toxin effector polypeptide comprises disruptions of at least five endogenous, B-cell and/or CD4+ T-cell epitope regions. For example in certain embodiments, the Shiga toxin effector polypeptide comprises disruptions of at least six endogenous, B-cell and/or CD4+ T-cell epitope regions. In certain further embodiments, the two, three, four, five, six, seven, eight or more disrupted epitope regions do not not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope, which may also disrupt an additional, different, endogenous, B-cell and/or CD4+ T-cell epitope region(s).

[108] In certain embodiments of Embodiment Sets #1 to #3, one or more disruptions comprises an amino acid residue substitution relative to a wild-type Shiga toxin A Subunit.

[109] In certain embodiments of Embodiment Sets #1 to #3, one or more endogenous, B-cell and/or T-cell epitope regions comprises a plurality of amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit. In certain embodiments, at least three, four, five or more of the B-cell and/or CD4+ T-cell epitope region disruptions comprise an amino acid residue substitution relative to a wild-type Shiga toxin A Subunit.

[110] In certain embodiments of Embodiment Sets #1 to #3, at least one, two, three, or four disruptions comprise a plurality of amino acid residue substitutions in the endogenous, B-cell and/or T-cell epitope region relative to a wild-type Shiga toxin A Subunit.

[111] In certain embodiments of Embodiment Sets #1 to #3, at least one disruption comprises at least one, two, three, four, five, six, seven, eight or more amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit, and optionally wherein at least one substitution occurs at the natively positioned Shiga toxin A Subunit amino acid residue selected from the group consisting of: 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 6 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 12 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 57 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 58 of SEQ

ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO: 1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; 251 of SEQ ID NO:1 or SEQ ID NO:2; 264 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 265 of SEQ ID NO:1 or SEQ ID NO:2; and 286 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent amino acid residue in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effectort polypeptide variants shown in SEQ ID NOs: 4–6 and in any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

[112] In certain embodiments of Embodiment Sets #1 to #3, at least one disruption comprises at least one, two, three, four, five, six, seven, eight, or more amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit, and optionally wherein at least one substitution (such as at least two, three, four, five, six, seven, eight or more amino acid residue substitutions) occurs at the natively positioned Shiga toxin A Subunit amino acid residue selected from the group consisting of: 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 6 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 12 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 57 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or

SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; and 251 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent amino acid residue in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18). In certain embodiments, the at least one substitution occurs at the natively positioned Shiga toxin A Subunit amino acid residues: 45 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1, SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1, SEQ ID NO:2; 59 of SEQ ID NO:1, SEQ ID NO:2; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 110 of SEQ ID NO:1 or SEQ ID NO:2; 141 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO:1 or SEQ ID NO:2; 248 of SEQ ID NO:1 or SEQ ID NO:2; and 251 of SEQ ID NO:1 or SEQ ID NO:2.

[113] In certain further embodiments, at least two disruptions each comprise at least one amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit selected form the group consisting of: 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 179 of SEQ ID NO:1,

SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 250 of SEQ ID NO:3; 264 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 265 of SEQ ID NO:1 or SEQ ID NO:2; and 286 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent amino acid residue in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

[114] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises disruption of at least three, endogenous, B-cell and/or CD4+ T-cell epitope regions selected from the group of consisting of: (i) 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; and 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18), wherein there is no disruption which is an amino-terminal truncation of amino acid residues that overlap with part or all of at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region; (ii) 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; and 210–218 of SEQ ID NO:3; and (iii) 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18), wherein there is no disruption which is a carboxy-terminal truncation of amino acid residues that overlap with part or all of at least one disrupted, endogenous, B-cell and/or CD4+ T-cell epitope and/or epitope region.

[115] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises disruptions of at least two, endogenous, B-cell and/or CD4+ T-cell epitope regions, wherein each disruption comprises one or more amino acid residue substitutions, and wherein the endogenous, B-cell and/or CD4+ T-cell epitope regions are selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID

NO:1, SEQ ID NO:2, or SEQ ID NO:3; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 effector polypeptide variants shown in SEQ ID NOs: 4–6 and any one of the Shiga-like toxin 2 effector polypeptide variants shown in SEQ ID NOs: 7–18).

5 [116] In certain embodiments of Embodiment Sets #1 to #3, the embedded or inserted, heterologous, T-cell epitope does not disrupt any endogenous, B-cell and/or CD4+ T-cell epitope region described herein.

[117] In certain embodiments of Embodiment Sets #1 to #3, at least one disruption comprises one or more amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit is selected from the group consisting of: D to A, D to G, D to V, D to L, D to I, D to F, D to S, D to Q, D to M, D to R, E to 10 A, E to G, E to V, E to L, E to I, E to F, E to S, E to Q, E to N, E to D, E to M, E to R, F to A, F to G, F to V, F to L, F to I, G to A, G to P, H to A, H to G, H to V, H to L, H to I, H to F, H to M, I to A, I to V, I to G, I to C, K to A, K to G, K to V, K to L, K to I, K to M, K to H, L to A, L to V, L to G, L to C, N to A, N to G, N to V, N to L, N to I, N to F, P to A, P to G, P to F, R to A, R to G, R to V, R to L, R to I, R to F, R to M, R to Q, R to S, R to K, R to H, S to A, S to G, S to V, S to L, S to I, S to F, S to M, T to A, 15 T to G, T to V, T to L, T to I, T to F, T to M, T to S, V to A, V to G, Y to A, Y to G, Y to V, Y to L, Y to I, Y to F, Y to M, and Y to T. In certain further embodiments, the one or more amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit is selected from the group consisting of: D to A, D to G, D to V, D to L, D to I, D to F, D to S, D to Q, E to A, E to G, E to V, E to L, E to I, E to F, E to S, E to Q, E to N, E to D, E to M, E to R, G to A, H to A, H to G, H to V, H to L, H to I, H to F, H to 20 M, K to A, K to G, K to V, K to L, K to I, K to M, K to H, L to A, L to G, N to A, N to G, N to V, N to L, N to I, N to F, P to A, P to G, P to F, R to A, R to G, R to V, R to L, R to I, R to F, R to M, R to Q, R to S, R to K, R to H, S to A, S to G, S to V, S to L, S to I, S to F, S to M, T to A, T to G, T to V, T to L, T to I, T to F, T to M, T to S, Y to A, Y to G, Y to V, Y to L, Y to I, Y to F, and Y to M.

[118] In certain embodiments of Embodiment Sets #1 to #3, at least one of the disruption(s) comprises 25 one or more amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit selected from the group consisting of: K1 to A, G, V, L, I, F, M and H; T4 to A, G, V, L, I, F, M, and S; D6 to A, G, V, L, I, F, S, Q and R; T8 to A, G, V, I, L, F, and M; S8 to A, G, V, I, L, F, and M; T9 to A, G, V, I, L, F, M, and S; S9 to A, G, V, L, I, F, and M; K11 to A, G, V, L, I, F, M and H; T12 to A, G, V, I, L, F, M, S, and K; S12 to A, G, V, I, L, F, and M; S33 to A, G, V, L, I, F, M, and C; S43 to A, G, V, L, I, F, and M; 30 G44 to A or L; S45 to A, G, V, L, I, F, and M; T45 to A, G, V, L, I, F, and M; G46 to A and P; D47 to A, G, V, L, I, F, S, M, and Q; N48 to A, G, V, L, M and F; L49 to A, V, C, and G; Y49 to A, G, V, L, I, F, M, and T; F50 to A, G, V, L, I, and T; D53 to A, G, V, L, I, F, S, and Q; V54 to A, G, I, and L; R55 to A, G, V, L, I, F, M, Q, S, K, and H; G56 to A and P; I57 to A, G, V, and M; L57 to A, V, C, G, M, and F; D58 to A, G, V, L, I, F, S, and Q; P59 to A, G, and F; E60 to A, G, V, L, I, F, S, Q, N, D, M, T, and R; 35 E61 to A, G, V, L, I, F, S, Q, N, D, M, and R; G62 to A; R84 to A, G, V, L, I, F, M, Q, S, K, and H; V88 to A and G; I88 to A, V, C, and G; D94 to A, G, V, L, I, F, S, and Q; S96 to A, G, V, I, L, F, and M; T104 to A, G, V, L, I, F, M; and N; A105 to L; T107 to A, G, V, L, I, F, M, and P; S107 to A, G, V, L, I, F, M, and P; L108 to A, V, C, and G; S109 to A, G, V, I, L, F, and M; T109 to A, G, V, I, L, F, M, and S;

G110 to A; S112 to A, G, V, L, I, F, and M; D111 to A, G, V, L, I, F, S, Q, and T; S112 to A, G, V, L, I, F, and M; D141 to A, G, V, L, I, F, S, and Q; G147 to A; V154 to A and G; R179 to A, G, V, L, I, F, M, Q, S, K, and H; T180 to A, G, V, L, I, F, M, and S; T181 to A, G, V, L, I, F, M, and S; D183 to A, G, V, L, I, F, S, and Q; D184 to A, G, V, L, I, F, S, and Q; L185 to A, G, V and C; S186 to A, G, V, I, L, F, and M; G187 to A; R188 to A, G, V, L, I, F, M, Q, S, K, and H; S189 to A, G, V, I, L, F, and M; D197 to A, G, V, L, I, F, S, and Q; D198 to A, G, V, L, I, F, S, and Q; R204 to A, G, V, L, I, F, M, Q, S, K, and H; R205 to A, G, V, L, I, F, M, Q, S, K and H; C242 to A, G and V; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to A, G, V, L, I, F, M, Q, S, K, and H; R250 to A, G, V, L, I, F, M, Q, S, K, and H; R251 to A, G, V, L, I, F, M, Q, S, K, and H; D264 to A, G, V, L, I, F, S, and Q; G264 to A; and T286 to A, G, V, L, I, F, M, and S. In certain embodiments of Embodiment Sets #1 to #3, the one or more substitutions are selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of: K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, R188A, R188L, S189A, D197A, D198A, R204A, R205A, C242A, S247I, Y247A, R247A, R248A, R250A, R251A, D264A, G264A, T286A, and T286I. In certain embodiments, the one or more substitutions are selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of: K1A, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, G147A, R188A, C242S, R248A, and R251A. In certain further embodiments, the Shiga toxin effector polypeptide comprises one or more substitutions selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of: K1R and K11R. In certain further embodiments, the Shiga toxin effector polypeptide comprises all the following substitutions: S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, R188A, C242S, R248A, and R251A. In certain other further embodiments, the Shiga toxin effector polypeptide comprises all the following substitutions: K1A, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, G147A, C242S, R248A, and R251A. In certain other further embodiments, the Shiga toxin effector polypeptide comprises all the following substitutions: S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, R188A, C242S, R248A, and R251A. In certain further embodiments, the Shiga toxin effector polypeptide comprises all the following substitutions: K1R, K11R, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, R188A, C242S, R248A, and R251A. In certain embodiments, the Shiga toxin effector polypeptide further comprises one or more additional substitutions selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of: K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, A51V, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P,

L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, R188A, R188L, S189A, D197A, D198A, R204A, R205A, C242S, S247I, R247A, Y247A, R248A, R250A, and R251A.

5 [119] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention comprises the Shiga toxin effector polypeptide comprising, consisting essentially of, or consisting of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to an amino acid sequence selected from any one of SEQ ID NOs: 19–21 and 75–89. In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting 10 molecule of the present invention comprises the Shiga toxin effector polypeptide comprising, consisting essentially of, or consisting of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to an amino acid sequence selected from any one of SEQ ID NOs: 19–21. In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention comprises the Shiga toxin effector polypeptide comprising, consisting 15 essentially of, or consisting of the polypeptide shown in any one of SEQ ID NOs: 19–21 and 75–89. In certain embodiments, the cell-targeting molecule of the present invention comprises the Shiga toxin effector polypeptide comprising, consisting essentially of, or consisting of the polypeptide shown in any one of SEQ ID NOs: 19–21. For example, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:20.

20 [120] For certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention is capable when introduced to a chordate of exhibiting improved *in vivo* tolerability and/or stability compared to a reference molecule, such as, *e.g.*, a fourth cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment and/or wild-type Shiga toxin furin-cleavage site at the 25 carboxy terminus of its A1 fragment region. In certain further embodiments, the Shiga toxin effector polypeptide is not cytotoxic and the molecular moiety is cytotoxic.

[121] In certain embodiments of Embodiment Sets #1 to #3, the binding region and Shiga toxin effector polypeptide are linked together, either directly or indirectly.

[122] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide is 30 fused to the binding region, either directly or indirectly, such as, *e.g.*, via a linker known to the skilled worker. The binding region and Shiga toxin effector polypeptide may be fused by a proteinaceous linker comprising one or more amino acids. For example, the linker may comprise, consist essentially of, or consist of an amino acid sequence selected from GSTSGSGKPGSGEGS (SEQ ID NO:93), AHHSEDPSSKAPKAP (SEQ ID NO:95), SPSTPPTPSPSTPPA (SEQ ID NO:181), 35 EFPKPSTPPGSSGGAP (SEQ ID NO:90), and GSTSGSGKPGSGEGSTKG (SEQ ID NO:96). The binding region and the Shiga toxin effector polypeptide may be indirectly fused together by the presence of an intervening single amino acid residue, such as, *e.g.*, an alanine residue.

[123] In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises at least one peptide and/or polypeptide. In certain further embodiments, the binding region is or comprises an immunoglobulin or immunoglobulin-type binding region. In certain further embodiments, the binding region comprising a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), complementary determining region 3 fragment (CDR3), constrained FR3-CDR3-FR4 polypeptide (FR3-CDR3-FR4), Fd fragment, small modular immunopharmaceutical (SMIP) domain, antigen-binding fragment (Fab), Armadillo repeat polypeptide (ArmRP), fibronectin-derived 10th fibronectin type III domain (10Fn3), tenascin type III domain (TNfn3), ankyrin repeat motif domain, low-density-lipoprotein-receptor-derived A-domain (LDLR-A), lipocalin (anticalin), Kunitz domain, Protein-A-derived Z domain, gamma-B crystallin-derived domain, ubiquitin-derived domain, Sac7d-derived polypeptide (affitin), Fyn-derived SH2 domain, miniprotein, C-type lectin-like domain scaffold, engineered antibody mimic, and any genetically manipulated counterparts of any of the foregoing which retain binding functionality. In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises a polypeptide selected from the group consisting of: autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), complementary determining region 3 fragment (CDR3), constrained FR3-CDR3-FR4 polypeptide (FR3-CDR3-FR4), Fd fragment, and antigen-binding fragment (Fab). In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), Fd fragment, and antigen-binding fragment (Fab). In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises a single-chain variable fragment (scFv). The binding region may comprise a polypeptide selected from the group consisting of: an autonomous V_H domain, single-domain antibody fragment (sdAb), nanobody®, heavy chain-antibody domain derived from a camelid (V_HH or V_H domain fragment), heavy-chain antibody domain derived from a cartilaginous fish antibody (V_HH or V_H domain fragment), immunoglobulin new antigen receptor (IgNAR), V_{NAR} fragment, single-chain variable fragment (scFv), antibody variable fragment (Fv), complementary determining region 3 fragment (CDR3), constrained FR3-CDR3-FR4 polypeptide (FR3-CDR3-FR4), Fd fragment, and antigen-binding fragment (Fab). For example, the cell-targeting molecule of the present invention comprises a binding region comprising one or more of: an antibody variable

fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor. In a further example, the binding region comprises a single-chain variable fragment and/or a V_HH fragment derived from a camelid antibody. In yet a further example, the binding region comprises a single-chain variable fragment. In yet a further example, the binding region comprises a V_HH fragment derived from a camelid antibody.

[124] In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises an immunoglobulin binding region comprising at least one heavy-chain variable domain polypeptide linked to at least one light-chain variable domain polypeptide by a linker comprising a non-branched sequence of thirteen or more amino acid residues, optionally wherein the linker comprises an amino acid sequence selected from any one of $(G_4S)_3$ (SEQ ID NO:180), $(G_4S)_4$ (SEQ ID NO:177), $(G_4S)_5$ (SEQ ID NO:92), $(G_4S)_6$ (SEQ ID NO:178), or $(G_4S)_7$ (SEQ ID NO:179).

[125] For certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention is capable of exhibiting (i) a catalytic activity level comparable to a wild-type Shiga toxin A1 fragment or wild-type Shiga toxin effector polypeptide, (ii) a ribosome inhibition activity with a half-maximal inhibitory concentration (IC_{50}) value of 10,000 picomolar or less, and/or (iii) a significant level of Shiga toxin catalytic activity.

[126] For certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention and/or its Shiga toxin effector polypeptide is capable of exhibiting subcellular routing efficiency comparable to a reference cell-targeting molecule comprising a wild-type Shiga toxin A1 fragment or wild-type Shiga toxin effector polypeptide and/or capable of exhibiting a significant level of intracellular routing activity to the endoplasmic reticulum and/or cytosol from an endosomal starting location of a cell.

[127] For certain embodiments of Embodiment Sets #1 to #3, whereby administration of the cell-targeting molecule of the present invention to a cell physically coupled with the extracellular target biomolecule of the cell-targeting molecule's binding region, the cell-targeting molecule is capable of causing death of the cell. For certain further embodiments, administration of the cell-targeting molecule of the invention to two different populations of cell types which differ with respect to the presence or level of the extracellular target biomolecule, the cell-targeting molecule is capable of causing cell death to the cell-types physically coupled with an extracellular target biomolecule of the cytotoxic cell-targeting molecule's binding region at a CD_{50} at least three times or less than the CD_{50} to cell types which are not physically coupled with an extracellular target biomolecule of the cell-targeting molecule's binding region. For certain embodiments, whereby administration of the cell-targeting molecule of the present invention to a first population of cells whose members are physically coupled to extracellular target biomolecules of the cell-targeting molecule's binding region, and a second population of cells whose members are not physically coupled to any extracellular target biomolecule of the binding region, the cytotoxic effect of the cell-targeting molecule to members of said first population of cells relative to

members of said second population of cells is at least 3-fold greater. For certain embodiments, whereby administration of the cell-targeting molecule of the present invention to a first populations of cells whose members are physically coupled to a significant amount of the extracellular target biomolecule of the cell-targeting molecule's binding region, and a second population of cells whose members are not physically coupled to a significant amount of any extracellular target biomolecule of the binding region, the cytotoxic effect of the cell-targeting molecule to members of said first population of cells relative to members of said second population of cells is at least 3-fold greater. For certain embodiments, whereby administration of the cell-targeting molecule of the present invention to a first population of target biomolecule positive cells, and a second population of cells whose members do not express a significant amount of a target biomolecule of the cell-targeting molecule's binding region at a cellular surface, the cytotoxic effect of the cell-targeting molecule to members of the first population of cells relative to members of the second population of cells is at least 3-fold greater.

[128] For certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting a cytotoxicity with a half-maximal inhibitory concentration (CD_{50}) value of 300 nM or less and/or capable of exhibiting a significant level of Shiga toxin cytotoxicity.

[129] For certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention is capable of delivering an embedded or inserted, heterologous, CD8+ T-cell epitope to a MHC class I presentation pathway of a cell for cell-surface presentation of the epitope bound by a MHC class I molecule.

[130] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule comprises a molecular moiety associated with the carboxy-terminus of the Shiga toxin effector polypeptide. In certain embodiments, the molecular moiety comprises or consists of the binding region. In certain embodiments, the molecular moiety comprises at least one amino acid and the Shiga toxin effector polypeptide is linked to at least one amino acid residue of the molecular moiety. In certain further embodiments, the molecular moiety and the Shiga toxin effector polypeptide are fused forming a continuous polypeptide.

[131] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule further comprises a cytotoxic molecular moiety associated with the carboxy-terminus of the Shiga toxin effector polypeptide. For certain embodiments, the cytotoxic molecular moiety is a cytotoxic agent, such as, *e.g.*, a small molecule chemotherapeutic agent, anti-neoplastic agent, cytotoxic antibiotic, alkylating agent, antimetabolite, topoisomerase inhibitor, and/or tubulin inhibitor known to the skilled worker and/or described herein. For certain further embodiments, the cytotoxic molecular moiety is cytotoxic at concentrations of less than 10,000, 5,000, 1,000, 500, or 200 pM.

[132] In certain embodiments of Embodiment Sets #1 to #3, the binding region is linked, either directly or indirectly, to the Shiga toxin effector polypeptide by at least one covalent bond which is not a disulfide bond. In certain further embodiments, the binding region is fused, either directly or indirectly, to the carboxy-terminus of the Shiga toxin effector polypeptide to form a single, continuous polypeptide. In

certain further embodiments, the binding region is an immunoglobulin or immunoglobulin-type binding region. For example, in the cell-targeting molecule of the present invention, the binding region and the Shiga toxin effector polypeptide may be fused forming a continuous polypeptide such that the binding region is associated with the carboxy-terminus of the Shiga toxin A subunit effector polypeptide

[133] In certain embodiments of Embodiment Sets #1 to #3, the disrupted furin-cleavage motif comprises one or more mutations in the minimal, furin-cleavage site relative to a wild-type Shiga toxin A Subunit. In certain embodiments, the disrupted furin-cleavage motif is not an amino-terminal truncation of sequences that overlap with part or all of at least one amino acid residue of the minimal furin-cleavage site. In certain embodiments, the mutation in the minimal, furin-cleavage site is an amino acid deletion, insertion, and/or substitution of at least one amino acid residue in the R/Y-x-x-R furin cleavage motif. In certain further embodiments, the disrupted furin-cleavage motif comprises at least one mutation relative to a wild-type Shiga toxin A Subunit, the mutation altering at least one amino acid residue in the region natively positioned (1) at 248–251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 variant sequence (*e.g.* SEQ ID NOs: 4–6); or (2) at 247–250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant sequence (*e.g.* SEQ ID NOs: 7–18); or the equivalent amino acid sequence position in any Shiga toxin A Subunit. In certain further embodiments, the mutation is an amino acid residue substitution of an arginine residue with a non-positively charged, amino acid residue. In certain embodiments, the Shiga toxin effector polypeptide comprises a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment derived region, wherein said disrupted furin-cleavage motif comprises (i) a carboxy-terminal truncation of as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit and (ii) at least one amino acid substitution in the furin-cleavage site relative to a wild-type Shiga toxin A Subunit, at the natively positioned amino acid residues 248 and 251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 effector polypeptide variant (SEQ ID NOs: 4–6); or at the natively positioned amino acid residues 247 and 250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 effector polypeptide variant (SEQ ID NOs: 7–18). In certain embodiments, the disrupted furin-cleavage motif comprises a carboxy-terminal truncation as compared to a wild-type Shiga toxin A Subunit; and an amino acid substitution in the furin-cleavage motif relative to a wild-type Shiga toxin A Subunit, at the natively positioned amino acid residues 248 and 251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1) or Shiga toxin (SEQ ID NO:2); or at the natively positioned amino acid residues 247 and 250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3). In certain embodiments, the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with an alanine residue.

[134] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention comprises the Shiga toxin effector polypeptide comprising or consisting essentially of the polypeptide shown in any one of SEQ ID NOs: 19–21 and 75–89.

[135] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention is capable when introduced to cells of exhibiting cytotoxicity comparable to a cytotoxicity of a

reference molecule, such as, *e.g.*, a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment.

[136] In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises the peptide
5 or polypeptide shown in any one of SEQ ID NOs: 45–74, 91–92, or 94.

[137] In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ
10 ID NO:26; amino acids 269 to 500 of SEQ ID NO:27; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; amino acids 269 to 514 of SEQ ID NO:36; amino acids 268 to 500 of SEQ ID NO:97; amino acids 268 to 512 of SEQ ID NO:98; amino acids 268 to
15 498 of SEQ ID NO:99; amino acids 268 to 499 of SEQ ID NO:100; amino acids 268–519 of SEQ ID NO:101; or amino acids 268 to 518 of SEQ ID NO:102 or SEQ ID NO:103. In certain embodiments, the binding region comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID
20 NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 500 of SEQ ID NO:27; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; or amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments, the binding region
25 comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to
30 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; or amino acids 269 to 514 of SEQ ID NO:36.

[138] In certain embodiments of Embodiment Sets #1 to #3, the binding region comprises the peptide or polypeptide shown in any one of SEQ ID NOs: 45–74 and 90–96.

[139] In certain embodiments of Embodiment Set #1 to #3, the binding region comprises, consists
35 essentially of, or consists of the polypeptide represented by any of the following: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 500 of SEQ ID NO:27; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to

499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; amino acids 269 to 514 of SEQ ID NO:36; amino acids 268 to 500 of SEQ ID NO:97; amino acids 268 to 512 of SEQ ID NO:98; amino acids 268 to 498 of SEQ ID NO:99; amino acids 268 to 499 of SEQ ID NO:100; amino acids 268–519 of SEQ ID NO:101; and amino acids 268 to 518 of SEQ ID NO:102 or SEQ ID NO:103. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any of the following: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 500 of SEQ ID NO:27; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; and amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any of the following: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO 2; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; and amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29, amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; or amino acids 253 to 367 of SEQ ID NO:35. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29. In certain, embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 386 of SEQ ID NO:31. In certain, embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 370 of SEQ ID NO:34. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 367 of SEQ ID NO:35.

[140] In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 25–27 and 29–36. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 29, 31, 34 and 35. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:29. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:31. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:34. In

certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:35. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:102.

5 [141] In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists 10 essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 25–27 and 29–36. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 15 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 29, 31, 34 and 35. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO: 29. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting 20 molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO:31. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 25 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO:34. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO:35.

30 [142] In certain embodiments of Embodiment Set #1 to #3, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any of the following: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 500 of SEQ ID NO:27; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 35 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; amino acids 269 to 514 of SEQ ID NO:36; amino acids 268 to 500 of SEQ ID NO:97; amino acids 268 to 512 of SEQ ID NO:98; amino acids 268 to 498 of SEQ ID NO:99; amino acids 268 to 499 of SEQ ID NO:100; amino acids 268–519 of SEQ ID

NO:101; amino acids 268 to 518 of SEQ ID NO:102 or SEQ ID NO:103; amino acids 267 to 384 of SEQID NO:104; amino acids 268 to 498 of SEQ ID NO:105; amino acids 252 to 370 of SEQ ID NO:106; amino acids 252 to 366 of SEQ ID NO:107; and amino acids 268 to 513 of SEQ ID NO:108. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any of the following: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 500 of SEQ ID NO:27; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; and amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any of the following: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; and amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29, amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; or amino acids 253 to 367 of SEQ ID NO:35. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29. In certain, embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 386 of SEQ ID NO:31. In certain, embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 370 of SEQ ID NO:34. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 367 of SEQ ID NO:35.

[143] In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 25–27 and 29–36. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in any one of SEQ ID NOs: 29, 31, 34 and 35. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:29. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:31. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:34. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of the polypeptide shown in SEQ ID NO:35.

[144] In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 25–27 and 29–36. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 29, 31, 34 and 35. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO: 29. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO:31. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO:34. In certain embodiments of Embodiment Set #1 to #3, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of SEQ ID NO:35.

[145] In certain embodiments of Embodiment Sets #1 to #3, the binding region sterically covers the carboxy-terminus of the A1 fragment region.

[146] In certain embodiments of Embodiment Sets #1 to #3, the molecular moiety sterically covers the carboxy-terminus of the A1 fragment region. In certain further embodiments, the molecular moiety comprises the binding region.

[147] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention comprises a binding region and/or molecular moiety located carboxy-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region. In certain further embodiments, the mass of the binding region and/or molecular moiety is at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater.

[148] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule comprises a binding region with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the

appropriate level of the Shiga toxin biological activity noted herein (*e.g.*, cytotoxicity and/or intracellular routing).

[149] In certain embodiments of Embodiment Sets #1 to #3, the binding region is comprised within a relatively large, molecular moiety comprising such as, *e.g.*, a molecular moiety with a mass of at least 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, 50 kDa, 100 kDa, or greater, as long as the cell-targeting molecule retains the appropriate level of the Shiga toxin biological activity noted herein.

[150] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention comprises or consists essentially of the polypeptide shown in any one of SEQ ID NOs: 22–37 and 97–108, and optionally the cell-targeting molecule comprises an amino-terminal methionine residue.

[151] For certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention exhibits low cytotoxic potency (*i.e.* is not capable when introduced to certain positive target cell types of exhibiting a cytotoxicity greater than 1% cell death of a cell population at a cell-targeting molecule concentration of 1000 nM, 500nM, 100 nM, 75 nM, or 50 nM) and is capable when introduced to cells of exhibiting a greater subcellular routing efficiency from an extracellular space to a subcellular compartment of an endoplasmic reticulum and/or cytosol as compared to the cytotoxicity of a reference molecule, such as, *e.g.*, a fifth cell-targeting molecule having an amino-terminus and comprising the binding region and the Shiga toxin effector polypeptide which is not positioned at or proximal to the amino-terminus of the fifth cell-targeting molecule. In certain further embodiments, the fifth cell-targeting molecule does not comprise any carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif of the KDEL family.

[152] In certain embodiments of Embodiment Sets #1 to #3, the molecular moiety comprises a peptide and/or polypeptide derived from the Shiga toxin A2 fragment of a naturally occurring Shiga toxin.

[153] The embodiments of the present invention are not intended to cover any naturally-occurring Shiga holotoxin or Shiga toxin A Subunit. In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule of the present invention does not comprise a naturally occurring Shiga toxin B Subunit. In certain further embodiments, the cell-targeting molecule of the invention does not comprise any polypeptide comprising, consisting essentially of, or consisting of a functional binding domain of a native Shiga toxin B subunit. Rather, in certain embodiments of the cell-targeting molecules of the invention, the Shiga toxin A Subunit derived regions are functionally associated with heterologous binding regions to effectuate cell-targeting.

[154] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises at least two, embedded or inserted, heterologous epitopes.

[155] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide does not comprise the set of amino acid residue substitutions relative to a wild-type Shiga toxin A Subunit selected from the following sets: (1) R248H and R251H; (2) R248G and R251G; (3) A246G, S247A, A253G, and S254A; and (4) A246G, S247A, R248G, R251G, A253G, and S254A.

[156] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide does not comprise a deletion of the region natively positioned at 247-252 in a wild-type Shiga toxin A Subunit. In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide does not comprise deletions of the regions natively positioned at 245-247 and 253-255 in a wild-type Shiga toxin A Subunit.

[157] In certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide comprises one or more mutations relative to a naturally occurring (or wild-type) A Subunit of a member of the Shiga toxin family which changes an enzymatic activity of the Shiga toxin effector polypeptide, the mutation selected from at least one amino acid residue deletion, insertion, or substitution. In certain further embodiments, the mutation relative to the naturally occurring A Subunit reduces or eliminates a cytotoxic activity of the Shiga toxin effector polypeptide but the Shiga toxin effector polypeptide retains at least one other Shiga toxin effector function, such as, *e.g.*, promoting cellular internalization and/or directing intracellular routing to a certain subcellular compartment(s). In certain further embodiments, the mutation relative to the naturally occurring (or wild-type) A Subunit is selected from at least one amino acid residue substitution, such as, *e.g.*, A231E, R75A, Y77S, Y114S, E167D, R170A, R176K, and/or W203A in SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

[158] For certain embodiments of Embodiment Sets #1 to #3, the Shiga toxin effector polypeptide is capable of: (i) routing to a subcellular compartment of a cell in which the Shiga toxin effector polypeptide is present selected from the following: cytosol, endoplasmic reticulum, and lysosome; (ii) intracellular delivery of the epitope from an early endosomal compartment to a proteasome of a cell in which the Shiga toxin effector polypeptide is present; and/or (iii) intracellular delivery of the epitope to a MHC class I molecule from an early endosomal compartment of a cell in which the Shiga toxin effector polypeptide is present. In certain further embodiments, the Shiga toxin effector polypeptide is capable of intracellular delivery of the CD 8+ T-cell epitope for presentation by a MHC class I molecule on the surface of a cell in which the Shiga toxin effector polypeptide is present.

[159] In certain embodiments, the molecule of the present invention does not comprise, at a position carboxy-terminal of the Shiga toxin effector polypeptide and/or the carboxy-terminus of the Shiga toxin A1 fragment region, any additional exogenous material representing an antigen and/or heterologous, CD8+, T-cell epitope-peptide.

[160] In certain embodiments of Embodiment Sets #1 to #3, the binding region does not comprise a ligand.

[161] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule is de-immunized due to the embedded or inserted, heterologous, epitope, and exhibits reduced relative antigenicity and/or relative immunogenicity. The cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a seventh cell-targeting molecule consisting of the cell-targeting molecule except for it lacks one or more embedded or inserted epitopes present in the cell targeting molecule.

[162] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule is de-immunized due to the furin-cleavage motif disruption, and exhibits reduced relative antigenicity and/or relative immunogenicity. The cell-targeting molecule exhibits reduced relative antigenicity and/or relative immunogenicity as compared to a reference cell-targeting molecule consisting of the cell-targeting molecule except for the furin-cleavage motif is wild-type and/or all the Shiga toxin effector polypeptide components consist of a wild-type Shiga toxin A1 fragment.

[163] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule is de-immunized due to the plurality of disrupted B-cell and/or CD4+ T-cell epitope regions and exhibits reduced relative B-cell and/or CD4+ T-cell antigenicity and/or reduced relative B-cell and/or CD4+ T-cell immunogenicity. In certain further embodiments, the cell-targeting molecule exhibits reduced relative B-cell antigenicity and/or relative B-cell immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A1 fragment or cell-targeting molecule comprising the aforementioned, such as a third cell-targeting molecule consisting of the cell-targeting molecule except for all of its Shiga toxin effector polypeptide component(s) each comprise a wild-type Shiga toxin A1 fragment. In certain further embodiments, the cell-targeting molecule exhibits reduced relative CD4+ T-cell antigenicity and/or relative CD4+ T-cell immunogenicity as compared to a reference cell-targeting molecule consisting of the cell-targeting molecule except for the Shiga toxin effector polypeptide component(s) comprises a wild-type Shiga toxin A1 fragment sequence.

[164] In certain embodiments of Embodiment Sets #1 to #3, the cell-targeting molecule is in the form of a pharmaceutically acceptable salt or solvate.Among certain embodiments of the present invention is a pharmaceutical composition comprising any one of the above Shiga toxin effector polypeptides of the present invention and/or any one of the above cell-targeting molecules of the present invention; and at least one pharmaceutically acceptable excipient or carrier. The at least one pharmaceutically acceptable carrier may include a solvent, a dispersion medium, a coating, an antimicrobial agent, an isotonic agent, or an absorption delaying agent; and/or wherein the pharmaceutical composition further comprises an aqueous or non-aqueous carrier; a surfactant; a stabilizer, a preservative, a buffer, an antioxidant, a wetting agent, an emulsifying agent, a dispersing agent; an isotonic agent; and/or an antibacterial or antifungal agent.

[165] Among certain embodiments of the present invention is a diagnostic composition comprising any one of the above cell-targeting molecules of the present invention and a detection promoting agent. Certain further embodiments are cell-targeting molecules of the present invention wherein the detection promoting agent is a heterologous epitope and the cell-targeting molecule comprises the heterologous epitope.

[166] Beyond the Shiga toxin effector polypeptides of the present invention, cell-targeting molecules of the present invention, and compositions thereof, polynucleotides capable of encoding a cell-targeting molecule of the present invention are within the scope of the present invention, as well as expression vectors which comprise a polynucleotide of the present invention and host cells comprising any polynucleotide and/or expression vector of the present invention. Host cells comprising an expression

vector may be used, *e.g.*, in methods for producing a molecule of the present invention or a polypeptide component or fragment thereof by recombinant expression.

[167] Among certain embodiments of the present invention is a method of killing a cell (*e.g.* a HER2-expressing cell), the method comprising the step of contacting the cell with any of the above cell-targeting molecules of the present invention or the above pharmaceutical compositions of the present invention. In certain embodiments, the step of contacting the cell(s) occurs *in vitro*. In certain embodiments, the cell expresses muc-4 and/or CD44. In certain embodiments, the cell is resistant to cytotoxicity caused by T-DM1 (trastuzumab emtansine) and/or trastuzumab. In further embodiments of the cell-killing methods, the method is capable of selectively killing cell(s) and/or cell types preferentially over other cell(s) and/or cell types when contacting a mixture of cells which differ with respect to the extracellular presence and/or expression level of a target HER2/neu/ErbB2 of the binding region of the cell-targeting molecule. In certain further embodiments the cell(s) are in the presence of pertuzumab, T-DM1 (trastuzumab emtansine), and/or lapatinib and/or had previously been contacted with pertuzumab, T-DM1 (trastuzumab emtansine), and/or lapatinib. In certain embodiments, the step of contacting the cell(s) occurs or *in vivo*. In further embodiments of the cell-killing methods, the method is capable of selectively killing cell(s) and/or cell types preferentially over other cell(s) and/or cell types when contacting a mixture of cells which differ with respect to the extracellular presence and/or expression level of an extracellular target biomolecule of the binding region of the cell-targeting molecule.

[168] Among certain embodiments of the present invention is a method of killing a cell (*e.g.* a HER2-expressing cell), the method comprising the step of contacting the cell with any of the above cell-targeting molecules of the present invention or the above pharmaceutical compositions of the present invention. In certain embodiments, the step of contacting the cell(s) occurs *in vitro*. In certain other embodiments, the step of contacting the cell(s) occurs or *in vivo*. In certain embodiments, the cell expresses muc-4 and/or CD44. In certain embodiments, the cell is resistant to cytotoxicity caused by T-DM1 (trastuzumab emtansine) and/or trastuzumab. In further embodiments of the cell-killing methods, the method is capable of selectively killing cell(s) and/or cell types preferentially over other cell(s) and/or cell types when contacting a mixture of cells which differ with respect to the extracellular presence and/or expression level of an extracellular target biomolecule of the binding region of the cell-targeting molecule. In certain further embodiments the cell(s) are in the presence of pertuzumab, T-DM1 (trastuzumab emtansine), and/or lapatinib and/or had previously been contacted with pertuzumab, T-DM1 (trastuzumab emtansine), and/or lapatinib.

[169] Among certain embodiments of the present invention is a method of killing a cell (*e.g.* a HER2-expressing cell), the method comprising the step of contacting the cell with any of the above cell-targeting molecules of the present invention or the above pharmaceutical compositions of the present invention wherein the cell is in the presence of pertuzumab, T-DM1 (trastuzumab emtansine), and/or lapatinib and/or had previously been contacted with pertuzumab, T-DM1 (trastuzumab emtansine), and/or lapatinib. In certain embodiments, the step of contacting the cell(s) occurs *in vitro*. In certain

other embodiments, the step of contacting the cell(s) occurs or *in vivo*. In certain embodiments, the cell expresses muc-4 and/or CD44. In certain embodiments, the cell is resistant to cytotoxicity caused by T-DM1 (trastuzumab emtansine) and/or trastuzumab. In further embodiments of the cell-killing methods, the method is capable of selectively killing cell(s) and/or cell types preferentially over other cell(s) and/or cell types when contacting a mixture of cells which differ with respect to the extracellular presence and/or expression level of an extracellular target biomolecule of the binding region of the cell-targeting molecule.

[170] The present invention further provides methods of treating diseases, disorders, and/or conditions in patients, the methods each comprising the step of administering to a patient in need thereof a therapeutically effective amount of a cell-targeting molecule of the present invention and/or pharmaceutical composition of the present invention. For certain embodiments, the method of treating diseases, disorders, and/or conditions in a patient in need thereof further comprises administering to the patient in need thereof a therapeutically effective amount of one or more additional HER2-targeting therapeutic agent as described herein. For certain embodiments, the patient in need thereof has been previously treated with one or more additional HER2-targeting therapeutic agent and/or does not respond to, or does not benefit from, treatment with one or more additional HER2-targeting therapeutic agent. For certain embodiments, the disease, disorder, or condition to be treated using a method of the invention is selected from: a cancer, tumor, growth abnormality, immune disorder, or microbial infection. In certain embodiments of these methods, the cancer to be treated is selected from the group consisting of: bone cancer, breast cancer, central/peripheral nervous system cancer, gastrointestinal cancer, germ cell cancer, glandular cancer, head-neck cancer, hematological cancer, kidney-urinary tract cancer, liver cancer, lung/pleura cancer, prostate cancer, sarcoma, skin cancer, and uterine cancer, such as, *e.g.*, breast cancer, gastric cancer, urothelial cancer, bladder cancer, urothelial bladder cancer, serous uterine cancer, extrahepatic biliary tract cancer, or biliary carcinoma. For certain embodiments, the cancer being treated is breast cancer and/or gastrointestinal cancer. For certain embodiments of these methods, the immune disorder to be treated is an immune disorder associated with a disease selected from the group consisting of: amyloidosis, ankylosing spondylitis, asthma, Crohn's disease, diabetes, graft rejection, graft-versus-host disease, Hashimoto's thyroiditis, hemolytic uremic syndrome, HIV-related disease, lupus erythematosus, multiple sclerosis, polyarteritis nodosa, polyarthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, scleroderma, septic shock, Sjögren's syndrome, ulcerative colitis, and vasculitis.

[171] The use of any composition of matter of the present invention for the treatment or prevention of a cancer, tumor, growth abnormality, and/or immune disorder is within the scope of the present invention. Among certain embodiments of the present invention is a cell-targeting molecule of the present invention and/or a pharmaceutical composition of the invention for use in the treatment or prevention of a disease, disorder or condition in a patient in need thereof. Furthermore, the diagnostic composition, polynucleotide, expression vector, and host cell of the present invention are for use in the the treatment or prevention of a disease, disorder or condition in a patient in need thereof. Among certain embodiments of the present invention is a cell-targeting molecule of the present invention and/or a pharmaceutical

composition thereof for the treatment or prevention of a cancer, tumor, growth abnormality, immune disorder, and/or microbial infection. Among certain embodiments of the present invention is the use of a cell-targeting molecule of the present invention and/or pharmaceutical composition of the present invention in the manufacture of a medicament for the treatment or prevention of a disease, disorder or condition in a patient in need thereof. Furthermore, the present invention provides the use of the diagnostic composition, polynucleotide, expression vector, and host cell of the present invention in the manufacture of a medicament for the treatment or prevention of a disease, disorder or condition in a patient in need thereof. Among certain embodiments of the present invention is the use of a cell-targeting molecule of the present invention and/or pharmaceutical composition thereof in the manufacture of a medicament for the treatment or prevention of a cancer, tumor, growth abnormality, immune disorder, or microbial infection. Furthermore, the present invention provides the use of the diagnostic composition, polynucleotide, expression vector, and host cell of the present invention in the manufacture of a medicament for the treatment or prevention of a cancer, tumor, growth abnormality, immune disorder, or microbial infection. The "disease, disorder or condition" or the "cancer, tumor, growth abnormality, immune disorder, or microbial infection" may be characterized by cells that are physically coupled with HER2/neu/ErbB2. The HER2/neu/ErbB2 target biomolecule can be physically coupled to the surface of the cells. In certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2/neu/ErbB2 target biomolecule (including cells that overexpress HER2). The HER2/neu/ErbB2 can be expressed (including overexpressed) at the surface of the cells.

[172] Certain embodiments of the cell-targeting molecules of the present invention may be utilized for the delivery of additional exogenous material into a cell physically coupled with an extracellular target biomolecule of the cell-targeting molecule of the present invention. Additionally, the present invention provides a method for delivering exogenous material to the inside of a cell(s) comprising contacting the cell(s), either *in vitro* or *in vivo*, with a cell-targeting molecule, pharmaceutical composition, and/or diagnostic composition of the present invention. The present invention further provides a method for delivering exogenous material to the inside of a cell(s) (*e.g.* a HER2-expressing cell) in a patient, the method comprising the step of administering to the patient a cell-targeting molecule of the present invention (with or without cytotoxic activity), wherein the target cell(s) is physically coupled with an extracellular target biomolecule of the cell-targeting molecule.

[173] Among certain embodiments of the present invention is a method of delivering into a cell (*e.g.* a HER2-expressing cell), the method a T-cell epitope capable of being presented by a MHC class I molecule of the cell, the method comprising the step of contacting the cell with the cell-targeting molecule of the present invention which is associated with a heterologous, T-cell epitope and/or a composition thereof (*e.g.*, a pharmaceutical or diagnostic composition of the present invention).

[174] Among certain embodiments of the present invention is a method for "seeding" a tissue locus within a chordate, the method comprising the step of: administering to the chordate a cell-targeting molecule of the present invention, a pharmaceutical composition of the present invention, and/or a

diagnostic composition of the present invention (*see e.g.* WO 2017/019623; WO 2018/140427). For certain further embodiments, the methods of the invention for "seeding" a tissue locus are for "seeding" a tissue locus which comprises a malignant, diseased, or inflamed tissue. The malignant, diseased, or inflamed tissue may be characterized by cells that are physically coupled with HER2/neu/ErbB2. The HER2/neu/ErbB2 target biomolecule can be physically coupled to the surface of the cells. For certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2/neu/ErbB2 target biomolecule (including cells that overexpress HER2). The HER2/neu/ErbB2 can be expressed (including overexpressed) at the surface of the cells. For certain further embodiments, the methods of the invention for "seeding" a tissue locus are for "seeding" a tissue locus which comprises the tissue selected from the group consisting of: diseased tissue, tumor mass, cancerous growth, tumor, infected tissue, or abnormal cellular mass. For certain further embodiments, the methods of the invention for "seeding" a tissue locus comprises administering to the chordate the cell-targeting molecule of the invention, the pharmaceutical composition of the invention, or the diagnostic composition of the invention comprising the heterologous, T-cell epitope selected from the group consisting of: peptides not natively presented by the target cells of the cell-targeting molecule in MHC class I complexes, peptides not natively present within any protein expressed by the target cell, peptides not natively present within the proteome of the target cell, peptides not natively present in the extracellular microenvironment of the site to be seeded, and peptides not natively present in the tumor mass or infected tissue site to be targeted. The diseased tissue, tumor mass, cancerous growth, tumor, infected tissue, or abnormal cellular mass may be characterized by cells that are physically coupled with HER2/neu/ErbB2. The HER2/neu/ErbB2 target biomolecule can be physically coupled to the surface of the cells. For certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2/neu/ErbB2 target biomolecule (including cells that overexpress HER2). The HER2/neu/ErbB2 can be expressed (including overexpressed) at the surface of the cells.

[175] The use of any composition of matter of the present invention for the diagnosis, prognosis, and/or characterization of a disease, disorder, and/or condition is within the scope of the present invention. For example, the use of the cell-targeting molecule, pharmaceutical composition, diagnostic composition, polynucleotide, expression vector, and host cell of the present inventyion for the diagnosis, prognosis, and/or characterization of a disease, disorder, and/or condition is within the scope of the present invention. Among certain embodiments of the present invention is a method of using a cell-targeting molecule of the present invention comprising a detection promoting agent and/or composition of the present invention (*e.g.* a diagnostic composition) for the collection of information useful in the diagnosis, prognosis, or characterization of a disease, disorder, or condition. Among certain embodiments of the present invention is the method of detecting a cell (or subcellular compartment thereof) using a cell-targeting molecule and/or diagnostic composition of the present invention, the method comprising the steps of contacting a cell with the cell-targeting molecule and/or diagnostic composition and detecting the presence of said cell-targeting molecule and/or diagnostic composition. In certain embodiments, the step of contacting the cell(s) occurs *in vitro*. In certain embodiments, the step of contacting the cell(s) occurs

in vivo. In certain embodiments, the step of detecting the cell(s) occurs *in vitro*. In certain embodiments, the step of detecting the cell(s) occurs *in vivo*. In certain further embodiments, the method involves the detection of the location of the cell-targeting molecule in an organism using one or more imaging procedures after the administration of the cell-targeting molecule to said organism. For example, cell-targeting molecules of the invention which incorporate detection promoting agents as described herein may be used to characterize diseases as potentially treatable by a related pharmaceutical composition of the present invention. For example, certain cell-targeting molecules of the present invention and compositions thereof (*e.g.* pharmaceutical compositions and diagnostic compositions of the present invention), and methods of the present invention may be used to determine if a patient belongs to a group that responds to a pharmaceutical composition of the present invention. For example, certain cell-targeting molecules of the present invention and compositions thereof may be used to identify cells which present a delivered heterologous epitope-peptide on a cellular surface and/or to identify subjects containing cells which present a heterologous epitope-peptide delivered by a cell-targeting molecule of the present invention. The "disease, disorder or condition" may be characterized by cells that are physically coupled with HER2/neu/ErbB2. The HER2/neu/ErbB2 target biomolecule can be physically coupled to the surface of the cells. In certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2/neu/ErbB2 target biomolecule (including cells that overexpress HER2). The HER2/neu/ErbB2 can be expressed (including overexpressed) at the surface of the cells.

[176] Among certain embodiments of the present invention is a method of producing a molecule of the present invention, the method comprising the step of purifying the molecule of the present invention using a bacterial cell-wall protein domain interaction, such as, *e.g.*, protein L from *P. magnus* or derivatives and binding domain fragments thereof or protein A from *S. areus* or derivatives and binding domain fragments thereof. For certain further embodiments, the purifying step of the method involves the cell-targeting molecule comprising, consisting essentially of, or consisting of any one of the polypeptides shown in SEQ ID NOs: 22–36 or 97–108.

[177] Among certain embodiments of the present invention are kits comprising a composition of matter of the present invention, and optionally, instructions for use, additional reagent(s), and/or pharmaceutical delivery device(s). For example, the present invention provides a kit comprising: (i) a cell-targeting molecule of the present invention, (ii) a pharmaceutical composition of the present invention, (iii) a diagnostic composition of the present invention, (iv) a polynucleotide of the present invention, (v) an expression vector of the present invention and/or (vi) a host cell of the present invention; and optionally, instructions for use, additional reagent(s), and/or pharmaceutical delivery device(s). The kit may further comprise reagents and other tools for detecting a cell type (*e.g.* a tumor cell) in a sample or in a subject.

[178] These and other features, aspects and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying figures. The aforementioned elements of the invention may be individually combined or removed freely in order to

make other embodiments of the invention, without any statement to object to such synthesis or removal hereinafter.

BRIEF DESCRIPTION OF THE FIGURES

[179] **Figure 1** depicts exemplary HER2-targeting molecules comprising one or more de-immunized Shiga toxin A Subunit effector polypeptides and one or more HER2 binding regions. These exemplary cell-targeting molecules each comprise a Shiga toxin effector polypeptide having de-immunizing mutations and a disrupted furin cleavage site near the carboxy terminus of the Shiga toxin effector polypeptide. A dashed, vertical, gray line depicts a disrupted furin-cleavage site at the carboxy-terminus of an A1 fragment derived region of the Shiga toxin effector polypeptide. The "N" and "C" denote an amino-terminus and carboxy-terminus, respectively, of a polypeptide component of a cell-targeting molecule. In one exemplary HER2-targeting molecule, the HER2 binding region is a scFv, and the scFv is shown participating in intermolecular variable domain exchange with a neighboring scFv (bottom left). The depictions of exemplary molecules in Figure 1 are for illustrative purposes of certain, general arrangements of the structural features of a limited set of embodiments of the present invention. It is to be understood that these exemplary molecules do not intend, nor should any be construed, to be wholly definitive as to the arrangement of any structural features and/or components of a molecule of the present invention. The relative size, location, or number of features shown in the schematics of Figure 1 have been simplified. The schematics in Figure 1 are not intended to accurately portray any information regarding the relative sizes of molecular structures in any embodiment of the present invention.

[180] **Figure 2** shows a Coomassie-stained, sodium dodecyl sulfate, polyacrylamide gel (SDS-PAGE) after electrophoresis of samples of exemplary HER2-targeting molecules 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), 114791 (SEQ ID NO:26), and a molecular weight size marker, all prepared for gel-loading in reducing conditions. The samples of 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), and 114791 (SEQ ID NO:26) were prepared using chromatography involving a chitin binding interaction and then cleavage away from a chitin resin chromatography column by removal of the affinity tag (SEQ ID NO:43) and elution. Figure 2 shows that the sizes of the predominant protein in the reduced samples of the preparations of the molecules 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), and 114791 (SEQ ID NO:26) were all about 55 kiloDaltons (kDa).

[181] **Figure 3** graphically shows the results of a cell-kill assay investigating the activities of the HER2-targeting molecules 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), and 114791 (SEQ ID NO:26). Figure 3 shows that the exemplary HER2-targeting molecules 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), and 114791 (SEQ ID NO:26) exhibited cytotoxicity to two, different HER2-expressing cell-types: HCC1954 and NCI/ADR-RES-HER2+ cells. The percent viability of target positive cells for each cell type was plotted over the logarithm to base 10 of the HER2-targeting molecule concentrations administered to the respective cells.

[182] **Figure 4** shows a Coomassie-stained, SDS-PAGE gel after electrophoresis of samples of exemplary HER2-targeting molecules 114773 (SEQ ID NO:22) and 114791 (SEQ ID NO:26), and a

molecular weight size marker, all prepared for gel-loading in non-reducing conditions. The samples of 114773 (SEQ ID NO:22) and and 114791 (SEQ ID NO:26), both comprising a carboxy-terminal intein chitin binding domain (CBD) sequence (SEQ ID NO:43), were prepared using Protein-L affinity chromatography. Figure 4 shows that the size of the predominant protein in the sample of the preparations of the molecules 114773 (SEQ ID NO:22) was about 100 kiloDaltons (kDa) whereas the 114791 (SEQ ID NO:26) sample was devoid of protein signal in this assay, presumably due to a lack in Protein L binding affinity.

[183] **Figure 5** shows two Coomassie-stained, SDS-PAGE gels after electrophoresis of samples of exemplary HER2-targeting molecules 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), 115411 (SEQ ID NO:30), and a molecular weight size marker, all prepared for gel-loading in non-reducing conditions. The samples of 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), 115411 (SEQ ID NO:30) were prepared using Protein-L affinity chromatography and not using any chitin binding affinity tag. Figure 5 shows that the size of the predominant protein in the sample of the preparations of the molecules 115111 (SEQ ID NO:29), 115411 (SEQ ID NO:30), and 114912 (SEQ ID NO:28) for each was about 55 kiloDaltons (kDa).

[184] **Figure 6** graphically shows that the exemplary HER2-targeting molecules 114912 (SEQ ID NO:28) and 115111 (SEQ ID NO:29) exhibited cytotoxicity to five different HER2-expressing cell-types: HCC1954, NCI/ADR-RES-HER2+, JIMT-1, SK-OV-3, and HCC1419 cells. The percent viability of cells was plotted over the logarithm to base 10 of the administered HER2-targeting protein concentrations. Figure 6 graphically shows that 115111 (SEQ ID NO:29) often had more potent cytotoxicity than 114912 (SEQ ID NO:28). Figure 6 also shows that for most of the HER2-targeting molecule concentrations tested no cytotoxicity was observed for MCF7 cells, which express very low levels of HER2. The samples of 114912 (SEQ ID NO:28) and 115111 (SEQ ID NO:29) were prepared using Protein-L affinity chromatography without using any chitin binding affinity tag.

[185] **Figure 7** graphically shows that the exemplary HER2-targeting molecules 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115194 (SEQ ID NO:33), and 115195 (SEQ ID NO:32) exhibited cytotoxicity to four different HER2-expressing cell-types: HCC1954, NCI/ADR-RES-HER2+, JIMT-1, and HCC1569 cells. The percent viability of cells was plotted over the logarithm to base 10 of the administered HER2-targeting protein concentrations. Figure 7 graphically shows that 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115194 (SEQ ID NO:33), and 115195 (SEQ ID NO:32) exhibited similar cytotoxic activities in this assay under the conditions tested. Figure 6 also shows that no cytotoxicity was observed for HER2 negative ST486 cells for most of the HER-targeting molecule concentrations tested.

[186] **Figure 8** graphically shows the protein synthesis inhibition activities of exemplary HER2-targeting molecules of the present invention *in vitro* and over a range of concentrations. For each sample molecule, the luminescent intensity of luciferase expressed during the assay in relative luminescent units (RLU times e^3) was plotted over the logarithm to base 10 of the concentration of the HER2-targeting molecule tested in picomolar (pM). These exemplary HER2-targeting molecules 115111 (SEQ ID

NO:29), 115172 (SEQ ID NO:23), and 115411 (SEQ ID NO:30) exhibited ribosome inhibition activities comparable to a "control" molecule, a Shiga toxin effector polypeptide (SLTA-DI-2 (SEQ ID NO:20)) alone, not coupled with any targeting agent or binding region. Additionally, the protein synthesis inhibition activity of 115111 (SEQ ID NO:29) was similar to the activity of 115172 (SEQ ID NO:23), indicating that the same scFv fused with either SLTA-DI-2 (SEQ ID NO:20) or SLTA-FR (SEQ ID NO:37), resulted in similar ribosomal inhibtion activities.

[187] **Figure 9** graphically shows that the exemplary HER2-targeting molecules 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), and 115411 (SEQ ID NO:30) exhibited cytotoxicity to NCI/ADR-RES-HER2+ cells. The percent viability of cells was plotted over the logarithm to base 10 of the administered protein concentrations. Figure 9 also shows that an untargeted Shiga toxin effector polypeptide (SLTA-DI-2 (SEQ ID NO:20)) alone was not cytotoxic in this assay over the range of concentrations tested. Figure 9 graphically shows that 115111 (SEQ ID NO:29) and 115172 (SEQ ID NO:23) exhibited more potent cytotoxicity than 115411 (SEQ ID NO:30) at several concentrations.

[188] **Figure 10** graphically shows HER2 binding characteristics of exemplary HER2-targeting molecules of the present invention using HER2 positive HCC1954 cells and a flow cytometry method. For each sample molecule, the fluorescence signal of FITC measured as mean fluorescent intensity (total MFI) was plotted over the logarithm to base 10 of the concentration of the HER2-targeting molecule tested in μg/mL. The exemplary HER2-targeting molecules 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), 115195 (SEQ ID NO:32), 115645 (SEQ ID NO:34), and 115845 (SEQ ID NO:35) all exhibited binding to HER2 positive cells albeit with varying characteristics. 115111 (SEQ ID NO:29), 115195 (SEQ ID NO:32), and 115845 (SEQ ID NO:35) appeared to exhibit the highest affinity binding to HCC1954 cells under the conditions in this assay.

[189] **Figure 11** shows two pictorial representations of the human HER2 protein structure with certain residues marked for their involvement in being bound by HER2 binding proteins. On the left side of Figure 11, the HER2 residues involved in 115111 (SEQ ID NO:29) binding human HER2 are shown as red and blue atomic space filling spheres. On the right side of Figure 11, the same HER2 residues are shown just as blue atomic space filling spheres, the HER2 residues known to be critical for binding by certain approved anti-HER2 therapeutic monoclonal antibodies: the HER2 residues known to be critical for binding by pertuzumab binding are shown as magenta space filling spheres, and HER2 residues known to be critical for trastuzumab binding are shown as purple atomic space filling spheres. Figure 11 demonstrates that the HER2 epitope bound by 115111 (SEQ ID NO:29) was mapped within the HER2 extracellular domain (ECD) to domain I (on right in green); the HER2 epitope bound by pertzumab was mapped to domain II of the ECD, and the HER2 epitope bound by trastuzumab was mapped to domain IV of the ECD. Figure 11 highlights the HER2 epitopes bound by 115111 (SEQ ID NO:29), pertuzumab, and trastuzumab are distinct and distant from each other.

[190] **Figure 12** graphically shows the results of a human membrane proteome array assay used to test the specificity and selectivity of HER2 binding by the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29). The results shown in Figure 12 show that only HER2 was identified and validated as being

bound by 115111 (SEQ ID NO:29) from among about 5,300 different proteins. Flow cytometry was used to identify the bining signal for each individual protein, and data was normalized to background signal. Non-specific fluorescence was determined to be any value below three standard deviations above noise (dotted line).

5 [191] **Figure 13** graphically shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) is more cytotoxic than T-DM1 to two different HER2-expressing cell-types: HCC1954 and NCI/ADR-RES-HER2+. The percent viability of cells was plotted over the logarithm to base 10 of the administered HER2-targeting molecule concentrations. Figure 13 also shows that no cytotoxicity was observed for HER2 negative MDA-MB-468 cells contacted with HER2-targeting molecule 115111 (SEQ 10 ID NO:29) tested under the conditions of the assay.

[192] **Figure 14** graphically shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) is cytotoxic to two different HER2-expressing cell-types, HCC1419 and HCC1954, in either the presence or absence of lapatinib. The percent viability of cells was plotted for different conditions, including: HCC1419 cells treated with lapatinib only at 1 μM, 115111 (SEQ ID NO:29) at 20 nanogram 15 per milliliter (ng/mL), and both 115111 (SEQ ID NO:29) at 20 ng/mL and lapatinib at 1 μM; or HCC1954 cells treated with lapatinib only at 1 μM, 115111 (SEQ ID NO:29) at 2 ng/mL, and both 115111 (SEQ ID NO:29) at 2 ng/mL and lapatinib at 1 μM. Figure 14 also shows a control treatment using the Shiga toxin effector polypeptide SLTA-DI-2 (SEQ ID NO:20) alone, which lacks any specific targeting agent or binding region for cell-targeting, resulted in no alteration to cell viability in this assay.

20 [193] **Figure 15** graphically shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) is cytotoxic to HER2 postive HCC1954 cells in the presence of T-DM1. The cells were treated with 115111 (SEQ ID NO:29) alone T-DM1 alone, or both 115111 (SEQ ID NO:29) and T-DM1 mixed together at equal concentrations. The percent viability of cells was plotted over the logarithm to base 10 of the total administered protein concentration: either 115111 (SEQ ID NO:29) alone, T-DM1 alone, or 25 the total of both T-DM1 and 115111 (SEQ ID NO:29).

[194] **Figure 16** graphically shows the activity of HER2-targeting molecules in the presence of excess trastuzumab (20 μg/mL) pretreated for 1 hour prior to the addition of HER2-targeting molecules. The percent viability of HER2 positive HCC1954 cells was plotted over the logarithm to base 10 of the administered HER2-targeting molecule concentrations. Figure 16 shows the exemplary HER2-targeting 30 molecule 115111 (SEQ ID NO:29) is cytotoxic to cells in the presence of excess trastuzumab. The top graph of Figure 16 shows that the exemplary HER2-targeting molecule 114912 (SEQ ID NO:28) was not cytotoxic in the presence of excess trastuzumab under the conditions tested. The middle graph of Figure 16 shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was cytotoxic to HCC1954 cells pre-incubated with excess trastuzumab, with no significant loss in cytotoxicity. The 35 bottom graph shows that the cytotoxic activity of T-DM1 to HCC1954 cells was reduced by pre-incubation of the cells with excess trastuzumab (20 μg/mL).

[195] **Figure 17** graphically shows the cytotoxic activities of the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) in the presence of excess trastuzumab (100 μg/mL), pertuzumab (100 μg/mL),

or both (100 μg/mL of each antibody), pretreated for 1 hour prior to the addition of HER2-targeting molecules. The percent viability of HER2 positive cells was plotted over the logarithm to base 10 of the administered 115111 (SEQ ID NO:29) concentrations. Figure 17 shows the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) is cytotoxic to cells in the presence of excess trastuzumab, pertuzumab, or both trastuzumab and pertuzumab. The top graph of Figure 17 shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was cytotoxic to HCC1954 cells pre-incubated with excess trastuzumab, pertuzumab, or both. The bottom graph shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was cytotoxic to NCI-N87 cells pre-incubated with excess trastuzumab, pertuzumab, or both. Figure 17 also shows the cytotoxicity of treatment of the cells with 115111 (SEQ ID NO:29) alone. The cytotoxicity of 115111 (SEQ ID NO:29) alone appeared to be very similar to its cytotoxicity in the presence of excess trastuzumab, excess pertuzumab, or excess of both trastuzumab and pertuzumab.

[196] **Figure 18** graphically shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) is more potently cytotoxic than exemplary HER2-targeting molecule 114912 (SEQ ID NO:28) to HER2 expressing cells for shorter exposure durations. The percent viability of HER2 positive SKBR3 cells was plotted over the logarithm to base 10 of the administered HER2-targeting molecule concentrations. The top graph of Figure 18 shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was more cytotoxic to SKBR3 cells than 114912 (SEQ ID NO:28) at higher concentrations under the conditions of 1-hour exposures. The middle graph of Figure 18 shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was more cytotoxic to SKBR3 cells than 114912 (SEQ ID NO:28) at higher concentrations under the conditions of 4-hour exposures. The bottom graph shows that the exemplary HER2-targeting molecules 115111 (SEQ ID NO:29) and 114912 (SEQ ID NO:28) exhibited similar cytotoxicities under the conditions of continuous exposure.

[197] **Figure 19** graphically shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) is more potently cytotoxic than other exemplary HER2-targeting molecules for shorter exposure durations. The percent viability of HER2 positive HCC1954 cells was plotted over the logarithm to base 10 of the administered HER2-targeting molecule concentrations. The top graph of Figure 19 shows that the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was more cytotoxic to HCC1954 cells than 114898 (SEQ ID NO:31) under the conditions with 4-hour exposures. The middle graph of Figure 19 shows that the exemplary HER2-targeting molecules 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) exhibited similar cytotoxicities to each other under the conditions of both 4-hour exposures and continuous exposure and that these molecules exhibited the least difference in cytotoxic potency when comparing the 4 hour (short) incubation results with the continuous exposure results. The bottom graph of Figure 19 shows that the exemplary HER2-targeting molecule 115645 (SEQ ID NO:34) and 115845 (SEQ ID NO:35) exhibited similar cytotoxicities to each other under the conditions of both 4-hour exposures and continuous exposure and that the activity of both of these HER2-targeting molecules was reduced in cytotoxic potency under the shorter four hour incubation with the HER2-targeting molecule as compared to continuous exposure for days.

[198] **Figure 20** graphically shows the *in vitro* HER2 binding characteristics of exemplary HER2-targeting molecules of the present invention using recombinant HER2 proteins of human (SEQ ID NO:39), mouse (SEQ ID NO:42), or cynomolgus monkey (SEQ ID NO:40) origin. The top section of Figure 20 graphs the ELISA signal for 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), and 115195 (SEQ ID NO:32) tested over a series of HER2-targeting molecule concentrations. The background subtracted ELISA signal measured in absorbance at 450 nanometers (nm) is graphed on the Y-axis versus the HER2-targeting molecule concentration in ng/mL on the x-axis. The 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) bound both human HER2 ECD protein ("huHER2") and and cynomolgus monkey HER2 ECD protein ("cynoHER2") with similar binding characteristics, which appeared to be at slightly higher affinities at most concentrations in this assay than the HER2 binding exhibited by 114912 (SEQ ID NO:28), a trastuzumab binding domain-derived molecule. The bottom section of Figure 20 shows the background subtracted ELISA signal measured in absorbance at 450 nm for the binding of 115111 (SEQ ID NO:29) to human HER2, cynomolgus monkey HER2, or mouse HER2 tested at 10 μg/mL of HER2-targeting molecule. The exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) bound both recombinant human HER2 protein and recombinant cynomolgus monkey HER protein but did not exhibit appreciable binding to recombinant mouse HER2 protein in this assay.

[199] **Figure 21** graphically shows the body weight of immunocompetent mice administered repeat doses of exemplary HER2-targeting molecules of the present invention. The mean body weight change per treatment group calculated using the pre-dose weights of the mice in each group are graphed on the Y-axis versus the day of the study. Groups of BALB/c mice were intravenously administered a vehicle-only control or 1 milligram per kilogram (mg/kg) of body weight of one of these exemplary HER2-targeting molecules: 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115195 (SEQ ID NO:32), or 115194 (SEQ ID NO:33). In the 11594 (SEQ ID NO:33) treatment group, all mice had died by study Day 12. In the 115195 (SEQ ID NO:32) treatment group, all but one of the mice died by study Day 14. By contrast, the 115111 (SEQ ID NO:29) treatment group showed minimal weight changes similar to the vehicle only control group.

[200] **Figure 22** graphically shows the body weight of immunocompetent mice administered repeat doses of exemplary HER2-targeting molecules of the present invention. The mean body weight change per treatment group calculated using the pre-dose weights of the mice in each group are graphed on the Y-axis versus the day of the study. Groups of C57BL/6 mice were intravenously administered a vehicle-only control or 1 mg/kg of body weight of one of these exemplary HER2-targeting molecules: 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), or 115411 (SEQ ID NO:30). In the 115172 (SEQ ID NO:23) treatment group, all mice had died by study Day 23. By contrast, the mice in the 115111 (SEQ ID NO:29) treatment group tolerated 115111 (SEQ ID NO:29) dosing through study Day 45 (end of the study).

[201] **Figure 23** shows that administration of the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) comprising a de-immunized Shiga toxin effector polypeptide resulted in reduced, *in vivo*, antibody respone(s) by a mammalian immune system compared to 115172 (SEQ ID NO:23), which

comprised a less de-immunzed Shiga toxin effector polypeptide. BALB/c mice were administered either 115111 (SEQ ID NO:29) or 115172 (SEQ ID NO:23) at doses between 0.25 to 1 mg/kg body weight. The top graph of Figure 23 shows the amount of anti-drug antibodies measured in the blood sera of the 115111 (SEQ ID NO:29) treatment group as a percentage of the 115172 (SEQ ID NO:23) treatment group measured during different days of a study using BALB/c mice administered 0.25 mg/kg body weight of 115111 (SEQ ID NO:29) or 115172 (SEQ ID NO:23) by intraperitoneal injection (IP). The bottom graph of Figure 23 shows the ELISA signal measured as absorbance at 450 nm shows the amount of anti-drug antibodies measured in blood sera collected on study Day 22 of a study using groups of BALB/c mice intravenously (IV) administered 1 mg/kg body weight of 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), or a vehicle-only control. The sera from the 115111 (SEQ ID NO:29) treatment group exhibited much less anti-drug antibodies than the sera from 115172 (SEQ ID NO:23) treatment group collected on Day 22.

[202] **Figure 24** graphically shows the results from a subcutaneous HCC1954 xenograft murine model study of human breast cancer. The top section of Figure 24 graphs the change in human tumor burdens over time for groups of SCID Beige mice after receiving either the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) of the present invention or a vehicle-only control sample. The mean tumor volume measured in cubic millimeters for each group of mice was graphed versus time (days post-tumor implant). Administration of the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) delayed and reduced the increase in tumor burden observed for the vehicle only control group at all dosages displayed, 0.1 mg to 2 mg per kilogram body weight per dose in cycles over 31 to 33 days. The bottom section of Figure 24 graphs the survival of groups of mice in the same study as above until Day 84 using a Kaplan Meier estamitor plot. On the y-axis is the percent survival of mice within a dosage group, and the x-axis is in days of the study. The repeated administration of 115111 (SEQ ID NO:29) at 0.1 to 2 mg/kg body weight provided survival benefits compared to the vehicle-only control sample.

DETAILED DESCRIPTION

[203] The present invention is described more fully hereinafter using illustrative, non-limiting embodiments, and references to the accompanying figures. This invention may, however, be embodied in many different forms and should not be construed as to be limited to the embodiments set forth below. Rather, these embodiments are provided so that this disclosure is thorough and conveys the scope of the invention to those skilled in the art.

[204] In order that the present invention may be more readily understood, certain terms are defined below. Additional definitions may be found within the detailed description of the invention.

[205] As used in the specification and the appended claims, the terms "a," "an" and "the" include both singular and the plural referents unless the context clearly dictates otherwise.

[206] As used in the specification and the appended claims, the term "and/or" when referring to two species, A and B, means at least one of A and B. As used in the specification and the appended claims, the term "and/or" when referring to greater than two species, such as A, B, and C, means at least one of

A, B, or C, or at least one of any combination of A, B, or C (with each species in singular or multiple possibility).

[207] Throughout this specification, the word "comprise" or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer (or components) or group of integers (or components), but not the exclusion of any other integer (or components) or group of integers (or components).

[208] Throughout this specification, the term "including" is used to mean "including but not limited to". "Including" and "including but not limited to" are used interchangeably.

[209] As used herein, the term "a plurality of" means more than one; such as at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23.

[210] The term "amino acid residue" or "amino acid" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide. The term "polypeptide" includes any polymer of amino acids or amino acid residues. The term "polypeptide sequence" refers to a series of amino acids or amino acid residues which physically comprise a polypeptide. A "protein" is a macromolecule comprising one or more polypeptides or polypeptide "chains." A "peptide" is a small polypeptide of sizes less than about a total of 15 to 20 amino acid residues. The term "amino acid sequence" refers to a series of amino acids or amino acid residues which physically comprise a peptide or polypeptide depending on the length. Unless otherwise indicated, polypeptide and protein sequences disclosed herein are written from left to right representing their order from an amino-terminus to a carboxy-terminus.

[211] The terms "amino acid," "amino acid residue," "amino acid sequence," or polypeptide sequence include naturally occurring amino acids (including L and D isosteriomers) and, unless otherwise limited, also include known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids, such as selenocysteine, pyrrolysine, *N*-formylmethionine, gamma-carboxyglutamate, hydroxyprolinehypusine, pyroglutamic acid, and selenomethionine. The amino acids referred to herein are described by shorthand designations as follows in Table A:

TABLE A. Amino Acid Nomenclature

Name	**3-letter**	**1-letter**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid or Aspartate	Asp	D
Cysteine	Cys	C
Glutamic Acid or Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S

Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[212] The phrase "conservative substitution" with regard to an amino acid residue of a peptide, peptide region, polypeptide region, protein, or molecule refers to a change in the amino acid composition of the peptide, peptide region, polypeptide region, protein, or molecule that does not substantially alter the function and structure of the overall peptide, peptide region, polypeptide region, protein, or molecule (*see* Creighton, *Proteins: Structures and Molecular Properties* (W. H. Freeman and Company, New York (2nd ed., 1992))).

[213] As used herein, the term "HER2" is used interchangeably with the terms "neu" and "ErbB2".

[214] For purposes of the present invention, the phrase "derived from" when referring to a polypeptide or polypeptide region means that the polypeptide or polypeptide region comprises amino acid sequences originally found in a "parental" protein and which may now comprise certain amino acid residue additions, deletions, truncations, rearrangements, or other alterations relative to the original polypeptide or polypeptide region as long as a certain function(s) and a structure(s) of the "parental" molecule are substantially conserved. The skilled worker will be able to identify a parental molecule from which a polypeptide or polypeptide region was derived using techniques known in the art, *e.g.*, protein sequence alignment software.

[215] For purposes of the claimed invention and with regard to a Shiga toxin polypeptide sequence or Shiga toxin derived polypeptide, the term "wild-type" generally refers to a naturally occurring, Shiga toxin protein sequence(s) found in a living species, such as, *e.g.*, a pathogenic bacterium, wherein that Shiga toxin protein sequence(s) is one of the most frequently occurring variants. This is in contrast to infrequently occurring Shiga toxin protein sequences that, while still naturally occurring, are found in less than one percent of individual organisms of a given species when sampling a statistically powerful number of naturally occurring individual organisms of that species which comprise at least one Shiga toxin protein variant. A clonal expansion of a natural isolate outside its natural environment (regardless of whether the isolate is an organism or molecule comprising biological sequence information) does not alter the naturally occurring requirement as long as the clonal expansion does not introduce new sequence variety not present in naturally occurring populations of that species and/or does not change the relative proportions of sequence variants to each other.

[216] The terms "associated," "associating," "linked," or "linking" with regard to the claimed invention refers to the state of two or more components of a molecule being joined, attached, connected, or otherwise coupled to form a single molecule or the act of making two molecules associated with each other to form a single molecule by creating an association, linkage, attachment, and/or any other connection between the two molecules. For example, the term "linked" may refer to two or more components associated by one or more atomic interactions such that a single molecule is formed and wherein the atomic interactions may be covalent and/or non-covalent. Non-limiting examples of

covalent associations between two components include peptide bonds and cysteine-cysteine disulfide bonds. Non-limiting examples of non-covalent associations between two molecular components include ionic bonds.

[217] For purposes of the present invention, the term "linked" refer to two or more molecular components associated by one or more atomic interactions such that a single molecule is formed and wherein the atomic interactions includes at least one covalent bond. For purposes of the present invention, the term "linking" refers to the act of creating a linked molecule as described above.

[218] For purposes of the present invention, the term "fused" refers to two or more proteinaceous components associated by at least one covalent bond which is a peptide bond, regardless of whether the peptide bond involves the participation of a carbon atom of a carboxyl acid group or involves another carbon atom, such as, *e.g.*, the α-carbon, β-carbon, γ-carbon, σ-carbon, etc. Non-limiting examples of two proteinaceous components fused together include, *e.g.*, an amino acid, peptide, or polypeptide fused to a polypeptide via a peptide bond such that the resulting molecule is a single, continuous polypeptide. For purposes of the present invention, the term "fusing" refers to the act of creating a fused molecule as described above, such as, *e.g.*, a fusion protein generated from the recombinant fusion of genetic regions which when translated produces a single proteinaceous molecule.

[219] The symbol "::" means the polypeptide regions before and after it are physically linked together to form a continuous polypeptide.

[220] As used herein, the terms "expressed," "expressing," or "expresses," and grammatical variants thereof, refer to translation of a polynucleotide or nucleic acid into a protein. The expressed protein may remain intracellular, become a component of the cell surface membrane or be secreted into an extracellular space.

[221] As used herein, cells which express a significant amount of an extracellular target biomolecule at least one cellular surface are "target positive cells" or "target+ cells" and are cells physically coupled to the specified, extracellular target biomolecule.

[222] As used herein, the symbol "α" is shorthand for an immunoglobulin-type binding region capable of binding to the biomolecule following the symbol. The symbol "α" is used to refer to the functional characteristic of an immunoglobulin-type binding region based on its ability to bind to the biomolecule following the symbol with a binding affinity described by a dissociation constant (K_D) of 10^{-5} or less.

[223] As used herein, the term "heavy chain variable (V_H) domain" or "light chain variable (V_L) domain" respectively refer to any antibody V_H or V_L domain (*e.g.* a human V_H or V_L domain) as well as any derivative thereof retaining at least qualitative antigen binding ability of the corresponding native antibody (*e.g.* a humanized V_H or V_L domain derived from a native murine V_H or V_L domain). A V_H or V_L domain consists of a "framework" region interrupted by the three CDRs or ABRs. The framework regions serve to align the CDRs or ABRs for specific binding to an epitope of an antigen. From amino-terminus to carboxy-terminus, both V_H and V_L domains comprise the following framework (FR) and CDR regions or ABR regions: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4; or, similarly, FR1, ABR1, FR2, ABR2, FR3, ABR3, and FR4. As used herein, the terms "HCDR1," "HCDR2," or

"HCDR3" are used to refer to CDRs 1, 2, or 3, respectively, in a V_H domain, and the terms "LCDR1," "LCDR2," and "LCDR3" are used to refer to CDRs 1, 2, or 3, respectively, in a V_L domain. As used herein, the terms "HABR1," "HABR2," or "HABR3" are used to refer to ABRs 1, 2, or 3, respectively, in a V_H domain, and the terms "LABR1," "LABR2," or "LABR3" are used to refer to ABRs 1, 2, or 3,
5 respectively, in a V_L domain. For camelid V_HH fragments, IgNARs of cartilaginous fish, V_{NAR} fragments, certain single domain antibodies, and derivatives thereof, there is a single, heavy chain variable domain comprising the same basic arrangement: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. As used herein, the terms "HCDR1," "HCDR2," or "HCDR3" may be used to refer to CDRs 1, 2, or 3, respectively, in a single heavy chain variable domain.

10 [224] For purposes of the present invention, the term "effector" means providing a biological activity, such as cytotoxicity, biological signaling, enzymatic catalysis, subcellular routing, and/or intermolecular binding resulting in an allosteric effect(s) and/or the recruitment of one or more factors.

[225] For purposes of the present invention, the phrases "Shiga toxin A Subunit effector polypeptide", "Shiga toxin effector polypeptide," "Shiga toxin effector polypeptide region," and "Shiga toxin effector
15 region" refer to a polypeptide or polypeptide region derived from at least one Shiga toxin A Subunit of a member of the Shiga toxin family wherein the polypeptide or polypeptide region is capable of exhibiting at least one Shiga toxin function. For example, SEQ ID NOs: 19–21 are derived from StxA and SLT-1A.

[226] For purposes of the present invention, a Shiga toxin effector function is a biological activity conferred by a polypeptide region derived from a Shiga toxin A Subunit or an original Shiga toxin A
20 Subunit. Non-limiting examples of Shiga toxin effector functions include promoting cell entry; lipid membrane deformation; promoting cellular internalization; stimulating clathrin-mediated endocytosis; directing intracellular routing to various intracellular compartments such as, *e.g.*, the Golgi, endoplasmic reticulum, and cytosol; directing intracellular routing with a cargo; inhibiting a ribosome function(s); catalytic activities, such as, *e.g.*, N-glycosidase activity and catalytically inhibiting ribosomes; reducing
25 protein synthesis, inducing caspase activity, activating effector caspases, effectuating cytostatic effects, and cytotoxicity. Shiga toxin catalytic activities include, for example, ribosome inactivation, protein synthesis inhibition, N-glycosidase activity, polynucleotide:adenosine glycosidase activity, RNAase activity, and DNAase activity. Shiga toxins are ribosome inactivating proteins (RIPs). RIPs can depurinate nucleic acids, polynucleosides, polynucleotides, rRNA, ssDNA, dsDNA, mRNA (and polyA),
30 and viral nucleic acids (*see e.g.*, Barbieri L et al., *Biochem J* 286: 1-4 (1992); Barbieri L et al., *Nature* 372: 624 (1994); Ling J et al., *FEBS Lett* 345: 143-6 (1994); Barbieri L et al., *Biochem J* 319: 507-13 (1996); Roncuzzi L, Gasperi-Campani A, *FEBS Lett* 392: 16-20 (1996); Stirpe F et al., *FEBS Lett* 382: 309-12 (1996); Barbieri L et al., Nucleic Acids Res 25: 518-22 (1997); Wang P, Tumer N, *Nucleic Acids Res* 27: 1900-5 (1999); Barbieri L et al., *Biochim Biophys Acta* 1480: 258-66 (2000); Barbieri L et al., *J*
35 *Biochem* 128: 883-9 (2000); Brigotti M et al., *Toxicon* 39: 341-8 (2001); Brigotti M et al., *FASEB J* 16: 365-72 (2002); Bagga S et al., *J Biol Chem* 278: 4813-20 (2003); Picard D et al., *J Biol Chem* 280: 20069-75 (2005)). Some RIPs show antiviral activity and superoxide dismutase activity (Erice A et al., *Antimicrob Agents Chemother* 37: 835-8 (1993); Au T et al., *FEBS Lett* 471: 169-72 (2000); Parikh B,

Tumer N, *Mini Rev Med Chem* 4: 523-43 (2004); Sharma N et al., *Plant Physiol* 134: 171-81 (2004)). Shiga toxin catalytic activities have been observed both *in vitro* and *in vivo*. Non-limiting examples of assays for Shiga toxin effector activity measure various activities, such as, *e.g.*, protein synthesis inhibitory activity, depurination activity, inhibition of cell growth, cytotoxicity, supercoiled DNA relaxation activity, and nuclease activity.

[227] As used herein, the retention of Shiga toxin effector function refers to being capable of exhibiting a level of Shiga toxin functional activity, as measured by an appropriate quantitative assay with reproducibility, comparable to a wild-type, Shiga toxin effector polypeptide control (*e.g.* a Shiga toxin A1 fragment) or cell-targeting molecule comprising a wild-type Shiga toxin effector polypeptide (*e.g.* a Shiga toxin A1 fragment) under the same conditions. For the Shiga toxin effector function of ribosome inactivation or ribosome inhibition, retained Shiga toxin effector function is exhibiting an IC_{50} of 10,000 pM or less in an *in vitro* setting, such as, *e.g.*, by using an assay known to the skilled worker and/or described herein. For the Shiga toxin effector function of cytotoxicity in a target positive cell-kill assay, retained Shiga toxin effector function is exhibiting a CD_{50} of 1,000 nM or less, depending on the cell type and its expression of the appropriate extracellular target biomolecule, as shown, *e.g.*, by using an assay known to the skilled worker and/or described herein.

[228] For purposes of the claimed invention, the term "equivalent" with regard to ribosome inhibition means an empirically measured level of ribosome inhibitory activity, as measured by an appropriate quantitative assay with reproducibility, which is reproducibly within 10% or less of the activity of the reference molecule (*e.g.*, the second cell-targeting molecule, third cell-targeting molecule, etc.) under the same conditions.

[229] For purposes of the claimed invention, the term "equivalent" with regard to cytotoxicity means an empirically measured level of cytotoxicity, as measured by an appropriate quantitative assay with reproducibility, which is reproducibly within 10% or less of the activity of the reference molecule (*e.g.*, the second cell-targeting molecule, third cell-targeting molecule, etc.) under the same conditions.

[230] As used herein, the term "attenuated" with regard to cytotoxicity means a molecule exhibits or exhibited a CD_{50} between 10-fold to 100-fold of a CD_{50} exhibited by a reference molecule under the same conditions.

[231] Inaccurate IC_{50} and CD_{50} values should not be considered when determining a level of Shiga toxin effector function activity. For some samples, accurate values for either IC_{50} or CD_{50} might be unobtainable due to the inability to collect the required data points for an accurate curve fit. For example, theoretically, neither an IC_{50} nor CD_{50} can be determined if greater than 50% ribosome inhibition or cell death, respectively, does not occur in a concentration series for a given sample. Data insufficient to accurately fit a curve as described in the analysis of the data from exemplary Shiga toxin effector function assays, such as, *e.g.*, assays described in the Examples below, should not be considered as representative of actual Shiga toxin effector function.

[232] A failure to detect activity in Shiga toxin effector function may be due to improper expression, polypeptide folding, and/or protein stability rather than a lack of cell entry, subcellular routing, and/or

enzymatic activity. Assays for Shiga toxin effector functions may not require much polypeptide of the invention to measure significant amounts of Shiga toxin effector function activity. To the extent that an underlying cause of low or no effector function is determined empirically to relate to protein expression or stability, one of skill in the art may be able to compensate for such factors using protein chemistry and molecular engineering techniques known in the art, such that a Shiga toxin functional effector activity may be restored and measured. As examples, improper cell-based expression may be compensated for by using different expression control sequences; and improper polypeptide folding and/or stability may benefit from stabilizing terminal sequences, or compensatory mutations in non-effector regions which stabilize the three-dimensional structure of the molecule.

[233] Certain Shiga toxin effector functions are not easily measurable, *e.g.* subcellular routing functions. For example, there is no routine, quantitative assay to distinguish whether the failure of a Shiga toxin effector polypeptide to be cytotoxic and/or deliver a heterologous epitope is due to improper subcellular routing, but at a time when tests are available, then Shiga toxin effector polypeptides may be analyzed for any significant level of subcellular routing as compared to the appropriate wild-type Shiga toxin effector polypeptide. However, if a Shiga toxin effector polypeptide component of a cell-targeting molecule of the present invention exhibits cytotoxicity comparable or equivalent to a wild-type Shiga toxin A Subunit construct, then the subcellular routing activity level is inferred to be comparable or equivalent, respectively, to the subcellular routing activity level of a wild-type Shiga toxin A Subunit construct at least under the conditions tested.

[234] When new assays for individual Shiga toxin functions become available, Shiga toxin effector polypeptides and/or cell-targeting molecules comprising Shiga toxin effector polypeptides may be analyzed for any level of those Shiga toxin effector functions, such as a being within 1000-fold or 100-fold or less the activity of a wild-type Shiga toxin effector polypeptide or exhibiting 3-fold to 30-fold or greater activity as compared to a functional knockout, Shiga toxin effector polypeptide.

[235] Sufficient subcellular routing may be merely deduced by observing a molecule's cytotoxic activity levels in cytotoxicity assays, such as, *e.g.*, cytotoxicity assays based on T-cell epitope presentation or based on a toxin effector function involving a cytosolic and/or endoplasmic reticulum-localized, target substrate.

[236] As used herein, the retention of "significant" Shiga toxin effector function refers to a level of Shiga toxin functional activity, as measured by an appropriate quantitative assay with reproducibility comparable to a wild-type Shiga toxin effector polypeptide control (*e.g.* a Shiga toxin A1 fragment). For *in vitro* ribosome inhibition, significant Shiga toxin effector function is exhibiting an IC_{50} of 300 pM or less depending on the source of the ribosomes used in the assay (*e.g.* a bacterial, archaeal, or eukaryotic (algal, fungal, plant, or animal) source). This is significantly greater inhibition as compared to the approximate IC_{50} of 100,000 pM for the catalytically disrupted SLT-1A 1-251 double mutant (Y77S/E167D). For cytotoxicity in a target-positive cell-kill assay in laboratory cell culture, significant Shiga toxin effector function is exhibiting a CD_{50} of 100, 50, 30 nM, or less, depending on the target biomolecule(s) of the binding region and the cell type, particularly that cell type's expression and/or cell-

surface representation of the appropriate extracellular target biomolecule(s) and/or the extracellular epitope(s) targeted by the molecule being evaluated . This is significantly greater cytotoxicity to the appropriate, target-positive cell population as compared to a Shiga toxin A Subunit alone (or a wild-type Shiga toxin A1 fragment), without a cell targeting binding region, which has a CD_{50} of 100–10,000 nM, depending on the cell line.

[237] For purposes of the present invention and with regard to the Shiga toxin effector function of a molecule of the present invention, the term "reasonable activity" refers to exhibiting at least a moderate level (*e.g.* within 11-fold to 1,000-fold) of Shiga toxin effector activity as defined herein in relation to a molecule comprising a naturally occurring (or wild-type) Shiga toxin, wherein the Shiga toxin effector activity is selected from the group consisting of: internalization efficiency, subcellular routing efficiency to the cytosol, delivered epitope presentation by a target cell(s), ribosome inhibition, and cytotoxicity. For cytotoxicity, a reasonable level of Shiga toxin effector activity includes being within 1,000-fold of a wild-type, Shiga toxin construct, such as, *e.g.*, exhibiting a CD_{50} of 500 nM or less when a wild-type Shiga toxin construct exhibits a CD_{50} of 0.5 nM (*e.g.* a cell-targeting molecule comprising a wild-type Shiga toxin A1 fragment).

[238] For purposes of the present invention and with regard to the cytotoxicity of a molecule of the present invention, the term "optimal" refers to a level of Shiga toxin catalytic domain mediated cytotoxicity that is within 2, 3, 4, 5, 6, 7, 8, 9, or 10 -fold of the cytotoxicity of a molecule comprising wild-type Shiga toxin A1 fragment (*e.g.* a Shiga toxin A Subunit or certain truncated variants thereof) and/or a naturally occurring Shiga toxin.

[239] It should be noted that even if the cytotoxicity of a Shiga toxin effector polypeptide is reduced relative to a wild-type Shiga toxin A Subunit or fragment thereof, in practice, applications using attenuated, Shiga toxin effector polypeptides may be equally or more effective than using wild-type Shiga toxin effector polypeptides because the highest potency variants might exhibit undesirable effects which are minimized or reduced in reduced cytotoxic-potency variants. Wild-type Shiga toxins are very potent, being able to kill an intoxicated cell after only one toxin molecule has reached the cytosol of the intoxicated cell or perhaps after only forty toxin molecules have been internalized into the intoxicated cell. Shiga toxin effector polypeptides with even considerably reduced Shiga toxin effector functions, such as, *e.g.*, subcellular routing or cytotoxicity, as compared to wild-type Shiga toxin effector polypeptides may still be potent enough for practical applications, such as, *e.g.*, applications involving targeted cell-killing, heterologous epitope delivery, and/or detection of specific cells and their subcellular compartments. In addition, certain reduced-activity Shiga toxin effector polypeptides may be particularly useful for delivering cargos (*e.g.* an additional exogenous material or T-cell epitope) to certain intracellular locations or subcellular compartments of target cells.

[240] The term "selective cytotoxicity" with regard to the cytotoxic activity of a molecule refers to the relative level of cytotoxicity between a biomolecule target positive cell population (*e.g.* a targeted cell-type) and a non-targeted bystander cell population (*e.g.* a biomolecule target negative cell-type), which can be expressed as a ratio of the half-maximal cytotoxic concentration (CD_{50}) for a targeted cell type

over the CD_{50} for an untargeted cell type to provide a metric of cytotoxic selectivity or indication of the preferentiality of killing of a targeted cell versus an untargeted cell.

[241] The cell surface representation and/or density of a given extracellular target biomolecule (or extracellular epitope of a given target biomolecule) may influence the applications for which certain cell-targeting molecules of the present invention may be most suitably used. Differences in cell surface representation and/or density of a given target biomolecule between cells may alter, both quantitatively and qualitatively, the efficiency of cellular internalization and/or cytotoxicity potency of a given cell-targeting molecule of the present invention. The cell surface representation and/or density of a given target biomolecule can vary greatly among target biomolecule positive cells or even on the same cell at different points in the cell cycle or cell differentiation. The total cell surface representation of a given target biomolecule and/or of certain extracellular epitopes of a given target biomolecule on a particular cell or population of cells may be determined using methods known to the skilled worker, such as methods involving fluorescence-activated cell sorting (FACS) flow cytometry.

[242] As used herein, the terms "disrupted," "disruption," or "disrupting," and grammatical variants thereof, with regard to a polypeptide region or feature within a polypeptide refers to an alteration of at least one amino acid within the region or composing the disrupted feature. Amino acid alterations include various mutations, such as, *e.g.*, a deletion (such as a truncation), inversion, insertion, or substitution which alter the amino acid sequence of the polypeptide. Amino acid alterations also include chemical changes, such as, *e.g.*, the alteration one or more atoms in an amino acid functional group or the addition of one or more atoms to an amino acid functional group.

[243] As used herein, "de-immunized" means reduced antigenic and/or immunogenic potential after administration to a chordate as compared to a reference molecule, such as, *e.g.*, a wild-type peptide region, polypeptide region, or polypeptide. This includes a reduction in overall antigenic and/or immunogenic potential despite the introduction of one or more, de novo, antigenic and/or immunogenic epitopes as compared to a reference molecule. For certain embodiments, "de-immunized" means a molecule exhibited reduced antigenicity and/or immunogenicity after administration to a mammal as compared to a "parental" molecule from which it was derived, such as, *e.g.*, a wild-type Shiga toxin A1 fragment. In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention is capable of exhibiting a relative antigenicity compared to a reference molecule which is reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than the antigenicity of the reference molecule under the same conditions measured by the same assay, such as, *e.g.*, an assay known to the skilled worker and/or described herein like a quantitative ELISA or Western blot analysis. In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention is capable of exhibiting a relative immunogenicity compared to a reference molecule which is reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or greater than the immunogenicity of the reference molecule under the same conditions measured by the same assay, such as, *e.g.*, an assay known to the skilled worker and/or described herein like a quantitative measurement of anti-molecule

antibodies produced in a mammal(s) after receiving parenteral administration of the molecule at a given time-point.

[244] The relative immunogenicities of exemplary cell-targeting molecules were determined using an assay for *in vivo* antibody responses to the cell-targeting molecules after repeat, parenteral administrations over periods of time.

[245] For purposes of the present invention, the phrase "B-cell and/or CD4+ T-cell de-immunized" means that the molecule has a reduced antigenic and/or immunogenic potential after administration to a mammal regarding either B-cell antigenicity or immunogenicity and/or CD4+ T-cell antigenicity or immunogenicity. For certain embodiments, "B-cell de-immunized" means a molecule exhibited reduced B-cell antigenicity and/or immunogenicity after administration to a mammal as compared to a "parental" molecule from which it was derived, such as, *e.g.*, a wild-type Shiga toxin A1 fragment. For certain embodiments, "CD4+ T-cell de-immunized" means a molecule exhibited reduced CD4 T-cell antigenicity and/or immunogenicity after administration to a mammal as compared to a "parental" molecule from which it was derived, such as, *e.g.*, a wild-type Shiga toxin A1 fragment.

[246] The term "endogenous" with regard to a B-cell epitope, CD4+ T-cell epitope, B-cell epitope region, or CD4+ T-cell epitope region in a Shiga toxin effector polypeptide refers to an epitope present in a wild-type Shiga toxin A Subunit.

[247] For purposes of the present invention, the phrase "CD8+ T-cell hyper-immunized" means that the molecule, when present inside a nucleated, chordate cell within a living chordate, has an increased antigenic and/or immunogenic potential regarding CD8+ T-cell antigenicity or immunogenicity. Commonly, CD8+ T-cell immunized molecules are capable of cellular internalization to an early endosomal compartment of a nucleated, chordate cell due either to an inherent feature(s) or as a component of a cell-targeting molecule.

[248] For purposes of the present invention, the term "heterologous" means of a different source than an A Subunit of a naturally occurring Shiga toxin, *e.g.* a heterologous polypeptide is not naturally found as part of any A Subunit of a native Shiga toxin. The term "heterologous" with regard to T-cell epitope or T-cell epitope-peptide component of a polypeptide of the present invention refers to an epitope or peptide sequence which did not initially occur in the polypeptide to be modified, but which has been added to the polypeptide, whether added via the processes of embedding, fusion, insertion, and/or amino acid substitution as described herein, or by any other engineering means. The result is a modified polypeptide comprising a T-cell epitope foreign to the original, unmodified polypeptide, *i.e.* the T-cell epitope was not present in the original polypeptide.

[249] The term "embedded" and grammatical variants thereof with regard to a T-cell epitope or T-cell epitope-peptide component of a polypeptide of the present invention refers to the internal replacement of one or more amino acids within a polypeptide region with different amino acids in order to generate a new polypeptide sequence sharing the same total number of amino acid residues with the starting polypeptide region. Thus, the term "embedded" does not include any external, terminal fusion of any additional amino acid, peptide, or polypeptide component to the starting polypeptide nor any additional

internal insertion of any additional amino acid residues, but rather includes only substitutions for existing amino acids. The internal replacement may be accomplished merely by amino acid residue substitution or by a series of substitutions, deletions, insertions, and/or inversions. If an insertion of one or more amino acids is used, then the equivalent number of proximal amino acids must be deleted next to the
5 insertion to result in an embedded T-cell epitope. This is in contrast to use of the term "inserted" with regard to a T-cell epitope contained within a polypeptide of the present invention to refer to the insertion of one or more amino acids internally within a polypeptide resulting in a new polypeptide having an increased number of amino acid residues compared to the starting polypeptide.

[250] The term "inserted" and grammatical variants thereof with regard to a T-cell epitope contained
10 within a polypeptide of the present invention refers to the insertion of one or more amino acids within a polypeptide resulting in a new polypeptide sequence having an increased number of amino acid residues compared to the starting polypeptide. The "pure" insertion of a T-cell epitope-peptide is when the resulting polypeptide increased in length by the number of amino acid residues equivalent to the number of amino acid residues in the entire, inserted T-cell epitope-peptide. The phrases "partially inserted,"
15 "embedded and inserted," and grammatical variants thereof with regard to a T-cell epitope contained within a polypeptide of the present invention, refers to when the resulting polypeptide increased in length, but by less than the number of amino acid residues equivalent to the length of the entire, inserted T-cell epitope-peptide. Insertions, whether "pure" or "partial," include any of the previously described insertions even if other regions of the polypeptide not proximal to the insertion site within the
20 polypeptide are deleted thereby resulting in a decrease in the total length of the final polypeptide because the final polypeptide still comprises an internal insertion of one or more amino acids of a T-cell epitope-peptide within a polypeptide region.

[251] As used herein, the term "T-cell epitope delivering" when describing a functional activity of a molecule means that a molecule provides the biological activity of localizing within a cell to a subcellular
25 compartment that is competent to result in the proteasomal cleavage of a proteinaceous part of the molecule which comprises a T-cell epitope-peptide. The "T-cell epitope delivering" function of a molecule can be assayed by observing the MHC presentation of a T-cell epitope-peptide cargo of the molecule on a cell surface of a cell exogenously administered the molecule or in which the assay was begun with the cell containing the molecule in one or more of its endosomal compartments. Generally,
30 the ability of a molecule to deliver a T-cell epitope to a proteasome can be determined where the initial location of the "T-cell epitope delivering" molecule is an early endosomal compartment of a cell, and then, the molecule is empirically shown to deliver the epitope-peptide to the proteasome of the cell. However, a "T-cell epitope delivering" ability may also be determined where the molecule starts at an extracellular location and is empirically shown, either directly or indirectly, to deliver the epitope into a
35 cell and to proteasomes of the cell. For example, certain "T-cell epitope delivering "molecules pass through an endosomal compartment of the cell, such as, *e.g.* after endocytotic entry into that cell. Alternatively, "T-cell epitope delivering" activity may be observed for a molecule starting at an extracellular location whereby the molecule does not enter any endosomal compartment of a cell—

instead the "T-cell epitope delivering" molecule enters a cell and delivers a T-cell epitope-peptide to proteasomes of the cell, presumably because the "T-cell epitope delivering" molecule directed its own routing to a subcellular compartment competent to result in proteasomal cleavage of its T-cell epitope-peptide component.

[252] For purposes of the present invention, the phrase "proximal to an amino-terminus" with reference to the position of a Shiga toxin effector polypeptide region of a cell-targeting molecule of the present invention refers to a distance wherein at least one amino acid residue of the Shiga toxin effector polypeptide region is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more, *e.g.*, up to 18–20 amino acid residues, of an amino-terminus of the cell-targeting molecule as long as the cell-targeting molecule is capable of exhibiting the appropriate level of Shiga toxin effector functional activity noted herein (*e.g.*, a certain level of cytotoxic potency). Thus for certain embodiments of the present invention, any amino acid residue(s) fused amino-terminal to the Shiga toxin effector polypeptide should not reduce any Shiga toxin effector function (*e.g.*, by sterically hindering a structure(s) near the amino-terminus of the Shiga toxin effector polypeptide region) such that a functional activity of the Shiga toxin effector polypeptide is reduced below the appropriate activity level required herein.

[253] For purposes of the present invention, the phrase "more proximal to an amino-terminus" with reference to the position of a Shiga toxin effector polypeptide region within a cell-targeting molecule of the present invention as compared to another component (*e.g.*, a cell-targeting, binding region, molecular moiety, and/or additional exogenous material) refers to a position wherein at least one amino acid residue of the amino-terminus of the Shiga toxin effector polypeptide is closer to the amino-terminus of a linear, polypeptide component of the cell-targeting molecule of the present invention as compared to the other referenced component.

[254] For purposes of the present invention, the phrase "active enzymatic domain derived from one A Subunit of a member of the Shiga toxin family" refers to having the ability to inhibit protein synthesis via a catalytic ribosome inactivation mechanism. The enzymatic activities of naturally occurring (or wild-type) Shiga toxins may be defined by the ability to inhibit protein translation using assays known to the skilled worker, such as, *e.g.*, *in vitro* assays involving RNA translation in the absence of living cells or *in vivo* assays involving RNA translation in a living cell. Using assays known to the skilled worker and/or described herein, the potency of a Shiga toxin enzymatic activity may be assessed directly by observing N-glycosidase activity toward ribosomal RNA (rRNA), such as, *e.g.*, a ribosome nicking assay, and/or indirectly by observing inhibition of ribosome function and/or protein synthesis.

[255] For purposes of the present invention, the term "Shiga toxin A1 fragment region" refers to a polypeptide region consisting essentially of a Shiga toxin A1 fragment and/or derived from a Shiga toxin A1 fragment of a Shiga toxin.

[256] For purposes of the present invention, the terms "terminus," "amino-terminus," or "carboxy-terminus" with regard to a cell-targeting molecule refers generally to the last amino acid residue of a polypeptide chain of the cell-targeting molecule (*e.g.*, a single, continuous polypeptide chain). A cell-targeting molecule may comprise more than one polypeptides or proteins, and, thus, a cell-targeting

molecule of the present invention may comprise multiple amino-terminals and carboxy-terminals. For example, the "amino-terminus" of a cell-targeting molecule may be defined by the first amino acid residue of a polypeptide chain representing the amino-terminal end of the polypeptide, which is generally characterized by a starting, amino acid residue which does not have a peptide bond with any amino acid residue involving the primary amino group of the starting amino acid residue or involving the equivalent nitrogen for starting amino acid residues which are members of the class of N-alkylated alpha amino acid residues. Similarly, the "carboxy-terminus" of a cell-targeting molecule may be defined by the last amino acid residue of a polypeptide chain representing the carboxyl-terminal end of the polypeptide, which is generally characterized by a final, amino acid residue which does not have any amino acid residue linked by a peptide bond to the alpha-carbon of its primary carboxyl group.

[257] For purposes of the present invention, the terms "terminus," "amino-terminus," or "carboxy-terminus" with regard to a polypeptide region refers to the regional boundaries of that region, regardless of whether additional amino acid residues are linked by peptide bonds outside of that region. In other words, the terminals of the polypeptide region regardless of whether that region is fused to other peptides or polypeptides. For example, a fusion protein comprising two proteinaceous regions, *e.g.*, a binding region comprising a peptide or polypeptide and a Shiga toxin effector polypeptide, may have a Shiga toxin effector polypeptide region with a carboxy-terminus ending at amino acid residue 251 of the Shiga toxin effector polypeptide region despite a peptide bond involving residue 251 to an amino acid residue at position 252 representing the beginning of another proteinaceous region, *e.g.*, the binding region. In this example, the carboxy-terminus of the Shiga toxin effector polypeptide region refers to residue 251, which is not a terminus of the fusion protein but rather represents an internal, regional boundary. Thus, for polypeptide regions, the terms "terminus," "amino-terminus," and "carboxy-terminus" are used to refer to the boundaries of polypeptide regions, whether the boundary is a physically terminus or an internal, position embedded within a larger polypeptide chain.

[258] For purposes of the present invention, the phrase "carboxy-terminus region of a Shiga toxin A1 fragment" refers to a polypeptide region derived from a naturally occurring (or wild-type) Shiga toxin A1 fragment, the region beginning with a hydrophobic residue (*e.g.*, V236 of StxA-A1 and SLT-1A1, and V235 of SLT-2A1) that is followed by a hydrophobic residue and the region ending with the furin-cleavage site conserved among Shiga toxin A1 fragment polypeptides and ending at the junction between the A1 fragment and the A2 fragment in native, Shiga toxin A Subunits. For purposes of the present invention, the carboxy-terminal region of a Shiga toxin A1 fragment includes a peptidic region derived from the carboxy-terminus of a Shiga toxin A1 fragment polypeptide, such as, *e.g.*, a peptidic region comprising, consisting essentially of, or consisting of the the carboxy-terminus of a Shiga toxin A1 fragment. Non-limiting examples of peptidic regions derived from the carboxy-terminus of a Shiga toxin A1 fragment include the amino acid residue sequences natively positioned from position 236 to position 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, or 251 in Stx1A (SEQ ID NO:2) or SLT-1A (SEQ ID NO:1); and from position 235 to position 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, or 250 in SLT-2A (SEQ ID NO:3).

[259] For purposes of the present invention, the phrase "proximal to the carboxy-terminus of an A1 fragment polypeptide" with regard to a linked molecular moiety and/or binding region refers to being within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acid residues from the amino acid residue defining the last residue of the Shiga toxin A1 fragment polypeptide.

[260] For purposes of the present invention, the phrase "sterically covers the carboxy-terminus of the A1 fragment-derived region" includes any molecular moiety of a size of 4.5 kDa or greater (*e.g.*, an immunoglobulin-type binding region) linked and/or fused to an amino acid residue in the carboxy-terminus of the A1 fragment-derived region, such as, *e.g.*, the amino acid residue derived from the amino acid residue natively positioned at any one of positions 236 to 251 in Stx1A (SEQ ID NO:2) or SLT-1A (SEQ ID NO:1) or from 235 to 250 in SLT-2A (SEQ ID NO:3). For purposes of the present invention, the phrase "sterically covers the carboxy-terminus of the A1 fragment-derived region" also includes any molecular moiety of a size of 4.5 kDa or greater (*e.g.*, an immunoglobulin-type binding region) linked and/or fused to an amino acid residue in the carboxy-terminus of the A1 fragment-derived region, such as, *e.g.*, the amino acid residue carboxy-terminal to the last amino acid A1 fragment-derived region and/or the Shiga toxin effector polypeptide. For purposes of the present invention, the phrase "sterically covers the carboxy-terminus of the A1 fragment-derived region" also includes any molecular moiety of a size of 4.5 kDa or greater (*e.g.*, an immunoglobulin-type binding region) physically preventing cellular recognition of the carboxy-terminus of the A1 fragment-derived region, such as, *e.g.* recognition by the ERAD machinery of a eukaryotic cell.

[261] For purposes of the present invention, a binding region, such as, *e.g.*, an immunoglobulin binding region or an immunoglobulin-type binding region, that comprises a polypeptide comprising at least forty amino acids and that is linked (*e.g.*, fused) to the carboxy-terminus of the Shiga toxin effector polypeptide region comprising an A1 fragment-derived region is a molecular moiety which is "sterically covering the carboxy-terminus of the A1 fragment-derived region."

[262] For purposes of the present invention, a binding region, such as, *e.g.*, an immunoglobulin binding region or an immunoglobulin-type binding region, that comprises a polypeptide comprising at least forty amino acids and that is linked (*e.g.*, fused) to the carboxy-terminus of the Shiga toxin effector polypeptide region comprising an A1 fragment-derived region is a molecular moiety "encumbering the carboxy-terminus of the A1 fragment-derived region."

[263] For purposes of the present invention, the term "A1 fragment of a member of the Shiga toxin family" refers to the remaining amino-terminal fragment of a Shiga toxin A Subunit after proteolysis by furin at the furin-cleavage site conserved among Shiga toxin A Subunits and positioned between the A1 fragment and the A2 fragment in wild-type Shiga toxin A Subunits.

[264] For purposes of the claimed invention, the phrase "furin-cleavage motif at the carboxy-terminus of the A1 fragment region" refers to a specific, furin-cleavage motif conserved among Shiga toxin A Subunits and bridging the junction between the A1 fragment and the A2 fragment in naturally occurring, Shiga toxin A Subunits.

[265] For purposes of the present invention, the phrase "furin-cleavage site proximal to the carboxy-terminus of the A1 fragment region" refers to any identifiable, furin-cleavage site having an amino acid residue within a distance of less than 1, 2, 3, 4, 5, 6, 7 or more amino acid residues of the amino acid residue defining the last amino acid residue in the A1 fragment region or A1 fragment derived region, including a furin-cleavage motif located carboxy-terminal of an A1 fragment region or A1 fragment derived region, such as, *e.g.*, at a position proximal to the linkage of the A1 fragment-derived region to another component of the molecule, such as, *e.g.*, a molecular moiety of a cell-targeting molecule of the present invention.

[266] For purposes of the present invention, the phrase "disrupted furin-cleavage motif" refers to (i) a specific furin-cleavage motif as described herein in Section I-B and (ii) which comprises a mutation and/or truncation that can confer a molecule with a reduction in furin-cleavage as compared to a reference molecule, such as, *e.g.*, a reduction in furin-cleavage reproducibly observed to be 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98%, 99%, or less (including 100% for no cleavage) than the furin-cleavage of a reference molecule observed in the same assay under the same conditions. The percentage of furin-cleavage as compared to a reference molecule can be expressed as a ratio of cleaved:uncleaved material of the molecule of interest divided by the cleaved:uncleaved material of the reference molecule (*see e.g.* WO 2015/191764; WO 2016/196344). Non-limiting examples of suitable reference molecules include certain molecules comprising a wild-type Shiga toxin furin-cleavage motif and/or furin-cleavage site as described herein and/or molecules used as reference molecules in the Examples below.

[267] For purposes of the present invention, the phrase "furin-cleavage resistant" means a molecule or specific polypeptide region thereof exhibits reproducibly less furin cleavage than (i) the carboxy-terminus of a Shiga toxin A1 fragment in a wild-type Shiga toxin A Subunit or (ii) the carboxy-terminus of the Shiga toxin A1 fragment derived region of construct wherein the naturally occurring furin-cleavage site natively positioned at the junction between the A1 and A2 fragments is not disrupted; as assayed by any available means to the skilled worker, including by using a method described herein.

[268] For purposes of the present invention, the phrase "active enzymatic domain derived form an A Subunit of a member of the Shiga toxin family" refers to a polypeptide structure having the ability to inhibit protein synthesis via catalytic inactivation of a ribosome based on a Shiga toxin enzymatic activity. The ability of a molecular structure to exhibit inhibitory activity of protein synthesis and/or catalytic inactivation of a ribosome may be observed using various assays known to the skilled worker, such as, *e.g.*, *in vitro* assays involving RNA translation assays in the absence of living cells or *in vivo* assays involving the ribosomes of living cells. For example, using assays known to the skilled worker, the enzymatic activity of a molecule based on a Shiga toxin enzymatic activity may be assessed directly by observing N-glycosidase activity toward ribosomal RNA (rRNA), such as, *e.g.*, a ribosome nicking assay, and/or indirectly by observing inhibition of ribosome function, RNA translation, and/or protein synthesis.

[269] As used herein with respect to a Shiga toxin effector polypeptide, a "combination" describes a Shiga toxin effector polypeptide comprising two or more sub-regions wherein each sub-region comprises at least one of the following: (1) a disruption in an endogenous epitope or epitope region; (2) an embedded, heterologous, T-cell epitope-peptide; (3) an inserted, heterologous, T-cell epitope-peptide; and (4) a disrupted furin-cleavage motif at the carboxy-terminus of an A1 fragment region.

[270] As used herein, the term "additional HER2-targeting therapeutic agent" means an additional therapeutic agent (*e.g.* a molecule) that targets HER2 to produce a therapeutic effect or benefit. This additional HER2-targeting therapeutic agent is complementary to the cell-targeting molecule of the present invention and does not compete directly with the cell-targeting molecule in its HER2-targeting activity. The additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of an anti-HER2 antibody or small molecule inhibitor that interferes with HER2 signaling. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consists of a dual tyrosine kinase inhibitor, such as lapatinib and/or neratinib. The additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of an anti-HER2 antibody therapy that binds to an antigenic determinant that does not overlap with the antigenic determinant bound by the cell-targeting molecule of the invention or that binds a HER2 molecule in such a manner that when bound the additional HER2-tageting therapeutic does not prevent the binding of that HER2 molecule by the cell-targeting molecule of the invention. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of anti-HER2 monoclonal antibody therapy and/or anti-HER2 antibody drug conjugate therapy, such as, *e.g.*, T-DM1 (trastuzumab emtansine), trastuzumab, and/ or pertuzumab. The additional HER2-targeting therapeutic agent may be selected from any one of or a combination of: lapatinib, neratinib, T-DM1 (trastuzumab emtansine), trastuzumab, and/or pertuzumab.

[271] As used herein with respect to a molecule of the present invention, a "cell-targeting molecule" is used interchangeably with a "HER2-targeting molecule" or "HER2-binding molecule". All of the aforementioned molecule types include various "HER2-binding proteins".

Introduction

[272] The present invention provides various cell-targeting molecules comprising one or more Shiga toxin effector polypeptides and at least one HER2-binding region. Certain embodiments of the cell-targeting molecules of the present invention comprise Shiga toxin effector polypeptides that combine structural elements resulting in two or more properties in a single molecule, such as, *e.g.*, the ability to 1) exhibit reduced antigenicity and/or immunogenicity as compared to molecular variants lacking that particular combination of elements, 2) exhibit reduced protease-cleavage as compared to molecular variants lacking that particular combination of elements, 3) exhibit reduced non-specific toxicity to a multicellular organism at certain dosages as compared to molecular variants lacking that particular combination of elements, and/or 5) exhibit potent cytotoxicity. The cell-targeting molecules of the present invention may serve as scaffolds to create various cell-targeting molecules, such as, *e.g.*, HER2-

targeted, cytotoxic, therapeutic molecules; HER2-targeted, nontoxic, delivery vehicles; and HER2-targeted diagnostic molecules.

I. The General Structures of the Cell-Targeting Molecules of the Present Invention

[273] The present invention provides various cell-targeting molecules, each comprising (1) a cell-targeting, binding region and (2) a Shiga toxin effector polypeptide component. The Shiga toxin effector polypeptides of the present invention may be associated with and/or coupled to various, diverse, cell-targeting components (*e.g.* a molecular moiety and/or agent) to create cell-targeting molecules of the present invention. A cell-targeting molecule of the present invention comprises (1) a binding region capable of specifically binding an extracellular part of a target biomolecule and (2) a Shiga toxin effector polypeptide capable of exhibiting one or more Shiga toxin A Subunit effector functions. The association of a cell-targeting binding region(s) with a Shiga toxin effector polypeptide of the present invention enables the engineering of therapeutic and diagnostic molecules with desirable characteristics, such as, *e.g.*, de-immunization, potent cytotoxicity, efficient intracellular routing, T-cell hyper-immunization, molecular stability, and *in vivo* tolerability at high dosages as compared to certain reference molecules.

[274] The present invention provides various HER2-targeting molecules, each comprising (1) a cell-targeting, binding region capable of binding HER2 and (2) a Shiga toxin A Subunit effector polypeptide capable of exhibiting a Shiga toxin effector function. The Shiga toxin effector polypeptide may be associated with and/or coupled to various, diverse, HER2-targeting components (*e.g.* a molecular moiety and/or agent) to create cell-targeting molecules of the present invention. A cell-targeting molecule of the present invention comprises (1) a binding region capable of specifically binding an extracellular part of a HER2 target biomolecule and (2) a Shiga toxin effector polypeptide region comprising a Shiga toxin effector polypeptide capable of exhibiting one or more Shiga toxin A Subunit effector functions, such as, *e.g.*, cytostasis, cytotoxicity, catalytic activity, promoting cellular internalization, directing intracellular routing to a certain subcellular compartment(s), and intracellular delivery of a material(s). For example, the cell-targeting molecules of the present invention may comprise a Shiga toxin A Subunit effector polypeptide component that comprises a Shiga toxin A1 fragment derived region, wherein the Shiga toxin A Subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region; and (b) a disruption of at least three, endogenous, B-cell and/or CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; wherein the Shiga toxin effector polypeptide comprises a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region, wherein said furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin A1 fragment region as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function.

[275] The Shiga toxin effector polypeptides of the present invention may be linked to one or more cell-targeting, binding regions that mediate cell-targeting via binding specificity to extracellular parts of target biomolecules, such as, *e.g.*, a HER2 target biomolecule physically coupled to a cellular surface of a cell.

One non-limiting example of a cell-targeting molecule of the present invention is a Shiga toxin effector polypeptide of the present invention fused to a proteinaceous, cell-targeting, binding region, such as, *e.g.*, an immunoglobulin or immunoglobulin-type binding region. For example, the cell-targeting molecules of the present invention may comprise an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising a polypeptide comprising one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor.

A. HER2/neu/ErbB2 Binding Regions

[276] In certain embodiments, a binding region of a cell-targeting molecule of the present invention is a cell-targeting component, such as, *e.g.*, a domain, molecular moiety, or agent, capable of binding specifically to an extracellular part of a HER2/neu/ErbB2 target biomolecule on a cell surface (*i.e.* an extracellular target biomolecule) with high affinity. There are numerous types of binding regions known to skilled worker or which may be discovered by the skilled worker using techniques known in the art. For example, any cell-targeting component that exhibits the requisite binding characteristics described herein may be used as the binding region in certain embodiments of the cell-targeting molecules of the present invention.

[277] An extracellular part of a target biomolecule refers to a portion of its structure exposed to the extracellular environment when the molecule is physically coupled to a cell, such as, *e.g.*, when the target biomolecule is expressed at a cellular surface by the cell. In this context, exposed to the extracellular environment means that part of the target biomolecule is accessible by, *e.g.*, an antibody or at least a binding moiety smaller than an antibody such as a single-domain antibody domain, a nanobody®, a heavy-chain antibody domain derived from camelids or cartilaginous fishes, a single-chain variable fragment, or any number of engineered alternative scaffolds to immunoglobulins (see below). The exposure to the extracellular environment of or accessibility to a part of target biomolecule physically coupled to a cell may be empirically determined by the skilled worker using methods well known in the art.

[278] HER2, also recognized in the art as receptor tyrosine-protein kinase erbB-2, is a transmembrane protein which functions as a cell surface receptor for transducing signals across the cellular membrane to intracellular regulators of cell proliferation and apoptosis. HER2 is also recognized in the art as *Neu*, erbB-2, p185, CD340, NGL, and HER2/neu (Coussens L et al., *Science* 230: 1132-39 (1985); King C et al., *Science* 229: 974-6 (1985); Semba K et al., *Proc Natl Acad Sci USA* 82: 6497-501 (1985); Yamamoto T et al., *Nature* 319:230-234 (1986); Kokai Y et al., *Proc Natl Acad Sci USA* 85: 5389-93 (1988); Disis M et al., *Cancer Res* 54: 16-20 (1994); Yoshino I et al., *J Immunol* 152: 2393-400 (1994) *see e.g.*, GenBank Acc. Nos. X03363; M17730; NM_004448; SEG_HUMHER20). While the name HER2 might refer to multiple proteins with related structures and polypeptide sequences from various species, for the

purposes of the structural examples of this section, the term "HER2" refers to the epidermal growth factor receptor proteins present in humans whose exact sequence might vary slightly based on the isoform and from individual to individual. For example, HER2 refers to the human protein represented by the exemplary polypeptide sequences UniProt P04626 and NCBI accessions NP_004439.2, NP_001005862.1, NP_001276865.1, NP_001276866.1, and NP_001276867.1; however, different isoforms and variants exist due to splicing, polymorphisms and/or mutations (*see e.g.* Siddig A et al., *Ann N Y Acad Sci* 1138: 84-94 (2008); Poole E et al., *Int J Mol Epidemiol Genet* 2: 300-15 (2011); WO 2000/020579). A skilled worker will be able to identify other HER2 proteins in humans, even if they differ from the referenced sequences.

[279] HER2 is overexpressed by many cancer cells, notably breast cancer cells, and its overexpression is strongly associated with increased metastasis, increased disease reoccurrence, and poor prognosis (*see e.g.* Slamon D et al., *Science* 235: 177-82 (1987)).

[280] There are numerous HER2 binding regions known to the skilled worker which may be associated with a Shiga toxin effector polypeptide of the present invention to create a cell-targeting molecule of the present invention. For purposes of the present invention, the term "HER2 binding region" refers to a molecular moiety (*e.g.* a proteinaceous molecule) or agent capable of specifically binding an extracellular part of a HER2 molecule with high affinity, such as, *e.g.*, having a dissociation constant with regard to HER2 of 10^{-5} to 10^{-12} moles per liter. As used herein, HER2 binding refers to the ability to bind to an extracellular part of an isoform or variant of human HER2 (also known as neu or ErbB2).

[281] A binding region of a cell-targeting molecule of the present invention may be, *e.g.*, a ligand, peptide, immunoglobulin-type binding region, monoclonal antibody, engineered antibody derivative, or engineered alternative to antibodies. For example, the binding region is an immunoglobulin binding region.

[282] In certain embodiments, the binding region of a cell-targeting molecule of the present invention is a proteinaceous moiety capable of binding specifically to an extracellular part of target biomolecule with high affinity. A binding region of a cell-targeting molecule of the present invention may comprise one or more various peptidic or polypeptide moieties, such as randomly generated peptide sequences, naturally occurring ligands or derivatives thereof, immunoglobulin derived domains, synthetically engineered scaffolds as alternatives to immunoglobulin domains, and the like (*see e.g.*, WO 2005/092917; WO 2007/033497; Cheung M et al., *Mol Cancer* 9: 28 (2010); US 2013/0196928; WO 2014/164693; WO 2015/113005; WO 2015/113007; WO 2015/138452; WO 2015/191764). In certain embodiments, a cell-targeting molecule of the present invention comprises a binding region comprising one or more polypeptides capable of selectively and specifically binding an extracellular target biomolecule.

[283] There are numerous binding regions known in the art that are useful for targeting molecules to extracellular portions of HER2 via their binding characteristics, such as certain monoclonal antibodies, engineered antibody derivatives, and engineered alternatives to antibodies.

[284] According to one specific, but non-limiting aspect, the binding region may comprise an immunoglobulin-type binding region. The term "immunoglobulin-type binding region" as used herein refers to a polypeptide region capable of binding one or more target biomolecules, such as an antigen or epitope. Binding regions may be functionally defined by their ability to bind to target molecules.
5 Immunoglobulin-type binding regions are commonly derived from antibody or antibody-like structures; however, alternative scaffolds from other sources are contemplated within the scope of the term. In certain embodiments, the binding region may comprise an immunoglobulin binding region derived from antibody or antibody-like structure.

[285] Immunoglobulin (Ig) proteins have a structural domain known as an Ig domain. Ig domains
10 range in length from about 70–110 amino acid residues and possess a characteristic Ig-fold, in which typically 7 to 9 antiparallel beta strands arrange into two beta sheets which form a sandwich-like structure. The Ig fold is stabilized by hydrophobic amino acid interactions on inner surfaces of the sandwich and highly conserved disulfide bonds between cysteine residues in the strands. Ig domains may be variable (IgV or V-set), constant (IgC or C-set) or intermediate (IgI or I-set). Some Ig domains
15 may be associated with a complementarity determining region (CDR), also called a "complementary determining region," which is important for the specificity of antibodies binding to their epitopes. Ig-like domains are also found in non-immunoglobulin proteins and are classified on that basis as members of the Ig superfamily of proteins. The HUGO Gene Nomenclature Committee (HGNC) provides a list of members of the Ig-like domain containing family.

20 [286] An immunoglobulin-type binding region may be a polypeptide sequence of an antibody or antigen-binding fragment thereof wherein the amino acid sequence has been varied from that of a native antibody or an Ig-like domain of a non-immunoglobulin protein, for example by molecular engineering or selection by library screening. Because of the relevance of recombinant DNA techniques and *in vitro* library screening in the generation of immunoglobulin-type binding regions, antibodies can be redesigned
25 to obtain desired characteristics, such as smaller size, cell entry, or other improvements for *in vivo* and/or therapeutic applications. The possible variations are many and may range from the changing of just one amino acid to the complete redesign of, for example, a variable region. Typically, changes in the variable region will be made in order to improve the antigen-binding characteristics, improve variable region stability, or reduce the potential for immunogenic responses.

30 [287] There are numerous immunoglobulin-type binding regions contemplated as components of the present invention. In certain embodiments, the immunoglobulin-type binding region is derived from an immunoglobulin binding region, such as an antibody paratope capable of binding an extracellular target biomolecule. In certain other embodiments, the immunoglobulin-type binding region comprises an engineered polypeptide not derived from any immunoglobulin domain but which functions like an
35 immunoglobulin binding region by providing high-affinity binding to an extracellular target biomolecule. This engineered polypeptide may optionally include polypeptide scaffolds comprising, consisting of, or consisting essentially of complementary determining regions from immunoglobulins as described herein.

[288] There are also numerous binding regions in the prior art that are useful for targeting polypeptides to specific cell-types via their high-affinity binding characteristics. In certain embodiments, the binding region of the cell-targeting molecule of the present invention is selected from the group which includes autonomous V_H domains, single-domain antibody domains (sdAbs), heavy-chain antibody domains derived from camelids (V_HH fragments or V_H domain fragments), heavy-chain antibody domains derived from camelid V_HH fragments or V_H domain fragments, heavy-chain antibody domains derived from cartilaginous fishes, immunoglobulin new antigen receptors (IgNARs), V_{NAR} fragments, single-chain variable (scFv) fragments, nanobodies®, Fd fragments consisting of the heavy chain and C_H1 domains, single chain Fv-C_H3 minibodies, dimeric C_H2 domain fragments (C_H2D), Fc antigen binding domains (Fcabs), isolated complementary determining region 3 (CDR3) fragments, constrained framework region 3, CDR3, framework region 4 (FR3-CDR3-FR4) polypeptides, small modular immunopharmaceutical (SMIP) domains, scFv-Fc fusions, multimerizing scFv fragments (diabodies, triabodies, tetrabodies), disulfide stabilized antibody variable (Fv) fragments, disulfide stabilized antigen-binding (Fab) fragments consisting of the V_L, V_H, C_L and C_H1 domains, bivalent nanobodies®, bivalent minibodies, bivalent $F(ab')_2$ fragments (Fab dimers), bispecific tandem V_HH fragments, bispecific tandem scFv fragments, bispecific nanobodies®, bispecific minibodies, and any genetically manipulated counterparts of the foregoing that retain its paratope and binding function (*see* Ward E et al., *Nature* 341: 544-6 (1989); Davies J, Riechmann L, *Biotechnology (NY)* 13: 475-9 (1995); Reiter Y et al., *Mol Biol* 290: 685-98 (1999); Riechmann L, Muyldermans S, *J Immunol Methods* 231: 25-38 (1999); Tanha J et al., *J Immunol Methods* 263: 97-109 (2002); Vranken W et al., *Biochemistry* 41: 8570-9 (2002); Jespers L et al., *J Mol Biol* 337: 893-903 (2004); Jespers L et al., *Nat Biotechnol* 22: 1161-5 (2004); To R et al., *J Biol Chem* 280: 41395-403 (2005); Saerens D et al., *Curr Opin Pharmacol* 8: 600-8 (2008); Dimitrov D, *MAbs* 1: 26-8 (2009); Weiner L, *Cell* 148: 1081-4 (2012); Ahmad Z et al., *Clin Dev Immunol* 2012: 980250 (2012)). For example, the cell-targeting molecule of the present invention may comprise a binding region that comprises, consists essentially of, or consists of one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor. In certain further embodiments, the binding region comprises, consists essentially of, or consists of a single-chain variable fragment and/or a V_HH fragment derived from a camelid antibody. In certain further embodiments, the binding region comprises, consists essentially of, or consists of a single-chain variable fragment. In certain further embodiments, the binding region comprises, consists essentially of, or consists of a V_HH fragment derived from a camelid antibody.

[289] There are a variety of binding regions comprising polypeptides derived from the constant regions of immunoglobulins, such as, *e.g.*, engineered dimeric Fc domains, monomeric Fcs (mFcs), scFv-Fcs, V_HH-Fcs, C_H2 domains, monomeric C_H3s domains (mC_H3s), synthetically reprogrammed immunoglobulin domains, and/or hybrid fusions of immunoglobulin domains with ligands (Hofer T et

al., *Proc Natl Acad Sci U. S. A.* 105: 12451-6 (2008); Xiao J et al., *J Am Chem Soc* 131: 13616–13618 (2009); Xiao X et al., *Biochem Biophys Res Commun* 387: 387-92 (2009); Wozniak-Knopp G et al., *Protein Eng Des Sel* 23 289-97 (2010); Gong R et al., *PLoS ONE* 7: e42288 (2012); Wozniak-Knopp G et al., *PLoS ONE* 7: e30083 (2012); Ying T et al., *J Biol Chem* 287: 19399-408 (2012); Ying T et al., *J*
5 *Biol Chem* 288: 25154-64 (2013); Chiang M et al., *J Am Chem Soc* 136: 3370-3 (2014); Rader C, *Trends Biotechnol* 32: 186-97 (2014); Ying T et al., *Biochimica Biophys Acta* 1844: 1977-82 (2014)).

[290] In accordance with certain other embodiments, the binding region comprises an engineered, alternative scaffold to immunoglobulin domains. Engineered alternative scaffolds are known in the art which exhibit similar functional characteristics to immunoglobulin-derived structures, such as high-
10 affinity and specific binding of target biomolecules, and may provide improved characteristics to certain immunoglobulin domains, such as, *e.g.*, greater stability or reduced immunogenicity. Generally, alternative scaffolds to immunoglobulins are less than 20 kilodaltons, consist of a single polypeptide chain, lack cysteine residues, and exhibit relatively high thermodynamic stability.

[291] In certain embodiments of the cell-targeting molecules of the present invention, the binding
15 region comprises an alternative scaffold selected from the group which includes autonomous V_H domains, single-domain antibody domains (sdAbs), heavy-chain antibody domains derived from camelids (V_HH fragments or V_H domain fragments), heavy-chain antibody domains derived from camelid V_HH fragments or V_H domain fragments, heavy-chain antibody domains derived from cartilaginous fishes, immunoglobulin new antigen receptors (IgNARs), V_{NAR} fragments, single-chain variable (scFv)
20 fragments, nanobodies®, Fd fragments consisting of the heavy chain and C_H1 domains, permutated Fvs (pFv), single chain Fv-C_H3 minibodies, dimeric C_H2 domain fragments (C_H2D), Fc antigen binding domains (Fcabs), isolated complementary determining region 3 (CDR3) fragments, constrained framework region 3, CDR3, framework region 4 (FR3-CDR3-FR4) polypeptides, small modular immunopharmaceutical (SMIP) domains, scFv-Fc fusions, multimerizing scFv fragments (diabodies,
25 triabodies, tetrabodies), disulfide stabilized antibody variable (Fv) fragments, disulfide stabilized antigen-binding (Fab) fragments consisting of the V_L, V_H, C_L and C_H1 domains, bivalent nanobodies®, bivalent minibodies, bivalent F(ab')$_2$ fragments (Fab dimers), bispecific tandem V_HH fragments, bispecific tandem scFv fragments, bispecific nanobodies®, bispecific minibodies, and any genetically manipulated counterparts of the foregoing that retains its binding functionality (Wörn A, Plückthun A, *J Mol Biol* 305:
30 989-1010 (2001); Xu L et al., *Chem Biol* 9: 933-42 (2002); Wikman M et al., *Protein Eng Des Sel* 17: 455-62 (2004); Binz H et al., *Nat Biotechnol* 23: 1257-68 (2005); Hey T et al., *Trends Biotechnol* 23 :514-522 (2005); Holliger P, Hudson P, *Nat Biotechnol* 23: 1126-36 (2005); Gill D, Damle N, *Curr Opin Biotech* 17: 653-8 (2006); Koide A, Koide S, *Methods Mol Biol* 352: 95-109 (2007); Byla P et al., *J Biol Chem* 285: 12096 (2010); Zoller F et al., *Molecules* 16: 2467-85 (2011); Alfarano P et al., *Protein Sci* 21:
35 1298-314 (2012); Madhurantakam C et al., *Protein Sci* 21: 1015-28 (2012); Varadamsetty G et al., *J Mol Biol* 424: 68-87 (2012); Reichen C et al., *J Struct Biol* 185: 147-62 (2014)).

[292] For example, numerous alternative scaffolds have been identified which bind to an extracellular part of the human cell-surface receptor HER2 (*see e.g.* Wikman M et al., *Protein Eng Des Sel* 17: 455-62

(2004); Orlova A et al. *Cancer Res* 66: 4339-8 (2006); Ahlgren S et al., *Bioconjug Chem* 19: 235-43 (2008); Feldwisch J et al., *J Mol Biol* 398: 232-47 (2010); U.S. patents 5,578,482; 5,856,110; 5,869,445; 5,985,553; 6,333,169; 6,987,088; 7,019,017; 7,282,365; 7,306,801; 7,435,797; 7,446,185; 7,449,480; 7,560,111; 7,674,460; 7,815,906; 7,879,325; 7,884,194; 7,993,650; 8,241,630; 8,349,585; 8,389,227; 8,501,909; 8,512,967; 8,652,474; and U.S. patent application 2011/0059090). In addition to alternative antibody formats, antibody-like binding abilities may be conferred by non-proteinaceous compounds, such as, *e.g*., oligomers, RNA molecules, DNA molecules, carbohydrates, and glycocalyxcalixarenes (*see e.g*. Sansone F, Casnati A, *Chem Soc Rev* 42: 4623-39 (2013)) or partially proteinaceous compounds, such as, *e.g*., phenol-formaldehyde cyclic oligomers coupled with peptides and calixarene-peptide compositions (*see e.g*. U.S. 5,770,380).

[293] In certain embodiments, the HER2 binding region is an immunoglobulin-type binding region. In certain embodiments, the immunoglobulin-type, HER2 binding region is derived from an immunoglobulin, HER2 binding region, such as an antibody paratope capable of binding an extracellular part of HER2. In certain other embodiments, the immunoglobulin-type, HER2 binding region comprises an engineered polypeptide not derived from any immunoglobulin domain but which functions like an immunoglobulin, HER2 binding region by providing high-affinity binding to an extracellular part of HER2. This engineered polypeptide may optionally include polypeptide scaffolds comprising, consisting of, or consisting essentially of complementary determining regions (such as, *e.g*., a heavy chain variable domain and/or light chain variable domain) and/or antigen binding regions from immunoglobulins as described herein.

[294] There are numerous HER2 binding regions contemplated as components of the present invention. Non-limiting examples of immunoglobulin-type, HER2 binding regions include HER2-binding monoclonal antibodies and derivatives thereof, such as, *e.g*., anti-ErbB2, 4D5, 2C4, 7F3, 7C2, mumAb 4D5, chmAb 4D5, (rhu)mAb 4D5, huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, huMAb4D5-8, trastuzumab, humanized 520C9, 4D5Fc8, hingeless rhu4D5, non-glycosylated rhu4D5 with mutated cysteine residues, pertuzumab, and humanized 2C4 (Hudziak R et al., *Mol Cell Biol* 9: 1165-72 (1989); McKenzie S et al., *Oncogene* 4:543-8 (1989); Bacus S et al., *Molecular Carcinogenesis* 3: 350-62 (1990); Hancock M et al., *Cancer Res* 51: 4575-80 (1991); Maier L et al., *Cancer Res* 51: 5361-5369 (1991); Stancovski I et al., *Proc Natl Acad Sci USA* 88: 8691-5 (1991); Tagliabue E et al., *Int J Cancer* 47: 933-937 (1991); Bacus S et al., *Cancer Res* 52: 2580-9 (1992); Carter P et al., *Proc Natl Acad Sci USA* 89: 4285-89 (1992); Harwerth I et al. *J Biol Chem* 267: 15160-7 (1992); Kasprzyk P et al., *Cancer Res* 52: 2771-6 (1992); Lewis G et al., *Cancer Immunol Immunother* 37: 255-63 (1993); Xu F et al., *Int J Cancer* 53: 401-8 (1993); Arteaga C et al., *Cancer Res* 54: 3758-65 (1994); Shawver L et al., *Cancer Res* 54: 1367-73 (1994); Klapper L et al. *Oncogene* 14: 2099-109 (1997); WO 1993/21319; WO 1994/00136; WO 1997/00271; WO 1998/77797; US 5,772,997; US 5,783,186; US 5,821,337; US 5,840,525; US 6,949,245; and US 7,625,859).

[295] In certain embodiments, the cell-targeting molecule of the present invention comprises a binding region comprising an immunoglobulin-type polypeptide (*e.g*. an immunoglobulin polypeptide) selected

for specific and high-affinity binding to human HER2 and/or the cellular surface of a HER2 positive cell. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises at least one heavy chain variable (V_H) domain; and/or at least one light chain variable (V_L) domain. As described herein, the at least one heavy-chain variable domain polypeptide may be linked to the at least one light-chain variable domain polypeptide by a linker (such as a linker or inter-domain linker described herein). In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises a single-domain antibody fragment, such as, *e.g.*, only a heavy chain variable (V_HH) domain (*e.g.* as derived from a camelid antibody).

[296] The binding region of the cell-targeting molecule of the present invention may be defined by reference to its CDRs, such as those defined in SEQ ID NOs: 45–74. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises a polypeptide(s) selected from the group consisting of: a) a heavy chain variable (VH) domain comprising (i) a HCDR1 comprising or consisting essentially of one of the amino acid sequences as shown in SEQ ID NO:45, SEQ ID NO:51, SEQ ID NO:57 or SEQ ID NO:63; (ii) a HCDR2 comprising or consisting essentially of one of the amino acid sequence as shown in SEQ ID NO:46, SEQ ID NO:52, SEQ ID NO:58, or SEQ ID NO:64; and (iii) a HCDR3 comprising or consisting essentially of one of the amino acid sequence as shown in SEQ ID NO:47, SEQ ID NO:53, SEQ ID NO:59, or SEQ ID NO:65; and/or b) a light chain variable (VL) domain comprising (i) a LCDR1 comprising or consisting essentially of one of the amino acid sequence as shown in SEQ ID NO:48, SEQ ID NO:54, SEQ ID NO:60, or SEQ ID NO:66; (ii) a LCDR2 comprising or consisting essentially of one of the amino acid sequence as shown in SEQ ID NO:49, SEQ ID NO:55, SEQ ID NO:61 or SEQ ID NO:67; and (iii) a LCDR3 comprising or consisting essentially of one of the amino acid sequence as shown in SEQ ID NO:50, SEQ ID NO:56, SEQ ID NO:62, or SEQ ID NO:68. In certain embodiments, the binding region comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, SEQ ID NO:52, and SEQ ID NO:53, respectively; (ii) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NO:57, SEQ ID NO:58, and SEQ ID NO:59, respectively; or (iii) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NO:63, SEQ ID NO:64, and SEQ ID NO:65, respectively. In certain embodiments, the binding region comprises at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:54, SEQ ID NO:55, and SEQ ID NO:56, respectively; (ii) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively; or (iii) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:68, respectively.

[297] In certain embodiments, the binding region comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, SEQ ID NO:52, and SEQ ID NO:53, respectively; (ii) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NO:57, SEQ ID NO:58, and SEQ ID NO:59, respectively; or (iii) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NO:63, SEQ ID NO:64,

and SEQ ID NO:65, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:54, SEQ ID NO:55, and SEQ ID NO:56, respectively; (ii) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively; or (iii) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:68, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, SEQ ID NO:52, and SEQ ID NO:53, respectively; and at least one light-chain variable domain polypeptide comprising: (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:54, SEQ ID NO:55, and SEQ ID NO:56, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 57, SEQ ID NO:58, and SEQ ID NO:59, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively. For example, the binding region may comprises at least one heavy-chain variable domain polypeptide comprising (i) the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 63, SEQ ID NO:64, and SEQ ID NO:65, respectively; and at least one light-chain variable domain polypeptide comprising (i) the LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:68, respectively. The binding region having these CDRs may be an immunoglobulin binding region comprising a single-chain variable fragment.

[298] In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises a polypeptide(s) selected from the group consisting of: a) a heavy chain only variable (V_HH) domain comprising (i) a HCDR1 comprising or consisting essentially of the amino acid sequences as shown in SEQ ID NO:69 or SEQ ID NO:72; (ii) a HCDR2 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:70 or SEQ ID NO:73; and/or (iii) a HCDR3 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:71 or SEQ ID NO:74. In certain further embodiments, the binding region comprises a polypeptide(s) selected from the group consisting of: a) a heavy chain only variable (V_HH) domain comprising (i) a HCDR1 comprising or consisting essentially of the amino acid sequences as shown in SEQ ID NO:69 or SEQ ID NO:72; (ii) a HCDR2 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:70 or SEQ ID NO:73; and (iii) a HCDR3 comprising or consisting essentially of the amino acid sequence as shown in SEQ ID NO:71 or SEQ ID NO:74. The binding region having these CDRs may be an immunoglobulin binding region comprising a heavy chain only variable (V_HH) domain derived from a camelid antibody (*see e.g.* Example 1, *infra*).

[299] In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25;

amino acids 269 to 499 of SEQ ID NO: 26 or SEQ ID NO:27; amino acids; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; amino acids 269 to 514 of SEQ ID NO:36; amino acids 268 to 498 of SEQ ID NO:99; amino acids 268 to 499 of SEQ ID NO:100; amino acids 268 to 500 of SEQ ID NO:97; amino acids 268 to 512 of SEQ ID NO:98; amino acids 268 to 518 of SEQ ID NO:102 or SEQ ID NO:103; amino acids 268–519 of SEQ ID NO:101; amino acids 267 to 384 of SEQID NO:104; amino acids 268 to 498 of SEQ ID NO:105; amino acids 252 to 370 of SEQ ID NO:106; amino acids 252 to 366 of SEQ ID NO:107; and amino acids 268 to 513 of SEQ ID NO:108. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence of: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; or amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30.

[300] In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any one of the following polypeptide sequences: amino acids 269 to 501 of SEQ ID NO:24; amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO: 26 or SEQ ID NO:27; amino acids; amino acids 269–520 of SEQ ID NO:28; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 499 of SEQ ID NO:32; amino acids 269 to 499 of SEQ ID NO:33; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; amino acids 269 to 514 of SEQ ID NO:36 amino acids 268 to 498 of SEQ ID NO:99; amino acids 268 to 499 of SEQ ID NO:100; amino acids 268 to 500 of SEQ ID NO:97; amino acids 268 to 512 of SEQ ID NO:98; amino acids 268 to 518 of SEQ ID NO:102 or SEQ ID NO:103; amino acids 268–519 of SEQ ID NO:101; amino acids 267 to 384 of SEQID NO:104; amino acids 268 to 498 of SEQ ID NO:105; amino acids 252 to 370 of SEQ ID NO:106; amino acids 252 to 366 of SEQ ID NO:107; and amino acids 268 to 513 of SEQ ID NO:108. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of the polypeptide represented by any one of the following polypeptide sequences: amino acids 269 to 513 of SEQ ID NO:25; amino acids 269 to 499 of SEQ ID NO:26; amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30; amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; amino acids 253 to 367 of SEQ ID NO:35; and amino acids 269 to 514 of SEQ ID NO:36. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of the polypeptide

represented by amino acids 269 to 519 of SEQ ID NO:29 or SEQ ID NO:30. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29, amino acids 268 to 386 of SEQ ID NO:31; amino acids 253 to 370 of SEQ ID NO:34; or amino acids 253 to 367 of SEQ ID NO:35. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 519 of SEQ ID NO:29. In certain, embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 268 to 386 of SEQ ID NO:31. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 370 of SEQ ID NO:34. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 253 to 367 of SEQ ID NO:35. In certain embodiments, the binding region comprises, consists essentially of, or consists of the polypeptide represented by amino acids 269 to 514 of SEQ ID NO:36.

[301] In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of: amino acids 253 to 367 of SEQ ID NO:35; amino acids 253 to 370 of SEQ ID NO:34; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 387 of SEQ ID NO: 26, 29, 30 or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; and amino acids 401 to 520 of SEQ ID NO:28. In certain further embodiments, the binding region comprises at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of: amino acids 253 to 367 of SEQ ID NO:35; amino acids 253 to 370 of SEQ ID NO:34; amino acids 268 to 386 of SEQ ID NO:31; amino acids 269 to 387 of SEQ ID NO: 26, 29, 30 or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; and amino acids 401 to 520 of SEQ ID NO:28. In certain embodiments of the cell-targeting molecule of the present invention, the binding region comprises at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of: amino acids 269 to 387 of SEQ ID NO: 26, 29, 30 or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; and amino acids 401 to 520 of SEQ ID NO:28. In certain further embodiments of the cell-targeting molecule of the present invention, the binding region comprises at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of: amino acids 269 to 375 of SEQ ID NO: 24, 27, or 28; amino acids 393 to 499 of SEQ ID NO:26; amino acids 403 to 513 of SEQ ID NO:25; amino acids 408 to 514 of SEQ ID NO:36; and amino acids 413 to 519 of SEQ ID NO: 29 or 30. In certain further embodiments of the cell-targeting molecule of the present invention, the binding region comprises at least one light

chain variable (V_L) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 375 of SEQ ID NO: 24, 27, or 28; amino acids 393 to 499 of SEQ ID NO:26; amino acids 403 to 513 of SEQ ID NO:25; amino acids 408 to 514 of SEQ ID NO:36; and amino acids 413 to 519 of SEQ ID NO: 29 or 30. Any of heavy chain variable domain polypeptides described herein may be used in combination with any of the light chain variable domain polypeptides described herein.

[302] In certain embodiments, the binding region may comprise: (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 387 of SEQ ID NOs: 26, 29, 30, or 36; amino acids 269 to 397 of SEQ ID NO:25; amino acids 381 to 500 of SEQ ID NO: 24 or 27; amino acids 401 to 522 of SEQ ID NO:36, or amino acids 401 to 520 of SEQ ID NO:28; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of: amino acids 269 to 375 of SEQ ID NO: 24, 27, or 28; amino acids 393 to 499 of SEQ ID NO:26; amino acids 403 to 513 of SEQ ID NO:25; amino acids 408 to 514 of SEQ ID NO:36; and amino acids 413 to 519 of SEQ ID NO: 29 or 30. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 381 to 500 of SEQ ID NO:24; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 269 to 375 of SEQ ID NO:24. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 397 of SEQ ID NO:25; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of: amino acids 403 to 513 of SEQ ID NO:25. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:26; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 393 to 499 of SEQ ID NO:26. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 381 to 500 of SEQ ID NO:27; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 269 to 375 of SEQ ID NO:27. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 401 to 520 of SEQ ID NO:28; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 269 to 375 of SEQ ID NO:28. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:29; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 413 to 519 of SEQ ID NO:29. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:30; and (b) at least one light chain variable (V_L) domain comprising, consisting essentially of, or consisting of amino acids 413 to 519 of SEQ ID NO:30. For example, the binding region may comprise (a) at least one heavy chain variable (V_H) domain comprising, consisting essentially of, or consisting of amino acids 269 to 387 of SEQ ID NO:36; and (b) at least one light chain variable

(V_L) domain comprising, consisting essentially of, or consisting of amino acids 408 to 514 of SEQ ID NO:36.

[303] In certain embodiments, the binding region comprises or consists essentially of amino acids 269–520 of SEQ ID NO:102.

5 [304] In certain embodiments, the binding region comprises the heavy chain variable domain comprising or consisting essentially of amino acids 269 to 387 of SEQ ID NO:26, 29–30, or 36; 269 to 397 of SEQ ID NO:25; 381 to 500 of SEQ ID NO:27; or 401 to 522 of SEQ ID NO:36. In certain further embodiments, the binding region comprises the light chain variable domain comprising or consisting essentially of amino acids 269 to 375 of SEQ ID NO:27; 393 to 499 of SEQ ID NO:26; 403 to 513 of
10 SEQ ID NO:25; 408 to 514 of SEQ ID NO:36; 413 to 519 of SEQ ID NO:29 or 30. In certain further embodiments, the binding region comprises or consists essentially of amino acids 269 to 513 of SEQ ID NO:25; 269 to 499 of SEQ ID NO:26; 269 to 519 of SEQ ID NO:29; 269 to 519 of SEQ ID NO:30; 268 to 386 of SEQ ID NO:31; 269 to 499 of SEQ ID NO:32; 269 to 499 of SEQ ID NO:33; 253 to 370 of SEQ ID NO:34; 253 to 367 of SEQ ID NO:35; or 269 to 514 of SEQ ID NO:36.

15 [305] A natural ligand or derivative thereof may be utilized as the HER2 binding region for a cell-targeting molecule of the present invention. Native HER2 is known to heterodimerize with other members of the ErbB family upon binding ligands such as epidermal growth factors like epiregulin and heregulin (Moasser M, *Oncogene* 26: 6469-87 (2007); Riese D, Cullum R, *Semin Cell Dev Biol* 28: 49-56 (2014); Sollome J et al., *Cell Signal* 26: 70-82 (2014)). ErbB ligands which bind members of the
20 ErbB family include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF, epiregulin, HER2-68 and HER2-100, heregulins, herstatin, NRG-2, NRG-3, and NRG-4 (Justman Q et al., *J Biol Chem* 277: 20618-24 (2002); Jhabvala-Romero F., et al., *Oncogene* 22: 8178-86 (2003)). Examples of an ErbB ligand include the heregulins (HRG), such as the prototype heregulin disclosed in U.S. Patent 5,641,869 and Marchionni M et al., *Nature* 362: 312-8 (1993). Examples of heregulins include heregulin-α, heregulin-β1,
25 heregulin-β2 and heregulin-β3 (Holmes W et al., *Science* 256: 1205-10 (1992); US 5,641,869); neu differentiation factor (NDF) (Peles et al., *Cell* 69: 205-16 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls D et al., *Cell* 72: 801-15 (1993)); glial growth factors (GGFs) (Marchionni M et al., *Nature* 362: 312-8 (1993)); sensory and motor neuron derived factor (SMDF) (Ho W et al., *J Biol Chem* 270: 14523-32 (1995)); γ-heregulin (Schaefer G et al., *Oncogene* 15: 1385-94 (1997)).

30 [306] An ErbB ligand according to the present invention may also be a synthetic ErbB ligand. The synthetic ligand may be specific for a particular ErbB receptor or may recognize particular ErbB receptor complexes. An example of a synthetic ligand is the synthetic heregulin/EGF chimera biregulin (Jones J et al., *FEBS Lett,* 447: 227-31 (1999)) and the EGF-like domain fragment HRGβ1177-244. ErbB ligands or a part of an ErbB ligand that interacts with HER2 or a derivative thereof may be fused to Shiga toxin
35 effector polypeptides of the invention to construct HER2-targeting, cell-targeting molecules of the invention that bind an extracellular part of HER2.

[307] Synthetic peptides which bind an extracellular part of HER2 may be utilized as the binding region for targeting. Many peptides have been described which are capable of binding to HER2 (*see e.g.*

U.S. patents 5,578,482; 5,856,110; 5,869,445; 5,985,553; 6,333,169; 6,987,088; 7,019,017; 7,282,365; 7,306,801; 7,435,797; 7,446,185; 7,449,480; 7,560,111; 7,674,460; 7,815,906, 7,879,325; 7,884,194; 7,993,650; 8,241,630; 8,349,585; 8,389,227; 8,501,909; 8,512,967; 8,652,474; and US 2011/0059090).

[308] In certain embodiments, small molecules which bind an extracellular part of HER2 may be utilized as the binding region for targeting. Many small molecules have been described which are capable of binding to HER2 such as tyrosine kinase inhibitors, AZD8931, lapatinib, neratinib (HKI-272), dacomitinib (PF-00299804), afatinib (BIBW 2992) (Barlaam B et al., *ACS Med Chem Lett* 4: 742-6 (2013); Yu H, Riley G, J *Natl Compr Canc Netw* 11: 161-9 (2013); Roskoski R, *Pharmacol Res* 87C: 42-59 (2014)). Other small molecules which bind to an extracellular part of HER2 may be identified using methods well known to those of skill in the art, such as by derivatizing known EGFR binders like gefitinib, erlotinib, AEE788, AG1478, AG1571 (SU-5271), AP26113, CO-1686, XL647, vandetanib, and BMS-690514 (Kurokawa H, Arteaga C, *Clin Cancer Res* 7: 4436s-4442s (2001); Yigitbasi O et al., *Cancer Res* 64: 7977-84 (2004); Yu H, Riley G, J *Natl Compr Canc Netw* 11: 161-9 (2013); Roskoski R, *Pharmacol Res* 87C: 42-59 (2014)).

[309] Any of the aforementioned HER2 binding molecules may be suitable for use as a HER2 binding region or modified to create one or more HER2 binding regions for use in a cell-targeting molecule of the present invention. Any of the above binding region structures may be used as a component of a molecule of the present invention as long as the binding region component has a dissociation constant of 10^{-5} to 10^{-12} moles per liter, preferably less than 200 nanomolar (nM), towards an extracellular part of a HER2 molecule.

HER2/neu/ErbB2 Target Biomolecules Bound by the Binding Regions

[310] In certain embodiments, the binding region of a cell-targeting molecules of the present invention comprises a proteinaceous region capable of binding specifically to an extracellular part of a HER2 biomolecule or an extracellular HER2 biomolecule, preferably which is physically coupled to the surface of a cell type of interest, such as, *e.g.*, a cancer cell and/or tumor cell.

[311] The term "target biomolecule" refers to a biological molecule, commonly a proteinaceous molecule or a protein modified by post-translational modifications, such as glycosylation, that is bound by a binding region of a cell-targeting molecule of the present invention resulting in the targeting of the cell-targeting molecule to a specific cell, cell-type, and/or location within a multicellular organism.

[312] For purposes of the present invention, the term "extracellular" with regard to a target biomolecule refers to a biomolecule that has at least a portion of its structure exposed to the extracellular environment. The exposure to the extracellular environment of or accessibility to a part of target biomolecule coupled to a cell may be empirically determined by the skilled worker using methods well known in the art. Non-limiting examples of extracellular target biomolecules include cell membrane components, transmembrane spanning proteins, cell membrane-anchored biomolecules, cell-surface-bound biomolecules, and secreted biomolecules.

[313] With regard to the present invention, the phrase "physically coupled" when used to describe a target biomolecule means covalent and/or non-covalent intermolecular interactions couple the target biomolecule, or a portion thereof, to the outside of a cell, such as a plurality of non-covalent interactions between the target biomolecule and the cell where the energy of each single interaction is on the order of at least about 1–5 kiloCalories (*e.g.*, electrostatic bonds, hydrogen bonds, ionic bonds, Van der Walls interactions, hydrophobic forces, etc.). All integral membrane proteins can be found physically coupled to a cell membrane, as well as peripheral membrane proteins. For example, an extracellular target biomolecule might comprise a transmembrane spanning region, a lipid anchor, a glycolipid anchor, and/or be non-covalently associated (*e.g.* via non-specific hydrophobic interactions and/or lipid binding interactions) with a factor comprising any one of the foregoing.

[314] Extracellular parts of target biomolecules may include various epitopes, including unmodified polypeptides, polypeptides modified by the addition of biochemical functional groups, and glycolipids (*see e.g.* US 5,091,178; EP2431743).

[315] The binding regions of the cell-targeting molecules of the present invention may be designed or selected based on numerous criteria, such as the cell-type specific expression of their HER2 target, the physical localization of their HER2 target biomolecules with regard to specific cell types, and/or the properties of their target HER2 biomolecules. For example, certain cell-targeting molecules of the present invention comprise binding regions capable of binding a cell-surface HER2 target biomolecule that is expressed at a cellular surface exclusively by only one cell-type of a species or only one cell-type within a multicellular organism. It is desirable, but not necessary, that an extracellular target HER2 biomolecule be intrinsically internalized or be readily forced to internalize upon interacting with a cell-targeting molecule of the present invention.

[316] Among certain embodiments of the cell-targeting molecules of the present invention, the binding region is derived from an immunoglobulin-type polypeptide selected for specific and high-affinity binding to a HER2 antigen on the cell surface of a cancer or tumor cell, where the antigen is restricted in expression to cancer or tumor cells (*see* Glokler J et al., *Molecules* 15: 2478-90 (2010); Liu Y et al., *Lab Chip* 9: 1033-6 (2009). In accordance with other embodiments, the binding region is selected for specific and high-affinity binding to an extracellular part of HER2 on the cell surface of a cancer cell, where the HER2 is over-expressed or preferentially expressed by cancer cells as compared to non-cancer cells.

[317] It will be appreciated by the skilled worker that any desired target HER2 biomolecule may be used to design or select a suitable binding region to be associated and/or coupled with a Shiga toxin effector polypeptide to produce a cell-targeting molecule of the present invention.

[318] Any of the above binding regions described herein may be used alone or in combination with each individual embodiment of the present invention, including methods of the present invention.

[319] The general structure of the cell-targeting molecules of the present invention is modular, in that various, diverse, HER2-targeting binding regions may be associated with various, Shiga toxin effector polypeptides of the present invention to create different, cell-targeting molecules of the present invention which exhibit differences in their cell-targeting activities due to differences in their binding regions. This

enables a variety of cell-targeting activities to be exhibited by different embodiments of the cell-targeting molecules of the present invention such that different embodiments target different types of cells with Shiga toxin effector functions, such as, *e.g.*, cytostasis, cytotoxicity, and intracellular delivery of exogenous materials. Furthermore, certain embodiments of the cell-targeting molecules of the present invention exhibit certain characteristics due to differences in their respective Shiga toxin effector polypeptide regions, such as, *e.g.*, low antigenicity and/or immunogenicity when administered to a chordate, resistance to proteolytic cleavage by certain proteases, high stability when administered to a multicellular organism, *in vivo* tolerability at high dosages, ability to deliver a cargo to an intracellular location, and/or ability to deliver a T-cell epitope to a MHC class I molecule for presentation on a cellular surface.

[320] For the purposes of the present invention, the specific order or orientation of the Shiga toxin effector polypeptide region and the cell-targeting, HER2-binding region is not fixed in relation to each other or within the cell-targeting molecule of the present invention unless expressly noted. For example, when the cell-targeting molecule of the present invention is a fusion protein with an amino-terminal(s) and carboxy-terminal(s), various arrangements of the components of the invention may be suitable (*see e.g.* Figure 1). In certain embodiments of the cell-targeting molecules of the present invention, the arrangement of their components in relation to each other or within the cell-targeting molecule are limited as described herein. For example, certain endoplasmic reticulum retention/retrieval signal motifs (*see e.g.* WO 2015/138435) are commonly positioned on a carboxy-terminus of a cell-targeting molecule of the present invention and/or a carboxy-terminus of a protein component of a cell-targeting molecule of the present invention.

B. The General Structures of the Shiga Toxin A Subunit Effector Polypeptides

[321] The cell-targeting molecules of the present invention comprise at least one, Shiga toxin effector polypeptide derived from wild-type Shiga toxin A Subunits that further comprise one or more structural modifications, such as, *e.g.*, a mutation like a truncation and/or amino acid residue substitution(s). For certain embodiments, the present invention involves the engineering of improved, Shiga toxin A Subunit effector polypeptides comprising the combination of two or more of the following Shiga toxin effector polypeptide sub-regions: (1) a de-immunized sub-region, (2) a protease-cleavage resistant sub-region near the carboxy-terminus of a Shiga toxin A1 fragment region, and (3) a T-cell epitope-peptide embedded or inserted sub-region. For example, the Shiga toxin effector polypeptide of the present invention may comprise the combination of: (1) a de-immunized sub-region, (2) a protease-cleavage resistant sub-region near the carboxy-terminus of a Shiga toxin A1 fragment region, and (3) a T-cell epitope-peptide embedded or inserted sub-region that does not overlap with the de-immunized sub-region.

[322] In certain embodiments, the cell-targeting molecule of the invention comprises a Shiga toxin effector polypeptide that comprises a Shiga toxin A1 fragment region, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which

disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region (such as a region within the Shiga toxin A1 fragment region); (b) a disruption of at least three, endogenous, B-cell and/or CD4+ T-cell epitope regions (such as a three or more regions within the Shiga toxin A1 fragment region) which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; and (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function. In certain further embodiments, the Shiga toxin A subunit effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions. In certain further embodiments, the furin-cleavage motif comprises a carboxy-terminal truncation as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit. In certain further embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit. For certain embodiments, the cell-targeting molecule is capable of exhibiting less relative antigenicity and/or relative immunogenicity as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin A effector polypeptide comprising a Shiga toxin A1 fragment region, a reference cell-targeting molecule comprising a wild-type Shiga toxin A effector polypeptide comprising a Shiga toxin A1 fragment region, or a reference cell-targeting molecule consisting of the cell-targeting molecule except for it lacks any combination of the following featues present in the cell targeting molecule: (1) an embedded or inserted, CD8+ T-cell epitope, (2) a disruption of at least three, endogenous, B-cell and/or CD4+ T-cell epitope regions, and/or (3) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region.

[323] For purposes of the present invention, a Shiga toxin effector polypeptide is a polypeptide derived from a Shiga toxin A Subunit member of the Shiga toxin family that is capable of exhibiting one or more Shiga toxin functions (*see e.g.*, Cheung M et al., *Mol Cancer* 9: 28 (2010); WO 2014/164693; WO 2015/113005; WO 2015/113007; WO 2015/138452; WO 2015/191764) and comprises a Shiga toxin A1 fragment derived region having a carboxy-terminus. Shiga toxin functions include, *e.g.*, increasing cellular internalization, directing subcellular routing from an endosomal compartment to the cytosol, avoiding intracellular degradation, catalytically inactivating ribosomes, and effectuating cytostatic and/or cytotoxic effects.

[324] The Shiga toxin family of protein toxins is composed of various naturally occurring toxins which are structurally and functionally related, *e.g.*, Shiga toxin, Shiga-like toxin 1, and Shiga-like toxin 2 (Johannes L, Römer W, *Nat Rev Microbiol* 8: 105-16 (2010)). Holotoxin members of the Shiga toxin family contain targeting domains that preferentially bind a specific glycosphingolipid present on the surface of some host cells and an enzymatic domain capable of permanently inactivating ribosomes once inside a cell (Johannes L, Römer W, *Nat Rev Microbiol* 8: 105-16 (2010)). Members of the Shiga toxin family share the same overall structure and mechanism of action (Engedal N et al., *Microbial Biotech* 4: 32-46 (2011)). For example, Stx, SLT-1 and SLT-2 display indistinguishable enzymatic activity in cell free systems (Head S et al., *J Biol Chem* 266: 3617-21 (1991); Tesh V et al., *Infect Immun* 61: 3392-402 (1993); Brigotti M et al., *Toxicon* 35:1431–1437 (1997)).

[325] The Shiga toxin family encompasses true Shiga toxin (Stx) isolated from *S. dysenteriae* serotype 1, Shiga-like toxin 1 A Subunit variants (SLT1 or Stx1 or SLT-1 or Slt-I) isolated from serotypes of enterohemorrhagic *E. coli*, and Shiga-like toxin 2 variants (SLT2 or Stx2 or SLT-2) isolated from serotypes of enterohemorrhagic *E. coli*. SLT1 differs by only one amino acid residue from Stx, and both have been referred to as Verocytotoxins or Verotoxins (VTs) (O'Brien A, *Curr Top Microbiol Immunol* 180: 65-94 (1992)). Although SLT1 and SLT2 variants are only about 53–60% similar to each other at the primary amino acid sequence level, they share mechanisms of enzymatic activity and cytotoxicity common to the members of the Shiga toxin family (Johannes L, Römer W, *Nat Rev Microbiol* 8: 105-16 (2010)). Over 39 different Shiga toxins have been described, such as the defined subtypes Stx1a, Stx1c, Stx1d, and Stx2a–g (Scheutz F et al., *J Clin Microbiol* 50: 2951-63 (2012)). Members of the Shiga toxin family are not naturally restricted to any bacterial species because Shiga-toxin-encoding genes can spread among bacterial species via horizontal gene transfer (Strauch E et al., *Infect Immun* 69: 7588-95 (2001); Bielaszewska M et al., *Appl Environ Micrbiol* 73: 3144-50 (2007); Zhaxybayeva O, Doolittle W, *Curr Biol* 21: R242-6 (2011)). As an example of interspecies transfer, a Shiga toxin was discovered in a strain of *A. haemolyticus* isolated from a patient (Grotiuz G et al., *J Clin Microbiol* 44: 3838-41 (2006)). Once a Shiga toxin encoding polynucleotide enters a new subspecies or species, the Shiga toxin amino acid sequence is presumed to be capable of developing slight sequence variations due to genetic drift and/or selective pressure while still maintaining a mechanism of cytotoxicity common to members of the Shiga toxin family (*see* Scheutz F et al., *J Clin Microbiol* 50: 2951-63 (2012)).

1. De-Immunized, Shiga Toxin A Subunit Effector Polypeptides

[326] In certain embodiments, the Shiga toxin effector polypeptide of the present invention is de-immunized, such as, *e.g.*, as compared to a wild-type Shiga toxin, wild-type Shiga toxin polypeptide, and/or Shiga toxin effector polypeptide comprising only wild-type polypeptide sequences. The de-immunized, Shiga toxin effector polypeptides of the present invention each comprise a disruption of at least one (such as, *e.g.*, at least two, three, four, five, six, seven, eight, nine or more), putative, endogenous, epitope region in order to reduce the antigenic and/or immunogenic potential of the Shiga toxin effector polypeptide after administration of the polypeptide to a chordate. A Shiga toxin effector polypeptide and/or Shiga toxin A Subunit polypeptide, whether naturally occurring or not, can be de-immunized by a method described herein, described in WO 2015/113005 and/or WO 2015/113007, and/or known to the skilled worker, wherein the resulting molecule retains or exhibits one or more Shiga toxin A Subunit functions.

[327] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption of an endogenous epitope or epitope region, such as, *e.g.*, a B-cell and/or CD4+ T-cell epitope. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption of at least one (such as at least two, three, four, five, six, seven, eight or more) endogenous, B-cell and/or CD4+ T-cell epitope region. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption of at least one (such as at least two, three, four, five, six,

seven, eight or more), endogenous, epitope region described herein, wherein the disruption reduces the antigenic and/or immunogenic potential of the Shiga toxin effector polypeptide after administration of the polypeptide to a chordate, and wherein the Shiga toxin effector polypeptide is capable of exhibiting one or more Shiga toxin A Subunit functions, such as, *e.g.*, a significant level of Shiga toxin cytotoxicity. For example, the Shiga toxin effector polypeptide of the present invention comprises a disruption of at least three, endogenous, B-cell and/or CD4+ T-cell epitope regions (such as, *e.g.*, due to two or more mutations and one or more truncations relative to a wild-type Shiga toxin A Subunit).

[328] The term "disrupted" or "disruption" as used herein with regard to an epitope region refers to the deletion of at least one (such as at least two, three, four, five, six, seven, eight or more) amino acid residue in an epitope region, inversion of two or more amino acid residues where at least one of the inverted amino acid residues is in an epitope region, insertion of at least one (such as at least two, three, four, five, six, seven, eight or more) amino acid into an epitope region, and a substitution of at least one amino acid residue in an epitope region. An epitope region disruption by mutation includes amino acid substitutions with non-standard amino acids and/or non-natural amino acids. Epitope regions may alternatively be disrupted by mutations comprising the modification of an amino acid by the addition of a covalently-linked chemical structure which masks at least one amino acid in an epitope region, *see*, *e.g.* PEGylation (*see* Zhang C et al., *BioDrugs* 26: 209-15 (2012), small molecule adjuvants (Flower D, *Expert Opin Drug Discov* 7: 807-17 (2012), and site-specific albumination (Lim S et al., *J Control Release* 207-93 (2015)).

[329] Certain epitope regions and disruptions are indicated herein by reference to specific amino acid positions of native Shiga toxin A Subunits provided in the Sequence Listing, noting that naturally occurring Shiga toxin A Subunits may comprise precursor forms containing signal sequences of about 22 amino acids at their amino-terminals which are removed to produce mature Shiga toxin A Subunits and are recognizable to the skilled worker. Further, certain epitope region disruptions are indicated herein by reference to specific amino acids (*e.g.* S for a serine residue) natively present at specific positions within native Shiga toxin A Subunits (*e.g.* S33 for the serine residue at position 33 from the amino-terminus) followed by the amino acid with which that residue has been substituted in the particular mutation under discussion (*e.g.* S33I represents the amino acid substitution of isoleucine for serine at amino acid residue 33 from the amino-terminus).

[330] In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises a disruption of at least one (such as at least two, three, four, five, six, seven, eight or more) epitope region provided herein. For example, the de-immunized, Shiga toxin effector polypeptide of the present invention may comprise a disruption of at least three epitope regions provided herein. In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises a disruption of at least four epitope regions provided herein. In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises a disruption of at least five epitope regions provided herein. In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises a disruption of at least one epitope region described in

WO 2015/113005 or WO 2015/113007. As described herein, when the Shiga toxin effector polypeptide also comprises an embedded or inserted, heterologous, CD8+ T-cell epitope, at least some number of disrupted, endogenous, B-cell and/or CD4+ T-cell epitope region does not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope.

5 [331] In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises, consists of, or consists essentially of a full-length Shiga toxin A Subunit (*e.g.* SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), or SLT-2A (SEQ ID NO:3)) comprising at least one disruption of the amino acid sequence selected from the group of natively positioned amino acids consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 10 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3; 240–258 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 15 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent position in a Shiga toxin A Subunit polypeptide, conserved Shiga toxin effector polypeptide sub-region, and/or non-native, Shiga toxin effector polypeptide sequence (such as the Shiga toxin effector polypeptides shown in SEQ ID NOs: 4–18).

20 [332] In certain embodiments, the de-immunized Shiga toxin effector polypeptide of the present invention comprises, consists essentially of, or consists of a full-length or truncated Shiga toxin A Subunit (*e.g.* SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), SLT-2A (SEQ ID NO:3), or any one of SEQ ID NOs: 7–18 further comprising a disruption of at least one (such as at least two, three, four, five, six, seven, eight or more) endogenous, B-cell and/or CD4+ T-cell epitope region, wherein the B-cell 25 region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 30 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2, and 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2; 262–278 of SEQ ID NO:3; 281–297 of SEQ ID NO:3; and 285–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 A Subunit 35 variants shown in SEQ ID NOs: 4–6 and the Shiga-like toxin 2 A Subunit variants shown in SEQ ID NOs: 7–18); and the CD4+ T-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID

NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; 236–258 of SEQ ID NO:1 or SEQ ID NO:2; and 274–293 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 A Subunit variants shown in SEQ ID NOs: 4–6 and the Shiga-like toxin 2 A Subunit variants shown in SEQ ID NOs: 7–18). In certain embodiments, the B-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3 and 243–257 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 A Subunit variants shown in SEQ ID NOs: 4–6 and the Shiga-like toxin 2 A Subunit variants shown in SEQ ID NOs: 7–18); and the CD4+ T-cell epitope region is selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2;or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 A Subunit variants shown in SEQ ID NOs: 4–6 and the Shiga-like toxin 2 A Subunit variants shown in SEQ ID NOs: 7–18).

[333] In certain embodiments, the de-immunized Shiga toxin effector polypeptide of the present invention comprises, consists essentially of, or consists of a full-length or truncated Shiga toxin A Subunit (*e.g.* SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), Shiga toxin 1 A Subunit variant effector polypeptide (SEQ ID NOs: 4–6), SLT-2A (SEQ ID NO:3), or Shiga-like toxin 2 A Subunit variant effector polypeptide (SEQ ID NOs: 7–18)) comprising a disruption of at least three, endogenous, B-cell and/or CD4+ T-cell epitope regions, wherein the disruption comprises a mutation, relative to a wild-type Shiga toxin A Subunit, in the B-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3 and 243–257 of SEQ ID NO:1 or SEQ ID NO:2; or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 A Subunit variants shown in SEQ ID NOs: 4–6 and Shiga-like toxin 2 A Subunit variants shown in SEQ ID NOs: 7–18); and/or the CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of: 4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1

or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2;or the equivalent region in a Shiga toxin A Subunit or derivative thereof (such as the equivalent region in any one of the Shiga toxin 1 A Subunit variants shown in SEQ ID NOs: 4–6 and the Shiga-like toxin 2 A Subunit variants shown in SEQ ID NOs: 7–18). In certain embodiments, each of the at least three of the B-cell and/or CD4+ T-cell epitope regions comprises a disruption comprising an amino acid residue substitution relative to a wild-type Shiga toxin A Subunit sequence.

[334] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises, consists of, or consists essentially of a truncated Shiga toxin A Subunit. Truncations of Shiga toxin A Subunits might result in the deletion of an entire epitope region(s) without affecting Shiga toxin effector function(s). The smallest, Shiga toxin A Subunit fragment shown to exhibit significant enzymatic activity was a polypeptide composed of residues 75–247 of StxA (Al-Jaufy A et al., *Infect Immun* 62: 956-60 (1994)). Truncating the carboxy-terminus of SLT-1A, StxA, or SLT-2A to amino acids 1–251 removes two predicted B-cell epitope regions, two predicted CD4 positive (CD4+) T-cell epitopes, and a predicted, discontinuous, B-cell epitope. Truncating the amino-terminus of SLT-1A, StxA, or SLT-2A to 75–293 removes at least three, predicted, B-cell epitope regions and three predicted CD4+ T-cell epitopes. Truncating both amino- and carboxy-terminals of SLT-1A, StxA, or SLT-2A to 75–251 deletes at least five, predicted, B-cell epitope regions; four, putative, CD4+ T-cell epitopes; and one, predicted, discontinuous, B-cell epitope.

[335] In certain embodiments, a Shiga toxin effector polypeptide of the invention may comprise, consist of, or consist essentially of a full-length or truncated Shiga toxin A Subunit with at least one (such as at least two, three, four, five, six, seven, eight or more) mutation, *e.g.* deletion, insertion, inversion, or substitution, in a provided epitope region. In certain further embodiments, the polypeptides comprise a disruption which comprises a deletion of at least one amino acid within the epitope region. In certain further embodiments, the polypeptides comprise a disruption which comprises an insertion of at least one amino acid within the epitope region. In certain further embodiments, the polypeptides comprise a disruption which comprises an inversion of amino acids, wherein at least one inverted amino acid is within the epitope region. In certain further embodiments, the polypeptides comprise a disruption which comprises a substitution of at least one (such as at least two, three, four, five, six, seven, eight or more) amino acid within the epitope region. In certain further embodiments, the polypeptides comprise a disruption which comprises a mutation, such as an amino acid substitution to a non-standard amino acid or an amino acid with a chemically modified side chain. Numerous examples of single amino acid substitutions are provided in the Examples below.

[336] In certain embodiments, the Shiga toxin effector polypeptides of the invention may comprise, consist of, or consist essentially of a full-length or truncated Shiga toxin A Subunit with one or more mutations as compared to the native sequence which comprises at least one amino acid substitution selected from the group consisting of: A, G, V, L, I, P, C, M, F, S, D, N, Q, H, and K. In certain further embodiments, the polypeptide may comprise, consist of, or consist essentially of a full-length or

truncated Shiga toxin A Subunit with a single mutation as compared to the native sequence wherein the substitution is selected from the group consisting of: D to A, D to G, D to V, D to L, D to I, D to F, D to S, D to Q, E to A, E to G, E to V, E to L, E to I, E to F, E to S, E to Q, E to N, E to D, E to M, E to R, G to A, H to A, H to G, H to V, H to L, H to I, H to F, H to M, K to A, K to G, K to V, K to L, K to I, K to M, K to H, L to A, L to G, N to A, N to G, N to V, N to L, N to I, N to F, P to A, P to G, P to F, R to A, R to G, R to V, R to L, R to I, R to F, R to M, R to Q, R to S, R to K, R to H, S to A, S to G, S to V, S to L, S to I, S to F, S to M, T to A, T to G, T to V, T to L, T to I, T to F, T to M, T to S, Y to A, Y to G, Y to V, Y to L, Y to I, Y to F, and Y to M.

[337] In certain embodiments, the Shiga toxin effector polypeptides of the invention comprise, consist of, or consist essentially of a full-length or truncated Shiga toxin A Subunit with one or more mutations as compared to the native amino acid residue sequence which comprises at least one amino acid substitution of an immunogenic residue and/or within an epitope region, wherein at least one substitution occurs at the natively positioned group of amino acids selected from the group consisting of: 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1 or SEQ ID NO:2; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1 or SEQ ID NO:2; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1 or SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1 or SEQ ID NO:2; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1 or SEQ ID NO:2; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1 or SEQ ID NO:2; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1 or SEQ ID NO:2; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1 or SEQ ID NO:2; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 241 of SEQ ID NO:3; 242 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; 251 of SEQ ID NO:1 or SEQ ID NO:2; 264 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3;

265 of SEQ ID NO:1 or SEQ ID NO:2; and 286 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent position in a Shiga toxin A Subunit polypeptide, conserved Shiga toxin effector polypeptide sub-region, and/or non-native, Shiga toxin effector polypeptide sequence (such as the Shiga toxin 1 A Subunit variant effector polypeptides shown in SEQ ID NOs: 4–6 or the Shiga-like toxin 2 A Subunit variant effector polypeptides shown in SEQ ID NOs: 7–18).

[338] In certain further embodiments, the Shiga toxin effector polypeptides of the invention comprise, consist of, or consist essentially of a full-length or truncated Shiga toxin A Subunit with at least one substitution of an immunogenic residue and/or within an epitope region, wherein at least one amino acid substitution is to a non-conservative amino acid (*see*, *e.g.*, Table C, *infra*) relative to a natively occurring amino acid positioned at one of the following native positions: 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1 or SEQ ID NO:2; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1 or SEQ ID NO:2; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1 or SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1 or SEQ ID NO:2; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1 or SEQ ID NO:2; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1 or SEQ ID NO:2; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1 or SEQ ID NO:2; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1 or SEQ ID NO:2; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 241 of SEQ ID NO:3; 242 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; 251 of SEQ ID NO:1 or SEQ ID NO:2; 264 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 265 of SEQ ID NO:1 or SEQ ID NO:2; and 286 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent position in a Shiga toxin A Subunit polypeptide, conserved Shiga toxin effector polypeptide sub-region, and/or non-native, Shiga toxin

effector polypeptide sequence (such as the Shiga toxin effector polypeptide of any one of SEQ ID NOs: 4–18).

[339] In certain embodiments, the Shiga toxin effector polypeptides of the invention comprise, consist essentially of, or consist of a full-length or truncated Shiga toxin A Subunit with one or more mutations as compared to the native amino acid residue sequence which comprises at least one amino acid substitution of an immunogenic residue and/or within an epitope region, wherein at least one substitution occurs at the natively positioned amino acid position selected from the group consisting of: 1 of SEQ ID NO:1 or SEQ ID NO:2; 11 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1, SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1, SEQ ID NO:2; 59 of SEQ ID NO:1, SEQ ID NO:2; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 110 of SEQ ID NO:1 or SEQ ID NO:2; 141 of SEQ ID NO:1 or SEQ ID NO:2; 147 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO:1 or SEQ ID NO:2; 248 of SEQ ID NO:1 or SEQ ID NO:2; and 251 of SEQ ID NO:1 or SEQ ID NO:2.

[340] In certain further embodiments, the Shiga toxin effector polypeptides of the invention comprise or consist essentially of a full-length or truncated Shiga toxin A Subunit with at least one amino acid substitution selected from the group consisting of: K1 to A, G, V, L, I, F, M and H; T4 to A, G, V, L, I, F, M, and S; D6 to A, G, V, L, I, F, S, and Q; S8 to A, G, V, I, L, F, and M; T8 to A, G, V, I, L, F, M, and S; T9 to A, G, V, I, L, F, M, and S; S9 to A, G, V, L, I, F, and M; K11 to A, G, V, L, I, F, M and H; T12 to A, G, V, I, L, F, M, and S; S33 to A, G, V, L, I, F, and M; S43 to A, G, V, L, I, F, and M; G44 to A and L; S45 to A, G, V, L, I, F, and M; T45 to A, G, V, L, I, F, and M; G46 to A and P; D47 to A, G, V, L, I, F, S, and Q; N48 to A, G, V, L, and M; L49 to A or G; F50; A51 to V; D53 to A, G, V, L, I, F, S, and Q; V54 to A, G, and L; R55 to A, G, V, L, I, F, M, Q, S, K, and H; G56 to A and P; I57 to A, G, M, and F; L57 to A, G, M, and F; D58 to A, G, V, L, I, F, S, and Q; P59 to A, G, and F; E60 to A, G, V, L, I, F, S, Q, N, D, M, and R; E61 to A, G, V, L, I, F, S, Q, N, D, M, and R; G62 to A; D94 to A, G, V, L, I, F, S, and Q; R84 to A, G, V, L, I, F, M, Q, S, K, and H; V88 to A and G; I88 to A, G, and V; D94; S96 to A, G, V, I, L, F, and M; T104 to A, G, V, I, L, F, M, and S; A105 to L; T107 to A, G, V, I, L, F, M, and S; S107 to A, G, V, L, I, F, and M; L108 to A, G, and M; S109 to A, G, V, I, L, F, and M; T109 to A, G, V, I, L, F, M, and S; G110 to A; D111 to A, G, V, L, I, F, S, and Q; S112 to A, G, V, L, I, F, and M; D141 to A, G, V, L, I, F, S, and Q; G147 to A; V154 to A and G; R179 to A, G, V, L, I, F, M, Q, S, K, and H; T180 to A, G, V, L, I, F, M, and S; T181 to A, G, V, L, I, F, M, and S; D183 to A, G, V, L, I, F, S, and Q; D184 to A, G, V, L, I, F, S, and Q; L185 to A, G, and V; S186 to A, G, V, I, L, F, and M; G187 to A; R188 to A, G, V, L, I, F, M, Q, S, K, and H; S189 to A, G, V, I, L, F, and M; D197 to A, G, V, L, I, F, S, and Q; D198 to A, G, V, L, I, F, S, and Q; R204 to A, G, V, L, I, F, M, Q, S, K, and H; R205 to A, G, V, L, I, F, M, Q, S, K and H; C242 to A, G, V, and S; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to A, G, V, L, I, F, M, Q, S, K, and H; R250 to A, G, V, L, I, F, M, Q, S, K, and H; R251 to A, G, V, L, I, F, M, Q, S, K, and H; C262 to A, G, V, and S; D264 to A, G, V, L, I, F, S, and Q; G264 to A; and T286 to A, G, V, L, I, F, M, and S.

[341] In certain further embodiments, the Shiga toxin effector polypeptides of the invention comprise, consist of, or consist essentially of a full-length or truncated Shiga toxin A Subunit with at least one (such as at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen or more) of the following amino acid substitutions: K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, A51V, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, R188A, R188L, S189A, D198A, R204A, R205A, C242S, S247I, Y247A, R247A, R248A, R250A, R251A, or D264A, G264A, T286A, and/or T286I. In certain further embodiments, the Shiga toxin effector polypeptides of the invention comprise, consist essentially of, or consist of a full-length or truncated Shiga toxin A Subunit with at least one (such as at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen or more) of the following amino acid substitutions: K1A, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, G147A, R188A, C242S, R248A, and R251A. These epitope disrupting substitutions may be combined to form a de-immunized, Shiga toxin effector polypeptide with multiple substitutions per epitope region and/or multiple epitope regions disrupted while still retaining Shiga toxin effector function. For example, substitutions at the natively positioned K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, A51V, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, R188A, R188L, S189A, D198A, R204A, R205A, C242S, S247I, Y247A, R247A, R248A, R250A, R251A, or D264A, G264A, T286A, and/or T286I may be combined, where possible, with substitutions at the natively positioned residues K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, A51V, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, R188A, R188L, S189A, D198A, R204A, R205A, C242S, S247I, Y247A, R247A, R248A, R250A, R251A, or D264A, G264A, T286A, and/or T286I to create de-immunized, Shiga toxin effector polypeptides of the invention. For example, the Shiga toxin effector polypeptides of the invention may comprise, consist essentially of, or consist of a full-length or truncated Shiga toxin A Subunit comprising the following substitutions at native positions in a Shiga toxin A Subunit: K1A, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, G147A, C242S, R248A, and R251A. These substitutions correspond to those present in the Shiga toxin effector

polypeptide of the exemplary cell-targeting molecule shown in any one of SEQ ID NOs: 24–27 and 97–100. For example, the Shiga toxin effector polypeptides of the invention may comprise, consist essentially of, or consist of a full-length or truncated Shiga toxin A Subunit comprising the following substitutions at native positions in a Shiga toxin A Subunit: S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, R188A, C242S, R248A, and R251A. These substitutions correspond to those present in the Shiga toxin effector polypeptide of the exemplary cell-targeting molecule shown in any one of SEQ ID NOs: 28–29, 31–32, 34, 36, 101–102, 104–105, 106, and 108. For example, the Shiga toxin effector polypeptides of the invention may comprise, consist essentially of, or consist of a full-length or truncated Shiga toxin A Subunit comprising the following substitutions at native positions in a Shiga toxin A Subunit: S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, R188A, C242S, R248A, and R251A. These substitutions correspond to those present in the Shiga toxin effector polypeptide of the exemplary cell-targeting molecule shown in any one of SEQ ID NOs: 30 or 103.

[342] Any of the de-immunized, Shiga toxin effector polypeptide sub-regions and/or epitope disrupting mutations described herein may be used alone or in combination with each individual embodiment of the present invention, including methods of the present invention.

2. Protease-Cleavage Resistant, Shiga Toxin A Subunit Effector Polypeptides

[343] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises (1) a Shiga toxin A1 fragment derived region having a carboxy-terminus and (2) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region. Improving the stability of connections between the Shiga toxin component and other components of cell-targeting molecules, *e.g.*, cell-targeting binding regions, can improve their toxicity profiles after administration to organisms by reducing non-specific toxicities caused by the breakdown of the connection and loss of cell-targeting, such as, *e.g.*, as a result of proteolysis. In certain embodiments, the protease-cleavage resistant Shiga toxin effector polypeptide of the present invention has a carboxy-terminal truncation as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit.

[344] Shiga toxin A Subunits of members of the Shiga toxin family comprise a conserved, furin-cleavage site at the carboxy-terminal of their A1 fragment regions important for Shiga toxin function. Furin-cleavage site motifs and furin-cleavage sites can be identified by the skilled worker using standard techniques and/or by using the information herein.

[345] The model of Shiga toxin cytotoxicity is that intracellular proteolytic processing of Shiga toxin A Subunits by furin in intoxicated cells is essential for 1) liberation of the A1 fragment from the rest of the Shiga holotoxin, 2) escape of the A1 fragment from the endoplasmic reticulum by exposing a hydrophobic domain in the carboxy-terminus of the A1 fragment, and 3) enzymatic activation of the A1 fragment (*see* Johannes L, Römer W, *Nat Rev Microbiol* 8: 105-16 (2010)). The efficient liberation of the Shiga toxin A1 fragment from the A2 fragment and the rest of the components of the Shiga holotoxin in the endoplasmic reticulum of intoxicated cells is essential for efficient intracellular routing to the

cytosol, maximal enzymatic activity, efficient ribosome inactivation, and achieving optimal cytotoxicity, *i.e.* comparable to a wild-type Shiga toxin (*see e.g.* WO 2015/191764 and references therein).

[346] During Shiga toxin intoxication, the A Subunit is proteolytically cleaved by furin at the carboxy bond of a conserved arginine residue (*e.g.* the arginine residue at position 251 in StxA and SLT-1A and the arginine residue at position 250 in Stx2A and SLT-2A). Furin cleavage of Shiga toxin A Subunits occurs in endosomal and/or Golgi compartments. Furin is a specialized serine endoprotease which is expressed by a wide variety of cell types, in all human tissues examined, and by most animal cells. Furin cleaves polypeptides comprising accessible motifs often centered on the minimal, dibasic, consensus motif R-x-(R/K/x)-R. The A Subunits of members of the Shiga toxin family comprise a conserved, surface-exposed, extended loop structure (*e.g.* 242-261 in StxA and SLT-1A, and 241-260 in SLT-2) with a conserved S-R/Y-x-x-R motif which is cleaved by furin. The surface exposed, extended loop structure positioned at amino acid residues 242–261 in StxA is required for furin-induced cleavage of StxA, including features flanking the minimal, furin-cleavage motif R-x-x-R.

[347] Furin-cleavage motifs and furin-cleavage sites in Shiga toxin A Subunits and Shiga toxin effector polypeptides can be identified by the skilled worker using standard methods and/or by using the information herein. Furin cleaves the minimal, consensus motif R-x-x-R (Schalken J et al., *J Clin Invest* 80: 1545-9 (1987); Bresnahan P et al., *J Cell Biol* 111: 2851-9 (1990); Hatsuzawa K et al., *J Biol Chem* 265: 22075-8 (1990); Wise R et al., *Proc Natl Acad Sci USA* 87: 9378-82 (1990); Molloy S et al., *J Biol Chem* 267: 16396-402 (1992)). Consistent with this, many furin inhibitors comprise peptides comprising the motif R-x-x-R. An example of a synthetic inhibitor of furin is a molecule comprising the peptide R-V-K-R (SEQ ID NO:157) (Henrich S et al., *Nat Struct Biol* 10: 520-6 (2003)). In general, a peptide or protein comprising a surface accessible, dibasic amino acid motif with two positively charged, amino acids separated by two amino acid residues may be predicted to be sensitive to furin-cleavage with cleavage occurring at the carboxy bond of the last basic amino acid in the motif.

[348] Consensus motifs in substrates cleaved by furin have been identified with some degree of specificity. A furin-cleavage site motif has been described that comprises a region of twenty, continuous, amino acid residues, which can be labeled P14 through P6' (Tian S et al., *Int J Mol Sci* 12: 1060-5 (2011)) using the nomenclature described in Schechter I, Berger, A, *Biochem Biophys Res Commun* 32: 898-902 (1968). According to this nomenclature, the furin-cleavage site is at the carboxy bond of the amino acid residue designated P1, and the amino acid residues of the furin-cleavage motif are numbered P2, P3, P4, etc., in the direction going toward the amino-terminus from this reference P1 residue. The amino acid residues of the motif going toward the carboxy-terminus from the P1 reference residue are numbered with the prime notation P2', P3', P4', etc. Using this nomenclature, the P6 to P2' region delineates the core substrate of the furin cleavage motif which is bound by the enzymatic domain of furin. The two flanking regions P14 to P7 and P3' to P6' are often rich in polar, amino acid residues to increase the accessibility to the core furin cleavage site located between them.

[349] A general, furin-cleavage site is often described by the consensus motif R-x-x-R which corresponds to P4-P3-P2-P1; where "R" represents an arginine residue (*see* Table A, *supra*), a dash "-"

represents a peptide bond, and a lowercase “x” represents any amino acid residue. However, other residues and positions may help to further define furin-cleavage motifs. A slightly more refined furin-cleavage site, consensus motif is often reported as the consensus motif R-x-[K/R]-R (where a forward slash “/” means “or” and divides alternative amino acid residues at the same position), which corresponds to P4-P3-P2-P1, because it was observed that furin has a strong preference for cleaving substrates containing this motif.

[350] In addition to the minimal, furin-cleavage site R-x-x-R, a larger, furin-cleavage motif has been described with certain amino acid residue preferences at certain positions. By comparing various known furin substrates, certain physicochemical properties have been characterized for the amino acid residues in a 20 amino acid residue long, furin-cleavage site motif. The P6 to P2’ region of the furin-cleavage motif delineates the core furin-cleavage site which physically interacts with the enzymatic domain of furin. The two flanking regions P14 to P7 and P3’ to P6’ are often hydrophilic being rich in polar, amino acid residues to increase the surface accessibility of the core furin-cleavage site located between them.

[351] In general, the furin-cleavage motif region from position P5 to P1 tends to comprise amino acid residues with a positive charge and/or high isoelectric points. In particular, the P1 position, which marks the position of furin proteolysis, is generally occupied by an arginine but other positively charged, amino acid residues may occur in this position. Positions P2 and P3 tend to be occupied by flexible, amino acid residues, and in particular P2 tends to be occupied by arginine, lysine, or sometimes by very small and flexible amino acid residues like glycine. The P4 position tends to be occupied by positively charged, amino acid residues in furin substrates. However, if the P4 position is occupied by an aliphatic, amino acid residue, then the lack of a positively charged, functional group can be compensated for by a positively charged residue located at position(s) P5 and/or P6. Positions P1’ and P2’ are commonly occupied by aliphatic and/or hydrophobic amino acid residues, with the P1’ position most commonly being occupied by a serine.

[352] The two, hydrophilic, flanking regions tend to be occupied by amino acid residues which are polar, hydrophilic, and have smaller amino acid functional groups; however, in certain verified furin substrates, the flanking regions do not contain any hydrophilic, amino acid residues (*see* Tian S, *Biochem Insights* 2: 9-20 (2009)).

[353] The twenty amino acid residue, furin-cleavage motif and furin-cleavage site found in native, Shiga toxin A Subunits at the junction between the Shiga toxin A1 fragment and A2 fragment is well characterized in certain Shiga toxins. For example in StxA (SEQ ID NO:2) and SLT-1A (SEQ ID NO:1) or another Shiga toxin 1 A Subunit effector polypeptide (*e.g.* SEQ ID NOs: 4–6), this furin-cleavage motif is natively positioned from L238 to F257, and in SLT-2A (SEQ ID NO:3 or Shiga toxin effector polypeptides based on Shiga-like toxin 2 A Subunit variants (*e.g.* SEQ ID NOs: 7–18), this furin-cleavage motif is natively positioned from V237 to Q256. Based on amino acid homology, experiment, and/or furin-cleavage assays described herein, the skilled worker can identify furin-cleavage motifs in other native, Shiga toxin A Subunits or Shiga toxin effector polypeptides, where the motifs are actual

furin-cleavage motifs or are predicted to result in the production of A1 and A2 fragments after furin cleavage of those molecules within a eukaryotic cell.

[354] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises (1) a Shiga toxin A1 fragment derived polypeptide having a carboxy-terminus and (2) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment derived polypeptide. The carboxy-terminus of a Shiga toxin A1 fragment derived polypeptide may be identified by the skilled worker by using techniques known in the art, such as, *e.g.*, by using protein sequence alignment software to identify (i) a furin-cleavage motif conserved with a naturally occurring Shiga toxin, (ii) a surface exposed, extended loop conserved with a naturally occurring Shiga toxin, and/or (iii) a stretch of amino acid residues which are predominantly hydrophobic (*i.e.* a hydrophobic “patch”) that may be recognized by the ERAD system.

[355] A protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention (1) may be completely lacking any furin-cleavage motif at a carboxy-terminus of its Shiga toxin A1 fragment region and/or (2) comprise a disrupted furin-cleavage motif at the carboxy-terminus of its Shiga toxin A1 fragment region and/or region derived from the carboxy-terminus of a Shiga toxin A1 fragment. A disruption of a furin-cleavage motif include various alterations to an amino acid residue in the furin-cleavage motif, such as, *e.g.*, a post-translation modification(s), an alteration of one or more atoms in an amino acid functional group, the addition of one or more atoms to an amino acid functional group, the association to a non-proteinaceous moiety(ies), and/or the linkage to an amino acid residue, peptide, polypeptide such as resulting in a branched proteinaceous structure.

[356] Protease-cleavage resistant, Shiga toxin effector polypeptides may be created from a Shiga toxin effector polypeptide and/or Shiga toxin A Subunit polypeptide, whether naturally occurring or not, using a method described herein, described in WO 2015/191764, and/or known to the skilled worker, wherein the resulting molecule still retains one or more Shiga toxin A Subunit functions.

[357] For purposes of the present invention with regard to a furin-cleavage site or furin-cleavage motif, the term “disruption” or “disrupted” refers to an alteration from the naturally occurring furin-cleavage site and/or furin-cleavage motif, such as, *e.g.*, a mutation, that results in a reduction in furin-cleavage proximal to the carboxy-terminus of a Shiga toxin A1 fragment region, or identifiable region derived thereof, as compared to the furin-cleavage of a wild-type Shiga toxin A Subunit or a polypeptide derived from a wild-type Shiga toxin A Subunit comprising only wild-type polypeptide sequences. An alteration to an amino acid residue in the furin-cleavage motif includes a mutation in the furin-cleavage motif, such as, *e.g.*, a deletion, insertion, inversion, substitution, and/or carboxy-terminal truncation of the furin-cleavage motif, as well as a post-translation modification, such as, *e.g.*, as a result of glycosylation, albumination, and the like which involve conjugating or linking a molecule to the functional group of an amino acid residue. Because the furin-cleavage motif is comprised of about twenty, amino acid residues, in theory, alterations, modifications, mutations, deletions, insertions, and/or truncations involving one or more amino acid residues of any one of these twenty positions might result in a reduction of furin-cleavage sensitivity (Tian S et al., *Sci Rep* 2: 261 (2012)). The disruption of a furin-cleavage site and/or

furin-cleavage motif may or may not increase resistance to cleavage by other proteases, such as, *e.g.*, trypsin and extracellular proteases common in the vascular system of mammals. The effects of a given disruption to cleavage sensitivity of a given protease may be tested by the skilled worker using techniques known in the art.

5 [358] For purposes of the present invention, a "disrupted furin-cleavage motif" is furin-cleavage motif comprising an alteration to one or more amino acid residues derived from the 20 amino acid residue region representing a conserved, furin-cleavage motif found in native, Shiga toxin A Subunits at the junction between the Shiga toxin A1 fragment and A2 fragment regions and positioned such that furin cleavage of a Shiga toxin A Subunit results in the production of the A1 and A2 fragments; wherein the 10 disrupted furin-cleavage motif exhibits reduced furin cleavage in an experimentally reproducible way as compared to a reference molecule comprising a wild-type, Shiga toxin A1 fragment region fused to a carboxy-terminal polypeptide of a size large enough to monitor furin cleavage using the appropriate assay known to the skilled worker and/or described herein.

[359] Examples of types of mutations which can disrupt a furin-cleavage site and furin-cleavage motif 15 are amino acid residue deletions, insertions, truncations, inversions, and/or substitutions, including substitutions with non-standard amino acids and/or non-natural amino acids. In addition, furin-cleavage sites and furin-cleavage motifs can be disrupted by mutations comprising the modification of an amino acid by the addition of a covalently-linked structure which masks at least one amino acid in the site or motif, such as, *e.g.*, as a result of PEGylation, the coupling of small molecule adjuvants, and/or site-20 specific albumination.

[360] If a furin-cleavage motif has been disrupted by mutation and/or the presence of non-natural amino acid residues, certain disrupted furin-cleavage motifs may not be easily recognizable as being related to any furin-cleavage motif; however, the carboxy-terminus of the Shiga toxin A1 fragment derived region will be recognizable and will define where the furin-cleavage motif would be located were 25 it not disrupted. For example, a disrupted furin-cleavage motif may comprise less than the twenty, amino acid residues of the furin-cleavage motif due to a carboxy-terminal truncation as compared to a Shiga toxin A Subunit and/or Shiga toxin A1 fragment.

[361] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises (1) a Shiga toxin A1 fragment derived polypeptide having a carboxy-terminus and (2) a disrupted furin-30 cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment polypeptide region; wherein the Shiga toxin effector polypeptide (and any cell-targeting molecule comprising it) is more furin-cleavage resistant as compared to a reference molecule, such as, *e.g.*, a wild-type Shiga toxin polypeptide comprising the carboxy-terminus of an A1 fragment and/or the conserved, furin-cleavage motif between A1 and A2 fragments. For example, a reduction in furin cleavage of one molecule compared to a 35 reference molecule may be determined using an *in vitro*, furin-cleavage assay described in the Examples below, conducted using the same conditions, and then performing a quantitation of the band density of any fragments resulting from cleavage to quantitatively measure in change in furin cleavage.

[362] In certain embodiments, the Shiga toxin effector polypeptide is more resistant to furin-cleavage *in vitro* and/or *in vivo* as compared to a wild-type, Shiga toxin A Subunit.

[363] In general, the protease-cleavage sensitivity of a cell-targeting molecule of the present invention is tested by comparing it to the same molecule having its furin-cleavage resistant, Shiga toxin effector polypeptide replaced with a wild-type, Shiga toxin effector polypeptide comprising a Shiga toxin A1 fragment. In certain embodiments, the molecules of the present invention comprising a disrupted furin-cleavage motif exhibits a reduction in *in vitro* furin cleavage of 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98% or greater compared to a reference molecule comprising a wild-type, Shiga toxin A1 fragment fused at its carboxy-terminus to a peptide or polypeptide, such as, *e.g.*, the reference molecule SLT-1A-WT::scFv-1 described in Example 2, below.

[364] Several furin-cleavage motif disruptions have been described. For example, mutating the two conserved arginines to alanines in the minimal R-x-x-R motif completely blocked processing by furin and/or furin-like proteases (*see e.g* Duda A et al., *J Virology* 78: 13865-70 (2004)). Because the furin-cleavage site motif is comprised of about twenty amino acid residues, in theory, certain mutations involving one or more of any one of these twenty, amino acid residue positions might abolish furin cleavage or reduce furin cleavage efficiency (*see e.g.* Tian S et al., *Sci Rep* 2: 261 (2012)).

[365] In certain embodiments, the molecules of the present invention comprise a Shiga toxin effector polypeptide derived from at least one A Subunit of a member of the Shiga toxin family wherein the Shiga toxin effector polypeptide comprises a disruption in one or more amino acids derived from the conserved, highly accessible, protease-cleavage sensitive loop of Shiga toxin A Subunits. For example, in StxA and SLT-1A, this highly accessible, protease-sensitive loop is natively positioned from amino acid residues 242 to 261, and in SLT-2A, this conserved loop is natively positioned from amino acid residues 241 to 260. Based on polypeptide sequence homology, the skilled worker can identify this conserved, highly accessible loop structure in other Shiga toxin A Subunits. Certain mutations to the amino acid residues in this loop can reduce the accessibility of certain amino acid residues within the loop to proteolytic cleavage and this might reduce furin-cleavage sensitivity.

[366] In certain embodiments, a molecule of the present invention comprises a Shiga toxin effector polypeptide comprising a disrupted furin-cleavage motif comprising a mutation in the surface-exposed, protease sensitive loop conserved among Shiga toxin A Subunits. In certain further embodiments, a molecule of the present invention comprises a Shiga toxin effector polypeptide comprising a disrupted furin-cleavage motif comprising a mutation in this protease-sensitive loop of Shiga toxin A Subunits, the mutation which reduce the surface accessibility of certain amino acid residues within the loop such that furin-cleavage sensitivity is reduced.

[367] In certain embodiments, the disrupted furin-cleavage motif of a Shiga toxin effector polypeptide of the present invention comprises a disruption in terms of existence, position, or functional group of one or both of the consensus amino acid residues P1 and P4, such as, *e.g.*, the amino acid residues in positions 1 and 4 of the minimal furin-cleavage motif R/Y-x-x-R. For example, mutating one or both of the two arginine residues in the minimal, furin consensus site R-x-x-R to alanine will disrupt a furin-

cleavage motif and prevent furin-cleavage at that site. Similarly, amino acid residue substitutions of one or both of the arginine residues in the minimal furin-cleavage motif R-x-x-R to any non-conservative amino acid residue known to the skilled worker will reduced the furin-cleavage sensitivity of the motif. In particular, amino acid residue substitutions of arginine to any non-basic amino acid residue which lacks a positive charge, such as, *e.g.*, A, G, P, S, T, D, E, Q, N, C, I, L, M, V, F, W, and Y, will result in a disrupted furin-cleavage motif.

[368] In certain embodiments, the disrupted furin-cleavage motif of a Shiga toxin effector polypeptide of the present invention comprises a disruption in the spacing between the consensus amino acid residues P4 and P1 in terms of the number of intervening amino acid residues being other than two, and, thus, changing either P4 and/or P1 into a different position and eliminating the P4 and/or P1 designations. For example, deletions within the furin-cleavage motif of the minimal furin-cleavage site or the core, furin-cleavage motif will reduce the furin-cleavage sensitivity of the furin-cleavage motif.

[369] In certain embodiments, the disrupted furin-cleavage motif comprises one or more amino acid residue substitutions, as compared to a wild-type, Shiga toxin A Subunit. In certain further embodiments, the disrupted furin-cleavage motif comprises one or more amino acid residue substitutions within the minimal furin-cleavage site R/Y-x-x-R, such as, *e.g.*, for StxA and SLT-1A (and other Shiga toxin 1 A Subunit variants) derived Shiga toxin effector polypeptides, the natively positioned amino acid residue R248 substituted with any non-positively charged, amino acid residue and/or R251 substituted with any non-positively charged, amino acid residue; and for SLT-2A (and other Shiga-like toxin 2 A Subunit variants) derived Shiga toxin effector polypeptides, the natively positioned amino acid residue R/Y247 substituted with any non-positively charged, amino acid residue and/or R250 substituted with any non-positively charged, amino acid residue. In certain further embodiments, the disrupted furin-cleavage motif comprises one or more amino acid residue substitutions within the minimal furin-cleavage site R/Y-x-x-R, such as, *e.g.*, for StxA and SLT-1A derived Shiga toxin effector polypeptides (and other Shiga toxin 1 A Subunit variants), the natively positioned amino acid residues R248 and R251 are substituted with an alanine residue; and for SLT-2A derived Shiga toxin effector polypeptides (and other Shiga-like toxin 2 A Subunit variants), the natively positioned amino acid residues R/Y247 and R250 substituted with an alanine residue.

[370] In certain embodiments, the disrupted furin-cleavage motif comprises an un-disrupted, minimal furin-cleavage site R/Y-x-x-R but instead comprises a disrupted flanking region, such as, *e.g.*, amino acid residue substitutions in one or more amino acid residues in the furin-cleavage motif flanking regions natively position at, *e.g.*, 241–247 and/or 252–259. In certain further embodiments, the disrupted furin cleavage motif comprises a substitution of one or more of the amino acid residues located in the P1–P6 region of the furin-cleavage motif; mutating P1' to a bulky amino acid, such as, *e.g.*, R, W, Y, F, and H; and mutating P2' to a polar and hydrophilic amino acid residue; and substituting one or more of the amino acid residues located in the P1'–P6' region of the furin-cleavage motif with one or more bulky and hydrophobic amino acid residues

[371] In certain embodiments, the disruption of the furin-cleavage motif comprises a deletion, insertion, inversion, and/or mutation of at least one amino acid residue within the furin-cleavage motif. In certain embodiments, a protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention may comprise a disruption of the amino acid sequence natively positioned at 248–251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 A Subunit variant (*e.g.* SEQ ID NOs: 4–6) or at 247–250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant (*e.g.* SEQ ID NOs: 7–18) or the equivalent position in a conserved Shiga toxin effector polypeptide and/or non-native Shiga toxin effector polypeptide sequence. In certain further embodiments, protease-cleavage resistant, Shiga toxin effector polypeptides comprise a disruption which comprises a deletion of at least one amino acid within the furin-cleavage motif. In certain further embodiments, protease-cleavage resistant, Shiga toxin effector polypeptides comprise a disruption which comprises an insertion of at least one amino acid within the protease-cleavage motif region. In certain further embodiments, the protease-cleavage resistant, Shiga toxin effector polypeptides comprise a disruption which comprises an inversion of amino acids, wherein at least one inverted amino acid is within the protease motif region. In certain further embodiments, the protease-cleavage resistant, Shiga toxin effector polypeptides comprise a disruption which comprises a mutation, such as an amino acid substitution to a non-standard amino acid or an amino acid with a chemically modified side chain. Examples of single amino acid substitutions are provided in the Examples below.

[372] In certain embodiments of the molecules of the present invention, the disrupted furin-cleavage motif comprises the deletion of nine, ten, eleven or more of the carboxy-terminal amino acid residues within the furin-cleavage motif. In these embodiments, the disrupted furin-cleavage motif will not comprise a furin-cleavage site or a minimal furin-cleavage motif. In other words, certain embodiments lack a furin-cleavage site at the carboxy-terminus of the A1 fragment region.

[373] In certain embodiments, the disrupted furin-cleavage motif comprises both an amino acid residue deletion and an amino acid residue substitution as compared to a wild-type, Shiga toxin A Subunit. In certain further embodiments, the disrupted furin-cleavage motif comprises one or more amino acid residue deletions and substitutions within the minimal furin-cleavage site R/Y-x-x-R, such as, *e.g.*, for StxA and SLT-1A (and other Shiga toxin 1 A Subunit variants) derived Shiga toxin effector polypeptides, the natively positioned amino acid residue R248 substituted with any non-positively charged, amino acid residue and/or R251 substituted with any non-positively charged, amino acid residue; and for SLT-2A (and other Shiga-like toxin A Subunit 2 variants) derived Shiga toxin effector polypeptides, the natively positioned amino acid residue R/Y247 substituted with any non-positively charged, amino acid residue and/or R250 substituted with any non-positively charged, amino acid residue.

[374] In certain embodiments, the disrupted furin-cleavage motif comprises an amino acid residue deletion and an amino acid residue substitution as well as a carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit. In certain further embodiments, the disrupted furin-cleavage motif comprises one or more amino acid residue deletions and substitutions within the minimal furin-cleavage

site R/Y-x-x-R, such as, *e.g.*, for StxA and SLT-1A (and other Shiga toxin 1 A Subunit variants) derived Shiga toxin effector polypeptides, the natively positioned amino acid residue R248 substituted with any non-positively charged, amino acid residue and/or R251 substituted with any non-positively charged, amino acid residue; and for SLT-2A (and other Shiga-like toxin A Subunit 2 variants) derived Shiga toxin effector polypeptides, the natively positioned amino acid residue R/Y247 substituted with any non-positively charged, amino acid residue and/or R250 substituted with any non-positively charged, amino acid residue.

[375] In certain further embodiments, the disrupted furin-cleavage motif comprises both an amino acid substitution within the minimal furin-cleavage site R/Y-x-x-R and a carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit, such as, *e.g.*, for StxA and SLT-1A (and other Shiga toxin 1 A Subunit variants) derived Shiga toxin effector polypeptides, truncations ending at the natively amino acid position 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater and comprising the natively positioned amino acid residue R248 and/or R251 substituted with any non-positively charged, amino acid residue where appropriate; and for SLT-2A (and other Shiga-like toxin A Subunit 2 variants) derived Shiga toxin effector polypeptides, truncations ending at the natively amino acid position 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater and comprising the natively positioned amino acid residue R/Y247 and/or R250 substituted with any non-positively charged, amino acid residue where appropriate. In certain further embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin A1 fragment region as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit; wherein the carboxy-terminal truncation ends at the natively amino acid position 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater; and wherein the disrupted furin-cleavage motif comprises the natively positioned amino acid residue R248 and/or R251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2) or another Shiga toxin 1 A Subunit variant (*see e.g.* SEQ ID NOs: 4–6), or the natively positioned amino acid residue R/Y247 and/or R250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 A Subunit effector polypeptide variant (*e.g.* SEQ ID NOs: 7–18) substituted with an alanine residue. In certain further embodiments, the furin-cleavage motif is disrupted by a carboxy-terminal truncation of the Shiga toxin A1 fragment region as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit; wherein the carboxy-terminal truncation ends at the natively amino acid position 250, 249, 248, 247, or less. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, or 261. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 250. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 251. In certain embodiments, the carboxy-terminal truncation ends at the

natively amino acid position 252. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 253. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 254. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 255. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 256. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 257. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 258. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 259. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 260. In certain embodiments, the carboxy-terminal truncation ends at the natively amino acid position 261.

[376] In certain embodiments, the disrupted furin-cleavage motif comprises an insertion of one or more amino acid residues as compared to a wild-type, Shiga toxin A Subunit as long as the inserted amino residue(s) does not create a *de novo* furin-cleavage site. In certain embodiments, the insertion of one or more amino acid residues disrupts the natural spacing between the arginine residues in the minimal, furin-cleavage site R/Y-x-x-R, such as, *e.g.*, StxA and SLT-1A (and other Shiga toxin 1 A Subunit variants) derived polypeptides comprising an insertion of one or more amino acid residues at 249 or 250 and thus between R248 and R251; or SLT-2A derived polypeptides (and other Shiga-like toxin 2 A Subunit variants) comprising an insertion of one or more amino acid residues at 248 or 249 and thus between R/Y247 and R250.

[377] In certain embodiments, the disrupted furin-cleavage motif comprises both an amino acid residue insertion and a carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit. In certain embodiments, the disrupted furin-cleavage motif comprises both an amino acid residue insertion and an amino acid residue substitution as compared to a wild-type, Shiga toxin A Subunit. In certain embodiments, the disrupted furin-cleavage motif comprises both an amino acid residue insertion and an amino acid residue deletion as compared to a wild-type, Shiga toxin A Subunit.

[378] In certain embodiments, the disrupted furin-cleavage motif comprises an amino acid residue deletion, an amino acid residue insertion, and an amino acid residue substitution as compared to a wild-type, Shiga toxin A Subunit.

[379] In certain embodiments, the disrupted furin-cleavage motif comprises an amino acid residue deletion, insertion, substitution, and carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit.

[380] In certain embodiments, the Shiga toxin effector polypeptide comprising a disrupted furin-cleavage motif is directly fused by a peptide bond to a molecular moiety comprising an amino acid, peptide, and/or polypeptide wherein the fused structure involves a single, continuous polypeptide. In these fusion embodiments, the amino acid sequence following the disrupted furin-cleavage motif should not create a *de novo*, furin-cleavage site at the fusion junction.

[381] Any of the above protease-cleavage resistant, Shiga toxin effector polypeptide sub-regions and/or disrupted furin-cleavage motifs may be used alone or in combination with each individual embodiment of the present invention, including methods of the present invention.

5 3. T-Cell Hyper-Immunized, Shiga Toxin A Subunit Effector Polypeptides

[382] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises an embedded or inserted epitope-peptide and a Shiga toxin A1 fragment derived region. In certain further embodiments, the epitope-peptide is a heterologous, T-cell epitope-peptide, such as, *e.g.*, an epitope considered heterologous to Shiga toxin A Subunits. In certain further embodiments, the Shiga 10 toxin effector polypeptide of the present invention comprises an embedded or inserted epitope-peptide within the Shiga toxin A1 fragment region. In certain further embodiments, the epitope-peptide is a CD8+ T-cell epitope. In certain further embodiments, the CD8+ T-cell epitope-peptide has a binding affinity to a MHC class I molecule characterized by a dissociation constant (K_D) of 10^{-4} molar or less and/or the resulting MHC class I-epitope-peptide complex has a binding affinity to a T-cell receptor 15 (TCR) characterized by a dissociation constant (K_D) of 10^{-4} molar or less.

[383] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises an embedded or inserted, heterologous, T-cell epitope, such as, *e.g.*, a human CD8+ T-cell epitope. In certain further embodiments, the heterologous, T-cell epitope is embedded or inserted so as to disrupt an endogenous epitope or epitope region (*e.g.* a B-cell epitope and/or CD4+ T-cell epitope) identifiable in a 20 naturally occurring Shiga toxin polypeptide or parental Shiga toxin effector polypeptide from which the Shiga toxin effector polypeptide of the present invention is derived. For example, the Shiga toxin effector polypeptide of the present invention may comprise an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region within the Shiga toxin A1 fragment derived region.

25 [384] For certain embodiments of the present invention, the Shiga toxin effector polypeptide (and any cell-targeting molecule comprising it) is CD8+ T-cell hyper-immunized, such as, *e.g.*, as compared to a wild-type Shiga toxin polypeptide. The CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptides of the present invention each comprise an embedded or inserted T-cell epitope-peptide. Hyper-immunized, Shiga toxin effector polypeptides can be created from Shiga toxin effector 30 polypeptides and/or Shiga toxin A Subunit polypeptides, whether naturally occurring or not, using a method described herein, described in WO 2015/113005, and/or known to the skilled worker, wherein the resulting molecule still retains one or more Shiga toxin A Subunit functions.

[385] For purposes of the claimed invention, a T-cell epitope is a molecular structure which is comprised by an antigenic peptide and can be represented by a linear, amino acid sequence. Commonly, 35 T-cell epitopes are peptides of sizes of eight to eleven amino acid residues (Townsend A, Bodmer H, *Annu Rev Immunol* 7: 601-24 (1989)); however, certain T-cell epitope-peptides have lengths that are smaller than eight or larger than eleven amino acids long (*see e.g.* Livingstone A, Fathman C, *Annu Rev Immunol* 5: 477-501 (1987); Green K et al., *Eur J Immunol* 34: 2510-9 (2004)). In certain embodiments,

the embedded or inserted epitope is at least seven amino acid residues in length. In certain embodiments, the embedded or inserted epitope is bound by a TCR with a binding affinity characterized by a K_D less than 10 mM (*e.g.* 1–100 μM) as caluclated using the formula in Stone J et al., *Immunology* 126: 165-76 (2009). However, it should be noted that the binding affinity within a given range between the MHC-epitope and TCR may not correlate with antigenicity and/or immunogenicity (*see e.g.* Al-Ramadi B et al., *J Immunol* 155: 662-73 (1995)), such as due to factors like MHC-peptide-TCR complex stability, MHC-peptide density and MHC-independent functions of TCR cofactors such as CD8 (Baker B et al., *Immunity* 13: 475-84 (2000); Hornell T et al., *J Immunol* 170: 4506-14 (2003); Woolridge L et al., *J Immunol* 171: 6650-60 (2003)).

[386] A heterologous, T-cell epitope is an epitope not already present in a wild-type Shiga toxin A Subunit; a naturally occurring Shiga toxin A Subunit; and/or a parental, Shiga toxin effector polypeptide used as a source polypeptide for modification by a method described herein, described in WO 2015/113005, and/or known to the skilled worker.

[387] A heterologous, T-cell epitope-peptide may be incorporated into a source polypeptide via numerous methods known to the skilled worker, including, *e.g.*, the processes of creating one or more amino acid substitutions within the source polypeptide, fusing one or more amino acids to the source polypeptide, inserting one or more amino acids into the source polypeptide, linking a peptide to the source polypeptide, and/or a combination of the aforementioned processes. The result of such a method is the creation of a modified variant of the source polypeptide which comprises one or more embedded or inserted, heterologous, T-cell epitope-peptides.

[388] T-cell epitopes may be chosen or derived from a number of source molecules for use in the present invention. T-cell epitopes may be created or derived from various naturally occurring proteins. T-cell epitopes may be created or derived from various naturally occurring proteins foreign to mammals, such as, *e.g.*, proteins of microorganisms. T-cell epitopes may be created or derived from mutated human proteins and/or human proteins aberrantly expressed by malignant human cells. T-cell epitopes may be synthetically created or derived from synthetic molecules (*see e.g.*, Carbone F et al., *J Exp Med* 167: 1767-9 (1988); Del Val M et al., *J Virol* 65: 3641-6 (1991); Appella E et al., *Biomed Pept Proteins Nucleic Acids* 1: 177-84 (1995); Perez S et al., *Cancer* 116: 2071-80 (2010)).

[389] Although any T-cell epitope-peptide is contemplated as being used as a heterologous, T-cell epitope of the present invention, certain epitopes may be selected based on desirable properties. One objective of the present invention is to create CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptides for administration to vertebrates, meaning that the heterologous, T-cell epitope is highly immunogenic and can elicit robust immune responses *in vivo* when displayed complexed with a MHC class I molecule on the surface of a cell. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises one or more, embedded or inserted, heterologous, T-cell epitopes which are CD8+ T-cell epitopes. A Shiga toxin effector polypeptide of the present invention that comprises a heterologous, CD8+ T-cell epitope is considered a CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptide.

[390] T-cell epitope components of the present invention may be chosen or derived from a number of source molecules already known to be capable of eliciting a vertebrate immune response. T-cell epitopes may be derived from various naturally occurring proteins foreign to vertebrates, such as, *e.g.*, proteins of pathogenic microorganisms and non-self, cancer antigens. In particular, infectious microorganisms may contain numerous proteins with known antigenic and/or immunogenic properties. Further, infectious microorganisms may contain numerous proteins with known antigenic and/or immunogenic sub-regions or epitopes.

[391] For example, the proteins of intracellular pathogens with mammalian hosts are sources for T-cell epitopes. There are numerous intracellular pathogens, such as viruses, bacteria, fungi, and single-cell eukaryotes, with well-studied antigenic proteins or peptides. T-cell epitopes can be selected or identified from human viruses or other intracellular pathogens, such as, *e.g.*, bacteria like mycobacterium, fungi like toxoplasmae, and protists like trypanosomes.

[392] For example, there are many immunogenic, viral peptide components of viral proteins from viruses that are infectious to humans. Numerous, human T-cell epitopes have been mapped to peptides within proteins from influenza A viruses, such as peptides in the proteins HA glycoproteins FE17, S139/1, CH65, C05, hemagglutin 1 (HA1), hemagglutinin 2 (HA2), nonstructural protein 1 and 2 (NS1 and NS 2), matrix protein 1 and 2 (M1 and M2), nucleoprotein (NP), neuraminidase (NA)), and many of these peptides have been shown to elicit human immune responses, such as by using *ex vivo* assay. Similarly, numerous, human T-cell epitopes have been mapped to peptide components of proteins from human cytomegaloviruses (HCMV), such as peptides in the proteins pp65 (UL83), UL128-131, immediate-early 1 (IE-1; UL123), glycoprotein B, tegument proteins, and many of these peptides have been shown to elicit human immune responses, such as by using *ex vivo* assays.

[393] Another example is there are many immunogenic, cancer antigens in humans. The CD8+ T-cell epitopes of cancer and/or tumor cell antigens can be identified by the skilled worker using techniques known in the art, such as, *e.g.*, differential genomics, differential proteomics, immunoproteomics, prediction then validation, and genetic approaches like reverse-genetic transfection (*see e.g.*, Admon A et al., *Mol Cell Proteomics* 2: 388-98 (2003); Purcell A, Gorman J, *Mol Cell Proteomics* 3: 193-208 (2004); Comber J, Philip R, *Ther Adv Vaccines* 2: 77-89 (2014)). There are many antigenic and/or immunogenic T-cell epitopes already identified or predicted to occur in human cancer and/or tumor cells. For example, T-cell epitopes have been predicted in human proteins commonly mutated or overexpressed in neoplastic cells, such as, *e.g.*, ALK, CEA, N-acetylglucosaminyl-transferase V (GnT-V), HCA587, HER2/neu, MAGE, Melan-A/MART-1, MUC-1, p53, and TRAG-3 (*see e.g.*, van der Bruggen P et al., *Science* 254: 1643-7 (1991); Kawakami Y et al., *J Exp Med* 180: 347-52 (1994); Fisk B et al., *J Exp Med* 181: 2109-17 (1995); Guilloux Y et al., *J Exp Med* 183: 1173 (1996); Skipper J et al., *J Exp Med* 183: 527 (1996); Brossart P et al., 93: 4309-17 (1999); Kawashima I et al., *Cancer Res* 59: 431-5 (1999); Papadopoulos K et al., *Clin Cancer Res* 5: 2089-93 (1999); Zhu B et al., *Clin Cancer Res* 9: 1850-7 (2003); Li B et al., *Clin Exp Immunol* 140: 310-9 (2005); Ait-Tahar K et al., *Int J Cancer* 118: 688-95 (2006); Akiyama Y et al., *Cancer Immunol Immunother* 61: 2311-9 (2012)). In addition, synthetic variants of T-cell epitopes

from human cancer cells have been created (*see e.g.*, Lazoura E, Apostolopoulos V, *Curr Med Chem* 12: 629-39 (2005); Douat-Casassus C et al., *J Med Chem* 50: 1598-609 (2007)).

[394] While any T-cell epitope may be used in the polypeptides and molecules of the present invention, certain T-cell epitopes may be preferred based on their known and/or empirically determined characteristics. For example, in many species, the MHC alleles in its genome encode multiple MHC-I molecular variants. Because MHC class I protein polymorphisms can affect antigen-MHC class I complex recognition by CD8+ T-cells, T-cell epitopes may be chosen for use in the present invention based on knowledge about certain MHC class I polymorphisms and/or the ability of certain antigen-MHC class I complexes to be recognized by T-cells having different genotypes.

[395] There are well-defined peptide-epitopes that are known to be immunogenic, MHC class I restricted, and/or matched with a specific human leukocyte antigen (HLA) variant(s). For applications in humans or involving human target cells, HLA-class I-restricted epitopes can be selected or identified by the skilled worker using standard techniques known in the art. The ability of peptides to bind to human MHC class I molecules can be used to predict the immunogenic potential of putative T-cell epitopes. The ability of peptides to bind to human MHC class I molecules can be scored using software tools. T-cell epitopes may be chosen for use as a heterologous, T-cell epitope component of the present invention based on the peptide selectivity of the HLA variants encoded by the alleles more prevalent in certain human populations. For example, the human population is polymorphic for the alpha chain of MHC class I molecules due to the varied alleles of the HLA genes from individual to individual. In certain T-cell epitopes may be more efficiently presented by a specific HLA molecule, such as, *e.g.*, the commonly occurring HLA variants encoded by the HLA-A allele groups HLA-A2 and HLA-A3.

[396] When choosing T-cell epitopes for use as a heterologous, T-cell epitope component of the present invention, multiple factors may be considered that can influence epitope generation and transport to receptive MHC class I molecules, such as, *e.g.*, the presence and epitope specificity of the following factors in the target cell: proteasome, ERAAP/ERAP1, tapasin, and TAPs.

[397] When choosing T-cell epitopes for use as a heterologous, T-cell epitope component of the present invention, epitope may be selected which best match the MHC class I molecules present in the cell-type or cell populations to be targeted. Different MHC class I molecules exhibit preferential binding to particular peptide sequences, and particular peptide-MHC class I variant complexes are specifically recognized by the t-cell receptors (TCRs) of effector T-cells. The skilled worker can use knowledge about MHC class I molecule specificities and TCR specificities to optimize the selection of heterologous, T-cell epitopes used in the present invention.

[398] In addition, multiple, immunogenic, T-cell epitopes for MHC class I presentation may be embedded in the same Shiga toxin effector polypeptide of the present invention, such as, *e.g.*, for use in the targeted delivery of a plurality of T-cell epitopes simultaneously.

[399] Any of the protease-cleavage resistant, Shiga toxin effector polypeptide sub-regions and/or disrupted furin-cleavage motifs described herein may be used alone or in combination with each individual embodiment of the present invention, including methods of the present invention.

C. Additional Exogenous Materials

[400] In certain embodiments, the cell-targeting molecules of the present invention comprises an additional exogeouns material. An "additional exogenous material" as used herein refers to one or more atoms or molecules, often not generally present in both Shiga toxins and native target cells, where the cell-targeting molecule of the present invention can be used to specifically transport such material to the interior of a cell. In one sense, the entire cell-targeting molecule of the invention is an exogenous material which will enter the cell; thus, the "additional" exogenous materials are heterologous materials linked to but other than the core cell-targeting molecule itself. Non-limiting examples of additional exogenous materials are radionucleides, peptides, detection promoting agents, proteins, small molecule chemotherapeutic agents, and polynucleotides.

[401] In certain embodiments of the cell-targeting molecules of the present invention, the additional exogenous material is one or more radionucleides, such as, *e.g.*, ^{211}At, ^{131}I, ^{125}I, ^{90}Y, ^{111}In, ^{186}Re, ^{188}Re, ^{153}Sm, ^{212}Bi, ^{32}P, ^{60}C, and/or radioactive isotopes of lutetium.

[402] In certain embodiments, the additional exogenous material comprises a proapoptotic peptide, polypeptide, or protein, such as, *e.g.*, BCL-2, caspases (*e.g.* fragments of caspase-3 or caspase-6), cytochromes, granzyme B, apoptosis-inducing factor (AIF), BAX, tBid (truncated Bid), and proapoptotic fragments or derivatives thereof (*see e.g.*, Ellerby H et al., *Nat Med* 5: 1032-8 (1999); Mai J et al., *Cancer Res* 61: 7709-12 (2001); Jia L et al., *Cancer Res* 63: 3257-62 (2003); Liu Y et al., *Mol Cancer Ther* 2: 1341-50 (2003); Perea S et al., *Cancer Res* 64: 7127-9 (2004); Xu Y et al., *J Immunol* 173: 61-7 (2004); Dälken B et al., *Cell Death Differ* 13: 576-85 (2006); Wang T et al., *Cancer Res* 67: 11830-9 (2007); Kwon M et al., *Mol Cancer Ther* 7: 1514-22 (2008); Qiu X et al., *Mol Cancer Ther* 7: 1890-9 (2008); Shan L et al., *Cancer Biol Ther* 11: 1717-22 (2008); Wang F et al., *Clin Cancer Res* 16: 2284-94 (2010); Kim J et al., *J Virol* 85: 1507-16 (2011)).

[403] In certain embodiments, the additional exogenous material comprises a protein or polypeptide comprising an enzyme. In certain other embodiments, the additional exogenous material is a nucleic acid, such as, *e.g.* a ribonucleic acid that functions as a small inhibiting RNA (siRNA) or microRNA (miRNA). In certain embodiments, the additional exogenous material is an antigen, such as antigens derived from pathogens, bacterial proteins, viral proteins, proteins mutated in cancer, proteins aberrantly expressed in cancer, or T-cell complementary determining regions. For example, exogenous materials include antigens, such as those characteristic of antigen-presenting cells infected by bacteria, and T-cell complementary determining regions capable of functioning as exogenous antigens. Exogenous materials comprising polypeptides or proteins may optionally comprise one or more antigens whether known or unknown to the skilled worker.

[404] In certain embodiments of the cell-targeting molecules of the present invention, all heterologous antigens and/or epitopes associated with the Shiga toxin effector polypeptide are arranged in the cell-targeting molecule amino-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region of the Shiga toxin effector polypeptide. In certain further embodiments, all heterologous antigens and/or

epitopes associated with the Shiga toxin effector polypeptide are associated, either directly or indirectly, with the Shiga toxin effector polypeptide at a position amino-terminal to the carboxy-terminus of the Shiga toxin A1 fragment region of the Shiga toxin effector polypeptide. In certain further embodiments, all additional exogenous material(s) that is an antigen is arranged amino-terminal to the Shiga toxin effector polypeptide, such as, *e.g.*, fused directly or indirectly to the amino terminus of the Shiga toxin effector polypeptide.

[405] In certain embodiments of the cell-targeting molecules of the present invention, the additional exogenous material is a cytotoxic agent, such as, *e.g.*, a small molecule chemotherapeutic agent, anti-neoplastic agent, cytotoxic antibiotic, alkylating agent, antimetabolite, topoisomerase inhibitor, and/or tubulin inhibitor. Non-limiting examples of cytotoxic agents suitable for use with the present invention include aziridines, cisplatins, tetrazines, procarbazine, hexamethylmelamine, vinca alkaloids, taxanes, camptothecins, etoposide, doxorubicin, mitoxantrone, teniposide, novobiocin, aclarubicin, anthracyclines, actinomycin, amanitin, amatoxins, bleomycin, centanamycin (indolecarboxamide), plicamycin, mitomycin, daunorubicin, epirubicin, idarubicins, dolastatins, maytansines, maytansionoids, duromycin, docetaxel, duocarmycins, adriamycin, calicheamicin, auristatins, pyrrolobenzodiazepines, pyrrolobenzodiazepine dimers (PBDs), carboplatin, 5-fluorouracil (5-FU), capecitabine, mitomycin C, paclitaxel, 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), rifampicin, cisplatin, methotrexate, gemcitabine, aceglatone, acetogenins (*e.g.* bullatacin and bullatacinone), aclacinomysins, AG1478, AG1571, aldophosphamide glycoside, alkyl sulfonates (*e.g.*, busulfan, improsulfan, and piposulfan), alkylating agents (*e.g.* thiotepa and cyclosphosphamide), aminolevulinic acid, aminopterin, amsacrine, ancitabine, anthramycin, arabinoside, azacitidine, azaserine, aziridines (*e.g.*, benzodopa, carboquone, meturedopa, and uredopa), azauridine, bestrabucil, bisantrene, bisphosphonates (*e.g.* clodronate), bleomycins, bortezomib, bryostatin, cactinomycin, callystatin, carabicin, carminomycin, carmofur, carmustine, carzinophilin, CC-1065, chlorambucil, chloranbucil, chlornaphazine, chlorozotocin, chromomycinis, chromoprotein enediyne antibiotic chromophores, CPT-11, cryptophycins (*e.g.* cryptophycin 1 and cryptophycin 8), cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunomycin, defofamine, demecolcine, detorubicin, diaziquone, 6-diazo-5-oxo-L-norleucine, dideoxyuridine, difluoromethylornithine (DMFO), doxifluridine, doxorubicins (*e.g.*, morpholinodoxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolinodoxorubicin, and deoxydoxorubicin), dynemicins, edatraxate, edatrexate, eleutherobins, elformithine, elliptinium acetate, enediyne antibiotics (*e.g.* calicheamicins), eniluracil, enocitabine, epirubicins, epothilone, esorubicins, esperamicins, estramustine, ethylenimines, 2-ethylhydrazide, etoglucid, fludarabine, folic acid analogues (*e.g.*, denopterin, methotrexate, pteropterin, and trimetrexate), folic acid replenishers (*e.g.* frolinic acid), fotemustine, fulvestrant, gacytosine, gallium nitrate, gefitinib, gemcitabine, hydroxyurea, ibandronate, ifosfamide, imatinib mesylate, erlotinib, fulvestrant, letrozole, PTK787/ZK 222584 (Novartis, Basel, CH), oxaliplatin, leucovorin, rapamycin, lapatinib, lonafarnib, sorafenib, methylamelamines (*e.g.*, altretamine, triethy lenemelamine, triethy lenephosphoramide, triethylenethiophosphoramide and trimethylomelamine), pancratistatins, sarcodictyins, spongistatins, nitrogen mustards (*e.g.*, chlorambucil,

chlornaphazine, cyclophosphamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard), nitrosureas (*e.g.*, carmustine, fotemustine, lomustine, nimustine, and ranimnustine), dynemicins, neocarzinostatin chromophores, anthramycin, detorubicin, epirubicins, marcellomycins, mitomycins (*e.g.* mitomycin C), mycophenolic acid, nogalamycins, olivomycins, peplomycins, potfiromycins, puromycins, quelamycins, rodorubicins, ubenimex, zinostatins, zorubicins, purine analogs (*e.g.*, fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine), pyrimidine analogs (*e.g.*, ancitabine, azacitidine, 6-azauridine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine), aceglatone, lentinan, lonidainine, maytansinoids (*e.g.* maytansins and ansamitocins), mitoguazone, mitoxantrone, mopidanmol, nitraerine, pentostatin, phenamet, pirarubicin, podophyllinic acid, 2-ethylhydrazide, rhizoxin, sizofuran, spirogermanium, tenuazonic acid, triaziquone, 2,2',2"trichlorotriethylamine, trichothecenes (*e.g.*, T-2 toxin, verracurin A, roridin A, and anguidine), urethan, vindesine, mannomustine, mitobronitol, mitolactol, pipobroman, arabinoside, cyclophosphamide, toxoids (*e.g.* paclitaxel and doxetaxel), 6-thioguanine, mercaptopurine, platinum, platinum analogs (*e.g.* cisplatin and carboplatin), etoposide (VP-16), mitoxantrone, vinorelbine, novantrone, daunomycin, xeloda, topoisomerase inhibitor RFS 2000, retinoids (*e.g.* retinoic acid), capecitabine, lomustine, losoxantrone, mercaptopurines, nimustine, nitraerine, rapamycin, razoxane, roridin A, spongistatins, streptonigrins, streptozocins, sutent, T-2 toxin, thiamiprine, thiotepa, toxoids (*e.g.* paclitaxel and doxetaxel), tubercidins, verracurin A, vinblastine, vincristine, and structural analogs of any of the aforementioned (*e.g.* synthetic analogs), and/or derivatives of any of the aforementioned (*see e.g.*, Lindell T et al., *Science* 170: 447-9 (1970); Remillard S et al., *Science* 189: 1002-5 (1975); Ravry M et al., *Am J Clin Oncol* 8: 148-50 (1985); Ravry M et al., *Cancer Treat Rep* 69: 1457-8 (1985); Sternberg C et al., *Cancer* 64: 2448-58 (1989); Bai R et al., *Biochem Pharmacol* 39: 1941-9 (1990); Boger D, Johnson D, *Proc Natl Acad Sci USA* 92: 3642-9 (1995); Beck J et al., *Leuk Lymphoma* 41: 117-24 (2001); Cassady J et al., *Chem Pharm Bull* (Tokyo) 52: 1-26 (2004); Sapra P et al., *Clin Cancer Res* 11: 5257-64 (2005); Okeley N et al., *Clinc Cancer Res* 16: 888-97 (2010); Oroudjev E et al., *Mol Cancer Ther* 9: 2700-13 (2010); Ellestad G, *Chirality* 23: 660-71 (2011); Kantarjian H et al., Lancet Oncol 13: 403-11 (2012); Moldenhauer G et al., *J Natl Cancer Inst* 104: 622-34 (2012); Meulendijks D et al., *Invest New Drugs* 34: 119-28 (2016)).

D. Structure-Function Relationships of Cell-Targeting Molecules of the Invention

[406] For certain embodiments of the cell-targeting molecules of the present invention, there specific structure-function relationships that have been observed, such as, *e.g.*, component relative orientation effects on cytotoxic potency; furin-cleavage sensitivity effects on *in vivo* tolerability at certain dosages; furin-cleavage sensitivity effects on *in vitro* stability; furin-cleavage sensitivity effects on *in vivo* half-life; and furin-cleavage sensitivity effects on *in vivo*, non-specific toxicity in multicellular organisms.

[407] In certain embodiments of the cell-targeting molecules of the present invention, the specific order or orientation of the Shiga toxin effector polypeptide region and binding region is fixed such that the binding region is located within the cell-targeting molecules more proximal to the carboxy-terminus of

the Shiga toxin effector polypeptide region than to the amino-terminus of the Shiga toxin effector polypeptide region. In certain embodiments of the cell-targeting molecules of the present invention, the arrangement of the Shiga toxin effector polypeptide region within the cell-targeting molecule is limited to being at and/or proximal to the amino-terminus of a polypeptide component of the cell-targeting molecule (*see* Figure 1). For example, certain embodiments of the cell-targeting molecule of the present invention comprise 1) a binding region oriented within the cell-targeting molecule at a position carboxy-terminal to the Shiga toxin effector polypeptide region, 2) a binding region associated with the Shiga toxin effector polypeptide region at a position distal from the amino-terminus of the Shiga toxin effector polypeptide region (*e.g.* distances of 50, 100, 200, or 250 amino acid residues or greater), 3) a binding region not sterically covering the amino-terminus of the Shiga toxin effector polypeptide region, and/or 4) a binding region not sterically hindering a structure(s) near the amino-terminus of the Shiga toxin effector polypeptide region (*see e.g.* Figure 1; WO 2015/138452). In certain further embodiments, the cell-targeting molecules of the present invention are capable of exhibiting more optimal cytotoxic potency, such as, *e.g.*, exhibiting a CD_{50} value which is 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, or higher than a related cell-targeting reference molecule comprising the same Shiga toxin A Subunit effector polypeptide region(s) and binding region(s), wherein the binding region is 1) amino-terminal to the Shiga toxin A Subunit effector polypeptide region, 2) associated with the Shiga toxin effector polypeptide region at a position proximal to the amino-terminus of the Shiga toxin effector polypeptide region (*e.g.* distances of less than 50, 40, 30, 20, or 10 amino acid residues or less), 3) not sterically covering the amino-terminus of the Shiga toxin effector polypeptide region, and/or 4) not sterically hindering a structure(s) near the amino-terminus of the Shiga toxin effector polypeptide region (*see e.g.* Figure 1; WO 2015/138452).

[408] In certain embodiments, the Shiga toxin A Subunit effector polypeptide of the present invention comprises a Shiga toxin A1 fragment derived region comprising a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment derived region (such as a disrupted furin-cleavage site located at the carboxy-terminus of a Shiga toxin A1 fragment region) (*see e.g.* Figure 1; WO 2015/191764). In certain further embodiments, the Shiga toxin effector polypeptide is more furin-cleavage resistant as compared to a related reference molecule, such as, *e.g.*, a molecule comprising a wild-type, Shiga toxin A Subunit or Shiga toxin A1 fragment (*see e.g.* WO 2015/191764). In certain further embodiments, the Shiga toxin effector polypeptide of the present invention exhibits a reduction in furin-cleavage reproducibly observed to be 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98%, 99%, or less (including 100% for no cleavage) than the furin-cleavage of a reference molecule observed in the same assay under the same conditions. In certain further embodiments, the Shiga toxin effector polypeptide is more cleavage resistant to a protease other than furin as compared to a related reference molecule, such as, *e.g.*, a molecule comprising a wild-type, Shiga toxin A Subunit or Shiga toxin A1 fragment.

[409] Certain cell-targeting molecules of the present invention exhibit cytotoxic potencies within 100-fold, 20-fold, 10-fold, 5-fold, or less than a reference molecule comprising a wild-type Shiga toxin

effector polypeptide region despite the lack of any compensatory structural feature for the disrupted furin-cleavage motif in the Shiga toxin effector polypeptide. For cell-targeting molecules comprising Shiga toxin A Subunit derived regions which do not maintain the furin cleavage event, *i.e.* molecules comprising Shiga toxin A Subunit derived components which are not cleaved by furin inside target cells, one alternative for preserving maximal cytotoxicity is compensation. Compensation for the lack of furin cleavage of a Shiga toxin A Subunit region in cytotoxic molecule might be accomplished by presenting the Shiga toxin A Subunit region in a "pre-processed" form. For example, a cell-targeting molecule comprising a Shiga toxin A Subunit region may be constructed such that the carboxy-terminus of the Shiga toxin A Subunit derived polypeptide is 1) proximal to a carboxy-terminus of the molecule and 2) matches or resembles a native Shiga toxin A1 fragment after cleavage by furin (*see* WO 2015/191764). Such compensation is not required in certain cell-targeting molecules of the present invention, rather it is intentionally avoided in order to provide one or more function(s), such as, *e.g.*, improved *in vivo* tolerability at certain dosages; increased *in vitro* stability; increased *in vivo* half-life; and/or reduced *in vivo*, non-specific toxicity in multicellular organisms. For certain embodiments, these beneficial function(s) are present without any significant reduction in cytotoxic potency of the cell-targeting molecule of the present invention as compared to a reference molecule comprising a wild-type Shiga toxin effector polypeptide.

[410] In certain embodiments, the cell-targeting molecule of the present invention comprises a Shiga toxin A Subunit effector polypeptide comprising a Shiga toxin A1 fragment derived region comprising a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment derived region (such as a disrupted furin-cleavage site located at the carboxy-terminus of a Shiga toxin A1 fragment region) (*see e.g.* Figure 1; WO 2015/191764) but do not comprise any compensatory protease cleavage site proximal to the carboxy-terminus of the Shiga toxin A1 fragment derived region and/or oriented between the Shiga toxin effector polypeptide and a relatively large, molecule moiety (*e.g.* a binding region of a size greater than 4.5 kDa, 6, kDa, 9 kDa, 12 kDa, 15 kDa, 20 kDa, 25 kDa, 28 kDa, 30 kDa, 41 kDa, or 50 kDa). In certain further embodiments, the cell-targeting molecule of the present invention comprises a Shiga toxin effector polypeptide which is more furin-cleavage resistant as compared to a related reference molecule, such as, *e.g.*, a molecule comprising a wild-type, Shiga toxin A Subunit or Shiga toxin A1 fragment (*see e.g.* WO 2015/191764). In certain further embodiments, the cell-targeting molecule of the present invention exhibits a reduction in furin-cleavage of 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98%, 99%, or 100% less than the furin-cleavage of a reference molecule observed in the same assay under the same conditions while the cell-targeting molecule exhibits a cytotoxic potency within 100-fold, 20-fold, 10-fold, 5-fold, or less than a reference molecule comprising a wild-type Shiga toxin effector polypeptide region. In certain further embodiments, the cell-targeting molecule of the present invention exhibits an improvement in *in vivo* tolerability as compared to a related reference molecule comprising a Shiga toxin effector polypeptide having a wild-type furin cleavage motif and/or wild-type furin cleavage site at the carboxy-terminus of its Shiga toxin A1 fragment region (*see e.g.* WO 2015/191764). For example, an increase in *in vivo* tolerability may be determined by comparing

measurements of mortality, signs of morbidity, and/or certain clinical signs in groups of laboratory animals administered different molecules at the same dosages (*see e.g.* Examples, *infra*; WO 2015/191764; WO 2016/196344).

[411] In certain embodiments, the cell-targeting molecule of the present invention comprises a Shiga toxin A Subunit effector polypeptide comprising a Shiga toxin A1 fragment derived region comprising a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment derived region (such as a disrupted furin-cleavage site located at the carboxy-terminus of a Shiga toxin A1 fragment derived region) (*see e.g.* Figure 1; WO 2015/191764). For certain further embodiments, the cell-targeting molecule of the present invention that comprise a cytotoxic component, the cell-targeting molecule exhibits reduced non-specific toxicity as compared to more protease-cleavage sensitive variants, which have greater propensity to break apart and thereby release the cytotoxic component from the binding region, especially when administered to living materials, such as, *e.g.*, a population of cells, a tissue, and/or an organism. Furthermore, certain protease-cleavage resistant, cell-targeting molecules of the present invention may exhibit increased, *in vivo*, half-lives after administration to living materials (*e.g.*, certain chordates) as compared to more protease-cleavage sensitive variants based on the protease-cleavage resistance conferred to the cell-targeting molecule by the disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment derived region.

III. Linkages Connecting Components of the Invention and/or Their Subcomponents

[412] Individual cell-targeting binding regions, Shiga toxin effector polypeptides, and/or components of the cell-targeting molecules present invention may be suitably linked to each other via one or more linkers well known in the art and/or described herein. Individual polypeptide subcomponents of the binding regions, *e.g.* heavy chain variable regions (V_H), light chain variable regions (V_L), CDR, and/or ABR regions, may be suitably linked to each other via one or more linkers well known in the art and/or described herein. Proteinaceous components of the invention, *e.g.*, multi-chain binding regions, may be suitably linked to each other or other polypeptide components of the invention via one or more linkers well known in the art. Peptide components of the invention, *e.g.*, KDEL family endoplasmic reticulum retention/retrieval signal motifs, may be suitably linked to another component of the invention via one or more linkers, such as a proteinaceous linker, which are well known in the art.

[413] Suitable linkers are generally those which allow each polypeptide component of the present invention to fold with a three-dimensional structure very similar to the polypeptide components produced individually without any linker or other component. Suitable linkers include single amino acids, peptides, polypeptides, and linkers lacking any of the aforementioned, such as various non-proteinaceous carbon chains, whether branched or cyclic.

[414] Suitable linkers may be proteinaceous and comprise one or more amino acids, peptides, and/or polypeptides. Proteinaceous linkers are suitable for both recombinant fusion proteins and chemically linked conjugates. A proteinaceous linker typically has from about 2 to about 50 amino acid residues, such as, *e.g.*, from about 5 to about 30 or from about 6 to about 25 amino acid residues. The length of the

linker selected will depend upon a variety of factors, such as, *e.g.*, the desired property or properties for which the linker is being selected. In certain embodiments, the linker is proteinaceous and is linked near the terminus of a protein component of the present invention, typically within about 20 amino acids of the terminus.

5 [415] Suitable linkers may be non-proteinaceous, such as, *e.g.* chemical linkers. Various non-proteinaceous linkers known in the art may be used to link cell-targeting binding regions to the Shiga toxin effector polypeptide components of the cell-targeting molecules of the present invention, such as linkers commonly used to conjugate immunoglobulin polypeptides to heterologous polypeptides. For example, polypeptide regions may be linked using the functional side chains of their amino acid residues

10 and carbohydrate moieties such as, *e.g.*, a carboxy, amine, sulfhydryl, carboxylic acid, carbonyl, hydroxyl, and/or cyclic ring group. For example, disulfide bonds and thioether bonds may be used to link two or more polypeptides. In addition, non-natural amino acid residues may be used with other functional side chains, such as ketone groups. Examples of non-proteinaceous chemical linkers include but are not limited to N-succinimidyl (4-iodoacetyl)-aminobenzoate, *S*-(*N*-succinimidyl) thioacetate

15 (SATA), N-succinimidyl-oxycarbonyl-cu-methyl-a-(2-pyridyldithio) toluene (SMPT), N-succinimidyl 4-(2-pyridyldithio)-pentanoate (SPP), succinimidyl 4-(N-maleimidomethyl) cyclohexane carboxylate (SMCC or MCC), sulfosuccinimidyl (4-iodoacetyl)-aminobenzoate, 4-succinimidyl-oxycarbonyl-α-(2-pyridyldithio) toluene, sulfosuccinimidyl-6-(α-methyl-α-(pyridyldithiol)-toluamido) hexanoate, N-succinimidyl-3-(-2-pyridyldithio)-proprionate (SPDP), succinimidyl 6(3(-(-2-pyridyldithio)-

20 proprionamido) hexanoate, sulfosuccinimidyl 6(3(-(-2-pyridyldithio)-propionamido) hexanoate, maleimidocaproyl (MC), maleimidocaproyl-valine-citrulline-*p*-aminobenzyloxycarbonyl (MC-vc-PAB), 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), alpha-alkyl derivatives, sulfoNHS-ATMBA (sulfosuccinimidyl N-[3-(acetylthio)-3-methylbutyryl-beta-alanine]), sulfodichlorophenol, 2-iminothiolane, 3-(2-pyridyldithio)-propionyl hydrazide, Ellman’s reagent, dichlorotriazinic acid, and S-

25 (2-thiopyridyl)-L-cysteine.

[416] Suitable linkers, whether proteinaceous or non-proteinaceous, may include, *e.g.*, protease sensitive, environmental redox potential sensitive, pH sensitive, acid cleavable, photocleavable, and/or heat sensitive linkers.

[417] Proteinaceous linkers may be chosen for incorporation into recombinant fusion cell-targeting

30 molecules of the present invention. For recombinant fusion cell-targeting proteins of the invention, linkers typically comprise about 2 to 50 amino acid residues, preferably about 5 to 30 amino acid residues. Commonly, proteinaceous linkers comprise a majority of amino acid residues with polar, uncharged, and/or charged residues, such as, *e.g.*, threonine, proline, glutamine, glycine, and alanine. Non-limiting examples of proteinaceous linkers include alanine-serine-glycine-glycine-proline-glutamate

35 (ASGGPE (SEQ ID NO:158)), valine-methionine (VM), alanine-methionine (AM), AM$(G_{2\text{ to }4}S)_x$AM where G is glycine, S is serine, and x is an integer from 1 to 10 (SEQ ID NO:159).

[418] Proteinaceous linkers may be selected based upon the properties desired. Proteinaceous linkers may be chosen by the skilled worker with specific features in mind, such as to optimize one or more of

the fusion molecule's folding, stability, expression, solubility, pharmacokinetic properties, pharmacodynamic properties, and/or the activity of the fused domains in the context of a fusion construct as compared to the activity of the same domain by itself. For example, proteinaceous linkers may be selected based on flexibility, rigidity, and/or cleavability. The skilled worker may use databases and linker design software tools when choosing linkers. In certain linkers may be chosen to optimize expression. In certain linkers may be chosen to promote intermolecular interactions between identical polypeptides or proteins to form homomultimers or different polypeptides or proteins to form heteromultimers. For example, proteinaceous linkers may be selected which allow for desired non-covalent interactions between polypeptide components of the cell-targeting molecules of the invention, such as, *e.g.*, interactions related to the formation dimers and other higher order multimers.

[419] Flexible proteinaceous linkers are often greater than 12 amino acid residues long and rich in small, non-polar amino acid residues, polar amino acid residues, and/or hydrophilic amino acid residues, such as, *e.g.*, glycines, serines, and threonines. Flexible proteinaceous linkers may be chosen to increase the spatial separation between components and/or to allow for intramolecular interactions between components. For example, various "GS" linkers are known to the skilled worker and are composed of multiple glycines and/or one or more serines, sometimes in repeating units, such as, *e.g.*, $G_xS)_n$ (SEQ ID NO:160), $(S_xG)_n$ (SEQ ID NO:161), $(GGGGS)_n$ (SEQ ID NO:162), and $(G)_n$, in which x is 1 to 6 and n is 1 to 30 (SEQ ID NO:163). Non-limiting examples of flexible proteinaceous linkers include GKSSGSGSESKS (SEQ ID NO:164), EGKSSGSGSESKEF (SEQ ID NO:165), GSTSGSGKSSEGKG (SEQ ID NO:166), GSTSGSGKSSEGSGSTKG (SEQ ID NO:167), GSTSGSGKPGSGEGSTKG (SEQ ID NO:96), SRSSG (SEQ ID NO:168), and SGSSC (SEQ ID NO:169).

[420] Rigid proteinaceous linkers are often stiff alpha-helical structures and rich in proline residues and/or one or more strategically placed prolines. Rigid linkers may be chosen to prevent intramolecular interactions between linked components.

[421] Suitable linkers may be chosen to allow for *in vivo* separation of components, such as, *e.g.*, due to cleavage and/or environment-specific instability. *In vivo* cleavable proteinaceous linkers are capable of unlinking by proteolytic processing and/or reducing environments often at a specific site within an organism or inside a certain cell type. *In vivo* cleavable proteinaceous linkers often comprise protease sensitive motifs and/or disulfide bonds formed by one or more cysteine pairs. *In vivo* cleavable proteinaceous linkers may be designed to be sensitive to proteases that exist only at certain locations in an organism, compartments within a cell, and/or become active only under certain physiological or pathological conditions (such as, *e.g.*, involving proteases with abnormally high levels, proteases overexpressed at certain disease sites, and proteases specifically expressed by a pathogenic microorganism). For example, there are proteinaceous linkers known in the art which are cleaved by proteases present only intracellularly, proteases present only within specific cell types, and proteases present only under pathological conditions like cancer or inflammation, such as, *e.g.*, R-x-x-R motif and AMGRSGGGCAGNRVGSSLSCGGLNLQAM (SED ID NO:170).

[422] In certain embodiments of the cell-targeting molecules of the present invention, a linker may be used which comprises one or more protease sensitive sites to provide for cleavage by a protease present within a target cell. In certain embodiments of the cell-targeting molecules of the invention, a linker may be used which is not cleavable to reduce unwanted toxicity after administration to a vertebrate organism.

5 [423] Suitable linkers may include, *e.g.*, protease sensitive, environmental redox potential sensitive, pH sensitive, acid cleavable, photocleavable, and/or heat sensitive linkers, whether proteinaceous or non-proteinaceous (*see e.g.*, Doronina S et al., *Bioconjug Chem* 17: 114-24 (2003); Saito G et al., *Adv Drug Deliv Rev* 55: 199-215 (2003); Jeffrey S et al., J Med Chem 48: 1344-58 (2005); Sanderson R et al., *Clin Cancer Res* 11: 843-52 (2005); Erickson H et al., *Cancer Res* 66: 4426-33 (2006); Chen X et al., *Adv*
10 *Drug Deliv Rev* 65: 1357-69 (2013)). Suitable cleavable linkers may include linkers comprising cleavable groups which are known in the art.

[424] Suitable linkers may include pH sensitive linkers. For example, certain suitable linkers may be chosen for their instability in lower pH environments to provide for dissociation inside a subcellular compartment of a target cell (*see e.g.*, van Der Velden V et al., *Blood* 97: 3197-204 (2001); Ulbrich K,
15 Subr V, *Adv Drug Deliv Rev* 56: 1023-50 (2004)). For example, linkers that comprise one or more trityl groups, derivatized trityl groups, bismaleimideothoxy propane groups, adipic acid dihydrazide groups, and/or acid labile transferrin groups, may provide for release of components of the cell-targeting molecules of the invention, *e.g.* a polypeptide component, in environments with specific pH ranges. In certain linkers may be chosen which are cleaved in pH ranges corresponding to physiological pH
20 differences between tissues, such as, *e.g.*, the pH of tumor tissue is lower than in healthy tissues.

[425] Photocleavable linkers are linkers that are cleaved upon exposure to electromagnetic radiation of certain wavelength ranges, such as light in the visible range. Photocleavable linkers may be used to release a component of a cell-targeting molecule of the invention, *e.g.* a polypeptide component, upon exposure to light of certain wavelengths. Non-limiting examples of photocleavable linkers include a
25 nitrobenzyl group as a photocleavable protective group for cysteine, nitrobenzyloxycarbonyl chloride cross-linkers, hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer, and methylrhodamine copolymer. Photocleavable linkers may have particular uses in linking components to form cell-targeting molecules of the invention designed for treating diseases, disorders, and conditions that can be exposed to light using fiber optics.

30 [426] In certain embodiments of the cell-targeting molecules of the present invention, a cell-targeting binding region is linked to a Shiga toxin effector polypeptide of the present invention using any number of means known to the skilled worker, including both covalent and noncovalent linkages.

[427] In certain embodiments of the cell-targeting molecules of the present invention, the molecule comprises a binding region which is a scFv with a linker connecting a heavy chain variable (V_H) domain
35 and a light chain variable (V_L) domain. There are numerous linkers known in the art suitable for this purpose, such as, *e.g.*, the 15-residue $(Gly4Ser)_3$ peptide (SED ID NO:171). Suitable scFv linkers which may be used in forming non-covalent multivalent structures include GGS, (SEQ ID NO:172), GGGGS

(SEQ ID NO:94), GGGGSGGG (SEQ ID NO:173), GGSGGGG (SEQ ID NO:174), GSTSGGGSGGGSGGGGSS (SEQ ID NO:175), and GSTSGSGKPGSSEGSTKG (SEQ ID NO:176).

[428] Suitable methods for linkage of the components of the cell-targeting molecules of the present invention may be by any method presently known in the art for accomplishing such, so long as the attachment does not substantially impede the binding capability of the cell-targeting binding region, the cellular internalization of the Shiga toxin effector polypeptide component, and/or when appropriate the desired Shiga toxin effector function(s) as measured by an appropriate assay, including assays described herein.

[429] The components of the cell-targeting molecule, *e.g.* a Shiga toxin A Subunit effector polypeptide and/or immunoglobulin-type HER2-binding region, may be engineered to provide a suitable attachment moiety for the linkage of additional components, *e.g.* an additional exogenous material (*see e.g.* WO2018/106895).

[430] For the purposes of the cell-targeting molecules of the present invention, the specific order or orientation is not fixed for the components: the Shiga toxin effector polypeptide(s), the binding region(s), and any optional linker(s), in relation to each other or the entire cell-targeting molecule unless specifically noted. The components of the cell-targeting molecules of the present invention may be arranged in any order provided that the desired activity(ies) of the binding region and Shiga toxin effector polypeptide are not eliminated.

IV. Examples of Structural Variations of the Shiga Toxin Effector Polypeptides and Cell-Targeting Molecules of the Invention

[431] In certain embodiments, a Shiga toxin effector polypeptide of the present invention may comprise, consist of, or consist essentially of a truncated Shiga toxin A Subunit. Truncations of Shiga toxin A Subunits might result in the deletion of an entire epitope(s) and/or epitope region(s), B-cell epitopes, CD4+ T-cell epitopes, and/or furin-cleavage sites without affecting Shiga toxin effector functions, such as, *e.g.*, catalytic activity and cytotoxicity. The smallest Shiga toxin A Subunit fragment shown to exhibit full enzymatic activity was a polypeptide composed of residues 1–239 of Slt1A (LaPointe P et al., *J Biol Chem* 280: 23310-18 (2005)). The smallest Shiga toxin A Subunit fragment shown to exhibit significant enzymatic activity was a polypeptide composed of residues 75–247 of StxA (Al-Jaufy A et al., *Infect Immun* 62: 956-60 (1994)).

[432] Although Shiga toxin effector polypeptides of the present invention may commonly be smaller than the full-length Shiga toxin A Subunit, it is preferred that the Shiga toxin effector polypeptide region of a cell-targeting molecule of the present invention maintain the polypeptide region from amino acid position 77 to 239 (SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), or Shiga toxin 1 A Subunit variants, *e.g.* SEQ ID NOs: 4–6) or the equivalent in other A Subunits of members of the Shiga toxin family (*e.g.* 77 to 238 of SEQ ID NOs: 3 and 7–18)). For example, in certain embodiments of the molecules of the present invention, the Shiga toxin effector polypeptides of the present invention derived from SLT-1A may comprise, consist of, or consist essentially of amino acids 75 to 251 of SEQ ID NO:1, 1 to 241 of

SEQ ID NO:1, 1 to 251 of SEQ ID NO:1, or amino acids 1 to 261 of SEQ ID NO:1, further comprising relative to a wild-type Shiga toxin A Subunit at least one amino acid residue which is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region. Similarly, Shiga toxin effector polypeptide regions derived from Shiga toxin 1 A Subunit variants (such as Stx1cA, Stx1dA, and Stx1eA) may comprise, consist essentially of, or consist of amino acids 75 to 251 of SEQ ID NOs: 4–6, 1 to 241 of SEQ ID NOs: 4–6, or 1 to 251 of SEQ ID NOs: 4–6, further comprising relative to a wild-type Shiga toxin A Subunit at least one amino acid residue which is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region. Additionally, Shiga toxin effector polypeptide regions derived from SLT-2 may comprise, consist of, or consist essentially of amino acids 75 to 251 of SEQ ID NO:3, 1 to 241 of SEQ ID NO:3, 1 to 251 of SEQ ID NO:3, or amino acids 1 to 261 of SEQ ID NO:3, further comprising relative to a wild-type Shiga toxin A Subunit at least one amino acid residue which is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region. Likewise, Shiga toxin effector polypeptide regions derived from Shiga-like toxin 2 A Subunit variants (such as Stx2cA variant 1, Stx2cA variant 2, Stx2cA variant 3, Stx2cA variant 4, Stx2cA variant 5, Stx2cA variant 6, Stx2dA variant 1, Stx2dA variant 2, Stx2dA variant 3, Stx2eA variant 1, Stx2eA variant 2, and Stx2fA) may comprise, consist essentially of, or consist of amino acids 1 to 241 of SEQ ID NOs: 7–18, further comprising relative to a wild-type Shiga toxin A Subunit at least one amino acid residue which is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[433] In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6; and/or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6; and/or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3, wherein relative to a wild-type Shiga toxin A Subunit at least one amino acid residue is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[434] The invention further provides variants of Shiga toxin effector polypeptides and cell-targeting molecules of the present invention, wherein the Shiga toxin effector polypeptide differs from a naturally occurring Shiga toxin A Subunit by only or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40 or more amino acid residues (but by no more than that which retains at least 85%, 90%, 95%, 99% or more amino acid sequence identity). Thus, a molecule of the present invention derived from an A Subunit of a

member of the Shiga toxin family may comprise additions, deletions, truncations, or other alterations from the original sequence as long as at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more amino acid sequence identity is maintained to a naturally occurring Shiga toxin A Subunit and wherein relative to a wild-type Shiga toxin A Subunit at least one amino acid residue is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[435] Accordingly, in certain embodiments, the Shiga toxin effector polypeptide of a molecule of the present invention comprises, consists of, or consists essentially of amino acid sequences having at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, 99.5% or 99.7% overall sequence identity to a naturally occurring Shiga toxin A Subunit (or a fragment thereof), such as SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), Shiga toxin 1 A Subunit variants (*e.g.* SEQ ID NOs: 4–6), SLT-2A (SEQ ID NO:3), and/or Shiga-like toxin 2 A Subunit variants (*e.g.* SEQ ID NOs: 7–18), wherein relative to a wild-type Shiga toxin A Subunit at least one amino acid residue is mutated or has been deleted in an endogenous epitope and/or epitope region, and/or wherein there is a disrupted, furin-cleavage motif region at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.7% identical) to a wild-type Shiga toxin A Subunit amino acid sequence selected from: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6; and (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.7% identical) to a wild-type Shiga toxin A Subunit amino acid sequence selected from: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–3; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–3; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–3; or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.7% identical) to a wild-type Shiga toxin A Subunit amino acid sequence selected from: (i) amino acids 75 to 251 of SEQ ID NO:1; (ii) amino acids 1 to 241 of SEQ ID NO:1; (iii) amino acids 1 to 251 of SEQ ID NO:1; or (iv) amino acids 1 to 261 of any one of SEQ ID NO:1. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.7% identical) to a wild-type Shiga toxin A Subunit amino acid sequence selected from: (i) amino acids 75 to 251 of SEQ ID NO:2; (ii) amino acids 1 to 241 of SEQ ID NO:2; (iii) amino acids 1 to 251 of SEQ ID NO:2; or (iv) amino acids 1 to 261 of SEQ ID NO:2. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical (such as at least 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.7% identical) to a wild-type Shiga toxin A Subunit amino acid sequence selected from: (i) amino acids 75 to 251 of any one of SEQ ID NO:3; (ii) amino acids 1 to 241 of SEQ ID NO:3; (iii) amino acids 1 to 251 of SEQ ID NO:3; or (iv) amino acids 1 to 261 of SEQ ID NO:3.

5 [436] Optionally, either a full-length or a truncated version of the Shiga toxin A Subunit may comprise the Shiga toxin effector polypeptide region of a molecule of the present invention, wherein the Shiga toxin derived polypeptide comprises one or more mutations (*e.g.* substitutions, deletions, insertions, or inversions) as compared to a naturally occurring Shiga toxin. It is preferred in certain embodiments of the invention that the Shiga toxin effector polypeptides have sufficient sequence identity to a naturally 10 occurring (or wild-type) Shiga toxin A Subunit to retain cytotoxicity after entry into a cell, either by well-known methods of host cell transformation, transfection, infection or induction, or by internalization mediated by a cell-targeting binding region linked with the Shiga toxin effector polypeptide. The most critical residues for enzymatic activity and/or cytotoxicity in the Shiga toxin A Subunits have been mapped to the following residue-positions: asparagine-75, tyrosine-77, glutamate-167, arginine-170, and 15 arginine-176 among others (Di R et al., *Toxicon* 57: 525-39 (2011)). In any one of the embodiments of the invention, the Shiga toxin effector polypeptides may preferably but not necessarily maintain one or more conserved amino acids at positions, such as those found at positions 77, 167, 170, and 176 in StxA, SLT-1A, or the equivalent conserved position in other members of the Shiga toxin family which are typically required for cytotoxic activity. The capacity of a cytotoxic molecule of the invention to cause 20 cell death, *e.g.* its cytotoxicity, may be measured using any one or more of a number of assays well known in the art.

A. Examples of De-Immunized, Shiga Toxin Effector Polypeptides

[437] In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present 25 invention may consist essentially of a truncated Shiga toxin A Subunit having two or more mutations. Truncations of Shiga toxin A Subunits might result in the deletion of an entire epitope(s) and/or epitope region(s), B-cell epitopes, CD4+ T-cell epitopes, and/or furin-cleavage sites without affecting Shiga toxin effector functions, such as, *e.g.*, catalytic activity and cytotoxicity. Truncating the carboxy-terminus of SLT-1A, StxA, or SLT-2A to amino acids 1–251 removes two predicted B-cell epitope 30 regions, two predicted CD4 positive (CD4+) T-cell epitopes, and a predicted discontinuous B-cell epitope. These epitopes are also absent from the Shiga toxin effector polypeptides shown in SEQ ID NOs: 4–18. The Shiga toxin 1 A Subunit effector polypeptides shown in SEQ ID NOs: 4–6 relate to fragments of wild-type Shiga toxin A Subunit variants which have been truncated at position 251, and the Shiga-like toxin 2 A Subunit effector polypeptides shown in SEQ ID NOs: 7–18 relate to fragments of 35 the Shiga-like toxin 2 A Subunit variants which have been truncated at position 250. Truncating the amino-terminus of SLT-1A, StxA, or SLT-2A to 75–293 removes at least three predicted B-cell epitope regions and three predicted CD4+ T-cell epitopes. Truncating both amino- and carboxy-terminals of

SLT-1A, StxA, or SLT-2A to 75–251 deletes at least five predicted B-cell epitope regions, four putative CD4+ T-cell epitopes and one predicted discontinuous B-cell epitope.

[438] In certain embodiments, a de-immunized, Shiga toxin effector polypeptide of the present invention may comprise, consist of, or consist essentially of a full-length or truncated Shiga toxin A Subunit with at least one mutation (relative to a wild-type Shiga toxin polypeptide), *e.g.* deletion, insertion, inversion, or substitution, in a provided, endogenous, B-cell and/or CD4+ T-cell epitope region. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption which comprises a mutation (relative to a wild-type Shiga toxin polypeptide) which includes a deletion of at least one amino acid residue within the endogenous, B-cell and/or CD4+ T-cell epitope region. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption which comprises an insertion of at least one amino acid residue within the endogenous, B-cell and/or CD4+ T-cell epitope region. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption which comprises an inversion of amino acid residues, wherein at least one inverted amino acid residue is within the endogenous, B-cell and/or CD4+ T-cell epitope region. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a disruption which comprises a mutation (relative to a wild-type Shiga toxin polypeptide), such as, *e.g.*, an amino acid substitution, an amino acid substitution to a non-standard amino acid, and/or an amino acid residue with a chemically modified side chain. Non-limiting examples of de-immunized, Shiga toxin effector sub-regions suitable for use in the present invention are described in WO 2015/113005, WO 2015/113007 and WO 2015/191764. Numerous, non-limiting examples of Shiga toxin effector polypeptides of the present invention which comprise amino acid substitutions are provided in the Examples.

[439] In other embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises a truncated Shiga toxin A Subunit which is shorter than a full-length Shiga toxin A Subunit wherein at least one amino acid residue is disrupted in a natively positioned, B-cell and/or CD4+ T-cell epitope region.

[440] To create a de-immunized, Shiga toxin effector polypeptide, in principle modifying any amino acid residue in a provided epitope region by various means can result in a disruption of an epitope, such as, *e.g.*, a modification which represents a deletion, insertion, inversion, rearrangement, substitution, and chemical modification of a side chain relative to a wild-type Shiga toxin polypeptide. However, modifying certain amino acid residues and using certain amino acid modifications are more likely to successfully reduce antigenicity and/or immunogenicity while maintaining a certain level of a Shiga toxin effector function(s). For example, terminal truncations and internal amino acid substitutions are preferred because these types of modifications maintain the overall spacing of the amino acid residues in a Shiga toxin effector polypeptide and thus are more likely to maintain Shiga toxin effector polypeptide structure and function.

[441] Among certain embodiments of the present invention, the de-immunized, Shiga toxin effector polypeptide comprising, consisting of, or consisting essentially of amino acids 75 to 251 of SLT-1A

(SEQ ID NO:1), StxA (SEQ ID NO:2), and/or SLT-2A (SEQ ID NO:3) wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Among certain other embodiments are de-immunized, Shiga toxin effector polypeptides which comprise or consist essentially of amino acids 1 to 241 of SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), and/or SLT-2A (SEQ ID NO:3) wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Further embodiments are de-immunized, Shiga toxin effector polypeptides which comprise, consist of, or consist essentially of amino acids 1 to 251 of SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), and/or SLT-2A (SEQ ID NO:3) wherein at least one amino acid residue is disrupted in a natively positioned, epitope region provided. Further embodiments are Shiga toxin effector polypeptides comprising amino acids 1 to 261 of SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), and/or SLT-2A (SEQ ID NO:3) wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Among certain embodiments of the present invention, the de-immunized, Shiga toxin effector polypeptide comprises, consists essentially of, or consists of amino acids 75 to 251 of any one of SEQ ID NOs: 1–6, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Among certain other embodiments are de-immunized, Shiga toxin effector polypeptides which comprise, consist essentially of, or consist of amino acids 1 to 241 of SEQ ID NOs: 1–18, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Further embodiments are de-immunized, Shiga toxin effector polypeptides which comprise, consist essentially of, or consist of amino acids 1 to 251 of SEQ ID NOs: 1–6, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region provided. Further embodiments are Shiga toxin effector polypeptides comprising amino acids 1 to 261 of SEQ ID NOs: 1–3, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. In certain embodiments, the de-immunized, Shiga toxin effector polypeptide comprises, consists essentially of, or consists of amino acids 75 to 251 of any one of SEQ ID NOs: 1–6, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Among certain further embodiments are de-immunized, Shiga toxin effector polypeptides which comprise, consist essentially of, or consist of amino acids 1 to 241 of SEQ ID NOs: 1–6, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region. Further embodiments are de-immunized, Shiga toxin effector polypeptides which comprise, consist essentially of, or consist of amino acids 1 to 251 of SEQ ID NOs: 1–6, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region provided. Further embodiments are Shiga toxin effector polypeptides comprising amino acids 1 to 261 of SEQ ID NOs: 1–3, wherein at least one amino acid residue is disrupted in a natively positioned, epitope region.

[442] There are numerous, diverse, internal amino acid substitutions that can be used to create de-immunized, Shiga toxin effector polypeptides of the invention. Of the possible substitute amino acids to use within an epitope region, the following substitute amino acid residues are predicted to be the most likely to reduce the antigenicity and/or immunogenicity of an epitope — G, D, E, S, T, R, K, and H. Except for glycine, these amino acid residues may all be classified as polar and/or charged residues. Of the possible amino acids to substitute with, the following amino acids A, G, V, L, I, P, C, M, F, S, D, N, Q, H, and K are predicted to be the most likely to reduce antigenicity and/or immunogenicity while

providing the retention of a significant level of a Shiga toxin effector function(s), depending on the amino acid substituted for. Generally, the substitution should change a polar and/or charged amino acid residue to a non-polar and uncharged residue (*see e.g.* WO 2015/113007). In addition, it may be beneficial to epitope disruption to reduce the overall size and/or length of the amino acid residue's R-group functional side chain (*see e.g.* WO 2015/113007). However despite these generalities of substitutions most likely to confer epitope disruption, because the aim is to preserve significant Shiga toxin effector function(s), the substitute amino acid might be more likely to preserve Shiga toxin effector function(s) if it resembles the amino acid substituted for, such as, *e.g.*, a nonpolar and/or uncharged residue of similar size substituted for a polar and/or charged residue.

[443] In the Examples below and in WO 2015/113007, many mutations have been empirically tested for effect(s) on the Shiga toxin effector function of various Shiga toxin effector polypeptides and cell-targeting molecules. Table B summarizes the results described in WO 2015/113007 and WO 2016/196344 where an amino acid substitution, alone or in combination with one or more other substitutions, did not prevent the exhibition of a potent level of a Shiga toxin effector function(s). Table B uses the epitope region numbering scheme described in WO 2016/196344.

TABLE B. Amino Acid Substitutions in Shiga Toxin Effector Polypeptides

Epitope Region Disrupted	natively positioned amino acid positions		
	Substitution	B-Cell Epitope Region	T-Cell Epitope
1	K1A	1–15	
1	K1M	1–15	
1	T4I	1–15	4–33
1	D6R	1–15	4–33
1	S8I	1–15	4–33
1	T9V	1–15	4–33
1	T9I	1–15	4–33
1	K11A	1–15	4–33
1	K11H	1–15	4–33
1	T12K	1–15	4–33
2	S33I	27–37	4–33
2	S33C	27–37	4–33
3	S43N	39–48	34–78
3	G44L	39–48	34–78
3	T45V	39–48	34–78
3	T45I	39–48	34–78
3	S45V	39–48	34–78
3	S45I	39–48	34–78
3	G46P	39–48	34–78
3	D47G	39–48	34–78
3	D47M	39–48	34–78
3	N48V	39–48	34–78
3	N48F	39–48	34–78
-	L49A	immunogenic residue	34–78
-	F50T		34–78

-	A51V		34–78
4	D53A	53–66	34–78
4	D53G	53–66	34–78
4	D53N	53–66	34–78
4	V54L	53–66	34–78
4	V54I	53–66	34–78
4	R55A	53–66	34–78
4	R55V	53–66	34–78
4	R55L	53–66	34–78
4	G56P	53–66	34–78
4	I57M	53–66	34–78
4	I57F	53–66	34–78
4	D58A	53–66	34–78
4	D58V	53–66	34–78
4	D58F	53–66	34–78
4	P59A	53–66	34–78
4	P59F	53–66	34–78
4	E60I	53–66	34–78
4	E60T	53–66	34–78
4	E60R	53–66	34–78
4	E61A	53–66	34–78
4	E61V	53–66	34–78
4	E61L	53–66	34–78
4	G62A	53–66	34–78
-	R84A		77–103
-	V88A		77–103
5	D94A	94–115	77–103
5	S96I	94–115	77–103
5	T104N	94–115	
5	A105L	94–115	
5	T107P	94–115	
5	L108M	94–115	
5	S109V	94–115	
5	G110A	94–115	
5	D111T	94–115	
5	S112V	94–115	
6	D141A	141–153	128–168
6	G147A	141–153	128–168
-	V154A		128–168
7	R179A	179–190	160–183
7	T180G	179–190	160–183
7	T181I	179–190	160–183
7	D183A	179–190	160–183
7	D183G	179–190	160–183
7	D184A	179–190	
7	D184F	179–190	
7	L185V	179–190	

7	S186A	179–190	
7	S186F	179–190	
7	G187A	179–190	
7	G187T	179–190	
7	R188A	179–190	
7	R188L	179–190	
7	S189A	179–190	
-	D198A	immunogenic residue	
-	R205A	immunogenic residue	
-	C242S		236–258
8	R248A	243–257	236–258
8	R251A	243–257	236–258

[444] Based on the empirical evidence in WO 2015/113007 and WO 2016/196344, certain amino acid positions in the A Subunits of Shiga toxins are predicted to tolerate epitope disruptions while still retaining significant Shiga toxin effector functions. For example, the following natively occurring positions tolerate amino acid substitutions, either alone or in combination, while retaining a Shiga toxin effector function(s) such as cytotoxicity — 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1 or SEQ ID NO:2; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1 or SEQ ID NO:2; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1 or SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1 or SEQ ID NO:2; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1 or SEQ ID NO:2; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1 or SEQ ID NO:2; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1 or SEQ ID NO:2; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1 or SEQ ID NO:2; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 241 of SEQ ID NO:3; 242 of SEQ

ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; 251 of SEQ ID NO:1 or SEQ ID NO:2; 264 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 265 of SEQ ID NO:1 or SEQ ID NO:2; and 286 of SEQ ID NO:1 or SEQ ID NO:2.

5 [445] The empirical data in WO 2015/113007 and WO 2016/196344 point towards other epitope disrupting substitutions and combinations of epitope disrupting substitutions that can reduce antigenicity and/or immunogenicity of a Shiga toxin effector polypeptide while retaining the ability of the Shiga toxin effector polypeptide to exhibit a significant Shiga toxin effector function such as, *e.g.*, new combinations of the aforementioned truncations and positions tolerating substitutions as well as new substitutions at 10 identical positions or conserved positions in related Shiga toxin A Subunits.

[446] It is predictable that other amino acid substitutions to amino acid residues of a conservative functional group of a substitution tested herein may reduce antigenicity and/or immunogenicity while preserving a significant Shiga toxin effector function. For example, other substitutions known to the skilled worker to be similar to any of K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, 15 S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, A51V, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, 20 R188A, R188L, S189A, D198A, R204A, R205A, C242S, R247A, S247I, Y247A, R248A, R250A, R251A, or D264A, G264A, T286A, and/or T286I may disrupt an endogenous epitope while maintaining at least one Shiga toxin effector function. In particular, amino acid substitutions to conservative amino acid residues similar to K1A, K1M, T4I, S8I, T8V, T9I, S9I, K11A, K11H, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, N48V, N48F, L49A, A51V, D53A, D53N, V54L, V54I, R55A, 25 R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, E60I, E60T, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184F, L185V, S186A, S186F, G187A, R188A, R188L, S189A, D198A, R204A, R205A, C242S, S247I, Y247A, R247A, R248A, R250A, R251A, D264A, G264A, T286A, and T286I may have the same or similar effects. In certain 30 embodiments, a Shiga toxin effector polypeptide of the invention may comprise similar conservative amino acid substitutions to empirically tested ones, such as, *e.g.*, K1 to G, V, L, I, F, and H; T4 to A, G, V, L, F, M, and S; S8 to A, G, V, L, F, and M; T8 to A, G, V, I, L, F, and M; T9 to A, G, L, F, M, and S; S9 to A, G, L, I, F, and M; K11 to G, V, L, I, F, and M; S33 to A, G, V, L, F, and M; S43 to A, G, V, L, I, F, and M; S45 to A, G, L, F, and M; T45 to A, G, L, F, and M; D47 to A, V, L, I, F, S, and Q; N48 to 35 A, G, L, and M; L49 to G; Y49 to A; D53 to V, L, I, F, S, and Q; R55 to G, I, F, M, Q, S, K, and H; D58 to G, L, I, S, and Q; P59 to G; E60 to A, G, V, L, F, S, Q, N, D, and M; E61 to G, I, F, S, Q, N, D, M, and R; R84 to G, V, L, I, F, M, Q, S, K, and H; V88 to G; I88 to G; D94 to G, V, L, I, F, S, and Q; S96 to A, G, V, L, F, and M; T107 to A, G, V, L, I, F, M, and S; S107 to A, G, V, L, I, F, and M; S109 to A, G,

I, L, F, and M; T109 to A, G, I, L, F, M, and S; S112 to A, G, L, I, F, and M; D141 to V, L, I, F, S, and Q; V154 to G; R179 to G, V, L, I, F, M, Q, S, K, and H; T180 to A, V, L, I, F, M, and S; T181 to A, G, V, L, F, M, and S; D183 to V, L, I, F, S, and Q; D184 to G, V, L, I, S, and Q; S186 to G, V, I, L, and M; R188 to G, V, I, F, M, Q, S, K, and H; S189 to G, V, I, L, F, and M; D197 to V, L, I, F, S, and Q; D198 to A, V, L, I, F, S, and Q; R204 to G, V, L, I, F, M, Q, S, K, and H; R205 to G, V, L, I, F, M, Q, S, K and H; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to G, V, L, I, F, M, Q, S, K, and H; R250 to G, V, L, I, F, M, Q, S, K, and H; R251 to G, V, L, I, F, M, Q, S, K, and H; D264 to A, G, V, L, I, F, S, and Q; and T286 to A, G, V, L, I, F, M, and S.

[447] Similarly, amino acid substitutions which remove charge, polarity, and/or reduce side chain length can disrupt an epitope while maintaining at least one Shiga toxin effector function. In certain embodiments, a Shiga toxin effector polypeptide of the invention may comprise one or more epitopes disrupted by substitutions such that side chain charge is removed, polarity is removed, and/or side chain length is reduced such as, *e.g.*, substituting the appropriate amino acid selected from the following group A, G, V, L, I, P, C, M, F, S, D, N, Q, H, or K for the amino acid residue at position 1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 6 of SEQ ID NO:1 or SEQ ID NO:2; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 12 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1 or SEQ ID NO:2; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1 or SEQ ID NO:2; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1 or SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1 or SEQ ID NO:2; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1 or SEQ ID NO:2; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 241 of SEQ ID NO:3; 242 of SEQ ID

NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; 251 of SEQ ID NO:1 or SEQ ID NO:2; 264 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 265 of SEQ ID NO:1 or SEQ ID NO:2; and 286 of SEQ ID NO:1 or SEQ ID NO:2. In certain embodiments, a Shiga toxin effector polypeptide of the present invention may comprise one or more of the following amino acid substitutions: K1 to A, G, V, L, I, F, M and H; T4 to A, G, V, L, I, F, M, and S; D6 to A, G, V, L, I, F, S, and Q; S8 to A, G, V, I, L, F, and M; T8 to A, G, V, I, L, F, M, and S; T9 to A, G, V, I, L, F, M, and S; S9 to A, G, V, L, I, F, and M; K11 to A, G, V, L, I, F, M and H; T12 to A, G, V, I, L, F, M, and S; S33 to A, G, V, L, I, F, and M; S43 to A, G, V, L, I, F, and M; G44 to A and L; S45 to A, G, V, L, I, F, and M; T45 to A, G, V, L, I, F, and M; G46 to A and P; D47 to A, G, V, L, I, F, S, and Q; N48 to A, G, V, L, and M; L49 to A or G; F50; A51 to V; D53 to A, G, V, L, I, F, S, and Q; V54 to A, G, and L; R55 to A, G, V, L, I, F, M, Q, S, K, and H; G56 to A and P; I57 to A, G, M, and F; L57 to A, G, M, and F; D58 to A, G, V, L, I, F, S, and Q; P59 to A, G, and F; E60 to A, G, V, L, I, F, S, Q, N, D, M, and R; E61 to A, G, V, L, I, F, S, Q, N, D, M, and R; G62 to A; D94 to A, G, V, L, I, F, S, and Q; R84 to A, G, V, L, I, F, M, Q, S, K, and H; V88 to A and G; I88 to A, G, and V; D94; S96 to A, G, V, I, L, F, and M; T104 to A, G, V, I, L, F, M, and S; A105 to L; T107 to A, G, V, I, L, F, M, and S; S107 to A, G, V, L, I, F, and M; L108 to A, G, and M; S109 to A, G, V, I, L, F, and M; T109 to A, G, V, I, L, F, M, and S; G110 to A; D111 to A, G, V, L, I, F, S, and Q; S112 to A, G, V, L, I, F, and M; D141 to A, G, V, L, I, F, S, and Q; G147 to A; V154 to A and G; R179 to A, G, V, L, I, F, M, Q, S, K, and H; T180 to A, G, V, L, I, F, M, and S; T181 to A, G, V, L, I, F, M, and S; D183 to A, G, V, L, I, F, S, and Q; D184 to A, G, V, L, I, F, S, and Q; L185 to A, G, and V; S186 to A, G, V, I, L, F, and M; G187 to A; R188 to A, G, V, L, I, F, M, Q, S, K, and H; S189 to A, G, V, I, L, F, and M; D197 to A, G, V, L, I, F, S, and Q; D198 to A, G, V, L, I, F, S, and Q; R204 to A, G, V, L, I, F, M, Q, S, K, and H; R205 to A, G, V, L, I, F, M, Q, S, K and H; C242 to A, G, V, and S; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to A, G, V, L, I, F, M, Q, S, K, and H; R250 to A, G, V, L, I, F, M, Q, S, K, and H; R251 to A, G, V, L, I, F, M, Q, S, K, and H; C262 to A, G, V, and S; D264 to A, G, V, L, I, F, S, and Q; G264 to A; and T286 to A, G, V, L, I, F, M, and S.

[448] In addition, any amino acid substitution in one epitope region of a Shiga toxin effector polypeptide which disrupts an epitope while retaining significant Shiga toxin effector function is combinable with any other amino acid substitution in the same or a different epitope region which disrupts an epitope while retaining significant Shiga toxin effector function to form a de-immunized, Shiga toxin effector polypeptide with multiple epitope regions disrupted while still retaining a significant level of Shiga toxin effector function. In certain embodiments, a Shiga toxin effector polypeptide of the invention may comprise combinations of two or more of the aforementioned substitutions and/or the combinations of substitutions described in WO 2015/113007 and/or WO 2016/196344.

[449] Based on the empirical evidence in the Examples and in WO 2015/113007 and WO 2016/196344, certain amino acid regions in the A Subunits of Shiga toxins are predicted to tolerate epitope disruptions while still retaining significant Shiga toxin effector functions. For example, the

epitope regions natively positioned at 1–15, 39–48, 53–66, 55–66, 94–115, 180–190, 179–190, and 243–257 tolerated multiple amino acid substitution combinations simultaneously without compromising Shiga toxin enzymatic activity and cytotoxicity.

[450] In certain embodiments, the de-immunized, Shiga toxin effector polypeptide of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to an amino acid sequence selected from any one of SEQ ID NOs: 19–21 and 75–89. For example, the de-immunized Shiga toxin effector polypeptide of the present invention comprises any of the following sets of substitutions: (i) K1A, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, G147A, C242S, R248A, and R251A; (ii) S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, R188A, C242S, R248A, and R251A; or (iii) S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, R188A, C242S, R248A, and R251A.

B. Examples of Furin-Cleavage Resistant, Shiga Toxin Effector Polypeptides

[451] In certain embodiments, the Shiga toxin effector polypeptide of the present invention may comprise a disrupted, furin cleavage motif and/or furin cleavage site at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain further embodiments, the Shiga toxin effector polypeptide does not comprise any known compensatory structure which may provide furin cleavage proximal to the carboxy-terminus of the Shiga toxin A1 fragment derived region. Non-limiting examples of disrupted furin cleavage motifs and furin cleave sites suitable for use in the present invention are described in WO 2015/191764.

[452] Certain furin-cleavage motif disruptions are indicated herein by reference to specific amino acid positions of native Shiga toxin A Subunits provided in the Sequence Listing, noting that naturally occurring Shiga toxin A Subunits includes precursor forms containing signal sequences of about 22 amino acids at their amino-terminals which are removed to produce mature Shiga toxin A Subunits and are recognizable to the skilled worker. Further, certain furin-cleavage motif disruptions comprising mutations are indicated herein by reference to specific amino acids (*e.g.* R for an arginine residue) natively present at specific positions within native Shiga toxin A Subunits (*e.g.* R251 for the arginine residue at position 251 from the amino-terminus) followed by the amino acid with which that residue has been substituted in the particular mutation under discussion (*e.g.* R251A represents the amino acid substitution of alanine for arginine at amino acid residue 251 from the amino-terminus).

[453] In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises a Shiga toxin A1 fragment derived region, wherein the Shiga toxin A1 fragment derived region comprises a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region, and such embodiments are referred to herein as “furin-cleavage resistant” or “protease-cleavage resistant,” Shiga toxin effector polypeptides to describe their property(ies) relative to wild-type, Shiga toxin A Subunits and/or wild-type, Shiga toxin A1 fragment fusion proteins.

[454] In certain embodiments, the protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention consists essentially of a truncated Shiga toxin A Subunit having two or more mutations.

[455] In certain embodiments, the protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention comprises the disrupted furin-cleavage motif comprising the amino acid residue substitution (relative to a wild-type Shiga toxin polypeptide) of one or both of the arginine residues in the minimal, furin-cleavage site consensus motif with A, G, or H. In certain embodiments, the protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention comprises a disruption which comprises an amino acid substitution within a furin-cleavage motif region, wherein the substitution occurs at the natively positioned amino acid selected from the group consisting of: 247 of SEQ ID NO:3, 248 of SEQ ID NO:1 or SEQ ID NO:2, 250 of SEQ ID NO:3, 251 of SEQ ID NO:1 or SEQ ID NO:2, or the equivalent position in a conserved Shiga toxin effector polypeptide and/or non-native Shiga toxin effector polypeptide sequence, such as, *e.g.*, position 247 of SEQ ID NOs: 7–18, 248 of SEQ ID NOs: 4–6, 250 of SEQ ID NOs: 7–18, or 251 of SEQ ID NOs: 4–6. In certain further embodiments, the substitution is to any non-conservative amino acid and the substitution occurs at the natively positioned amino acid residue position. In certain further embodiments, the mutation comprises an amino acid substitution selected from the group consisting of: R247A, R248A, R250A R251A, or the equivalent position in a conserved Shiga toxin effector polypeptide and/or non-native Shiga toxin effector polypeptide sequence.

[456] In certain embodiments, the protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention comprises the disrupted furin-cleavage motif comprising the mutation which is a deletion. In certain further embodiments, the disrupted furin-cleavage motif comprises a mutation which is a deletion of the region natively positioned at 247–252 in StxA (SEQ ID NO:2), SLT-1A (SEQ ID NO:1), and other Shiga toxin 1 A Subunit variants (*e.g.* SEQ ID NOs: 4–6), or the region natively positioned at 246–251 in SLT-2A (SEQ ID NO:3) and Shiga-like toxin 2 A Subunit variants (*e.g.* SEQ ID NOs: 7–18); a deletion of the region natively positioned at 244–246 in StxA (SEQ ID NO:2), SLT-1A (SEQ ID NO:1), and other Shiga toxin 1 A Subunit variants (*e.g.* SEQ ID NOs: 4–6), or the region natively positioned at 243–245 in SLT-2A (SEQ ID NO:3) and Shiga-like toxin 2 A Subunit variants (*e.g.* SEQ ID NOs: 7–18); or a deletion of the region natively positioned at 253–259 in StxA (SEQ ID NO:2) and SLT-1A (SEQ ID NO:3), or the region natively positioned at 252–258 in SLT-2A (SEQ ID NO:3).

[457] In certain embodiments of the protease-cleavage resistant, Shiga toxin effector polypeptide of the present invention comprises a Shiga toxin A1 fragment region comprising a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region that is disrupted by a carboxy-terminal truncation as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit, and wherein the truncation results in the deletion of one or more amino acid residues within the furin-cleavage motif a compard to the wild-type Shiga toxin A Subnit. In certain further embodiments, the disrupted furin-cleavage motif comprises the carboxy-terminal truncation which deletes one or more amino acid residues within the minimal cleavage site Y/R-x-x-R, such as, *e.g.*, for StxA and SLT-1A

derived Shiga toxin effector polypeptides, truncations ending at the natively amino acid residue position 250, 249, 248, 247, 246, 245, 244, 243, 242, 241, 240, or less; and for SLT-2A derived Shiga toxin effector polypeptides, truncations ending at the natively amino acid residue position 249, 248, 247, 246, 245, 244, 243, 242, 241, or less. Certain further embodiments comprise the disrupted furin-cleavage motif comprising a combination of any of the aforementioned mutations, where possible.

[458] In certain embodiments, the disrupted furin-cleavage motif comprises the mutation(s) that is a partial, carboxy-terminal truncation of the furin-cleavage motif; however, certain molecules of the present invention do not comprise the disrupted furin-cleavage motif which is a complete, carboxy-terminal truncation of the entire 20 amino acid residue, furin-cleavage motif. For example, certain, Shiga toxin effector polypeptides of the present invention comprise the disrupted furin-cleavage motif comprising a partial, carboxy-terminal truncation of the Shiga toxin A1 fragment region up to native position 240 in StxA (SEQ ID NO:2), SLT-1A (SEQ ID NO:1), or another Shiga toxin 1 A Subunit variant (*e.g.* SEQ ID NOs: 4–6) but not a carboxy-terminal truncation at position 239 or less. Similarly, certain, certain, Shiga toxin effector polypeptides of the present invention comprise the disrupted furin-cleavage motif comprising a partial, carboxy-terminal truncation of the Shiga toxin A1 fragment region up to native position 239 in SLT-2A (SEQ ID NO:3) or a Shiga-like toxin 2 A Subunit variant (*e.g.* SEQ ID NOs: 7–18) but not a carboxy-terminal truncation at position 238 or less. In the largest carboxy-terminal truncation of the furin-cleavage resistant, Shiga toxin effector polypeptide of the present invention, mutations comprising the disrupted furin-cleavage motif, positions P14 and P13 of the furin-cleavage motif are still present.

[459] In certain embodiments, the disrupted furin-cleavage motif comprises both an amino acid residue substitution within the furin-cleavage motif and a carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit. In certain further embodiments, the disrupted furin-cleavage motif comprises both an amino acid residue substitution within the minimal furin-cleavage site R/Y-x-x-R and a carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit, such as, *e.g.*, for StxA and SLT-1A derived Shiga toxin effector polypeptides (and Shiga toxin 1 A Subunit variants), truncations ending at the natively amino acid residue position 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater and comprising the natively positioned amino acid residue R248 and/or R251 substituted with any non-positively charged, amino acid residue where appropriate; and for SLT-2A derived Shiga toxin effector polypeptides (and Shiga-like toxin 2 A Subunit variants), truncations ending at the natively amino acid residue position 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater and comprising the natively positioned amino acid residue R/Y247 and/or R250 substituted with any non-positively charged, amino acid residue where appropriate. In certain embodiments, the truncated Shiga toxin effector polypeptide comprising a disrupted furin-cleavage motif

also comprises the furin-cleavage motif, amino acid residues at positions P9, P8, and/or P7 in order to maintain optimal cytotoxicity.

[460] In certain embodiments, the disrupted furin-cleavage motif comprises a mutation(s) which is one or more internal, amino acid residue deletions, as compared to a wild-type, Shiga toxin A Subunit. In certain further embodiments, the disrupted furin-cleavage motif comprises a mutation(s) which has one or more amino acid residue deletions within the minimal furin-cleavage site R/Y-x-x-R. For example, StxA and SLT-1A derived Shiga toxin effector polypeptides (and other Shiga toxin 1 A Subunit variants) comprising internal deletions of the natively positioned amino acid residues R248 and/or R251, which may be combined with deletions of surrounding residues such as, *e.g.*, 249, 250, 247, 252, etc.; and SLT-2A derived Shiga toxin effector polypeptides (and Shiga-like toxin 2 A Subunit variants) comprising internal deletions of the natively positioned amino acid residues R/Y247 and/or R250, which may be combined with deletions of surrounding residues such as, *e.g.*, 248, 249, 246, 251, etc. In certain further embodiments, the disrupted furin-cleavage motif comprises a mutation which is a deletion of four, consecutive, amino acid residues which deletes the minimal furin-cleavage site R/Y-x-x-R, such as, *e.g.*, StxA and SLT-1A derived Shiga toxin effector polypeptides (and other Shiga toxin 1 A Subunit variants) lacking R248–R251 and SLT-2A derived Shiga toxin effector polypeptides (and Shiga-like toxin 2 A Subunit variants) lacking R/Y247–R250. In certain further embodiments, the disrupted furin-cleavage motif comprises a mutation(s) having one or more amino acid residue deletions in the amino acid residues flanking the core furin-cleavage motif, such as, *e.g.*, a deletion of 244–247 and/or 252–255 in SLT-1A, StxA, or another Shiga toxin 1 A Subunit variant. In certain further embodiments, the disrupted furin-cleavage motif comprises a mutation which is an internal deletion of the entire surface-exposed, protease-cleavage sensitive loop as compared to a wild-type, Shiga toxin A Subunit, such as, *e.g.*, for StxA and SLT-1A derived Shiga toxin effector polypeptides (and other Shiga toxin 1 A Subunit variants), a deletion of natively positioned amino acid residues 241–262; and for SLT-2A derived Shiga toxin effector polypeptides, a deletion of natively positioned amino acid residues 240–261.

[461] In certain embodiments, the disrupted furin-cleavage motif comprises both a mutation which is an internal, amino acid residue deletion within the furin-cleavage motif and a mutation which is carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit. In certain further embodiments, the disrupted furin-cleavage motif comprises both a mutation which is an amino acid residue deletion within the minimal furin-cleavage site R/Y-x-x-R and a mutation which is a carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit. For example, protease-cleavage resistant, Shiga toxin effector polypeptides may comprise a disrupted furin-cleavage motif comprising mutation(s) which are deletions of the natively positioned amino acid residues 248–249 and/or 250–251 in a truncated StxA or SLT-1A polypeptide (or another Shiga toxin 1 A Subunit variant) which still has amino acid residue 247 and/or 252, or the amino acid residues 247–248 and/or 249–250 in a truncated SLT-2A (or a Shiga-like toxin 2 A Subunit variant) which still has amino acid residue 246 and/or 251. In certain further embodiments, the disrupted furin-cleavage motif comprises a mutation having a deletion of four, consecutive, amino acid residues which deletes the minimal furin-cleavage site R/Y-x-x-R and a

carboxy-terminal truncation as compared to a wild-type, Shiga toxin A Subunit, such as, *e.g.*, for StxA and SLT-1A (and other Shiga toxin 1 A Subunit variants) derived Shiga toxin effector polypeptides, truncations ending at the natively amino acid residue position 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater and lacking R248–R251; and for SLT-2A derived Shiga toxin effector polypeptides (and Shiga toxin 2 A Subunit variants), truncations ending at the natively amino acid residue position 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, or greater and lacking R/Y247–R250.

C. Examples of Shiga Toxin Effector Polypeptides Having an Embedded Epitope

[462] In certain embodiments, the Shiga toxin effector polypeptide of the present invention may comprise one or more embedded or inserted, heterologous, T-cell epitopes for purposes of de-immunization and/or delivery to a MHC class I presentation pathway of a target cell. For certain embodiments and/or certain Shiga toxin effector polypeptide sub-regions, embedding or partial embedding a T-cell epitope may be preferred over inserting a T-cell epitope because, *e.g.*, embedding-type modifications are more likely to be successful in diverse sub-regions of a Shiga toxin effector polypeptide whereas successful insertions may be more limited to a smaller subset of Shiga toxin effector polypeptide sub-regions. The term "successful" is used here to mean the modification to the Shiga toxin effector polypeptide (*e.g.* introduction of a heterologous, T-cell epitope) results in a modified Shiga toxin effector polypeptide which retains one or more Shiga toxin effector functions at the requisite level of activity either alone or as a component of a cell-targeting molecule.

[463] Any of the Shiga toxin effector polypeptide sub-regions described in WO 2015/113007 may be suitable for certain embodiments of the present invention, and any of the Shiga toxin effector polypeptides described in WO 2015/113007 may be modified into a Shiga toxin effector polypeptide of the present invention, *e.g.*, by the addition of one or more new epitope region disruptions for de-immunization (such one as described herein) and/or a furin-cleavage motif disruption (such as one described herein).

[464] In certain embodiments, the Shiga toxin effector polypeptide of the present invention consists essentially of a truncated Shiga toxin A Subunit comprising an embedded or inserted, heterologous, T-cell epitope and one or more other mutations. In certain embodiments, the Shiga toxin effector polypeptide of the present invention comprises an embedded or inserted, heterologous, T-cell epitope and is smaller than a full-length, Shiga toxin A Subunit, such as, *e.g.*, derived from the polypeptide represented by amino acids 77 to 239 of SLT-1A (SEQ ID NO:1) or StxA (SEQ ID NO:2) or the equivalent in other A Subunits of members of the Shiga toxin family (*e.g.* amino acids 77 to 238 of SLT-2A (SEQ ID NO:3)). For example, the Shiga toxin effector polypeptide of the present invention comprising an embedded or inserted, heterologous, T-cell epitope may comprise, consist essentially of, or consist of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%,

96%, 97%, 98%, 99% or more) identical to an amino acid sequence selected from any one of SEQ ID NOs: 19–21 and 75–89. For example, the Shiga toxin effector polypeptide of the present invention comprising an embedded, heterologous epitope comprises: V54I, R55L, I57F, P59F, E60T, and E61L.

5 D. Examples of Combination Shiga Toxin Effector Polypeptides

[465] A combination Shiga toxin effector polypeptide of the present invention comprises two or more sub-regions (*i.e.* non-overlapping sub-regions) wherein each sub-region comprises at least one of the following: (1) a disruption in an endogenous epitope or epitope region; (2) an embedded, heterologous, T-cell epitope-peptide; (3) an inserted, heterologous, T-cell epitope-peptide; and (4) a disrupted furin-
10 cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain further embodiments, the combination Shiga toxin effector polypeptide comprises a carboxy-terminal truncation relative to a wild-type Shiga toxin A Subunit. In certain further embodiments, the carboxy-terminal truncation results in the removal of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions present in an untruncated, wild-type Shiga toxin A Subunit.

15 [466] Certain embodiments of the combination Shiga toxin effector polypeptides of the present invention comprise both (1) a disruption in an endogenous epitope or epitope region and (2) a disrupted furin-cleavage motif at the carboxy-terminus of an A1 fragment derived region. It is predicted that any of the individual, de-immunized, Shiga toxin effector sub-regions described in WO 2015/113007 and WO 2016/196344 (*see e.g.* Table B, *supra*) may generally be combined with any Shiga toxin effector
20 sub-region comprising a disrupted furin-cleavage motif described herein, described in WO 2015/191764, and/or known in the art in order to create a Shiga toxin effector polypeptide of the present invention.

[467] In certain embodiments of the present invention, the Shiga toxin effector polypeptide consists essentially of the polypeptide shown in SEQ ID NO:37 which further comprises a disruption of at least one, endogenous, B-cell and/or T-cell epitope region which does not overlap with an embedded or
25 inserted, heterologous, CD8+ T-cell epitope; wherein the disruption comprises one or more amino acid residue substitutions relative to a wild-type Shiga toxin. In certain further embodiments the substitution is selected from the group consisting of: K1 to A, G, V, L, I, F, M and H; T4 to A, G, V, L, I, F, M, and S; D6 to A, G, V, L, I, F, S, Q and R; S8 to A, G, V, I, L, F, and M; T8 to A, G, V, I, L, F, and M; T9 to A, G, V, I, L, F, M, and S; S9 to A, G, V, L, I, F, and M; K11 to A, G, V, L, I, F, M and H; T12 to A, G,
30 V, I, L, F, M, S, and K; S12 to A, G, V, I, L, F, and M; S33 to A, G, V, L, I, F, M, and C; S43 to A, G, V, L, I, F, and M; G44 to A or L; S45 to A, G, V, L, I, F, and M; T45 to A, G, V, L, I, F, and M; G46 to A and P; D47 to A, G, V, L, I, F, S, M, and Q; N48 to A, G, V, L, M and F; L49 to A, V, C, and G; Y49 to A, G, V, L, I, F, M, and T; F50 to A, G, V, L, I, and T; D53 to A, G, V, L, I, F, S, and Q; V54 to A, G, I, and L; R55 to A, G, V, L, I, F, M, Q, S, K, and H; G56 to A and P; I57 to A, G, V, and M; L57 to A, V,
35 C, G, M, and F; D58 to A, G, V, L, I, F, S, and Q; P59 to A, G, and F; E60 to A, G, V, L, I, F, S, Q, N, D, M, T, and R; E61 to A, G, V, L, I, F, S, Q, N, D, M, and R; G62 to A; R84 to A, G, V, L, I, F, M, Q, S, K, and H; V88 to A and G; I88 to A, V, C, and G; D94 to A, G, V, L, I, F, S, and Q; S96 to A, G, V, I, L, F, and M; T104 to A, G, V, L, I, F, M; and N; A105 to L; T107 to A, G, V, L, I, F, M, and P; S107 to A,

G, V, L, I, F, M, and P; L108 to A, V, C, and G; S109 to A, G, V, I, L, F, and M; T109 to A, G, V, I, L, F, M, and S; G110 to A; S112 to A, G, V, L, I, F, and M; D111 to A, G, V, L, I, F, S, Q, and T; S112 to A, G, V, L, I, F, and M; D141 to A, G, V, L, I, F, S, and Q; G147 to A; V154 to A and G. R179 to A, G, V, L, I, F, M, Q, S, K, and H; T180 to A, G, V, L, I, F, M, and S; T181 to A, G, V, L, I, F, M, and S;
5 D183 to A, G, V, L, I, F, S, and Q; D184 to A, G, V, L, I, F, S, and Q; L185 to A, G, V and C; S186 to A, G, V, I, L, F, and M; G187 to A; R188 to A, G, V, L, I, F, M, Q, S, K, and H; S189 to A, G, V, I, L, F, and M; D198 to A, G, V, L, I, F, S, and Q; R204 to A, G, V, L, I, F, M, Q, S, K, and H; R205 to A, G, V, L, I, F, M, Q, S, K and H; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to A, G, V, L, I, F, M, Q, S, K, and H; R250 to A, G, V, L, I, F,
10 M, Q, S, K, and H; R251 to A, G, V, L, I, F, M, Q, S, K, and H; D264 to A, G, V, L, I, F, S, and Q; G264 to A; and T286 to A, G, V, L, I, F, M, and S. In certain further embodiments, there are multiple disruptions of multiple, endogenous B-cell and/or CD8+ T-cell epitope regions wherein each disruption involves at least one amino acid residue substitution selected from the group consisting of: K1 to A, G, V, L, I, F, M and H; T4 to A, G, V, L, I, F, M, and S; D6 to A, G, V, L, I, F, S, Q and R; S8 to A, G, V, I,
15 L, F, and M; T9 to A, G, V, I, L, F, M, and S; S9 to A, G, V, L, I, F, and M; K11 to A, G, V, L, I, F, M and H; T12 to A, G, V, I, L, F, M, S, and K; S12 to A, G, V, I, L, F, and M; S33 to A, G, V, L, I, F, M, and C; S43 to A, G, V, L, I, F, and M; G44 to A or L; S45 to A, G, V, L, I, F, and M; T45 to A, G, V, L, I, F, and M; G46 to A and P; D47 to A, G, V, L, I, F, S, M, and Q; N48 to A, G, V, L, M and F; L49 to A, V, C, and G; Y49 to A, G, V, L, I, F, M, and T; F50 to A, G, V, L, I, and T; A51 to V; D53 to A, G,
20 V, L, I, F, S, and Q; V54 to A, G, I, and L; R55 to A, G, V, L, I, F, M, Q, S, K, and H; G56 to A and P; I57 to A, G, V, and M; L57 to A, V, C, G, M, and F; D58 to A, G, V, L, I, F, S, and Q; P59 to A, G, and F; E60 to A, G, V, L, I, F, S, Q, N, D, M, T, and R; E61 to A, G, V, L, I, F, S, Q, N, D, M, and R; G62 to A; R84 to A, G, V, L, I, F, M, Q, S, K, and H; V88 to A and G; I88 to A, V, C, and G; D94 to A, G, V, L, I, F, S, and Q; S96 to A, G, V, I, L, F, and M; T104 to A, G, V, L, I, F, M; and N; A105 to L; T107 to A,
25 G, V, L, I, F, M, and P; S107 to A, G, V, L, I, F, M, and P; L108 to A, V, C, and G; S109 to A, G, V, I, L, F, and M; T109 to A, G, V, I, L, F, M, and S; G110 to A; S112 to A, G, V, L, I, F, and M; D111 to A, G, V, L, I, F, S, Q, and T; S112 to A, G, V, L, I, F, and M; D141 to A, G, V, L, I, F, S, and Q; G147 to A; V154 to A and G. R179 to A, G, V, L, I, F, M, Q, S, K, and H; T180 to A, G, V, L, I, F, M, and S; T181 to A, G, V, L, I, F, M, and S; D183 to A, G, V, L, I, F, S, and Q; D184 to A, G, V, L, I, F, S, and Q; L185
30 to A, G, V and C; S186 to A, G, V, I, L, F, and M; G187 to A; R188 to A, G, V, L, I, F, M, Q, S, K, and H; S189 to A, G, V, I, L, F, and M; D198 to A, G, V, L, I, F, S, and Q; R204 to A, G, V, L, I, F, M, Q, S, K, and H; R205 to A, G, V, L, I, F, M, Q, S, K and H; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to A, G, V, L, I, F, M, Q, S, K, and H; R250 to A, G, V, L, I, F, M, Q, S, K, and H; R251 to A, G, V, L, I, F, M, Q, S, K, and H; D264 to A, G,
35 V, L, I, F, S, and Q; G264 to A; and T286 to A, G, V, L, I, F, M, and S.

[468] Certain embodiments of the Shiga toxin effector polypeptides of the present invention comprise both (1) an embedded or inserted, heterologous, T-cell epitope-peptide and (2) a disrupted furin-cleavage motif at the carboxy-terminus of an A1 fragment derived region. Any of the Shiga toxin effector

polypeptide sub-regions comprising an embedded or inserted, heterologous, T-cell epitope described in the Examples below or in WO 2015/113007 may generally be combined with any protease-cleavage resistant, Shiga toxin effector polypeptide sub-region (*e.g.*, modified, Shiga toxin A Subunit sub-regions described herein, described in WO 2015/191764, and/or known in the art) in order to create a combination, Shiga toxin effector polypeptide which, as a component of a cell-targeting molecule, is both protease-cleavage resistant and capable of delivering a heterologous, T-cell epitope to the MHC class I presentation pathway of a target cell. Non-limiting examples of this type of combination Shiga toxin effector polypeptide are shown in SEQ ID NOs: 19–21 and 75–89.

[469] In certain embodiments of the present invention, the Shiga toxin effector polypeptide comprises an embedded or inserted, heterologous, T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. For example in certain embodiments, the Shiga toxin effector polypeptide of the present invention is derived from amino acids 75 to 251 of SEQ ID NO:1, 1 to 241 of SEQ ID NO:1, 1 to 251 of SEQ ID NO:1, or amino acids 1 to 261 of SEQ ID NO:1, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. Similarly in other embodiments, the Shiga toxin effector polypeptide of the present invention is derived from amino acids 75 to 251 of SEQ ID NO:2, 1 to 241 of SEQ ID NO:2, 1 to 251 of SEQ ID NO:2, or amino acids 1 to 261 of SEQ ID NO:2, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. Additionally, the Shiga toxin effector polypeptide may be derived from amino acids 75 to 251 of SEQ ID NO:3, 1 to 241 of SEQ ID NO:3, 1 to 251 of SEQ ID NO:3, or amino acids 1 to 261 of SEQ ID NO:3, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–18; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–18; and (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–18, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of SEQ ID NOs: 1–6, (ii) 1 to 241 of SEQ ID NOs: 1–18, (iii) 1 to 251 of SEQ ID NOs:1–6, or (iv) amino acids 1 to 261 of SEQ ID NOs: 1–3, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous, CD8+ T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18 and 75–89; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6 and 75–89; or (iv) amino acids 1 to 261 of any one

of SEQ ID NOs: 1–3; wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. In certain embodiments, the Shiga toxin effector polypeptide comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18 and 75–89; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6 and 75–89; or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3; wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[470] Certain embodiments of the combination Shiga toxin effector polypeptides of the present invention comprise both (1) a disruption in an endogenous epitope or epitope region and (2) an embedded, heterologous, T-cell epitope-peptide. However, the Shiga toxin effector sub-regions comprising inserted or embedded, heterologous, T-cell epitopes described herein or in WO 2015/191764 are generally not combinable with every de-immunized, Shiga toxin effector sub-regions described herein, except where empirically shown to be successfully combined such that the resulting combination molecule retained a sufficient level of a Shiga toxin effector function(s). The disclosure herein shows how such embodiments may be made and tested to empirically demonstrate success.

[471] The term "successful" is used here to mean two or more amino acid residue substitutions in a Shiga toxin effector polypeptide results in a functional feature, such as, *e.g.*, de-immunization, reduced furin-cleavage, and/or ability to deliver an embedded or inserted epitope, while the modified Shiga toxin effector polypeptide retains one or more Shiga toxin effector functions. The approaches and assays described herein show how to design, make and empirically test embodiments of the present invention, which represent combination, Shiga toxin effector polypeptides and cell-targeting molecules comprising the same.

[472] For example, in certain embodiments of the present invention, the Shiga toxin effector polypeptides is derived from amino acids 75 to 251 of SEQ ID NO:1, 1 to 241 of SEQ ID NO:1, 1 to 251 of SEQ ID NO:1, or amino acids 1 to 261 of SEQ ID NO:1, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region and wherein the disrupted amino acid does not overlap with the embedded or inserted epitope. Similarly in other embodiments, the Shiga toxin effector polypeptide of the present invention is derived from amino acids 75 to 251 of SEQ ID NO:2, 1 to 241 of SEQ ID NO:2, 1 to 251 of SEQ ID NO:2, or amino acids 1 to 261 of SEQ ID NO:2, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region and wherein the disrupted amino acid does not overlap with the embedded or inserted epitope. Additionally, the Shiga toxin effector polypeptide may be derived from amino acids 75 to 251 of SEQ ID NO:3, 1 to 241 of SEQ ID NO:3, 1 to 251 of SEQ ID NO:3, or amino acids 1 to 261 of SEQ ID NO:3, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted,

heterologous T-cell epitope and at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region and wherein the disrupted amino acid does not overlap with the embedded or inserted epitope. In certain embodiments, the Shiga toxin effector polypeptide is derived from the polypeptide that comprises, consists essentially of, or consists of (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6; and (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region and wherein the disrupted amino acid does not overlap with the embedded or inserted epitope. In certain embodiments, the Shiga toxin effector polypeptide is derived from the polypeptide that comprises, consists essentially of, or consists of: (i) amino acids 75 to 251 of SEQ ID NOs: 1–6, (ii) 1 to 241 of SEQ ID NOs: 1–18, (iii) 1 to 251 of SEQ ID NOs: 1–6, or (iv) amino acids 1 to 261 of SEQ ID NOs: 1–3, wherein the Shiga toxin effector polypeptide comprises at least one embedded or inserted, heterologous T-cell epitope and at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region and wherein the disrupted amino acid does not overlap with the embedded or inserted epitope and wherein the embedded or inserted, heterologous T-cell epitope disrupts an additional endogenous, B-cell and/or CD4+ T-cell epitope region.

[473] The combination, Shiga toxin effector polypeptides of the present invention combine the features of their respective sub-regions, such as, *e.g.*, a furin-cleavage motif disruption, individual epitope disruptions, and/or a heterologous T-cell epitope cargo, and these combinations sometimes result in Shiga toxin effector polypeptides with synergistic reductions in immunogenicity as compared to the sum of their partially de-immunized sub-regions. In particular, the exemplary, Shiga toxin effector polypeptides shown in SEQ ID NOs: 19–21 and 75–89 are synergistically de-immunized due to the combination of two or more sub-regions, one of which comprises an embedded, heterologous, T-cell epitope and another of which comprises an endogenous epitope disrupted by one or more amino acid residue substitutions.

[474] In certain embodiments, the combination, de-immunized, protease-cleavage resistant, Shiga toxin effector polypeptides comprising embedded, T-cell epitopes of the present invention comprises one or more substitutions selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of K1R and K11R.

[475] In certain embodiments, the combination, de-immunized, protease-cleavage resistant, Shiga toxin effector polypeptides comprising embedded, T-cell epitopes of the present invention comprise, consist of, or consist essentially of one of the polypeptides represented by the polypeptide sequence shown in any one of SEQ ID NOs: 19–21 and 75–89, represented by amino acids 2 to 252 of SEQ ID NO:35, or represented by amino acids 1 to 251 of SEQ ID NO:107.

[476] De-immunized, Shiga toxin effector polypeptides of the present invention which exhibit no cytotoxicity or reduced cytotoxicity at certain concentrations, *e.g.* Shiga toxin effector polypeptides comprising R179A, may still be useful as de-immunized, Shiga toxin effector polypeptides for delivering

exogenous materials into cells. Similarly, CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptides of the present invention which exhibit no cytotoxicity or reduced cytotoxicity at certain concentrations, *e.g.* a Shiga toxin effector polypeptide comprising an epitope embedded into its catalytic domain (*see e.g.* WO 2015/113005, Example 1-F), may still be useful for delivering a T-cell epitope(s) to a desired subcellular compartment of a cell in which the Shiga toxin effector polypeptide is present or as a component of a cell-targeting molecule for delivery of a T-cell epitope(s) into a target cell.

E. Examples of Cell-Targeting Molecules of the Present Invention

[477] The Shiga toxin effector polypeptides described herein may be used as components of cell-targeting molecules that target various HER2 target biomolecules and epitopes with the aforementioned. The following examples describe in more detail certain structures of exemplary cell-target molecules of the present invention which target cells physically coupled to HER2 at a cellular surface, *e.g.* cells which express HER2. The cell-targeting molecule of the present invention may be a HER2-targeting molecule comprising (i) an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising one or more of: an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous VH domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a VNAR fragment, and an immunoglobulin new antigen receptor; and (ii) a Shiga toxin A Subunit effector polypeptide comprising a Shiga toxin A1 fragment region, wherein the Shiga toxin A subunit effector polypeptide comprises: (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region within the Shiga toxin A1 fragment region; and (b) a disruption of a plurality of endogenous, B-cell and/or CD4+ T-cell epitope regions within the Shiga toxin A1 fragment region which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; wherein the Shiga toxin A1 fragment region comprises a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; wherein the Shiga toxin A subunit effector polypeptide comprises a carboxy-terminal truncation as compared to the carboxy-terminus of a wild-type Shiga toxin A Subunit; wherein the carboxy-terminal truncation results in the removal of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions described herein; wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function (*e.g.* catalytic activity); wherein the HER2-targeting molecule has reduced B-cell antigenicity or immunogenicity and/or reduced CD4+ T-cell antigenicity or immunogenicity; and wherein the binding region and the Shiga toxin effector polypeptide are fused forming a continuous polypeptide such that the binding region is associated with the carboxy-terminus of the Shiga toxin A subunit effector polypeptide.

Other Structural Variations

[478] It is within the scope of the present invention to use fragments, variants, and/or derivatives of the cell-targeting molecules of the present invention which contain a functional binding site to any

extracellular part of a target biomolecule, and even more preferably capable of binding a target biomolecule with high affinity (*e.g.* as shown by K_D). For example, any binding region which binds an extracellular part of a target biomolecule with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter, preferably less than 200 nM, may be substituted for use in making cell-targeting molecules of the invention and methods of the invention.

[479] The skilled worker will recognize that variations may be made to the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention, and polynucleotides encoding any of the former, without diminishing their biological activities, *e.g.*, by maintaining the overall structure and function of the Shiga toxin effector polypeptide, such as in conjunction with one or more 1) endogenous epitope disruptions which reduce antigenic and/or immunogenic potential, 2) furin-cleavage motif disruptions which reduce proteolytic cleavage, and/or 3) embedded or inserted epitopes which reduce antigenic and/or immunogenic potential or are capable of being delivered to a MHC I molecule for presentation on a cell surface. For example, some modifications may facilitate expression, facilitate purification, improve pharmacokinetic properties, and/or improve immunogenicity. Such modifications are well known to the skilled worker and include, for example, a methionine added at the amino-terminus to provide an initiation site, additional amino acids placed on either terminus to create conveniently located restriction sites or termination codons, and biochemical affinity tags fused to either terminus to provide for convenient detection and/or purification. A common modification to improve the immunogenicity of a polypeptide produced using a non-chordate system (*e.g.* a prokaryotic cell) is to remove, after the production of the polypeptide, the starting methionine residue, which may be formylated during production, such as, *e.g.*, in a bacterial host system, because, *e.g.*, the presence of N-formylmethionine (fMet) might induce undesirable immune responses in chordates.

[480] Also contemplated herein is the inclusion of additional amino acid residues at the amino and/or carboxy termini of a Shiga toxin effector polypeptide of the present invention, a cell-targeting molecule of the present invention, or a proteinaceous component of a cell-targeting molecules of the present invention, such as sequences for epitope tags or other moieties. The additional amino acid residues may be used for various purposes including, *e.g.*, facilitating cloning, facilitating expression, post-translational modification, facilitating synthesis, purification, facilitating detection, and administration. Non-limiting examples of epitope tags and moieties are chitin binding protein domains, enteropeptidase cleavage sites, Factor Xa cleavage sites, FIAsH tags, FLAG tags, green fluorescent proteins (GFP), glutathione-S-transferase moieties, HA tags, maltose binding protein domains, myc tags, polyhistidine tags, ReAsH tags, strep-tags, strep-tag II, TEV protease sites, thioredoxin domains, thrombin cleavage site, and V5 epitope tags.

[481] In certain of the above embodiments, the polypeptide sequence of the Shiga toxin effector polypeptides and/or cell-targeting molecules of the present invention are varied by one or more conservative amino acid substitutions introduced into the polypeptide region(s) as long as all required structural features are still present and the Shiga toxin effector polypeptide is capable of exhibiting any required function(s), either alone or as a component of a cell-targeting molecule. As used herein, the

term "conservative substitution" denotes that one or more amino acids are replaced by another, biologically similar amino acid residue. Examples include substitution of amino acid residues with similar characteristics, *e.g.* small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids (*see*, for example, Table C). An example of a conservative substitution with a residue normally not found in endogenous, mammalian peptides and proteins is the conservative substitution of an arginine or lysine residue with, for example, ornithine, canavanine, aminoethylcysteine, or another basic amino acid. For further information concerning phenotypically silent substitutions in peptides and proteins *see*, *e.g.*, Bowie J et al., *Science* 247: 1306-10 (1990).

TABLE C. Examples of Conservative Amino Acid Substitutions

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
A	D	H	C	F	N	A	C	F	A	C	A	A	D
G	E	K	I	W	Q	G	M	H	C	D	C	C	E
P	Q	R	L	Y	S	I	P	W	F	E	D	D	G
S	N		M		T	L		Y	G	H	G	E	K
T			V			V			H	K	N	G	P
									I	N	P	H	Q
									L	Q	S	K	R
									M	R	T	N	S
									R	S	V	Q	T
									T	T		R	
									V			S	
									W			P	
									Y			T	

[482] In the conservative substitution scheme in Table C, exemplary conservative substitutions of amino acids are grouped by physicochemical properties – I: neutral, hydrophilic; II: acids and amides; III: basic; IV: hydrophobic; V: aromatic, bulky amino acids, VI hydrophilic uncharged, VII aliphatic uncharged, VIII non-polar uncharged, IX cycloalkenyl-associated, X hydrophobic, XI polar, XII small, XIII turn-permitting, and XIV flexible. For example, conservative amino acid substitutions include the following: 1) S may be substituted for C; 2) M or L may be substituted for F; 3) Y may be substituted for M; 4) Q or E may be substituted for K; 5) N or Q may be substituted for H; and 6) H may be substituted for N.

[483] Additional conservative amino acid substitutions include the following: 1) S may be substituted for C; 2) M or L may be substituted for F; 3) Y may be substituted for M; 4) Q or E may be substituted for K; 5) N or Q may be substituted for H; and 6) H may be substituted for N.

[484] In certain embodiments, the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention may comprise functional fragments or variants of a polypeptide region of the present invention described herein that have, at most, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions compared to a polypeptide sequence recited herein (and which retain at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or more amino acid sequence identity to the polypeptide sequences recited herein), as long as it (1) comprises at least one embedded or inserted, heterologous T-cell epitope and at

least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region, wherein the disrupted amino acid does not overlap with the embedded or inserted epitope; (2) comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region; (3) comprises a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region and comprises at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region, wherein the disrupted amino acid does not overlap with the disrupted furin-cleavage motif; or (4) comprises at least one embedded or inserted, heterologous T-cell epitope, at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region, wherein the disrupted amino acid does not overlap with the embedded or inserted epitope, and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. Variants of the Shiga toxin effector polypeptides and cell-targeting molecules of the invention are within the scope of the present invention as a result of changing a polypeptide described herein by altering one or more amino acid residues or deleting or inserting one or more amino acid residues, such as within the binding region or Shiga toxin effector polypeptide region, in order to achieve desired properties, such as changed cytotoxicity, changed cytostatic effects, changed immunogenicity, and/or changed serum half-life. The Shiga toxin effector polypeptides and cell-targeting molecules of the present invention may further be with or without a signal sequence.

[485] Accordingly, in certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) identical to the amino acid sequence shown in any one of SEQ ID NOs: 22–36 and 97–108. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 25–31, 34–36, 97–104, and 106–108. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 29, 31, 34, 35, 36, 102, 104, and 106–108. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 29 or 102. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 31 or 104. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid

sequence shown in any one of SEQ ID NOs: 34 or 106. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 35 or 107. In certain embodiments, the cell-targeting molecule of the present invention comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% (such as at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more) identical to the amino acid sequence shown in any one of SEQ ID NOs: 36 or 108.

[486] Accordingly, in certain embodiments, the Shiga toxin effector polypeptides of the present invention comprise, consists essentially of, or consists of amino acid sequences having at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99%, overall sequence identity to a naturally occurring (*e.g.* a wild-type) Shiga toxin A Subunit or fragment thereof, such as, *e.g.*, Shiga toxin A Subunit, such as SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), SLT-2A (SEQ ID NO:3), Stx1cA (SEQ ID NO:4), Stx1dA (SEQ ID NO:5), Stx1eA (SEQ ID NO:6), Stx2cA variant 1 (SEQ ID NO:7), Stx2cA variant 2 (SEQ ID NO:8), Stx2cA variant 3 (SEQ ID NO:9), Stx2cA variant 4 (SEQ ID NO:10), Stx2cA variant 5 (SEQ ID NO:11), Stx2cA variant 6 (SEQ ID NO:12), Stx2dA variant 1 (SEQ ID NO:13), Stx2dA variant 2 (SEQ ID NO:14), Stx2dA variant 3 (SEQ ID NO:15), Stx2eA variant 1 (SEQ ID NO:16), Stx2eA variant 2 (SEQ ID NO:17), and/or Stx2fA (SED NO:18), wherein the Shiga toxin effector polypeptide (1) comprises at least one embedded or inserted, heterologous T-cell epitope and at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region, and wherein the disrupted amino acid does not overlap with the embedded or inserted epitope; (2) comprises at least one embedded or inserted, heterologous T-cell epitope and a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region; or (3) comprises a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region and comprises at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region, and wherein the disrupted amino acid does not overlap with the disrupted furin-cleavage motif; or (4) comprises (i) at least one embedded or inserted, heterologous T-cell epitope, (ii) at least one amino acid is disrupted in an endogenous, B-cell and/or CD4+ T-cell epitope region, wherein the disrupted amino acid does not overlap with the embedded or inserted epitope, and (iii) a disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region. As described herein, fragments of the Shiga toxin A Subunit may comprise, consist essentially of, or consists of: (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6, 37, and 75–89; (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18, 37, and 75–89; (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6, 37 and 75–89; or (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3. For example, the fragments of the Shiga toxin A Subunit may comprise, consist essentially of, or consists of amino acids: (i) 75 to 251 of any one SEQ ID NOs: 75–89, (ii) 1 to 241 of any one of SEQ ID NOs: 75–89, (iii) 1 to 251 of any one of SEQ ID NOs: 75–89, or (iv) 1 to 261 of any one of SEQ ID NOs: 1–3.

[487] In certain embodiments of the Shiga toxin effector polypeptides of the present invention, one or more amino acid residues may be mutated, inserted, or deleted in order to increase the enzymatic activity of the Shiga toxin effector polypeptide. In certain embodiments of the Shiga toxin effector polypeptides of the present invention, one or more amino acid residues may be mutated or deleted in order to reduce or eliminate catalytic and/or cytotoxic activity of the Shiga toxin effector polypeptide. For example, the catalytic and/or cytotoxic activity of the A Subunits of members of the Shiga toxin family may be diminished or eliminated by mutation or truncation.

[488] The cytotoxicity of the A Subunits of members of the Shiga toxin family may be altered, reduced, or eliminated by mutation and/or truncation. The positions labeled tyrosine-77, glutamate-167, arginine-170, tyrosine-114, and tryptophan-203 have been shown to be important for the catalytic activity of Stx, Stx1, and Stx2 (Hovde C et al., *Proc Natl Acad Sci USA* 85: 2568-72 (1988); Deresiewicz R et al., *Biochemistry* 31: 3272-80 (1992); Deresiewicz R et al., *Mol Gen Genet* 241: 467-73 (1993); Ohmura M et al., *Microb Pathog* 15: 169-76 (1993); Cao C et al., *Microbiol Immunol* 38: 441-7 (1994); Suhan M, Hovde C, *Infect Immun* 66: 5252-9 (1998)). Mutating both glutamate-167 and arginine-170 eliminated the enzymatic activity of Slt-I A1 in a cell-free ribosome inactivation assay (LaPointe P et al., *J Biol Chem* 280: 23310-18 (2005)). In another approach using *de novo* expression of Slt-I A1 in the endoplasmic reticulum, mutating both glutamate-167 and arginine-170 eliminated Slt-I A1 fragment cytotoxicity at that expression level (LaPointe P et al., *J Biol Chem* 280: 23310-18 (2005)). A truncation analysis demonstrated that a fragment of StxA from residues 75 to 268 still retains significant enzymatic activity *in vitro* (Haddad J et al., *J Bacteriol* 175: 4970-8 (1993)). A truncated fragment of Slt-I A1 containing residues 1-239 displayed significant enzymatic activity *in vitro* and cytotoxicity by *de novo* expression in the cytosol (LaPointe P et al., *J Biol Chem* 280: 23310-18 (2005)). Expression of a Slt-I A1 fragment truncated to residues 1-239 in the endoplasmic reticulum was not cytotoxic because it could not retrotranslocate to the cytosol (LaPointe P et al., *J Biol Chem* 280: 23310-18 (2005)).

[489] The most critical residues for enzymatic activity and/or cytotoxicity in the Shiga toxin A Subunits were mapped to the following residue-positions: asparagine-75, tyrosine-77, tyrosine-114, glutamate-167, arginine-170, arginine-176, and tryptophan-203 among others (Di R et al., *Toxicon* 57: 525-39 (2011)). In particular, a double-mutant construct of Stx2A containing glutamate-E167-to-lysine and arginine-176-to-lysine mutations was completely inactivated; whereas, many single mutations in Stx1 and Stx2 showed a 10-fold reduction in cytotoxicity. Further, truncation of Stx1A to 1-239 or 1–240 reduced its cytotoxicity, and similarly, truncation of Stx2A to a conserved hydrophobic residue reduced its cytotoxicity. The most critical residues for binding eukaryotic ribosomes and/or eukaryotic ribosome inhibition in the Shiga toxin A Subunit have been mapped to the following residue-positions arginine-172, arginine-176, arginine-179, arginine-188, tyrosine-189, valine-191, and leucine-233 among others (McCluskey A et al., *PLoS One* 7: e31191 (2012). However, certain modification may increase a Shiga toxin functional activity exhibited by a Shiga toxin effector polypeptide of the present invention. For example, mutating residue-position alanine-231 in Stx1A to glutamate increased Stx1A's enzymatic activity *in vitro* (Suhan M, Hovde C, *Infect Immun* 66: 5252-9 (1998)).

[490] In certain embodiments of Shiga toxin effector polypeptides of the present invention derived from SLT-1A (SEQ ID NO:1) or StxA (SEQ ID NO:2), the one or more amino acid residues mutated include substitution of the asparagine at position 75, tyrosine at position 77, tyrosine at position 114, glutamate at position 167, arginine at position 170, arginine at position 176, and/or substitution of the tryptophan at position 203. Examples of such substitutions will be known to the skilled worker based on the prior art, such as asparagine at position 75 to alanine, tyrosine at position 77 to serine, substitution of the tyrosine at position 114 to serine, substitution of the glutamate position 167 to glutamate, substitution of the arginine at position 170 to alanine, substitution of the arginine at position 176 to lysine, substitution of the tryptophan at position 203 to alanine, and/or substitution of the alanine at 231 with glutamate. Other mutations which either enhance or reduce Shiga toxin enzymatic activity and/or cytotoxicity are within the scope of the invention and may be determined using well known techniques and assays disclosed herein.

[491] In certain embodiments, the cell-targeting molecule of the present invention may be monovalent and/or monomeric. In certain embodiments, the cell-targeting molecule of the present invention may not be multivalent and/or multimeric. As demonstrated by the Examples of the application, monovalent amd/or monomeric forms of certain cell-targeting molecules may exhibit low levels of toxicity when used *in vivo* while still exhibiting potent cytotoxic to HER2-expressing cells.

[492] The Shiga toxin effector polypeptides and cell-targeting molecules of the present invention may optionally be conjugated to one or more additional agents, which may include therapeutic agents, diagnostic agents, and/or other additional exogenous materials known in the art, including such agents as described herein. In certain embodiments, the Shiga toxin effector polypeptide or cell-targeting molecule of the present invention is PEGylated or albuminated, such as, *e.g.*, to provide de-immunization, disrupt furin-cleavage by masking the extended loop and/or the furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region, improve pharmacokinetic properties, and/or improve immunogenicity (*see e.g.*, Wang Q et al., *Cancer Res* 53: 4588-94 (1993); Tsutsumi Y et al., *Proc Natl Acad Sci USA* 97: 8548-53 (2000); Buse J, El-Aneed A, *Nanomed* 5: 1237-60 (2010); Lim S et al., *J Control Release* 207-93 (2015)).

V. General Functions of the Cell-Targeting Molecules of the Present Invention

[493] The functional association of Shiga toxin effector polypeptides of the present invention with cell-targeting binding regions enables the creation of cell-targeting molecules which selectively kill, inhibit the growth of, deliver exogenous material to, and/or detect specific cell types. The properties of the Shiga toxin effector polypeptide of the present invention enable the creation of cell-targeting molecules with improved therapeutic windows in chordates as compared to prior Shiga toxin effector polypeptides.

[494] For certain embodiments, the cell-targeting molecule of the present invention provides, after administration to a chordate, one or more of the following: 1) potent and selective killing of targeted cells, *e.g.*, infected or malignant cells, at low administration doses, 2) linkage stability between the cell-targeting binding region and the Shiga toxin effector polypeptide region while the cell-targeting molecule

is present in extracellular spaces, 3) low levels of off-target cell deaths and/or unwanted tissue damage, and 4) cell-targeted delivery of heterologous, CD8+ T-cell epitopes for presentation by target cells in order to initiate desirable, T-cell mediated, immune responses, such as, *e.g.*, the recruitment of CD8+ T-cells and the localized release of cytokines at a tissue locus.

5 [495] The Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are useful in diverse applications involving, *e.g.*, cell-killing; cell growth inhibition; intracellular, cargo delivery; biological information gathering; immune response stimulation, and/or remediation of a health condition. The Shiga toxin effector polypeptides of the present invention are useful as components of various therapeutic and/or diagnostic molecules, such as, *e.g.* ligand-toxin fusions, immunotoxins, and/or

10 immuno-conjugates. The cell-targeting molecules of the present invention are useful as therapeutic and/or diagnostic molecules, such as, *e.g.*, as cell-targeting, cytotoxic, therapeutic molecules; cell-targeting, nontoxic, delivery vehicles; and/or cell-targeting, diagnostic molecules; for examples in applications involving the *in vivo* targeting of specific cell types for the diagnosis or treatment of a variety of diseases, including cancers, immune disorders, and microbial infections.

15 [496] Depending on the embodiment, a Shiga toxin effector polypeptide or cell-targeting molecule of the present invention may have or provide one or more of the following characteristics or functionalities: (1) de-immunization, (2) protease-cleavage resistance, (3) potent cytotoxicity at certain concentrations, (4) intracellular delivery of a cargo consisting of an additional material (*e.g.* a heterologous, T-cell epitope), (4) selective cytotoxicity, (6) low off-target toxicity in multicellular organisms at certain doses

20 or dosages, (7) delivery of a heterologous, T-cell epitope to the MHC class I presentation pathway of a target cell, and/or (8) stimulation of CD8+ T-cell immune response(s). Certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are multi-functional because the molecules have two or more of the characteristics or functionalities described herein. Certain further embodiments of the cell-targeting molecules of the present invention provide all

25 of the aforementioned characteristics and functionalities in a single molecule.

[497] The associating, coupling, and/or linking of a cell-targeting binding region(s) with a Shiga toxin effector polypeptide(s) of the present invention enables the engineering of cell-targeting molecules with Shiga toxin function(s) that can produce less adverse effects after administration at certain doses or dosages to a multicellular organism such as a mammal. Non-limiting examples of adverse effects include

30 off-target toxicities, untargeted cytotoxicities, and/or unwanted immune responses. Certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are particularly useful in applications involving administration of a Shiga toxin effector polypeptide and/or cell-targeting molecule to a chordate because of functional properties, such as, *e.g.*, de-immunization, reduced off-target toxicities, and/or targeted stimulation of desirable immune responses such as via cell-

35 surface presentation of a cell-targeting molecule delivered, CD8+ T-cell epitope.

[498] In certain embodiments, the cell-targeting molecules of the present invention are capable of binding extracellular target biomolecules associated with the cell surface of particular cell types and entering those cells. Once internalized within a targeted cell type, certain embodiments of the cell-

targeting molecules of the invention are capable of routing an enzymatically active, cytotoxic, Shiga toxin effector polypeptide fragment into the cytosol of the target cell and eventually killing the cell. Alternatively, nontoxic or reduced-toxicity variants of the cell-targeting molecules of the present invention may be used to deliver additional exogenous materials into target cells, such as epitopes,
5 peptides, proteins, polynucleotides, and detection promoting agents. This system is modular, in that any number of diverse binding regions can be used to target a Shiga toxin effector polypeptide of the present invention to various, diverse cell types.

A. De-Immunization for Applications Involving Administration to a Chordate

10 [499] The de-immunization of the Shiga toxin effector polypeptides of the present invention is accomplished by engineering disruptions of one or more, endogenous, B-cell and/or CD4+ T-cell epitopes regions of a Shiga toxin A Subunit or Shiga toxin effector polypeptide, including via mutation and/or truncation or via the conjugation of a covalently-linked chemical structure. Because B-cell epitopes often coincide or overlap with epitopes of mature CD4+ T-cells, the disruption of an
15 endogenous, B-cell epitope region often simultaneously disrupts an endogenous, CD4+ T-cell epitope or vice versa.

[500] Certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are de-immunized with respect to one or more B-cell and/or CD4+ T-cell epitopes meaning that these molecules exhibit reduced antigenic and/or immunogenic potential as compared to
20 prior, Shiga toxin effector polypeptides and cell-targeting molecules lacking identical disruptions to the same B-cell and/or CD4+ T-cell epitope or epitope regions and/or lacking any disruption to the same B-cell and/or CD4+ T-cell epitope(s) or epitope region(s). Certain further embodiments exhibit potent if not wild-type levels of Shiga toxin A Subunit catalytic domain dependent cytotoxicity despite the presence of multiple mutations providing the de-immunized property. The de-immunized, Shiga toxin
25 effector polypeptides and cell-targeting molecules of the present invention are useful for applications involving the parenteral administration of a Shiga toxin effector polypeptide and/or cell-targeting molecule to a chordate such as, *e.g.*, a mammal, amphibian, bird, fish, reptiles, or shark, because of the reduced likelihood of producing undesirable immune responses invoked by the administrated molecule.

[501] The various de-immunized, Shiga toxin effector polypeptides of the present invention might
30 differ in their antigenicity profiles when administered to various chordate species, but all the de-immunized polypeptides of the invention exhibit reduced antigenicity and/or immunogenicity in at least one organism as measured by at least one quantitative assay. In particular, certain embodiments of the cell-targeting molecules of the present invention are de-immunized with respect to a mammalian recipient, such as, *e.g.*, the molecule invokes lower quantities and/or frequencies of "anti-cell-targeting
35 molecule" antibodies when administered to that mammal as compared to a reference molecule (*e.g.* a related cell-targeting molecule comprising a wild-type Shiga toxin A1 fragment). In addition, Shiga toxin effector polypeptides of the present invention having disruptions of multiple, endogenous, epitope

regions are expected to more greatly reduced the probability of the occurrence of undesirable immune responses in a chordate recipient of such a polypeptide.

[502] For certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention, the de-immunization property(ies) is a result of the structural change(s) which include the disrupted furin-cleavage motif at the carboxy-terminus of a Shiga toxin A1 fragment derived region.

[503] For certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention, the de-immunization property(ies) is a result of the structural change(s) which include the embedding and/or inserting of a T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region.

[504] For certain embodiments, the desired biological function(s) of the parental, Shiga toxin polypeptide from which the de-immunized, Shiga toxin effector polypeptide was derived are preserved, such as, *e.g.*, the Shiga toxin A Subunit functions of promoting cellular internalization, directing intracellular routing, and potent cytotoxicity. Preservation refers to the retention of a minimal level of activity as described herein.

B. Reduced Protease-Cleavage Sensitivity

[505] Certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention exhibit reduced protease-cleavage sensitivity as compared to related molecules comprising wild-type, Shiga toxin A1 fragment regions. Certain further embodiments exhibit potent if not optimal, Shiga toxin A Subunit catalytic domain dependent cytotoxicity despite this reduced protease-cleavage sensitivity and lack of a canonical furin-cleavage event within an intoxicated cell.

[506] Certain embodiments of the protease-cleavage resistant, cell-targeting molecules of the present invention (*i.e.* a cell-targeting molecule comprising a Shiga toxin effector polypeptide comprising a disrupted furin-cleavage motif at the carboxy-terminus of its Shiga toxin A1 fragment region) exhibit improved *in vivo* tolerability as compared to related molecules comprising a wild-type, Shiga toxin A1 fragment region. Certain further embodiments exhibit potent if not optimal, Shiga toxin A Subunit catalytic domain dependent cytotoxicity despite this reduced protease-cleavage sensitivity and lack of a canonical furin-cleavage event within an intoxicated cell.

[507] Previously, it was believed that cytotoxic, Shiga toxin A Subunit constructs comprising Shiga toxin A1 fragment catalytic regions must maintain or somehow compensate for the naturally occurring proteolytic processing by furin within intoxicated cells in order to preserve the Shiga toxin's natural adaptations for efficient and potent cytotoxicity. It was unexpectedly discovered that the furin cleavage event was not required for potent cytotoxicity because potent Shiga toxin cytotoxicity at the level of a wild-type Shiga toxin control construct was achieved in the absence of any furin cleavage event at the carboxy-terminus of the Shiga toxin A1 fragment despite the presence of a carboxy-terminal moiety (*see e.g.* WO 2015/191764; WO 2016/196344). The lack of a furin-cleavage event within the intoxicated cell may prevent the efficient liberation of a Shiga toxin A1 fragment-like region and, thus, result in the

continued linkage of a relatively large moiety (*e.g.* greater than 28 kDa in size) to the Shiga toxin A1 fragment region. However despite this possibility, potent, Shiga toxin cytotoxicity was achieved with furin-cleavage deficient constructs comprising a Shiga toxin effector polypeptide region and lacking any known compensatory feature(s), such as, *e.g.*, providing intracellular cleavage proximal to the carboxy-terminus of a Shiga toxin A1 fragment derived region (*see e.g.* WO 2015/191764; WO 2016/196344).

[508] This suggests that the persistence and/or inefficient release of a relatively large, molecular moiety linked to the A1 fragment region did not necessarily reduce the potency of Shiga toxin cytotoxicity. This was surprising because the optimal Shiga toxin intoxication process was thought to require liberation of the Shiga toxin A1 fragments from all other large molecular moieties to efficiently retrotranslocate liberated A1 fragments from the endoplasmic reticulum to the cytosol where the A1 fragments can form an enzymatically active structure that catalytically inactivates the intoxicated cell's ribosomes. In particular, the persistence and/or inefficient release of a relatively large molecular moiety covering the carboxy-terminus of the Shiga toxin A1 fragment was expected to interfere with the Shiga toxin A1 fragment's natural mechanism of efficiently gaining access to the cytosol, which involves the exposure of the A1 fragment's, hydrophobic, carboxy-terminal domain and recognition of this domain by the ERAD system (*see* Di R et al., *Toxicon* 57: 525-39 (2011); Li S et al., *PLoS One* 7: e41119 (2012)).

[509] The lack of an intoxicated-cell-mediated, furin-cleavage event for a molecule comprising a Shiga toxin A Subunit derivative may be hypothetically compensated for. Non-limiting examples of potential, compensatory approaches include 1) terminating one carboxy-terminus of the construct with the carboxy-terminus of a Shiga toxin A1 fragment-like polypeptide region, 2) producing the Shiga toxin derived construct such that the Shiga toxin A Subunit polypeptide is already nicked near the carboxy-terminus of its Shiga toxin A1 fragment-like polypeptide, 3) engineering a heterologous and/or ectopic protease site that can functionally substitute for the lack of the native, Shiga toxin, furin-cleavage event, and 4) a combination of approach 3 and 4.

[510] In the first approach, the carboxy-terminus of the Shiga toxin A1 fragment-like polypeptide is not covered by any carboxy-terminal moiety, and, thus, the carboxy-terminus of the Shiga toxin A1 fragment-like polypeptide is permanently exposed for recognition by the ERAD machinery in the endoplasmic reticulum. In the last three approaches, the Shiga toxin A1 fragment-like polypeptide can be designed to intracellularly dissociate from one or more other components of the construct by the time the molecule reaches the endoplasmic reticulum of an intoxicated cell such that in the endoplasmic reticulum the carboxy-terminus of the Shiga toxin A1 fragment-like polypeptide becomes exposed for recognition by the ERAD machinery. For example, a cytotoxic molecule comprising a Shiga toxin A Subunit effector polypeptide could be pretreated with a protease to nick the polypeptide region near the carboxy terminus of the A1 fragment-like region prior to contacting a target cell. Alternatively, the cytotoxic molecule could be engineered to comprise a protease site which is cleaved by an intracellular protease of the target cell.

[511] These hypothetical approaches for designing Shiga toxin A Subunit effector polypeptides which compensate for the lack of an intoxicated-cell-mediated, furin-cleavage event may significantly alter the

efficiency and potency of cytotoxicity as compared to a wild-type Shiga holotoxin or Shiga toxin A Subunit construct comprising only wild-type sequences which include the optimal, naturally occurring, furin-cleavage site. For example, currently no compensatory approach relying on a target cell endoprotease other than furin is known which can provide fully compensatory cytotoxicity equivalent to furin cleavage and alternative cellular proteases to furin like calpains have been shown to be less efficient in facilitating Shiga toxin cytotoxicity (Garred O et al., *Exp Cell Res* 218: 39-49 (1995); Garred O et al., *J Biol Chem* 270: 10817-21 (1995); Kurmanova A et al., *Biochem Biophys Res Commun* 357: 144-9 (2007)).

[512] The present invention provides furin-cleavage resistant Shiga toxin A Subunit effector polypeptides which are potently cytotoxic, whether due to compensation for a lack of a furin cleavage event within the intoxicated cell or due to some unexplained reason. Certain cell-targeting molecules of the present invention are at least as efficiently and potently cytotoxic as cell-targeting molecules comprising protease-cleavage sensitive, wild-type Shiga toxin effector polypeptide regions (*see e.g.* WO 2016/196344).

C. Improved Stability and *In Vivo* Tolerability

[513] In certain embodiments, the molecules of the present invention (*e.g.* cell-targeting molecules of the invention) exhibit increased stability and/or improved *in vivo* tolerability as compared to more furin-cleavage sensitive analogs and/or less de-immunized analogs (an analog being a closely related molecule lacking one or more structural features of the present invention).

[514] The increased stability of a cell-targeting molecule compared to a reference molecule can be exhibited *in vitro* and/or *in vivo*. The stability of a therapeutic or diagnostic molecule over time is an important feature and can affect for which applications the molecule may be practically employed. Molecular stability includes *in vitro* and *in vivo*, such as, *e.g.*, stability within an organism after administration and during storage over a range of temperatures and concentrations. For certain immunotoxins or ligand-toxin fusions, the stability of the linkage between the toxin and other components can affect the amount of non-specific toxicity caused by the presence and/or quantity of untargeted toxin over time within the organism.

[515] Certain cell-targeting molecules of the present invention exhibit reduced non-specific toxicity *in vivo*, manifested as increased *in vivo* tolerability as compared to more protease-cleavage sensitive variants. *In vivo* tolerability can be determined by the skilled worker using techniques known in the art and/or described herein. In addition to assessing *in vivo* tolerability using mortality, signs of morbidity may be used for assessing *in vivo* tolerability, such as, *e.g.*, aspects of body weight, physical appearance, measureable clinical signs, unprovoked behavior, and responses to external stimuli (*see e.g.* Morton D, Griffiths P, *Vet Rec* 116: 431-43 (1985); Montgomery C, *Cancer Bull* 42: 230-7 (1990); Ullman-Culleré M, Foltz C, *Lab Anim Sci* 49: 319-23 (1999); Clingerman K, Summers L, *J Am Assoc Lab Anim Sci* 51: 31-6 (2012)). Euthanasia may be used in response to signs of morbidity and/or morbundity and, thus, create a mortality time-point. For example, a decrease in body weight of 15–20% in 2–3 days can be

used as a sign of morbidity in rodents and as a justification for euthanization (*see e.g.* Institute of Laboratory Animal Research 2011. *Guide for the care and use of laboratory animals*, 8th ed., Washington, DC, U.S.: National Academies Press).

[516] The improved *in vivo* tolerability observed for exemplary, cell-targeting molecules of the present invention as compared to more furin-cleavage sensitive analogs suggests that much higher doses of these cell-targeting molecules of the invention may be safely administered to mammals as compared to the doses of related molecules comprising a furin-cleavage sensitive, Shiga toxin effector polypeptide region. Certain cell-targeting molecules of the invention might exhibit reduced non-specific toxicity as compared to more protease sensitive variants because the protease resistance serves to protect and preserve the linkage between the Shiga toxin effector component and the cell-targeting moiety component.

[517] In addition, *in vivo* tolerability for cell-targeting molecules of the present invention may be related to the de-immunization properties of a given cell-targeting molecule. Thus, higher doses of such de-immunized, cell-targeting molecules of the invention may be safely administered to mammals as compared to the doses of related molecules comprising an "un-de-immunized" or less de-immunized, Shiga toxin effector polypeptide (*e.g.* a wild-type Shiga toxin A1 fragment).

[518] In addition, certain molecules of the invention exhibit increased half-lives, both *in vitro* and/or *in vivo*, as compared to more protease-cleavage sensitive variants. Molecular stability can be assayed by determining the half-life of a molecule of interest with regard to the association of its components. Certain embodiments of the molecules of the invention will have longer half-lives as compared to furin-cleavage sensitive variants, especially with regard to the continued association of the Shiga toxin effector polypeptide component and one or more other components. For example, certain embodiments of the molecules of the invention will have longer half-lives with regard to the continued association of the Shiga toxin effector polypeptide component and another component, *e.g.* a cell-targeting binding region, as compared to a furin-cleavage sensitive variant wherein the furin-cleavage sensitive site(s) lies between those two components.

D. Cell-Kill via Shiga Toxin A Subunit Cytotoxicity

[519] Certain embodiments of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are cytotoxic. Certain further embodiments of the cell-targeting molecules of the present invention are cytotoxic only due to the presence of one or more Shiga toxin effector polypeptide components. The A Subunits of members of the Shiga toxin family each comprise an enzymatically active polypeptide region capable of killing a eukaryotic cell once in the cell's cytosol. Because members of the Shiga toxin family are adapted to killing eukaryotic cells, molecules derived from Shiga toxins, such as, *e.g.*, molecules comprising certain embodiments of the Shiga toxin effector polypeptides of the present invention can exhibit potent cell-kill activities.

[520] For certain embodiments of the cell-targeting molecules of the present invention, upon contacting a cell physically coupled with an extracellular target biomolecule of the binding region of the cell-targeting molecule (*e.g.* a target positive cell), the cell-targeting molecule is capable of causing death of

the cell. For certain further embodiments, the CD_{50} value of the cell-targeting molecule is less than 5, 2.5, 1, 0.5, or 0.25 nM, which is vastly more potent than an untargeted, wild-type, Shiga toxin effector polypeptide (*e.g.* SEQ ID NOs: 1–18).

[521] Cell-kill may be accomplished using a molecule of the present invention under varied conditions of target cells, such as, *e.g.*, an *ex vivo* manipulated target cell, a target cell cultured *in vitro*, a target cell within a tissue sample cultured *in vitro*, or a target cell in an *in vivo* setting like within a multicellular organism.

[522] In certain embodiments, the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention comprise (1) a de-immunized, Shiga toxin effector sub-region, (2) a protease-cleavage resistant region near the carboxy-terminus of a Shiga toxin A1 fragment derived region, (3) a carboxy-terminal, endoplasmic reticulum retention/retrieval signal motif; and/or (4) a heterologous, T-cell epitope embedded or inserted region; however, for certain further embodiments, these structural modifications do not significantly alter the potency of Shiga toxin cytotoxicity as compared to a reference molecules comprising a wild-type Shiga toxin A Subunit polypeptide, such as, *e.g.*, a wild-type Shiga toxin A1 fragment. Thus, Shiga toxin effector polypeptides and cell-targeting molecules of the present invention which are de-immunized, protease cleavage resistant, and/or carrying embedded or inserted, heterologous, epitopes can maintain potent cytotoxicity while providing one or more various other functionalities or properties.

[523] Already cytotoxic cell-targeting molecules comprising Shiga toxin effector polypeptides may be engineered by the skilled worker using the information and methods provided herein to be more cytotoxic and/or to have redundant, backup cytotoxicities operating via completely different mechanisms. These multiple cytotoxic mechanisms may complement each other by their diversity of functions (such as by providing potent killing via two mechanisms of cell-killing, direct and indirect, as well as mechanisms of immuno-stimulation to the local area), redundantly backup each other (such as by providing one cell-killing mechanism in the absence of the other mechanisms—like if a target cell is resistant to or acquires some immunity to a subset of previously active mechanisms), and/or protect against developed resistance (by limiting resistance to the less probable situation of the malignant or infected cell blocking multiple, different cell-killing mechanisms simultaneously).

E. Delivery of a T-Cell Epitope for MHC Class I Presentation on a Cell Surface

[524] In certain embodiments, the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention comprise a T-cell epitope, which enables the engineering of "T-cell epitope delivering" molecules with virtually unlimited choices of epitope-peptide cargos for delivery and cell-surface presentation by a nucleated, chordate cell. For certain embodiments, the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are each capable of delivering one or more T-cell epitopes, associated with the Shiga toxin effector polypeptides and/or cell-targeting molecules, to the proteasome of a cell. The delivered T-cell epitope are then proteolytic processed and presented by the MHC class I pathway on the surface of the cell. By engineering MHC class I epitopes

into cell-targeting molecules, the targeted delivery and presentation of immuno-stimulatory antigens may be accomplished in order to harness and direct a beneficial function(s) of a chordate immune system.

[525] For certain embodiments, the cell-targeting molecule of the present invention is capable of delivering a T-cell epitope to a MHC class I molecule of a cell for cell-surface presentation. In certain embodiments, the Shiga toxin effector polypeptide or cell-targeting molecule of the present invention comprises a heterologous, T-cell epitope, whether as an additional exogenous material or embedded or inserted within a Shiga toxin effector polypeptide. For certain further embodiments, the Shiga toxin effector polypeptide or cell-targeting molecule of the present invention is capable of delivering an embedded or inserted T-cell epitope to a MHC class I molecule for cell-surface presentation.

[526] For certain embodiments, the Shiga toxin effector polypeptide of the present invention is capable of delivering a T-cell epitope, which is embedded or inserted in the Shiga toxin effector polypeptide, to a MHC class I molecule of a cell in which the Shiga toxin effector polypeptide is present for presentation of the T-cell epitope by the MHC class I molecule on a surface of the cell. For certain further embodiments, the T-cell epitope is a heterologous, T-cell epitope. For certain further embodiments, the T-cell epitope functions as CD8+ T-cell epitope, whether already known or identified in the future using methods which are currently routine to the skilled worker.

[527] For certain embodiments, the cell-targeting molecule of the present invention is capable of delivering a T-cell epitope, which is associated with the cell-targeting molecule, to a MHC class I molecule of a cell for presentation of the T-cell epitope by the MHC class I molecule on a surface of the cell. For certain further embodiments, the T-cell epitope is a heterologous, T-cell epitope which is embedded or inserted in the Shiga toxin effector polypeptide. For certain further embodiments, the T-cell epitope functions as CD8+ T-cell epitope, whether already known or identified in the future using methods which are currently routine to the skilled worker.

[528] For certain embodiments, upon contacting a cell with the cell-targeting molecule of the present invention, the cell-targeting molecule is capable of delivering a T-cell epitope-peptide, which is associated with the cell-targeting molecule, to a MHC class I molecule of the cell for presentation of the T-cell epitope-peptide by the MHC class I molecule on a surface of the cell. For certain further embodiments, the T-cell epitope-peptide is a heterologous epitope which is embedded or inserted in a Shiga toxin effector polypeptide. For certain further embodiments, the T-cell epitope-peptide functions as CD8+ T-cell epitope, whether already known or identified in the future using methods which are currently routine to the skilled worker.

[529] The addition of a heterologous epitope into or presence of a heterologous epitope in a cell-targeting molecule of the present invention, whether as an additional exogenous material or embedded or inserted within a Shiga toxin effector polypeptide, enables methods of using such cell-targeting molecules for the cell-targeted delivery of a chosen epitope for cell-surface presentation by a nucleated, target cell within a chordate.

[530] One function of certain, CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptides and cell-targeting molecules of the present invention is the delivery of one or more T-cell epitope-peptides to

a MHC class I molecule for MHC class I presentation by a cell. Delivery of exogenous, T-cell epitope-peptides to the MHC class I system of a target cell can be used to induce the target cell to present the T-cell epitope-peptide in association with MHC class I molecules on the cell surface, which subsequently leads to the activation of CD8+ effector T-cells to attack the target cell.

5 [531] The skilled worker, using techniques known in the art, can associate, couple, and/or link certain, Shiga toxin effector polypeptides of the present invention to various other cell-targeting binding region to create cell-targeting molecules of the present invention which target specific, extracellular, target biomolecules physically coupled to cells and promote target-cell internalization of these cell-targeting molecules. All nucleated vertebrate cells are believed to be capable of presenting intracellular epitopes

10 using the MHC class I system. Thus, extracellular target biomolecules of the cell-targeting molecules of the invention may in principle target any nucleated vertebrate cell for T-cell epitope delivery to a MHC class I presentation pathway of such a cell.

[532] The epitope-delivering functions of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention can be detected and monitored by a variety of standard methods

15 known in the art to the skilled worker and/or described herein. For example, the ability of cell-targeting molecules of the present invention to deliver a T-cell epitope-peptide and drive presentation of the epitope-peptide by the MHC class I system of target cells may be investigated using various *in vitro* and *in vivo* assays, including, *e.g.*, the direct detection/visualization of MHC class I/peptide complexes, measurement of binding affinities for the heterologous, T-cell epitope-peptide to MHC class I molecules,

20 and/or measurement of functional consequences of MHC class I-peptide complex presentation on target cells by monitoring cytotoxic T-lymphocyte (CTL) responses (*see e.g.* Examples, *infra*).

[533] Certain assays to monitor this function of the polypeptides and molecules of the present invention involve the direct detection of a specific MHC class I/peptide antigen complex *in vitro* or *ex vivo*. Common methods for direct visualization and quantitation of peptide-MHC class I complexes

25 involve various immuno-detection reagents known to the skilled worker. For example, specific monoclonal antibodies can be developed to recognize a particular MHC/class I/peptide antigen complex. Similarly, soluble, multimeric T cell receptors, such as the TCR-STAR reagents (Altor Bioscience Corp., Mirmar, FL, U.S.) can be used to directly visualize or quantitate specific MHC I/antigen complexes (Zhu X et al., *J Immunol* 176: 3223-32 (2006)). These specific mAbs or soluble, multimeric T-cell receptors

30 may be used with various detection methods, including, *e.g.* immunohistochemistry, flow cytometry, and enzyme-linked immuno assay (ELISA).

[534] An alternative method for direct identification and quantification of MHC I/peptide complexes involves mass spectrometry analyses, such as, *e.g.*, the ProPresent Antigen Presentation Assay (ProImmune, Inc., Sarasota, FL, U.S.) in which peptide-MCH class I complexes are extracted from the

35 surfaces of cells, then the peptides are purified and identified by sequencing mass spectrometry (Falk K et al., *Nature* 351: 290–6 (1991)).

[535] In certain assays to monitor the T-cell epitope delivery and MHC class I presentation function of the polypeptides and molecules of the present invention involve computational and/or experimental

methods to monitor MHC class I and peptide binding and stability. Several software programs are available for use by the skilled worker for predicting the binding responses of peptides to MHC class I alleles, such as, *e.g.*, The Immune Epitope Database and Analysis Resource (IEDB) Analysis Resource MHC-I binding prediction Consensus tool (Kim Y et al., *Nucleic Acid Res* 40: W525-30 (2012). Several experimental assays have been routinely applied, such as, *e.g.*, cell surface binding assays and/or surface plasmon resonance assays to quantify and/or compare binding kinetics (Miles K et al., *Mol Immunol* 48: 728-32 (2011)). Additionally, other MHC-peptide binding assays based on a measure of the ability of a peptide to stabilize the ternary MHC-peptide complex for a given MHC class I allele, as a comparison to known controls, have been developed (*e.g.*, MHC-peptide binding assay from ProImmmune, Inc.).

[536] Alternatively, measurements of the consequence of MHC class I/peptide antigen complex presentation on the cell surface can be performed by monitoring the cytotoxic T-cell (CTL) response to the specific complex. These measurements by include direct labeling of the CTLs with MHC class I tetramer or pentamer reagents. Tetramers or pentamers bind directly to T cell receptors of a particular specificity, determined by the Major Histocompatibility Complex (MHC) allele and peptide complex. Additionally, the quantification of released cytokines, such as interferon gamma or interleukins by ELISA or enzyme-linked immunospot (ELIspot) is commonly assayed to identify specific CTL responses. The cytotoxic capacity of CTL can be measured using a number of assays, including the classical 51 Chromium (Cr) release assay or alternative non-radioactive cytotoxicity assays (*e.g.*, CytoTox96® non-radioactive kits and CellTox™ CellTiter-GLO® kits available from Promega Corp., Madison, WI, U.S.), Granzyme B ELISpot, Caspase Activity Assays or LAMP-1 translocation flow cytometric assays. To specifically monitor the killing of target cells, carboxyfluorescein diacetate succinimidyl ester (CFSE) can be used to easily and quickly label a cell population of interest for in vitro or *in vivo* investigation to monitor killing of epitope specific CSFE labeled target cells (Durward M et al., *J Vis Exp* 45 pii 2250 (2010)).

[537] *In vivo* responses to MHC class I presentation can be followed by administering a MHC class I/antigen promoting agent (*e.g.*, a peptide, protein or inactivated/attenuated virus vaccine) followed by challenge with an active agent (*e.g.* a virus) and monitoring responses to that agent, typically in comparison with unvaccinated controls. *Ex vivo* samples can be monitored for CTL activity with methods similar to those described previously (*e.g.* CTL cytotoxicity assays and quantification of cytokine release).

[538] HLA-A, HLA-B, and/or HLA-C molecules are isolated from the intoxicated cells after lysis using immune affinity (*e.g.*, an anti-MHC antibody "pulldown" purification) and the associated peptides (*i.e.*, the peptides presented by the isolated MHC molecules) are recovered from the purified complexes. The recovered peptides are analyzed by sequencing mass spectrometry. The mass spectrometry data is compared against a protein database library consisting of the sequence of the exogenous (non-self) peptide (T-cell epitope X) and the international protein index for humans (representing "self" or non-immunogenic peptides). The peptides are ranked by significance according to a probability database. All detected antigenic (non-self) peptide sequences are listed. The data is verified by searching against a

scrambled decoy database to reduce false hits (*see e.g.* Ma B, Johnson R, *Mol Cell Proteomics* 11: O111.014902 (2012)). The results will demonstrate that peptides from the T-cell epitope X are presented in MHC complexes on the surface of intoxicated target cells.

[539] The set of presented peptide-antigen-MHC complexes can vary between cells due to the antigen-specific HLA molecules expressed. T-cells can then recognize specific peptide-antigen-MHC complexes displayed on a cell surface using different TCR molecules with different antigen-specificities.

[540] Because multiple T-cell epitopes may be delivered by a cell-targeting molecule of the invention, such as, *e.g.*, by embedding two or more different T-cell epitopes in a single proteasome delivering effector polypeptide, a single cell-targeting molecule of the invention may be effective chordates of the same species with different MHC class variants, such as, *e.g.*, in humans with different HLA alleles. This may allow for the combining within a single molecule of different T-cell epitopes with different effectiveness in different sub-populations of subjects based on MHC complex protein diversity and polymorphisms. For example, human MHC complex proteins, HLA proteins, vary among humans based on genetic ancestry, *e.g.* African (sub-Saharan), Amerindian, Caucasiod, Mongoloid, New Guinean and Australian, or Pacific islander.

[541] The applications involving the T-cell epitope delivering polypeptides and molecules of the present invention are vast. Every nucleated cell in a mammalian organism may be capable of MHC class I pathway presentation of immunogenic, T-cell epitope-peptides on their cell outer surfaces complexed to MHC class I molecules. In addition, the sensitivity of T-cell epitope recognition is so exquisite that only a few MHC-I peptide complexes are required to be presented to result in an immune response, *e.g.*, even presentation of a single complex can be sufficient for recognition by an effector T-cell (Sykulev Y et al., *Immunity* 4: 565–71 (1996)).

[542] The activation of T-cell responses are desired characteristics of certain anti-cancer, anti-neoplastic, anti-tumor, and/or anti-microbial biologic drugs to stimulate the patient's own immune system toward targeted cells. Activation of a robust and strong T-cell response is also a desired characteristic of many vaccines. The presentation of a T-cell epitope by a target cell within an organism can lead to the activation of robust immune responses to a target cell and/or its general locale within an organism. Thus, the targeted delivery of a T-cell epitope for presentation may be utilized for as a mechanism for activating T-cell responses during a therapeutic regime.

[543] The presentation of a T-cell immunogenic epitope-peptide by the MHC class I system targets the presenting cell for killing by CTL-mediated lysis and also triggers immune stimulation in the local microenvironment. By engineering immunogenic epitope sequences within Shiga toxin effector polypeptide components of target-cell-internalizing therapeutic molecules, the targeted delivery and presentation of immuno-stimulatory antigens may be accomplished. The presentation of immuno-stimulatory non-self antigens, such as *e.g.* known viral antigens with high immunogenicity, by target cells signals to other immune cells to destroy the target cells as well as to recruit more immune cells to the area.

[544] The presentation of an immunogenic, T-cell epitope-peptide by the MHC class I complex targets the presenting cell for killing by CTL-mediated cytolysis. The presentation by targeted cells of immuno-stimulatory non-self antigens, such as, *e.g.*, known viral epitope-peptides with high immunogenicity, can signal to other immune cells to destroy the target cells and recruit more immune cells to the target cell site within a chordate.

[545] Thus, already cytotoxic molecules, such as *e.g.* therapeutic or potentially therapeutic molecules comprising Shiga toxin effector polypeptides, may be engineered using methods of the present invention into more cytotoxic molecules and/or to have an additional cytotoxic mechanism operating via delivery of a T-cell epitope, presentation, and stimulation of effector T-cells. These multiple cytotoxic mechanisms may complement each other (such as by providing both direct target-cell-killing and indirect (CTL-mediated) cell-killing, redundantly backup each other (such as by providing one mechanism of cell-killing in the absence of the other), and/or protect against the development of therapeutic resistance (by limiting resistance to the less probable situation of the malignant or infected cell evolving to block two different cell-killing mechanisms simultaneously).

[546] In addition, a cytotoxic molecule comprising a Shiga toxin effector polypeptide region that exhibits catalytic-based cytotoxicity may be engineered by the skilled worker using routine methods into enzymatically inactive variants. For example, the cytotoxic Shiga toxin effector polypeptide component of a cytotoxic molecule may be conferred with reduced activity and/or rendered inactive by the introduction of one or mutations and/or truncations such that the resulting molecule can still be cytotoxic via its ability to deliver a T-cell epitope to the MHC class I system of a target cell and subsequent presentation to the surface of the target cell. In another example, a T-cell epitope may be inserted or embedded into a Shiga toxin effector polypeptide such that the Shiga toxin effector polypeptide is inactivated by the added epitope (*see e.g.* WO 2015/113005). This approach removes one cytotoxic mechanism while retaining or adding another and may also provide a molecule capable of exhibiting immuno-stimulation to the local area of a target cell(s) within an organism via delivered T-cell epitope presentation or “antigen seeding.” Furthermore, non-cytotoxic variants of the cell-targeting molecules of the present invention which comprise embedded or inserted, heterologous, T-cell epitopes may be useful in applications involving immune-stimulation within a chordate and/or labeling of target cells within a chordate with MHC class I molecule displayed epitopes.

[547] The ability to deliver a T-cell epitope of certain Shiga toxin effector polypeptides and cell-targeting molecules of the present invention may be accomplished under varied conditions and in the presence of non-targeted bystander cells, such as, *e.g.*, an *ex vivo* manipulated target cell, a target cell cultured *in vitro*, a target cell within a tissue sample cultured *in vitro*, or a target cell in an *in vivo* setting like within a multicellular organism.

F. Cell-Kill via Targeted Cytotoxicity and/or Engagement of Cytotoxic T-Cells

[548] For certain embodiments, the cell-targeting molecule of the present invention can provide 1) delivery of a T-cell epitope for MHC class I presentation by a target cell and/or 2) potent cytotoxicity.

For certain embodiments of the cell-targeting molecules of the present invention, upon contacting a cell physically coupled with an extracellular target biomolecule of the cell-targeting binding region, the cell-targeting molecule of the invention is capable of causing death of the cell. The mechanism of cell-kill may be direct, *e.g.* via the enzymatic activity of a toxin effector polypeptide region, or indirect via CTL-
5 mediated cytolysis.

1. Indirect Cell-Kill via T-Cell Epitope Delivery and MHC Class I Presentation

[549] Certain embodiments of the cell-targeting molecules of the present invention are cytotoxic because they comprise a CD8+ T-cell epitope capable of being delivered to the MHC class I presentation
10 pathway of a target cell and presented on a cellular surface of the target cell. For example, T-cell epitope delivering, CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptides of the present invention, with or without endogenous epitope de-immunization, may be used as components of cell-targeting molecules for applications involving indirect cell-killing.

[550] In certain embodiments of the cell-targeting molecules of the present invention, upon contacting
15 a cell physically coupled with an extracellular target biomolecule of the cell-targeting binding region, the cell-targeting molecule of the invention is capable of indirectly causing the death of the cell, such as, *e.g.*, via the presentation of one or more T-cell epitopes by the target cell and the subsequent recruitment of CTLs which kill the target cell.

[551] The presentation of an antigenic peptide complexed with a MHC class I molecule by a cell
20 sensitizes the presenting cell to targeted killing by cytotoxic T-cells (CTLs) via the induction of apoptosis, lysis, and/or necrosis. In addition, the CTLs which recognize the target cell may release immuno-stimulatory cytokines, such as, *e.g.*, interferon gamma (IFN-gamma), tumor necrosis factor alpha (TNF), macrophage inflammatory protein-1 beta (MIP-1beta), and interleukins such as IL-17, IL-4, and IL-22. Furthermore, CTLs activated by recognition of a presented epitope may indiscriminately kill
25 other cells proximal to the presenting cell regardless of the peptide-MHC class I complex repertoire presented by those proximal cells (Wiedemann A et al., *Proc Natl Acad Sci USA* 103: 10985-90 (2006)).

[552] Because of MHC allele diversity within different species, a cell-targeting molecule of the present invention comprising only a single epitope may exhibit varied effectiveness to different patients or subjects of the same species. However, certain embodiments of the cell-targeting molecules of the
30 present invention may each comprise multiple, T-cell epitopes that are capable of being delivered to the MHC class I system of a target cell simultaneously. Thus, for certain embodiments of the cell-targeting molecules of the present invention, a cell-targeting molecule is used to treat different subjects with considerable differences in their MHC molecules' epitope-peptide binding affinities (*i.e.* considerable differences in their MHC alleles and/or MHC genotypes). In addition, certain embodiments of the cell-
35 targeting molecules of the present invention reduce or prevent target cell adaptations to escape killing (*e.g.* a target cancer cell mutating to escape therapeutic effectiveness or "mutant escape") by using multiple cell-killing mechanisms simultaneously (*e.g.* direct killing and indirect killing via multiple different T-cell epitopes simultaneously).

2. Direct Cell-Kill via Cell-Targeted, Shiga Toxin Cytotoxicity

[553] Certain embodiments of the cell-targeting molecules of the present invention are cytotoxic because they comprise a catalytically active, Shiga toxin effector polypeptide and regardless of the presence of an immunogenic, CD8+ T-cell epitope in the molecule. For example, CD8+ T-cell hyper-immunized, Shiga toxin effector polypeptides of the present invention, with or without endogenous epitope de-immunization, may be used as components of cell-targeting molecules for applications involving direct cell-killing, such as, *e.g.*, via the ribotoxic, enzymatic activity of a Shiga toxin effector polypeptide or ribosome binding and interference with ribosome function due to a non-catalytic mechanism(s).

[554] For certain embodiments of the CD8+ T-cell hyper-immunized, cell-targeting molecules of the present invention, upon contacting a cell physically coupled with an extracellular target biomolecule of the cell-targeting binding region, the cell-targeting molecule of the invention is capable of directly causing the death of the cell, such as, *e.g.*, without the involvement of an untargeted, cytotoxic T-cell (*see* Section V-D, *supra*).

G. Selective Cytotoxicity among Cell Types

[555] Certain cell-targeting molecules of the present invention have uses in the selective killing of specific target cells in the presence of untargeted, bystander cells. By targeting the delivery of Shiga toxin effector polypeptides of the present invention to specific cells via a cell-targeting binding region(s), the cell-targeting molecules of the present invention can exhibit cell-type specific, restricted cell-kill activities resulting in the exclusive or preferential killing selected cell types in the presence of untargeted cells. Similarly, by targeting the delivery of immunogenic T-cell epitopes to the MHC class I pathway of target cells, the subsequent presentation of T-cell epitopes and CTL-mediated cytolysis of target cells induced by the cell-targeting molecules of the invention can be restricted to exclusively or preferentially killing selected cell types in the presence of untargeted cells. In addition, both the cell-targeted delivery of a cytotoxic, Shiga toxin effector polypeptide region and an immunogenic, T-cell epitope can be accomplished by a single cell-targeting molecule of the present invention such that deliver of both potentially cytotoxic components is restricted exclusively or preferentially to target cells in the presence of untargeted cells.

[556] For certain embodiments, the cell-targeting molecule of the present invention is cytotoxic at certain concentrations. In certain embodiments, upon administration of the cell-targeting molecule of the present invention to a mixture of cell types, the cytotoxic cell-targeting molecule is capable of selectively killing those cells which are physically coupled with an extracellular target biomolecule compared to cell types not physically coupled with an extracellular target biomolecule. For certain embodiments, the cytotoxic cell-targeting molecule of the present invention is capable of selectively or preferentially causing the death of a specific cell type within a mixture of two or more different cell types. This enables targeting cytotoxic activity to specific cell types with a high preferentiality, such as a 3-fold cytotoxic

effect, over "bystander" cell types that do not express the target biomolecule. Alternatively, the expression of the target biomolecule of the binding region may be non-exclusive to one cell type if the target biomolecule is expressed in low enough amounts and/or physically coupled in low amounts with cell types that are not to be targeted. This enables the targeted cell-killing of specific cell types with a high preferentiality, such as a 3-fold cytotoxic effect, over "bystander" cell types that do not express significant amounts of the target biomolecule or are not physically coupled to significant amounts of the target biomolecule.

[557] For certain further embodiments, upon administration of the cytotoxic cell-targeting molecule to two different populations of cell types, the cytotoxic cell-targeting molecule is capable of causing cell death as defined by the half-maximal cytotoxic concentration (CD_{50}) on a population of target cells, whose members express an extracellular target biomolecule of the binding region of the cytotoxic cell-targeting molecule, at a dose at least three-times lower than the CD_{50} dose of the same cytotoxic cell-targeting molecule to a population of cells whose members do not express an extracellular target biomolecule of the binding region of the cytotoxic cell-targeting molecule.

[558] For certain embodiments, the cytotoxic activity of a cell-targeting molecule of the present invention toward populations of cell types physically coupled with an extracellular target biomolecule is at least 3-fold higher than the cytotoxic activity toward populations of cell types not physically coupled with any extracellular target biomolecule of the binding region. According to the present invention, selective cytotoxicity may be quantified in terms of the ratio (a/b) of (a) cytotoxicity towards a population of cells of a specific cell type physically coupled with a target biomolecule of the binding region to (b) cytotoxicity towards a population of cells of a cell type not physically coupled with a target biomolecule of the binding region. In certain embodiments, the cytotoxicity ratio is indicative of selective cytotoxicity which is at least 3-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 75-fold, 100-fold, 250-fold, 500-fold, 750-fold, or 1000-fold higher for populations of cells or cell types physically coupled with a target biomolecule of the binding region compared to populations of cells or cell types not physically coupled with a target biomolecule of the binding region.

[559] For certain embodiments, the preferential cell-killing function or selective cytotoxicity of a cell-targeting molecule of the present invention is due to an additional exogenous material (*e.g.* a cytotoxic material) and/or heterologous, T-cell epitope present in a Shiga toxin effector polypeptide of the present invention and not necessarily a result of the catalytic activity of a Shiga toxin effector polypeptide region.

[560] This preferential cell-killing function allows a targeted cell to be killed by certain cytotoxic, cell-targeting molecules of the present invention under varied conditions and in the presence of non-targeted bystander cells, such as *ex vivo* manipulated mixtures of cell types, *in vitro* cultured tissues with mixtures of cell types, or *in vivo* in the presence of multiple cell types (*e.g. in situ* or in a native location within a multicellular organism).

H. Delivery of Additional Exogenous Material into the Interior of Targeted Cells

[561] In addition to cytotoxic, cytostatic, and immune stimulation applications, cell-targeting molecules of the present invention optionally may be used for targeted intracellular delivery functions, such as, *e.g.*, in applications involving information gathering and diagnostic functions.

5 [562] Because the cell-targeting molecules of the invention, including reduced cytotoxicity and/or nontoxic forms thereof, are capable of entering cells physically coupled with an extracellular target biomolecule recognized by the cell-targeting molecule's binding region, certain embodiments of the cell-targeting molecules of the invention may be used to deliver additional exogenous materials into the interior of targeted cell types. For example, non-toxic variants of the cytotoxic, cell-targeting molecules

10 of the invention, or optionally cytotoxic variants, may be used to deliver additional exogenous materials to and/or label the interiors of cells physically coupled with an extracellular target biomolecule of the binding region of the cell-targeting molecule. Various types of cells and/or cell populations which express target biomolecules to at least one cellular surface may be targeted by the cell-targeting molecules of the invention for receiving exogenous materials. The functional components of the present

15 invention are modular, in that various Shiga toxin effector polypeptides, additional exogenous materials, and binding regions may be associated with each other to provide cell-targeting molecules suitable for diverse applications involving cargo delivery, such as, *e.g.*, non-invasive, *in vivo* imaging of tumor cells.

[563] This delivery of exogenous material function of certain cell-targeting molecules of the present invention may be accomplished under varied conditions and in the presence of non-targeted bystander

20 cells, such as, *e.g.*, an *ex vivo* manipulated target cell, a target cell cultured *in vitro*, a target cell within a tissue sample cultured *in vitro*, or a target cell in an *in vivo* setting like within a multicellular organism. Furthermore, the selective delivery of exogenous material to certain cells by certain cell-targeting molecules of the present invention may be accomplished under varied conditions and in the presence of non-targeted bystander cells, such as *ex vivo* manipulated mixtures of cell types, *in vitro* cultured tissues

25 with mixtures of cell types, or *in vivo* in the presence of multiple cell types (*e.g. in situ* or in a native location within a multicellular organism).

[564] Shiga toxin effector polypeptides and cell-targeting molecules which are not capable, such as a certain concentration ranges, of killing a target cell and/or delivering an embedded or inserted epitope for cell-surface presentation by a MHC molecule of a target cell may still be useful for delivering exogenous

30 materials into cells, such as, *e.g.*, detection promoting agents.

[565] For certain embodiments, the Shiga toxin effector polypeptides of the present invention exhibits low to zero cytotoxicity and thus are referred to herein as "noncytotoxic and/or reduced cytotoxic." For certain embodiments, the cell-targeting molecule of the present invention exhibits low to zero cytotoxicity and may be referred to as "noncytotoxic" and/or "reduced cytotoxic variants." For example,

35 certain embodiments of the molecules of the present invention do not exhibit a significant level of Shiga toxin based cytotoxicity wherein at doses of less than 1000 nM, 500nM, 100 nM, 75 nM, 50 nM, there is no significant amount of cell death as compared to the appropriate reference molecule, such as, *e.g.*, as measured by an assay known to the skilled worker and/or described herein. For certain further

embodiments, the molecules of the present invention do not exhibit any toxicity at dosages of 1-100 μg per kg of a mammalian recipient. Reduced-cytotoxic variants may still be cytotoxic at certain concentrations or dosages but exhibit reduced cytotoxicity, such as, *e.g.*, are not capable of exhibiting a significant level of Shiga toxin cytotoxicity in certain situations.

5 [566] Shiga toxin effector polypeptides of the present invention, and certain cell-targeting molecules comprising the same, can be rendered non-cytotoxic, such as, *e.g.*, via the addition of one or more amino acid substitutions known to the skilled worker to inactive a Shiga toxin A Subunit and/or Shiga toxin effector polypeptide, including exemplary substitutions described herein. The non-cytotoxic and reduced cytotoxic variants of the cell-targeting molecules of the present invention may be in certain situations 10 more suitable for delivery of additional exogenous materials than more cytotoxic variants.

Information Gathering for Diagnostic Functions

[567] In certain cell-targeting molecules of the present invention have uses in the *in vitro* and/or *in vivo* detection of specific cells, cell types, and/or cell populations, as well as specific subcellular 15 compartments of any of the aforementioned. Reduced-cytotoxicity and/or nontoxic forms of the cytotoxic, cell-targeting molecules of the invention that are conjugated to detection promoting agents optionally may be used for diagnostic functions, such as for companion diagnostics used in conjunction with a therapeutic regimen comprising the same or a related binding region, such as, *e.g.*, a binding region with high-affinity binding to the same target biomolecule, an overlapping epitope, and/or the same 20 epitope.

[568] In certain embodiments, the cell-targeting molecules described herein are used for both diagnosis and treatment, or for diagnosis alone. When the same cytotoxic cell-targeting molecule is used for both diagnosis and treatment, for certain embodiments of the present invention the cell-targeting molecule variant which incorporates a detection promoting agent for diagnosis may have its cytotoxicity reduced 25 or may be rendered nontoxic by catalytic inactivation of its Shiga toxin effector polypeptide region(s) via one or more amino acid substitutions, including exemplary substitutions described herein. For example, certain nontoxic variants of the cell-targeting molecules of the present invention exhibit less than 5%, 4%, 3%, 2%, or 1% death of target cells after administration of a dose less than 1 mg/kg. Reduced-cytotoxicity variants may still be cytotoxic at certain concentrations or dosages but exhibit reduced 30 cytotoxicity, such as, *e.g.*, are not capable of exhibiting a significant level of Shiga toxin cytotoxicity as described herein.

[569] The ability to conjugate detection promoting agents known in the art to various cell-targeting molecules of the present invention provides useful compositions for the detection of certain cells, such as, *e.g.*, cancer, tumor, immune, and/or infected cells. These diagnostic embodiments of the cell-targeting 35 molecules of the invention may be used for information gathering via various imaging techniques and assays known in the art. For example, diagnostic embodiments of the cell-targeting molecules of the invention may be used for information gathering via imaging of intracellular organelles (*e.g.* endocytotic,

Golgi, endoplasmic reticulum, and cytosolic compartments) of individual cancer cells, immune cells, and/or infected cells in a patient or biopsy sample.

[570] Various types of information may be gathered using the diagnostic embodiments of the cell-targeting molecules of the invention whether for diagnostic uses or other uses. This information may be useful, for example, in diagnosing neoplastic cell types, determining therapeutic susceptibilities of a patient's disease, assaying the progression of anti-neoplastic therapies over time, assaying the progression of immunomodulatory therapies over time, assaying the progression of antimicrobial therapies over time, evaluating the presence of infected cells in transplantation materials, evaluating the presence of unwanted cell types in transplantation materials, and/or evaluating the presence of residual tumor cells after surgical excision of a tumor mass.

[571] For example, subpopulations of patients might be ascertained using information gathered using the diagnostic variants of the cell-targeting molecules of the invention, and then individual patients could be further categorized into subpopulations based on their unique characteristic(s) revealed using those diagnostic embodiments. For example, the effectiveness of specific pharmaceuticals or therapies might be a criterion used to define a patient subpopulation. For example, a nontoxic diagnostic variant of a particular cytotoxic, cell-targeting molecule of the invention may be used to differentiate which patients are in a class or subpopulation of patients predicted to respond positively to a cytotoxic variant of that cell-targeting molecule of the invention. Accordingly, associated methods for patient identification, patient stratification, and diagnosis using cell-targeting molecules of the present invention, including non-toxic variants of cytotoxic, cell-targeting molecules of the present invention, are considered to be within the scope of the present invention.

[572] The expression of the target biomolecule by a cell need not be native in order for cell-targeting by a cell-targeting molecule of the present invention, such as, *e.g.*, for direct cell-kill, indirect cell-kill, delivery of exogenous materials like T-cell epitopes, and/or information gathering. Cell surface expression of the target biomolecule could be the result of an infection, the presence of a pathogen, and/ or the presence of an intracellular microbial pathogen. Expression of a target biomolecule could be artificial such as, for example, by forced or induced expression after infection with a viral expression vector, *see e.g.* adenoviral, adeno-associated viral, and retroviral systems. Expression of HER2 can be induced by exposing a cell to ionizing radiation (Wattenberg M et al., *Br J Cancer* 110: 1472-80 (2014)).

VI. Production, Manufacture, and Purification of Shiga Toxin Effector Polypeptides of the Invention and Cell-Targeting Molecules Comprising the Same

[573] The Shiga toxin effector polypeptides and certain cell-targeting molecules of the present invention may be produced using techniques well known to those of skill in the art. For example, Shiga toxin effector polypeptides and cell-targeting molecules of the invention may be manufactured by standard synthetic methods, by use of recombinant expression systems, or by any other suitable method. Thus, Shiga toxin effector polypeptides and cell-targeting molecules of the invention may be synthesized in a number of ways, including, *e.g.* methods comprising: (1) synthesizing a polypeptide or polypeptide

component of a cell-targeting molecule using standard solid-phase or liquid-phase methodology, either stepwise or by fragment assembly, and isolating and purifying the final polypeptide compound product; (2) expressing a polynucleotide that encodes a protein or protein component of a cell-targeting molecule of the invention in a host cell and recovering the expression product from the host cell or host cell culture; or (3) cell-free, *in vitro* expression of a polynucleotide encoding a polypeptide or polypeptide component of a cell-targeting molecule of the invention, and recovering the expression product; or by any combination of the methods of (1), (2) or (3) to obtain fragments of the protein component, subsequently joining (*e.g.* ligating) the peptide or polypeptide fragments to obtain a polypeptide component, and recovering the polypeptide component.

[574] It may be preferable to synthesize a Shiga toxin effector polypeptide of the present invention, cell-targeting molecule of the present invention, or a protein component of a cell-targeting molecule of the invention by means of solid-phase or liquid-phase peptide synthesis. Polypeptides and cell-targeting molecules of the present invention may suitably be manufactured by standard synthetic methods. Thus, peptides may be synthesized by, *e.g.* methods comprising synthesizing the peptide by standard solid-phase or liquid-phase methodology, either stepwise or by fragment assembly, and isolating and purifying the final peptide product. In this context, reference may be made to WO 1998/011125 or, *inter alia*, Fields G et al., *Principles and Practice of Solid-Phase Peptide Synthesis* (Synthetic Peptides, Grant G, ed., Oxford University Press, U.K., 2nd ed., 2002) and the synthesis examples therein.

[575] Shiga toxin effector polypeptides and cell-targeting molecules of the present invention may be prepared (produced and purified) using recombinant techniques well known in the art. In general, methods for preparing proteins by culturing host cells transformed or transfected with a vector comprising the encoding polynucleotide and purifying or recovering the protein from cell culture are described in, *e.g.*, Sambrook J et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, NY, U.S., 1989); Dieffenbach C et al., *PCR Primer: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, N.Y., U.S., 1995). Any suitable host cell may be used to produce a polypeptide and/or cell-targeting protein of the invention. Host cells may be cells stably or transiently transfected, transformed, transduced or infected with one or more expression vectors which drive expression of a polypeptide of the invention. In addition, a Shiga toxin effector polypeptide and/or cell-targeting molecule of the invention may be produced by modifying the polynucleotide encoding a polypeptide or cell-targeting protein of the invention that result in altering one or more amino acids or deleting or inserting one or more amino acids in order to achieve desired properties, such as changed cytotoxicity, changed cytostatic effects, and/or changed serum half-life.

[576] There are a wide variety of expression systems which may be chosen to produce a polypeptide or cell-targeting protein of the present invention. For example, host organisms for expression of cell-targeting proteins of the invention include prokaryotes, such as *E. coli* and *B. subtilis*, eukaryotic cells, such as yeast and filamentous fungi (like *S. cerevisiae*, *P. pastoris*, *A. awamori*, and *K. lactis*), algae (like *C. reinhardtii*), insect cell lines, mammalian cells (like CHO cells), plant cell lines, and eukaryotic organisms such as transgenic plants (like *A. thaliana* and *N. benthamiana*).

[577] Accordingly, the present invention also provides methods for producing a Shiga toxin effector polypeptide and/or cell-targeting molecule of the present invention according to above recited methods and using a polynucleotide encoding part or all of a polypeptide of the invention or a protein component of a cell-targeting protein of the invention, an expression vector comprising at least one polynucleotide of the invention capable of encoding part or all of a polypeptide or cell-targeting protein of the invention when introduced into a host cell, and/or a host cell comprising a polynucleotide or expression vector of the invention.

[578] When a protein is expressed using recombinant techniques in a host cell or cell-free system, it is advantageous to separate (or purify) the desired protein away from other components, such as host cell factors, in order to obtain preparations that are of high purity or are substantially homogeneous. Purification can be accomplished by methods well known in the art, such as centrifugation techniques, extraction techniques, chromatographic and fractionation techniques (*e.g.* size separation by gel filtration, charge separation by ion-exchange column, hydrophobic interaction chromatography, reverse phase chromatography, chromatography on silica or cation-exchange resins such as DEAE and the like, chromatofocusing, and Protein A Sepharose chromatography to remove contaminants), and precipitation techniques (*e.g.* ethanol precipitation or ammonium sulfate precipitation). Any number of biochemical purification techniques may be used to increase the purity of a polypeptide and/or cell-targeting molecule of the present invention. In certain embodiments, the polypeptides and cell-targeting molecules of the invention may optionally be purified in homo-multimeric forms (*e.g.* a molecular complex comprising two or more polypeptides or cell-targeting molecules of the invention).

[579] In the Examples below are descriptions of non-limiting examples of methods for producing exemplary, Shiga toxin effector polypeptides and cell-targeting molecules of the present invention, as well as specific but non-limiting aspects of production methods.

VII. Pharmaceutical and Diagnostic Compositions Comprising Cell-Targeting Molecules of the Present Invention

[580] The present invention provides Shiga toxin effector polypeptides and cell-targeting molecules for use, alone or in combination with one or more additional therapeutic agents, in a pharmaceutical composition, for treatment or prophylaxis of conditions, diseases, disorders, or symptoms described in further detail below (*e.g.* cancers, malignant tumors, non-malignant tumors, growth abnormalities, immune disorders, and microbial infections). For certain embodiments, the one or more additional therapeutic agents comprises one or more additional HER2-targeting therapeutic agent, as described herein. The additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of an anti-HER2 antibody therapy or small molecule inhibitor that interferes with HER2 signaling. The additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of a small molecule inhibitor that interferes with HER2 signaling. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consists of a dual tyrosine kinase inhibitor, such as lapatinib and/or neratinib. For example, the additional HER2-targeting therapeutic agent may

comprise, consist essentially of, or consist of lapatinib. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of neratinib. The additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of an anti-HER2 antibody therapy that binds to an antigenic determinant that does not overlap with the antigenic determinant bound 5 by the cell-targeting molecule of the invention or that binds a HER2 molecule in such a manner that when bound the additional HER2-tageting therapeutic does not prevent the binding of that HER2 molecule by the cell-targeting molecule of the invention. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of anti-HER2 monoclonal antibody therapy and/or anti-HER2 antibody drug conjugate therapy. For example, the additional HER2-targeting 10 therapeutic agent may comprise, consist essentially of, or consist of: T-DM1, trastuzumab, and/ or pertuzumab. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of: T-DM1. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of: trastuzumab, and/ or pertuzumab. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of 15 trastuzumab. For example, the additional HER2-targeting therapeutic agent may comprise, consist essentially of, or consist of pertuzumab.

[581] In certain embodiments, the pharmaceutical composition further comprises at least one pharmaceutically acceptable carrier, excipient, or vehicle, as described herein. The present invention further provides pharmaceutical compositions comprising a Shiga toxin polypeptide or cell-targeting 20 molecule of the present invention, or a pharmaceutically acceptable salt or solvate thereof, according to the invention, together with at least one pharmaceutically acceptable carrier, excipient, or vehicle. In certain embodiments, the pharmaceutically acceptable excipient includes a solvent, a dispersion medium, a coating, an antimicrobial agent, an isotonic agent, or an absorption delaying agent; and/or wherein the pharmaceutical composition further comprises an aqueous or non-aqueous carrier; a surfactant; a 25 stabilizer, a preservative, a buffer, an antioxidant, a wetting agent, an emulsifying agent, a dispersing agent; an isotonic agent; and/or an antibacterial or antifungal agent.

[582] In certain embodiments, the pharmaceutical composition of the present invention may comprise homo-multimeric and/or hetero-multimeric forms of a Shiga toxin effector polypeptides or cell-targeting molecule of the present invention. In certain embodiments, the pharmaceutical composition of the 30 present invention may comprise monomeric and/or monovalent forms of the cell-targeting molecule of the present invention. In certain embodiments, the pharmaceutical composition of the present invention may be enriched for monomeric and/or monovalent forms of a cell-targeting molecule of the present invention. As demonstrated by the Examples of the application, compositions comprising predominantly monovalent amd/or monomeric forms of certain cell-targeting molecules may exhibit low levels of 35 toxicity when used *in vivo* while still exhibiting potent cytotoxic to HER2-expressing cells. The pharmaceutical compositions of the invention are useful in methods of treating, ameliorating, or preventing a disease, condition, disorder, or symptom described in further detail below. The disease, disorder, or condition may be characterized by cells that are physically coupled with HER2. The HER2

target biomolecule can be physically coupled to the surface of the cells. In certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2 target biomolecule (including cells that overexpress HER2). The HER2 can be expressed (including overexpressed) at the surface of the cells. Each such disease, condition, disorder, or symptom is envisioned to be a separate embodiment with respect to uses of a pharmaceutical composition according to the invention. The invention further provides pharmaceutical compositions for use in at least one method of treatment according to the invention, as described in more detail below.

[583] As used herein, the terms "patient" and "subject" are used interchangeably to refer to any organism, commonly vertebrates such as humans and animals, which presents symptoms, signs, and/or indications of at least one disease, disorder, or condition. These terms include mammals such as the non-limiting examples of primates, livestock animals (*e.g.* cattle, horses, pigs, sheep, goats, *etc.*), companion animals (*e.g.* cats, dogs, *etc.*) and laboratory animals (*e.g.* mice, rabbits, rats, *etc.*).

[584] As used herein, "treat," "treating," or "treatment" and grammatical variants thereof refer to an approach for obtaining beneficial or desired clinical results. The terms may refer to slowing the onset or rate of development of a condition, disorder or disease, reducing or alleviating symptoms associated with it, generating a complete or partial regression of the condition, or some combination of any of the above. For the purposes of this invention, beneficial or desired clinical results include, but are not limited to, reduction or alleviation of symptoms, diminishment of extent of disease, stabilization (*e.g.* not worsening) of state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treat," "treating," or "treatment" can also mean prolonging survival relative to expected survival time if not receiving treatment. A subject (*e.g.* a human) in need of treatment may thus be a subject already afflicted with the disease or disorder in question. The terms "treat," "treating," or "treatment" includes inhibition or reduction of an increase in severity of a pathological state or symptoms relative to the absence of treatment, and is not necessarily meant to imply complete cessation of the relevant disease, disorder, or condition. With regard to tumors and/or cancers, treatment includes reduction in overall tumor burden and/or individual tumor size.

[585] As used herein, the terms "prevent," "preventing," "prevention" and grammatical variants thereof refer to an approach for preventing the development of, or altering the pathology of, a condition, disease, or disorder. Accordingly, "prevention" may refer to prophylactic or preventive measures. For the purposes of this invention, beneficial or desired clinical results include, but are not limited to, prevention or slowing of symptoms, progression or development of a disease, whether detectable or undetectable. A subject (*e.g.* a human) in need of prevention may thus be a subject not yet afflicted with the disease or disorder in question. The term "prevention" includes slowing the onset of disease relative to the absence of treatment, and is not necessarily meant to imply permanent prevention of the relevant disease, disorder or condition. Thus "preventing" or "prevention" of a condition may in certain contexts refer to reducing the risk of developing the condition, or preventing or delaying the development of symptoms associated with the condition.

[586] As used herein, an "effective amount" or "therapeutically effective amount" is an amount or dose of a composition (*e.g.* a therapeutic composition, compound, or agent) that produces at least one desired therapeutic effect in a subject, such as preventing or treating a target condition or beneficially alleviating a symptom associated with the condition. The most desirable therapeutically effective amount is an amount that will produce a desired efficacy of a particular treatment selected by one of skill in the art for a given subject in need thereof. This amount will vary depending upon a variety of factors understood by the skilled worker, including but not limited to the characteristics of the therapeutic composition (including activity, pharmacokinetics, pharmacodynamics, and bioavailability), the physiological condition of the subject (including age, sex, disease type, disease stage, general physical condition, responsiveness to a given dosage, and type of medication), the nature of the pharmaceutically acceptable carrier or carriers in the formulation, and the route of administration. One skilled in the clinical and pharmacological arts will be able to determine a therapeutically effective amount through routine experimentation, namely by monitoring a subject's response to administration of a composition and adjusting the dosage accordingly (*see e.g. Remington: The Science and Practice of Pharmacy* (Gennaro A, ed., Mack Publishing Co., Easton, PA, U.S., 19th ed., 1995)).

[587] Diagnostic compositions of the present invention comprise a cell-targeting molecule of the present invention and one or more detection promoting agents. When producing or manufacturing a diagnostic composition of the present invention, a cell-targeting molecule of the present invention may be directly or indirectly linked to one or more detection promoting agents. There are numerous standard techniques known to the skilled worker for incorporating, affixing, and/or conjugating various detection promoting agents to proteins or proteinaceous components of molecules, especially to immunoglobulins and immunoglobulin-derived domains.

[588] There are numerous detection promoting agents known to the skilled worker, such as isotopes, dyes, colorimetric agents, contrast enhancing agents, fluorescent agents, bioluminescent agents, and magnetic agents, which can be operably linked to the polypeptides or cell-targeting molecules of the invention for information gathering methods, such as for diagnostic and/or prognostic applications to diseases, disorders, or conditions of an organism (*see e.g.* Cai W et al., *J Nucl Med* 48: 304-10 (2007); Nayak T, Brechbiel M, *Bioconjug Chem* 20: 825-41 (2009); Paudyal P et al., *Oncol Rep* 22: 115-9 (2009); Qiao J et al., *PLoS ONE* 6: e18103 (2011); Sano K et al., *Breast Cancer Res* 14: R61 (2012)). These agents may be associated with, linked to, and/or incorporated within the polypeptide or cell-targeting molecule of the invention at any suitable position. For example, the linkage or incorporation of the detection promoting agent may be via an amino acid residue(s) of a molecule of the present invention or via some type of linkage known in the art, including via linkers and/or chelators. The incorporation of the agent is in such a way to enable the detection of the presence of the diagnostic composition in a screen, assay, diagnostic procedure, and/or imaging technique.

[589] Similarly, there are numerous imaging approaches known to the skilled worker, such as non-invasive *in vivo* imaging techniques commonly used in the medical arena, for example: computed tomography imaging (CT scanning), optical imaging (including direct, fluorescent, and bioluminescent

imaging), magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), ultrasound, and x-ray computed tomography imaging.

VIII. Production or Manufacture of Pharmaceutical and/or Diagnostic Compositions Comprising Cell-Targeting Molecules of the Present Invention

[590] Pharmaceutically acceptable salts or solvates of any of the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention are within the scope of the present invention.

[591] The term "solvate" in the context of the present invention refers to a complex of defined stoichiometry formed between a solute (*in casu*, a proteinaceous compound or pharmaceutically acceptable salt thereof according to the invention) and a solvent. The solvent in this connection may, for example, be water, ethanol or another pharmaceutically acceptable, typically small-molecular organic species, such as, but not limited to, acetic acid or lactic acid. When the solvent in question is water, such a solvate is normally referred to as a hydrate.

[592] Polypeptides and proteins of the present invention, or salts thereof, may be formulated as pharmaceutical compositions prepared for storage or administration, which typically comprise a therapeutically effective amount of a molecule of the present invention, or a salt thereof, in a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers. Pharmaceutically acceptable carriers for therapeutic molecule use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences* (Mack Publishing Co. (A. Gennaro, ed., 1985). As used herein, "pharmaceutically acceptable carrier" includes any and all physiologically acceptable, *i.e.* compatible, solvents, dispersion media, coatings, antimicrobial agents, isotonic, and absorption delaying agents, and the like. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, and transdermal) administration. Exemplary pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyloleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. In certain embodiments, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.* by injection or infusion). Depending on selected route of administration, the protein or other pharmaceutical component may be coated in a material intended to protect the compound from the action of low pH and other natural inactivating conditions to which the active protein may encounter when administered to a patient by a particular route of administration.

[593] The formulations of the pharmaceutical compositions of the invention may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. In such form, the composition is divided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of the preparations, for example, packeted tablets, capsules, and powders in vials or ampoules. The unit dosage form can also be a capsule, cachet, or tablet itself, or it can be the appropriate number of any of these packaged forms. It may be provided in single dose injectable form, for example in the form of a pen. Compositions may be formulated for any suitable route and means of administration. Subcutaneous or transdermal modes of administration may be particularly suitable for therapeutic proteins described herein.

[594] The pharmaceutical compositions of the present invention may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Preventing the presence of microorganisms may be ensured both by sterilization procedures, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. Isotonic agents, such as sugars, sodium chloride, and the like into the compositions, may also be desirable. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as, aluminum monostearate and gelatin.

[595] A pharmaceutical composition of the present invention also optionally includes a pharmaceutically acceptable antioxidant. Exemplary pharmaceutically acceptable antioxidants are water soluble antioxidants such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propylgallate, alpha-tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[596] In another aspect, the present invention provides pharmaceutical compositions comprising one or a combination of different polypeptides and/or cell-targeting molecules of the invention, or an ester, salt or amide of any of the foregoing, and at least one pharmaceutically acceptable carrier.

[597] Therapeutic compositions are typically sterile and stable under the conditions of manufacture and storage. The composition may be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier may be a solvent or dispersion medium containing, for example, water, alcohol such as ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), or any suitable mixtures. The proper fluidity may be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by use of surfactants according to formulation chemistry well known in the art. In certain embodiments, isotonic agents, *e.g.*, sugars and polyalcohols such as mannitol, sorbitol, or sodium chloride, may be desirable in the composition. Prolonged absorption of injectable compositions may be brought about by including in the composition an agent that delays absorption for example, monostearate salts and gelatin.

[598] Solutions or suspensions used for intradermal or subcutaneous application typically include one or more of: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates; and tonicity adjusting agents such as, *e.g.*, sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide, or buffers with citrate, phosphate, acetate and the like. Such preparations may be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[599] Sterile injectable solutions may be prepared by incorporating a polypeptide or cell-targeting molecule of the invention in the required amount in an appropriate solvent with one or a combination of ingredients described above, as required, followed by sterilization microfiltration. Dispersions may be prepared by incorporating the active compound into a sterile vehicle that contains a dispersion medium and other ingredients, such as those described above. In the case of sterile powders for the preparation of sterile injectable solutions, the methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient in addition to any additional desired ingredient from a sterile-filtered solution thereof.

[600] When a therapeutically effective amount of a polypeptide and/or cell-targeting molecule of the invention is designed to be administered by, *e.g.* intravenous, cutaneous or subcutaneous injection, the binding agent will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. Methods for preparing parenterally acceptable protein solutions, taking into consideration appropriate pH, isotonicity, stability, and the like, are within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection will contain, in addition to binding agents, an isotonic vehicle such as sodium chloride injection, Ringer's injection, dextrose injection, dextrose and sodium chloride injection, lactated Ringer's injection, or other vehicle as known in the art. A pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives well known to those of skill in the art.

[601] As described elsewhere herein, a polypeptide and/or cell-targeting molecule of the present invention may be prepared with carriers that will protect the active therapeutic agent against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art (*see e.g. Sustained and Controlled Release Drug Delivery Systems* (Robinson J, ed., Marcel Dekker, Inc., NY, U.S., 1978)).

[602] In certain embodiments, the pharmaceutical composition of the present invention comprises a buffer, such as *e.g.*, citrate, citric acid, histidine, phosphate, succinate, and/or succinic acid. In certain embodiments, the pharmaceutical composition of the present invention comprises a preservative,

antibacterial, or antifungal agent, such as *e.g.*, mannitol or sorbitol. In certain embodiments, the pharmaceutical composition of the present invention comprises a detergent such as, *e.g.*, polysorbate 20 or polysorbate 80. In certain embodiments, the pharmaceutical composition of the present invention comprises a cryoprotectant such as, *e.g.*, polysorbate 20 or polysorbate 80. In certain embodiments, the pharmaceutical composition of the present invention comprises an excipient, such as, *e.g.*, arginine, arginine sulfate, glycerol, mannitol, methionine, polysorbate 20, polysorbate 80, sorbitol, sucrose, and/or trehalose. In certain further embodiments, the pharmaceutical composition of the present invention comprises one or more of (including all of): citrate, polysorbate 20, sodium, sorbitol, and chloride. In certain further embodiments, the pharmaceutical composition comprises a 20 millimolar concentration of citrate, 200 millimolar concentration of sorbitol, and 0.2% polysorbate 20. In certain further embodiments, at room temperature (*e.g.* about 25 °C) the pharmaceutical composition has a pH of about 5.3 to 5.7, a pH between 5.4 and 5.6, and/or a pH of 5.5.

[603] In certain embodiments, the composition of the present invention (*e.g.* a pharmaceutical and/or diagnostic composition) may be formulated to ensure a desired *in vivo* distribution of a cell-targeting molecule of the present invention. For example, the blood-brain barrier excludes many large and/or hydrophilic compounds. To target a therapeutic molecule or composition of the present invention to a particular *in vivo* location, they can be formulated, for example, in liposomes which may comprise one or more moieties that are selectively transported into specific cells or organs, thus enhancing targeted drug delivery. Exemplary targeting moieties include folate or biotin; mannosides; antibodies; surfactant protein A receptor; p120 catenin and the like.

[604] Pharmaceutical compositions include parenteral formulations designed to be used as implants or particulate systems. Examples of implants are depot formulations composed of polymeric or hydrophobic components such as emulsions, ion exchange resins, and soluble salt solutions. Examples of particulate systems are microspheres, microparticles, nanocapsules, nanospheres, and nanoparticles (*see e.g.* Honda M et al., *Int J Nanomedicine* 8: 495-503 (2013); Sharma A et al., *Biomed Res Int* 2013: 960821 (2013); Ramishetti S, Huang L, *Ther Deliv* 3: 1429-45 (2012)). Controlled release formulations may be prepared using polymers sensitive to ions, such as, *e.g.* liposomes, polaxamer 407, and hydroxyapatite.

[605] The pharmaceutically acceptable carrier in the pharmaceutical compositions of the present invention may comprise: a physiologically acceptable solvent, dispersion medium, coating, antimicrobial agent, isotonic agent, absorption delaying agent, sterile aqueous solution or dispersion, or sterile powder; an aqueous or non-aqueous carrier, such as water, alcohol (*e.g.* ethanol), polyol (*e.g.* glycerol, propylene glycol, or polyethylene glycol), and mixtures thereof; vegetable oil; or an injectable organic ester, such as ethyloleate. The pharmaceutical composition of the invention may further comprises an adjuvant, such as a preservative, wetting agent, emulsifying agent, or dispersing agent; an antibacterial or antifungal agent, such as a paraben, chlorobutanol, phenol, or sorbic acid; an isotonic agent, such as a sugar, a polyalcohol such as mannitol or sorbitol, or sodium chloride; an absorption-delaying agent, such as aluminum monostearate or gelatin; a coating, such as lecithin; a pharmaceutically acceptable antioxidant;

a surfactant; a buffer; and/or a stabilizer. In certain embodiments, the pharmaceutically acceptable antioxidant is a water soluble antioxidant, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, or sodium sulfite; an oil-soluble antioxidant, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propylgallate, or alpha-tocopherol; or a metal chelating agent, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, or phosphoric acid.

IX. Polynucleotides, Expression Vectors, and Host Cells of the Present Invention

[606] Beyond the polypeptides and cell-targeting molecules of the present invention, the polynucleotides that encode the polypeptides and cell-targeting molecules of the invention, or functional portions thereof, are also encompassed within the scope of the present invention. The term "polynucleotide" is equivalent to the term "nucleic acid," each of which includes one or more of: polymers of deoxyribonucleic acids (DNAs), polymers of ribonucleic acids (RNAs), analogs of these DNAs or RNAs generated using nucleotide analogs, and derivatives, fragments and homologs thereof. The polynucleotide of the present invention may be single-, double-, or triple-stranded. Such polynucleotides are specifically disclosed to include all polynucleotides capable of encoding an exemplary protein, for example, taking into account the wobble known to be tolerated in the third position of RNA codons, yet encoding for the same amino acid as a different RNA codon (*see* Stothard P, *Biotechniques* 28: 1102-4 (2000)).

[607] In one aspect, the present invention provides polynucleotides which encode a Shiga toxin effector polypeptide and/or cell-targeting molecule of the present invention, or a fragment or derivative thereof. The polynucleotides may include, *e.g.*, a nucleic acid sequence encoding a polypeptide at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more, identical to a polypeptide comprising one of the amino acid sequences of a polypeptide or cell-targeting molecule of the present invention. The invention also includes polynucleotides comprising nucleotide sequences that hybridize under stringent conditions to a polynucleotide which encodes Shiga toxin effector polypeptide and/or cell-targeting molecule of the invention, or a fragment or derivative thereof, or the antisense or complement of any such sequence.

[608] Derivatives or analogs of the molecules of the present invention (*e.g.*, Shiga toxin effector polypeptides of the present invention and cell-targeting molecules comprising the same) include, *inter alia*, polynucleotide (or polypeptide) molecules having regions that are substantially homologous to the polynucleotides (or Shiga toxin effector polypeptides and cell-targeting molecules of the present invention), *e.g.* by at least about 45%, 50%, 70%, 80%, 85%, 90%, 95%, 98%, or even 99% identity (with a preferred identity of 80–99%) over a polynucleotide (or polypeptide) sequence of the same size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art. An exemplary program is the GAP program (Wisconsin Sequence Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison, WI, U.S.) using the default settings, which uses the algorithm of Smith T, Waterman M, *Adv Appl Math* 2: 482-9 (1981).

Also included are polynucleotides capable of hybridizing to the complement of a sequence encoding the cell-targeting proteins of the invention under stringent conditions (*see e.g.* Ausubel F et al., *Current Protocols in Molecular Biology* (John Wiley & Sons, New York, NY, U.S., 1993)), and below. Stringent conditions are known to those skilled in the art and may be found, *e.g.*, in *Current Protocols in Molecular Biology* (John Wiley & Sons, NY, U.S., Ch. Sec. 6.3.1-6.3.6 (1989)).

[609] The present invention further provides expression vectors that comprise the polynucleotides within the scope of the present invention. The polynucleotides capable of encoding the Shiga toxin effector polypeptides and/or cell-targeting molecules of the invention may be inserted into known vectors, including bacterial plasmids, viral vectors and phage vectors, using material and methods well known in the art to produce expression vectors. Such expression vectors will include the polynucleotides necessary to support production of contemplated Shiga toxin effector polypeptides and/or cell-targeting molecules of the invention within any host cell of choice or cell-free expression systems (*e.g.* pTxb1 and pIVEX2.3). The specific polynucleotides comprising expression vectors for use with specific types of host cells or cell-free expression systems are well known to one of ordinary skill in the art, can be determined using routine experimentation, and/or may be purchased.

[610] The term "expression vector," as used herein, refers to a polynucleotide, linear or circular, comprising one or more expression units. The term "expression unit" denotes a polynucleotide segment encoding a polypeptide of interest and capable of providing expression of the nucleic acid segment in a host cell. An expression unit typically comprises a transcription promoter, an open reading frame encoding the polypeptide of interest, and a transcription terminator, all in operable configuration. An expression vector contains one or more expression units. Thus, in the context of the present invention, an expression vector encoding a Shiga toxin effector polypeptide and/or cell-targeting molecule of the invention comprising a single polypeptide chain includes at least an expression unit for the single polypeptide chain, whereas a protein comprising, *e.g.* two or more polypeptide chains (*e.g.* one chain comprising a V_L domain and a second chain comprising a V_H domain linked to a toxin effector polypeptide) includes at least two expression units, one for each of the two polypeptide chains of the protein. For expression of multi-chain cell-targeting proteins of the invention, an expression unit for each polypeptide chain may also be separately contained on different expression vectors (*e.g.* expression may be achieved with a single host cell into which expression vectors for each polypeptide chain has been introduced).

[611] Expression vectors capable of directing transient or stable expression of polypeptides and proteins are well known in the art. The expression vectors generally include, but are not limited to, one or more of the following: a heterologous signal sequence or peptide, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is well known in the art. Optional regulatory control sequences, integration sequences, and useful markers that can be employed are known in the art.

[612] The term "host cell" refers to a cell which can support the replication or expression of the expression vector. Host cells may be prokaryotic cells, such as *E. coli* or eukaryotic cells (*e.g.* yeast,

insect, amphibian, bird, or mammalian cells). Creation and isolation of host cell lines comprising a polynucleotide of the invention or capable of producing a polypeptide and/or cell-targeting molecule of the present invention can be accomplished using standard techniques known in the art.

[613] Shiga toxin effector polypeptides and/or proteins within the scope of the present invention may be variants or derivatives of the polypeptides and molecules described herein that are produced by modifying the polynucleotide encoding a polypeptide and/or proteinaceous component of a cell-targeting molecule by altering one or more amino acids or deleting or inserting one or more amino acids that may render it more suitable to achieve desired properties, such as more optimal expression by a host cell.

X. Molecules of the Present Invention Immobilized on Solid Substrates

[614] Certain embodiments of the present invention include a molecule of the present invention (*e.g.* a Shiga toxin effector polypeptide, a cell-targeting molecule, fusion protein, or polynucleotide of the present invention), or any effector fragment thereof, immobilized on a solid substrate. Solid substrates contemplated herein include, but are not limited to, microbeads, nanoparticles, polymers, matrix materials, microarrays, microtiter plates, or any solid surface known in the art (*see e.g.* US 7,771,955). In accordance with these embodiments, a molecule of the present invention may be covalently or non-covalently linked to a solid substrate, such as, *e.g.*, a bead, particle, or plate, using techniques known to the skilled worker (*see e.g.* Jung Y et al., *Analyst* 133: 697-701 (2008)). Immobilized molecules of the present invention (*e.g.* a HER2-targeting molecule which comprises, consists of, or consists essentially of any one of SEQ ID NOs: 29, 36, 102, and 108) may be used for screening applications using techniques known in the art (*see e.g.* Bradbury A et al., *Nat Biotechnol* 29: 245-54 (2011); Sutton C, *Br J Pharmacol* 166: 457-75 (2012); Diamante L et al., *Protein Eng Des Sel* 26: 713-24 (2013); Houlihan G et al., *J Immunol Methods* 405: 47-56 (2014)).

[615] Non-limiting examples of solid substrates to which a molecule of the invention may be immobilized on include: microbeads, nanoparticles, polymers, nanopolymers, nanotubes, magnetic beads, paramagnetic beads, superparamagnetic beads, streptavidin coated beads, reverse-phase magnetic beads, carboxy terminated beads, hydrazine terminated beads, silica (sodium silica) beads and iminodiacetic acid (IDA) -modified beads, aldehyde-modified beads, epoxy-activated beads, diaminodipropylamine (DADPA) -modified beads (beads with primary amine surface group), biodegradable polymeric beads, polystyrene substrates, amino-polystyrene particles, carboxyl-polystyrene particles, epoxy-polystyrene particles, dimethylamino-polystyrene particles, hydroxy-polystyrene particles, colored particles, flow cytometry particles, sulfonate-polystyrene particles, nitrocellulose surfaces, reinforced nitrocellulose membranes, nylon membranes, glass surfaces, activated glass surfaces, activated quartz surfaces, polyvinylidene difluoride (PVDF) membranes, polyacrylamide-based substrates, poly-vinyl chloride substrates, poly-methyl methacrylate substrates, poly(dimethyl siloxane) substrates, and photopolymers which contain photoreactive species (such as nitrenes, carbenes, and ketyl radicals) capable of forming covalent linkages. Other examples of solid substrates to which a

molecule of the invention may be immobilized on are commonly used in molecular display systems, such as, *e.g.*, cellular surfaces, phages, and virus particles.

XI. Delivery Devices and Kits

5 [616] In certain embodiments, the invention relates to a device comprising one or more compositions of matter of the present invention, such as a pharmaceutical composition or diagnostic composition, for delivery to a subject in need thereof. Thus, a delivery device comprising one or more compositions of the present invention can be used to administer to a patient a composition of matter of the present invention by various delivery methods, including: intravenous, subcutaneous, intramuscular or 10 intraperitoneal injection; oral administration; transdermal administration; pulmonary or transmucosal administration; administration by implant, osmotic pump, cartridge or micro pump; or by other means recognized by a person of skill in the art.

[617] Also within the scope of the present invention are kits comprising at least one composition of matter of the invention, and optionally, packaging and instructions for use. For example, the present 15 invention provides a kit comprising: (i) a HER2-targeting molecule of the present invention, (ii) a pharmaceutical composition of the present invention, (iii) a diagnostic composition of the present invention, (iv) a polynucleotide of the present invention, (v) an expression vector of the present invention and/or (vi) a host cell of the present invention; and optionally, packaging and instructions for use. Kits may be useful for drug administration and/or diagnostic information gathering. A kit of the invention 20 may optionally comprise at least one additional reagent (*e.g.*, standards, markers and the like). Kits typically include a label indicating the intended use of the contents of the kit. The kit may further comprise reagents and other tools for detecting a cell type (*e.g.* a tumor cell) in a sample or in a subject, or for diagnosing whether a patient belongs to a group that responds to a therapeutic strategy which makes use of a compound, composition, or related method of the present invention, *e.g.*, such as a 25 method described herein.

XII. Methods for Using Cell-Targeting Molecules of the Present Invention and/or Pharmaceutical and/or Diagnostic Compositions Thereof

[618] Generally, it is an object of the present invention to provide pharmacologically active agents, as 30 well as compositions comprising the same, that can be used in the prevention and/or treatment of diseases, disorders, and conditions, such as certain cancers, tumors, growth abnormalities, immune disorders, or further pathological conditions mentioned herein. Accordingly, the present invention provides methods of using the polypeptides, cell-targeting molecules, and pharmaceutical compositions of the invention for the targeted killing of cells, for delivering additional exogenous materials into 35 targeted cells, for labeling of the interiors of targeted cells, for collecting diagnostic information, for the delivering of T-cell epitopes to the MHC class I presentation pathway of target cells, and for treating diseases, disorders, and conditions as described herein. For example, the methods of the present

invention may be used to prevent or treat cancers, cancer initiation, tumor initiation, metastasis, and/or disease reoccurrence.

[619] In particular, it is an object of the invention to provide such pharmacologically active agents, compositions, and/or methods that have certain advantages compared to the agents, compositions, and/or methods that are currently known in the art. Accordingly, the present invention provides methods of using Shiga toxin effector polypeptides and cell-targeting molecules with specified protein sequences and pharmaceutical compositions thereof. For example, any of the amino acid sequences described herein may be specifically utilized as a component of the cell-targeting molecule used in the following methods or any method for using a cell-targeting molecule known to the skilled worker, such as, *e.g.*, various methods described in WO 2014/164680, WO 2014/164693, WO 2015/138435, WO 2015/138452, WO 2015/113005, WO 2015/113007, WO 2015/191764, US20150259428, WO 2016/196344, WO 2017/019623, and WO 2018/140427.

[620] The present invention provides methods of killing a cell comprising the step of contacting the cell, either *in vitro* or *in vivo*, with a Shiga toxin effector polypeptide, cell-targeting molecule, or pharmaceutical composition of the present invention. The Shiga toxin effector polypeptides, cell-targeting molecules, and pharmaceutical compositions of the present invention can be used to kill a specific cell type upon contacting a cell or cells with one of the claimed compositions of matter. For certain embodiments, the cell(s) is physically coupled with HER2. For certain embodiments, the cell(s) expresses (including over-expresses) HER2. The HER2 may be expressed (including overexpressed) at the surface of the cells. For certain embodiments, a cell-targeting molecule or pharmaceutical composition of the present invention can be used to kill specific cell types in a mixture of different cell types, such as mixtures comprising cancer cells, infected cells, and/or hematological cells. For certain embodiments, a cell-targeting molecule, or pharmaceutical composition of the present invention can be used to kill cancer cells in a mixture of different cell types. For certain embodiments, a cytotoxic Shiga cell-targeting molecule, or pharmaceutical composition of the present invention can be used to kill specific cell types in a mixture of different cell types, such as pre-transplantation tissues. For certain embodiments, a Shiga toxin effector polypeptide, cell-targeting molecule, or pharmaceutical composition of the present invention can be used to kill specific cell types in a mixture of cell types, such as pre-administration tissue material for therapeutic purposes. For certain embodiments, a cell-targeting molecule or pharmaceutical composition of the present invention can be used to selectively kill cells infected by viruses or microorganisms, or otherwise selectively kill cells expressing a particular extracellular target biomolecule, such as a cell surface localized HER2 variant. The Shiga toxin effector polypeptides, cell-targeting molecules, and pharmaceutical compositions of the present invention have varied applications, including, *e.g.*, uses in depleting unwanted cell types from tissues either *in vitro* or *in vivo*, uses as antiviral agents, and uses in purging transplantation tissues of unwanted cell types. For certain embodiments, the cell expresses muc-4 and/or CD44. For certain embodiments, the cell is resistant to cytotoxicity caused by T-DM1 (trastuzumab emtansine) and/or trastuzumab. For certain further embodiments the cell(s) are in the presence of pertuzumab, T-DM1 (trastuzumab emtansine),

lapatinib, and/or neratinib; and/or had previously been contacted with pertuzumab, T-DM1 (trastuzumab emtansine), lapatinib, and/or neratinib. Among certain embodiments of the present invention is a method of killing a cell (*e.g.* a HER2-expressing cell) comprising the step of contacting the cell with the cell-targeting molecule of the present invention or the pharmaceutical composition of the present invention wherein the cell is in the presence of pertuzumab, T-DM1 (trastuzumab emtansine), lapatinib, and/or neratinib; and/or had previously been contacted with pertuzumab, T-DM1 (trastuzumab emtansine), lapatinib and/or neratinib. For certain further embodiments the cell(s) are in the presence of T-DM1 (trastuzumab emtansine). For certain further embodiments the cell(s) are in the presence of pertuzumab. For certain further embodiments the cell(s) are in the presence of lapatinib. For certain further embodiments the cell(s) are in the presence of neratinib. For certain further embodiments the cell(s) had previously been contacted with pertuzumab. For certain further embodiments the cell(s) had previously been contacted with T-DM1 (trastuzumab emtansine). For certain further embodiments the cell(s) had previously been contacted with lapatinib. For certain further embodiments the cell(s) had previously been contacted with neratinib.

[621] For certain embodiments, certain Shiga toxin effector polypeptides, cell-targeting molecules, and pharmaceutical compositions of the present invention, alone or in combination with other compounds or pharmaceutical compositions, can show potent cell-kill activity when administered to a population of cells, *in vitro* or *in vivo* in a subject such as in a patient in need of treatment. By targeting the delivery of enzymatically active Shiga toxin A Subunit effector polypeptides and/or T-cell epitopes using high-affinity binding regions to specific cell types, cell-kill activities can be restricted to specifically and selectively killing certain cell types within an organism, such as certain cancer cells, neoplastic cells, malignant cells, non-malignant tumor cells, and/or infected cells.

[622] The present invention provides a method of killing a cell in a patient in need thereof, the method comprising the step of administering to the patient at least one cell-targeting molecule of the present invention or a pharmaceutical composition thereof.

[623] For certain embodiments, the cell-targeting molecule of the present invention or pharmaceutical compositions thereof can be used to kill a cancer cell in a patient by targeting an extracellular biomolecule found physically coupled with a cancer or tumor cell. The terms “cancer cell” or “cancerous cell” refers to various neoplastic cells which grow and divide in an abnormally accelerated and/or unregulated fashion and will be clear to the skilled person. The term “tumor cell” includes both malignant and non-malignant cells. Generally, cancers and/or tumors can be defined as diseases, disorders, or conditions that are amenable to treatment and/or prevention. The cancers and tumors (either malignant or non-malignant) which are comprised of cancer cells and/or tumor cells which may benefit from methods and compositions of the invention will be clear to the skilled person. Neoplastic cells are often associated with one or more of the following: unregulated growth, lack of differentiation, local tissue invasion, angiogenesis, and metastasis. The diseases, disorders, and conditions resulting from cancers and/or tumors (either malignant or non-malignant) which may benefit from the methods and compositions of the present invention targeting certain cancer cells and/or tumor cells will be clear to the

skilled person. For example, disease, disorder, or condition may be characterized by cells that are physically coupled with HER2. The HER2 target biomolecule may be physically coupled to the surface of the cells. For certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2 target biomolecule (including cells that overexpress HER2). The HER2 may be expressed (including overexpressed) at the surface of the cells.

[624] Certain embodiments of the cell-targeting molecules and compositions of the present invention may be used to treat diseases, disorders or conditions (such as, *e.g.*, HER2 positive cancers and/or tumors) in a patient after the patient has already received a HER2-targeted therapeutic agent. In many situations, cell-surface HER2 expression persists during disease progression after a therapeutic treatment such as, *e.g.*, a HER2-targeted therapy using an anti-HER2 monoclonal antibody therapy or anti-HER2 antibody drug conjugate therapy, or a chemotherapeutic agent therapy using a tyrosine kinase inhibitor. Thus, HER2 is still present as a target on the surfaces of malignant/target cells and available for targeting by a cell-targeting molecule of the present invention for cell-surface docking and cellular internalization. Furthermore, as demonstrated by the Examples, the cell-targeting molecules of the present invention (and compositions comprising the cell-targeting molecules) can be used in combination with other HER2-targeted therapeutic agents, such as the anti-HER2 antibody therapies that bind to non-overlapping antigenic determinants of HER2; or the tyrosine kinase inhibitors that have a different HER2-targeting activity to the cell-targeting molecules of the invention. Accordingly, the "patient in need thereof" that is administered with at least one cell-targeting molecule or a pharmaceutical composition thereof in the methods of the present invention, includes a patient(s) that has been previously treated with an additional HER2-targeting therapeutic agent; and/or is undergoing treatment with an additional HER2-targeting therapeutic agent. For certain embodiments, the patient(s) has been previously treated with an additional HER2-targeting therapeutic agent as described herein. For certain embodiments, the patient(s) is undergoing treatment with an additional HER2-targeting therapeutic agent as described herein. For certain embodiments, the "patient in need thereof" does not respond to, or does not benefit from, treatment with one or more additional HER2-targeting therapeutic agent. For example, this can be due to, *inter alia*, acquired and/or intrinsic resistance. For certain embodiments, the additional HER2-targeting therapeutic agent comprises a tyrosine kinase inhibitor, an anti-HER2 monoclonal antibody therapy or an anti-HER2 antibody drug conjugate therapy. For certain embodiments, the additional HER2-targeting therapeutic agent comprises one or more of: pertuzumab, trastuzumab, T-DM1 (trastuzumab emtansine), lapatinib and/or neratinib. For certain embodiments, the additional HER2-targeting therapeutic agent is pertuzumab. For certain embodiments, the additional HER2-targeting therapeutic agent is trastuzumab. For certain embodiments, additional HER2-targeting therapeutic agent is T-DM1 (trastuzumab emtansine). For certain embodiments, the additional HER2-targeting therapeutic agent is lapatinib. For certain embodiments, the additional HER2-targeting therapeutic agent is neratinib.

[625] As used herein, the reference to "a patient in need thereof" that "has been previously treated with an additional HER2-targeting therapeutic agent" includes patients that were last administered treatment with an additional HER2-targeting therapeutic agent at least 6 months (such as at least 5 months, 4

months, 3 months, 2 months or 1 month), at least 6 weeks (such as at least 5 weeks, 4 weeks, 3 weeks, 2 weeks or 1 week) or at least 144 hours (such as at least 120 hours, 96 hours , 72 hours, 48 hours, 24 hours, 12 hours, or 6 hours) prior to treatment with the cell-targeting molecule or pharmaceutical composition of the present invention.

[626] As used herein, the reference to "a patient in need thereof" that "is undergoing treatment with an additional HER2-targeting therapeutic agent" includes patients that are simultaneously or sequentially administered with the cell-targeting molecule or pharmaceutical composition of the present invention and an additional HER2-targeting therapeutic agent. The patient may be administered with the additional HER2-targeting therapeutic agent at least 1 hour (such as at least 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, or 144 hours), 1 week (such as at least 2 weeks, 3 weeks, 4 weeks, 5 weeks or 6 weeks) or 1 month (such as at least 2 months, 3 months, 4 months, 5 months or 6 months) prior to, or subsequent to, treatment with the cell-targeting molecule or pharmaceutical composition of the present invention.

[627] As used herein, the reference to a "patient in need thereof" that does not respond to, or does not benefit from, treatment with one or more additional HER2-targeting therapeutic agent includes patients that are resistant to or have developed resistance to the one or more additional HER2-targeting therapeutic agent. For examples, drug resistance may arise from the expression of drug efflux pumps or cytochrome P450 enzymes (*e.g.* CYP3A4) as well as obstacles preventing HER2 epitope binding, *e.g.* HER2 eptiope masking of the epitope bound by the HER2-targeting therapeutic. For example, drug resistance may arise from the existence of activated survival/proliferation pathways redundant to HER2 signaling or downstream of HER2 activity thereby bypassing HER2. For example, drug resistance may arise from the existence of mutations in HER2 that alter the drug's effectiveness, such as, *e.g.*, mutations in the ATP-binding pocket bound by a HER2 inhibitor. Resistance mechanisms tied to the additional HER2-targeting therapeutic agent mechanism of action can be avoided by HER2-targeting molecules of the present invention that effectuating a different mechanism of action.

[628] Certain embodiments of the cell-targeting molecules and compositions of the present invention may be used to treat cancers and/or tumors in a subject after the subject has already received a HER2-targeted therapy. In many situations HER2 persists during disease progression after a therapeutic treatment such as, *e.g.*, a HER2-targeted therapy using an anti-HER2 monoclonal antibody therapy or anti-HER2 antibody drug conjugate therapy, or a chemotherapeutic agent therapy using a tyrosine kinase inhibitor. Thus, HER2 is still present as a target on the surfaces of malignant/target cells and available for targeting by a cell-targeting molecule of the present invention.

[629] Certain embodiments of the cell-targeting molecules and compositions of the present invention may be used to kill cancer stem cells, tumor stem cells, pre-malignant cancer-initiating cells, and tumor-initiating cells, which commonly are slow dividing and resistant to cancer therapies like chemotherapy and radiation.

[630] Because of the Shiga toxin A Subunit based mechanism of action, compositions of matter of the present invention may be more effectively used in methods involving their combination with, or in

complementary fashion with other therapies, such as, *e.g.*, chemotherapies, immunotherapies, radiation, stem cell transplantation, and immune checkpoint inhibitors, and/or effective against chemoresistant/radiation-resistant and/or resting tumor cells/tumor initiating cells/stem cells. Similarly, compositions of matter of the present invention may be more effectively used in methods involving in combination with other cell-targeted therapies targeting other than the same epitope on, non-overlapping, or different targets for the same disease disorder or condition. These other therapies or other cell-targeted therapies include the additional HER2-targeting therapeutic agent(s) described herein.

[631] Certain embodiments of the cell-targeting molecules of the present invention, or pharmaceutical compositions thereof, can be used to kill an immune cell (whether healthy or malignant) in a patient by targeting an extracellular biomolecule found physically coupled with an immune cell.

[632] For certain embodiments of the cell-targeting molecule of the present invention, or pharmaceutical compositions thereof, can be used to kill an infected cell in a patient by targeting an extracellular biomolecule found physically coupled with an infected cell.

[633] For certain embodiments of the cell-targeting molecules of the present invention, or pharmaceutical compositions thereof, can be used to "seed" a locus within a chordate with non-self, T-cell epitope-peptide presenting cells in order to activate the immune system to enhance policing of the locus. For certain further embodiments of this "seeding" method of the present invention, the locus is a tumor mass or infected tissue site. In preferred embodiments of this "seeding" method of the present invention, the non-self, T-cell epitope-peptide is selected from the group consisting of: peptides not already presented by the target cells of the cell-targeting molecule, peptides not present within any protein expressed by the target cell, peptides not present within the proteome or transcriptome of the target cell, peptides not present in the extracellular microenvironment of the site to be seeded, and peptides not present in the tumor mass or infect tissue site to be targeting.

[634] This "seeding" method functions to label one or more target cells within a chordate with one or more MHC class I presented T-cell epitopes for recognition by effector T-cells and activation of downstream immune responses. By exploiting the cell internalizing, intracellularly routing, and T-cell epitope delivering functions of the cell-targeting molecules of the present invention, the target cells which display the delivered T-cell epitope are harnessed to induce recognition of the presenting target cell by host T-cells and induction of further immune responses including target-cell-killing by CTLs. This "seeding" method of using a cell-targeting molecule of the present invention can provide a temporary vaccination-effect by inducing adaptive immune responses to attack the cells within the seeded microenvironment, such as, *e.g.* a tumor mass or infected tissue site, whether presenting a cell-targeting molecule-delivered T-cell epitope(s) or not. This "seeding" method may also induce the breaking of immuno-tolerance to a target cell population, a tumor mass, and/or infected tissue site within a chordate.

[635] Certain methods of the present invention involving the seeding of a locus within a chordate with one or more antigenic and/or immunogenic epitopes may be combined with the administration of immunologic adjuvants, whether administered locally or systemically, to stimulate the immune response to certain antigens, such as, *e.g.*, the co-administration of a composition of the present invention with one

or more immunologic adjuvants like a cytokine, bacterial product, or plant saponin. Other examples of immunologic adjuvants which may be suitable for use in the methods of the present invention include aluminum salts and oils, such as, *e.g.*, alums, aluminum hydroxide, mineral oils, squalene, paraffin oils, peanut oils, and thimerosal.

[636] Additionally, the present invention provides a method of treating a disease, disorder, or condition in a patient, the method comprising the step of administering to a patient in need thereof a therapeutically effective amount of at least one of the cell-targeting molecules of the present invention, or a pharmaceutical composition thereof. The disease, disorder or condition may be characterized by cells that are physically coupled with HER2/neu/ErbB2. The HER2/neu/ErbB2 may be physically coupled to the surface of the cells. For certain embodiments, the disease, disorder or condition may be characterized by cells that express (including overexpress) HER2/neu/ErbB2. The HER2/neu/ErbB2 may be expressed (including overexpressed) at the surface of the cells. Contemplated diseases, disorders, and conditions that can be treated using this method include cancers, malignant tumors, non-malignant tumors, growth abnormalities, immune disorders, and microbial infections. The cancer, tumor, growth abnormality, immune disorder, or microbial infection may be characterized by cells that are physically coupled with HER2/neu/ErbB2. The HER2/neu/ErbB2 may be physically coupled to the surface of the cells. For certain embodiments, the cancer, tumor, growth abnormality, immune disorder, or microbial infection may be characterized by cells that express (including overexpress) HER2/neu/ErbB2. The HER2/neu/ErbB2 may be expressed (including overexpressed) at the surface of the cells. Administration of a "therapeutically effective dosage" of a composition of the present invention can result in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. The "patient in thereof" is as described herein. For certain embodiments, the "patient in need thereof" has been previously treated with one or more additional HER2-targeting therapeutic agent; and/or is undergoing treatment with one or more additional HER2-targeting therapeutic agent. For certain embodiments, the "patient in need thereof" has been previously treated with one or more additional HER2-targeting therapeutic agent is as described herein. For certain embodiments, the "patient in need thereof" is undergoing treatment with one or more additional HER2-targeting therapeutic agent is as described herein. For certain embodiments, the "patient in need thereof" does not respond to, or does not benefit from, treatment with one or more additional HER2-targeting therapeutic agent is as described herein. The one or more additional HER2-targeting therapeutic agent is as described herein. For example, the additional HER2-targeting therapeutic agent may comprise a dual tyrosine kinase inhibitor; such as *e.g.* lapatinib and/or neratinib. For example, the additional HER2-targeting therapeutic agent may comprise an anti-HER2 antibody that binds an antigenic determinant in HER2 that does not overlap with the antigenic determinant in HER2 bound by the HER2-targeting molecule; such as *e.g.* T-DM1, trastuzumab, and/or pertuzumab. For example, the additional HER2-targeting therapeutic agent may comprise anti-HER2 antibody drug conjugate therapy; such as T-DM1. For certain embodiments, the one or more additional

HER2-targeting therapeutic agent is selected from: lapatinib, neratinib, T-DM1, trastuzumab, and pertuzumab.

[637] The therapeutically effective amount of a composition of the present invention will depend on the route of administration, the type of organism being treated, and the physical characteristics of the specific patient under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical arts. This amount and the method of administration can be tailored to achieve optimal efficacy, and may depend on such factors as weight, diet, concurrent medication and other factors, well known to those skilled in the medical arts. The dosage sizes and dosing regimen most appropriate for human use may be guided by the results obtained by the present invention, and may be confirmed in properly designed clinical trials. An effective dosage and treatment protocol may be determined by conventional means, starting with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well. Numerous factors may be taken into consideration by a clinician when determining an optimal dosage for a given subject. Such considerations are known to the skilled person.

[638] An acceptable route of administration may refer to any administration pathway known in the art, including but not limited to aerosol, enteral, nasal, ophthalmic, oral, parenteral, rectal, vaginal, or transdermal (*e.g.* topical administration of a cream, gel or ointment, or by means of a transdermal patch). "Parenteral administration" is typically associated with injection at or in communication with the intended site of action, including infraorbital, infusion, intraarterial, intracapsular, intracardiac, intradermal, intramuscular, intraperitoneal, intrapulmonary, intraspinal, intrasternal, intrathecal, intrauterine, intravenous, subarachnoid, subcapsular, subcutaneous, transmucosal, or transtracheal administration.

[639] For administration of a pharmaceutical composition of the present invention, the dosage range will generally be from about 0.001 to 10 milligrams per kilogram (mg/kg), and more, usually 0.001 to 0.5 mg/kg, of the subject's body weight. Exemplary dosages may be 0.001 mg/kg body weight, 0.005 mg/kg body weight, 0.0075 mg/kg body weight, 0.015 mg/kg body weight, 0.020 mg/kg body weight, or 0.025 mg/kg body weight or within the range of 0.001 to 0.030 mg/kg. Exemplary dosages may be 0.01 mg/kg body weight, 0.03 mg/kg body weight, 0.05 mg/kg body weight, 0.075 mg/kg body weight, or 0.1 mg/kg body weight or within the range of 0.01 to 0.1 mg/kg. An exemplary treatment regime is a once or twice daily administration, or a once or twice weekly administration, once every two weeks, once every three weeks, once every four weeks, once a month, once every two or three months or once every three to 6 months. Dosages may be selected and readjusted by the skilled health care professional as required to maximize therapeutic benefit for a particular patient.

[640] Pharmaceutical compositions of the present invention will typically be administered to the same patient on multiple occasions. Intervals between single dosages can be, for example, two to five days, weekly, monthly, every two or three months, every six months, or yearly. Intervals between administrations can also be irregular, based on regulating blood levels or other markers in the subject or patient. Dosage regimens for a composition of the present invention include intravenous administration

to a subject of 1 to 50 μg of HER2-targeting molecule per kilogram (kg) body weight with the composition administered once or twice a week for three or more consecutive weeks, such as for four or five weeks. Exemplary dosage regimens for a composition of the present invention include intravenous administration to a subject of 1 to 25 μg of HER2-targeting molecule per kg body weight with the composition administered once or twice a week for three or more consecutive weeks, such as for four or five weeks. Dosage regimens for a composition of the present invention include intravenous administration to a subject of 10 to 50 μg of HER2-targeting molecule per kg body weight with the composition administered once or twice a week for three or more consecutive weeks, such as for four or five weeks. Dosage regimens for a composition of the present invention include intravenous administration of 0.01 to 1 mg/kg body weight or 0.03 to 3 mg/kg body weight with the composition administered every two to four weeks for six dosages, then every three months at 0.01 to 3 mg/kg body weight or 0.01 to 0.03 mg/kg body weight.

[641] A pharmaceutical composition of the present invention may be administered via one or more routes of administration, using one or more of a variety of methods known in the art. As will be appreciated by the skilled worker, the route and/or mode of administration will vary depending upon the desired results. Routes of administration for cell-targeting molecules and pharmaceutical compositions of the present invention include, *e.g.* intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal, or other parenteral routes of administration, for example by injection or infusion. For other embodiments, a cell-targeting molecule or pharmaceutical composition of the invention may be administered by a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually, or topically.

[642] Therapeutic cell-targeting molecules or pharmaceutical compositions of the present invention may be administered with one or more of a variety of medical devices known in the art. For example, in one embodiment, a pharmaceutical composition of the invention may be administered with a needleless hypodermic injection device. Examples of well-known implants and modules useful in the present invention are in the art, including *e.g.*, implantable micro-infusion pumps for controlled rate delivery; devices for administering through the skin; infusion pumps for delivery at a precise infusion rate; variable flow implantable infusion devices for continuous drug delivery; and osmotic drug delivery systems. These and other such implants, delivery systems, and modules are known to those skilled in the art.

[643] The cell-targeting molecule or pharmaceutical composition of the present invention may be administered alone or in combination with one or more other therapeutic or diagnostic agents. A combination therapy may include a cell-targeting molecule of the present invention, or pharmaceutical composition thereof, combined with at least one other therapeutic agent selected based on the particular patient, disease or condition to be treated. Examples of other such agents include, *inter alia*, a cytotoxic, anti-cancer or chemotherapeutic agent, an anti-inflammatory or anti-proliferative agent, an antimicrobial or antiviral agent, growth factors, cytokines, an analgesic, a therapeutically active small molecule or polypeptide, a single chain antibody, a classical antibody or fragment thereof, or a nucleic acid molecule

which modulates one or more signaling pathways, and similar modulating therapeutic molecules which may complement or otherwise be beneficial in a therapeutic or prophylactic treatment regimen.

[644] The cell-targeting molecule or pharmaceutical composition of the present invention may be administered alone or in combination with one or more other HER2-targeting therapeutic agents. The cell-targeting molecule or pharmaceutical composition of the present invention may be administered alone or in combination with one or more additional HER2-targeting therapeutic agents, such as, *e.g.*, T-DM1 (trastuzumab emtansine), trastuzumab, pertuzumab, and/or lapatinib. A combination therapy may include a cell-targeting molecule of the present invention, or pharmaceutical composition thereof, combined with at least one other therapeutic agent selected based on the particular patient, disease or condition to be treated. Examples of other such agents include, *inter alia*, a cytotoxic, anti-cancer or chemotherapeutic agent, an anti-inflammatory or anti-proliferative agent, an antimicrobial or antiviral agent, growth factors, cytokines, an analgesic, a therapeutically active small molecule or polypeptide, a single chain antibody, a classical antibody or fragment thereof, or a nucleic acid molecule which modulates one or more signaling pathways, and similar modulating therapeutic molecules which may complement or otherwise be beneficial in a therapeutic or prophylactic treatment regimen. For certain embodiments, the methods of the invention for treating a disease, disorder, or condition in a patient in need thereof may further comprise administering to the patient a therapeutically effective amount of one or more additional HER2-targeting therapeutic agent. The additional HER2-targeting therapeutic agent is as described herein. For example, the additional HER2-targeting therapeutic agent may comprise a dual tyrosine kinase inhibitor; such as lapatinib and/or neratinib. For example, the additional HER2-targeting therapeutic agent may comprise an anti-HER2 antibody that binds an antigenic determinant in HER2 that does not overlap with the antigenic determinant in HER2 bound by the HER2-targeting molecule; such as T-DM1, trastuzumab, and/or pertuzumab for the HER2-targeting molecule which comprises, consists of, or consists essentially of any one of SEQ ID NOs: 29, 36, 102, and 108. For example, the additional HER2-targeting therapeutic agent may comprise anti-HER2 antibody drug conjugate therapy; such as T-DM1.

[645] Treatment of a patient with cell-targeting molecule or pharmaceutical composition of the present invention preferably leads to cell death of targeted cells and/or the inhibition of growth of targeted cells. As such, cytotoxic, cell-targeting molecules of the present invention, and pharmaceutical compositions comprising them, will be useful in methods for treating a variety of pathological disorders in which killing or depleting target cells may be beneficial, such as, *inter alia*, cancer, tumors, other growth abnormalities, immune disorders, and infected cells. The present invention provides methods for suppressing cell proliferation, and treating cell disorders involving HER2-expressing cells, including neoplasia.

[646] In certain embodiments, the cell-targeting molecules and pharmaceutical compositions of the present invention are for use in the treatment or prevention of a disease, disorder, or condition in a patient in need thereof. The disease, disorder or condition may be characterized by cells that are physically coupled with HER2 (*e.g.* the cells express HER2 such that HER2 is expressed on the surfaces of the

cells). In certain embodiments, the cell-targeting molecules and pharmaceutical compositions of the present invention are for use in the treatment or prevention of a cancer, tumor (malignant and non-malignant), growth abnormality, immune disorder, and/or microbial infection in a patient in need thereof. In certain embodiments, the cell-targeting molecules and pharmaceutical compositions of the present invention are for use in the treatment or prevention of a cancer, tumor (malignant and non-malignant), and/or growth abnormality in a patient in need thereof. In certain embodiments, the cell-targeting molecules and pharmaceutical compositions of the present invention are for use in the treatment or prevention of a cancer and/or tumor (malignant and non-malignant) in a patient in need thereof. The cancer, tumor, growth abnormality, immune disorder, and/or microbial infection may be characterized by cells that are physically coupled with HER2 (*e.g.* the cells express HER2 such that HER2 is expressed on the surfaces of the cells). The "patient in thereof" is as described herein. I n certain embodiments, the "patient in need thereof" has been previously treated with one or more additional HER2-targeting therapeutic agent; and/or is undergoing treatment with one or more additional HER2-targeting therapeutic agent. For certain embodiments, the "patient in need thereof" has been previously treated with one or more additional HER2-targeting therapeutic agent is as described herein. For certain embodiments, the "patient in need thereof" is undergoing treatment with one or more additional HER2-targeting therapeutic agent is as described herein. For certain embodiments, the "patient in need thereof" does not respond to, or does not benefit from, treatment with one or more additional HER2-targeting therapeutic agent is as described herein. For certain embodiments, the treatment or prevention of a disease, disorder, or condition in a patient in need thereof may further comprise a step of administering to the patient a therapeutically effective amount of one or more additional HER2-targeting therapeutic agent. The additional HER2-targeting therapeutic agent is as described herein.

[647] In certain embodiments, the present invention provides methods for treating malignancies or neoplasms and other blood cell associated cancers in a mammalian subject, such as a human, the method comprising the step of administering to a subject in need thereof a therapeutically effective amount of a cytotoxic cell-targeting molecule or pharmaceutical composition of the present invention.

[648] The cell-targeting molecules and pharmaceutical compositions of the present invention have varied applications. The cell-targeting molecules and pharmaceutical compositions of the present invention are commonly anti-neoplastic agents – meaning they are capable of treating and/or preventing the development, maturation, or spread of neoplastic or malignant cells by inhibiting the growth and/or causing the death of cancer or tumor cells. However, certain embodiments of the cell-targeting molecule or pharmaceutical composition of the present invention is used to treat an immune disorder, such as, *e.g.*, a T-cell-, B-cell-, plasma cell- or antibody- mediated disease or disorder.

[649] Certain embodiments of the cell-targeting molecules and pharmaceutical compositions of the present invention can be utilized in a method of treating cancer comprising administering to a patient, in need thereof, a therapeutically effective amount of a cell-targeting molecule and/or pharmaceutical composition of the present invention. For certain embodiments of the methods of the present invention, the cancer being treated is selected from the group consisting of: bone cancer (such as multiple myeloma

or Ewing's sarcoma), breast cancer, central/peripheral nervous system cancer (such as brain cancer, neurofibromatosis, or glioblastoma), gastrointestinal cancer (such as stomach cancer or colorectal cancer), germ cell cancer (such as ovarian cancers and testicular cancers, glandular cancer (such as pancreatic cancer, parathyroid cancer, pheochromocytoma, salivary gland cancer, or thyroid cancer), head-neck cancer (such as nasopharyngeal cancer, oral cancer, or pharyngeal cancer), hematological cancers (such as leukemia, lymphoma, or myeloma), kidney-urinary tract cancer (such as renal cancer and bladder cancer), liver cancer, lung/pleura cancer (such as mesothelioma, small cell lung carcinoma, or non-small cell lung carcinoma), prostate cancer, sarcoma (such as angiosarcoma, fibrosarcoma, Kaposi's sarcoma, or synovial sarcoma), skin cancer (such as basal cell carcinoma, squamous cell carcinoma, or melanoma), and uterine cancer. For certain embodiments, the cancer to be treated is selected from the group consisting of: breast cancer, gastric cancer, urothelial cancer, bladder cancer, urothelial bladder cancer, serous uterine cancer, extrahepatic biliary tract cancer, and biliary carcinoma. For certain embodiments, the cancer being treated is breast cancer and/or gastrointestinal cancer.

[650] Among certain embodiments of the present invention is using the Shiga toxin effector polypeptide or cell-targeting molecule of the present invention as a component of a pharmaceutical composition or medicament for the treatment or prevention of a cancer, tumor, other growth abnormality, immune disorder, and/or microbial infection. For example, skin tumors may be treated with such a medicament in efforts to reduce tumor size or eliminate the tumor completely.

[651] Among certain embodiment of the present invention is a method of using a Shiga toxin effector polypeptide, cell-targeting molecule, pharmaceutical composition, and/or diagnostic composition of the present invention to label or detect the interiors of neoplastic cells. This method may be based on the ability of certain cell-targeting molecules of the present invention to enter specific cell types and route within cells via retrograde intracellular transport, to the interior compartments of specific cell types are labeled for detection. This can be performed on cells *in situ* within a patient or on cells and tissues removed from an organism, *e.g.* biopsy material.

[652] Among certain embodiment of the present invention is a method of using a Shiga toxin effector polypeptide, cell-targeting molecule, pharmaceutical composition, and/or diagnostic composition of the present invention to detect the presence of a cell type for the purpose of information gathering regarding diseases, conditions and/or disorders. The disease, disorder, or condition may be characterized by cells that are physically coupled with HER2. The HER2 target biomolecule may be physically coupled to the surface of the cells. For certain embodiments, the disease, disorder or condition may be characterized by cells that express the HER2 target biomolecule (including cells that overexpress HER2). The HER2 may be expressed (including overexpressed) at the surface of the cells. The method comprises contacting a cell with a diagnostically sufficient amount of a cell-targeting molecule of the present invention in order to detect the molecule by an assay or diagnostic technique. The phrase "diagnostically sufficient amount" refers to an amount that provides adequate detection and accurate measurement for information gathering purposes by the particular assay or diagnostic technique utilized. Generally, the diagnostically sufficient amount for whole organism *in vivo* diagnostic use will be a non-cumulative dose of between

0.001 to 10 milligrams of the detection promoting agent linked cell-targeting molecule of the invention per kg of subject per subject. Typically, the amount of cell-targeting molecule of the invention used in these information gathering methods will be as low as possible provided that it is still a diagnostically sufficient amount. For example, for *in vivo* detection in an organism, the amount of Shiga toxin effector polypeptide, cell-targeting molecule, or pharmaceutical composition of the invention administered to a subject will be as low as feasibly possible.

[653] The cell-type specific targeting of cell-targeting molecules of the present invention combined with detection promoting agents provides a way to detect and image cells physically coupled with an extracellular target biomolecule of a binding region of the molecule of the invention. Imaging of cells using the cell-targeting molecules of the present invention may be performed *in vitro* or *in vivo* by any suitable technique known in the art. Diagnostic information may be collected using various methods known in the art, including whole body imaging of an organism or using *ex vivo* samples taken from an organism. The term "sample" used herein refers to any number of things, but not limited to, fluids such as blood, urine, serum, lymph, saliva, anal secretions, vaginal secretions, and semen, and tissues obtained by biopsy procedures. For example, various detection promoting agents may be utilized for non-invasive *in vivo* tumor imaging by techniques such as magnetic resonance imaging (MRI), optical methods (such as direct, fluorescent, and bioluminescent imaging), positron emission tomography (PET), single-photon emission computed tomography (SPECT), ultrasound, x-ray computed tomography, and combinations of the aforementioned (*see*, Kaur S et al., *Cancer Lett* 315: 97-111 (2012), *for review*).

[654] Among certain embodiment of the present invention is a method of using a Shiga toxin effector polypeptide, cell-targeting molecule, or pharmaceutical composition of the present invention in a diagnostic composition to label or detect the interiors of a hematologic cell, cancer cell, tumor cell, infected cell, and/or immune cell (*see e.g.*, Koyama Y et al., *Clin Cancer Res* 13: 2936-45 (2007); Ogawa M et al., *Cancer Res* 69: 1268-72 (2009); Yang L et al., *Small* 5: 235-43 (2009)). Based on the ability of certain cell-targeting molecules of the invention to enter specific cell types and route within cells via retrograde intracellular transport, the interior compartments of specific cell types are labeled for detection. This can be performed on cells *in situ* within a patient or on cells and tissues removed from an organism, *e.g.* biopsy material.

[655] Diagnostic compositions of the present invention may be used to characterize a disease, disorder, or condition as potentially treatable by a related pharmaceutical composition of the present invention. Certain compositions of matter of the present invention may be used to determine whether a patient belongs to a group that responds to a therapeutic strategy which makes use of a compound, composition or related method of the present invention as described herein or is well suited for using a delivery device of the invention.

[656] Diagnostic compositions of the present invention may be used after a disease, *e.g.* a cancer, is detected in order to better characterize it, such as to monitor distant metastases, heterogeneity, and stage of cancer progression. The phenotypic assessment of disease disorder or infection can help prognostic

and prediction during therapeutic decision making. In disease reoccurrence, certain methods of the invention may be used to determine if local or systemic problem.

[657] Diagnostic compositions of the present invention may be used to assess responses to therapies regardless of the type of the type of therapy, *e.g.* small molecule drug, biological drug, or cell-based therapy. For example, certain embodiments of the diagnostics of the invention may be used to measure changes in tumor size, changes in antigen positive cell populations including number and distribution, or monitoring a different marker than the antigen targeted by a therapy already being administered to a patient (*see* Smith-Jones P et al., *Nat. Biotechnol* 22: 701-6 (2004); Evans M et al., *Proc. Natl. Acad. Sci. USA* 108: 9578-82 (2011)).

[658] For certain embodiments of the method used to detect the presence of a cell type may be used to gather information regarding diseases, disorders, and conditions, such as, for example bone cancer (such as multiple myeloma or Ewing's sarcoma), breast cancer, central/peripheral nervous system cancer (such as brain cancer, neurofibromatosis, or glioblastoma), gastrointestinal cancer (such as stomach cancer or colorectal cancer), germ cell cancer (such as ovarian cancers and testicular cancers, glandular cancer (such as pancreatic cancer, parathyroid cancer, pheochromocytoma, salivary gland cancer, or thyroid cancer), head-neck cancer (such as nasopharyngeal cancer, oral cancer, or pharyngeal cancer), hematological cancers (such as leukemia, lymphoma, or myeloma), kidney-urinary tract cancer (such as renal cancer and bladder cancer), liver cancer, lung/pleura cancer (such as mesothelioma, small cell lung carcinoma, or non-small cell lung carcinoma), prostate cancer, sarcoma (such as angiosarcoma, fibrosarcoma, Kaposi's sarcoma, or synovial sarcoma), skin cancer (such as basal cell carcinoma, squamous cell carcinoma, or melanoma), uterine cancer, acute lymphoblastic leukemia (ALL), T acute lymphocytic leukemia/lymphoma (ALL), acute myelogenous leukemia, acute myeloid leukemia (AML), B-cell chronic lymphocytic leukemia (B-CLL), B-cell prolymphocytic lymphoma, Burkitt's lymphoma (BL), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML-BP), chronic myeloid leukemia (CML), diffuse large B-cell lymphoma, follicular lymphoma, hairy cell leukemia (HCL), Hodgkin's Lymphoma (HL), intravascular large B-cell lymphoma, lymphomatoid granulomatosis, lymphoplasmacytic lymphoma, MALT lymphoma, mantle cell lymphoma, multiple myeloma (MM), natural killer cell leukemia, nodal marginal B-cell lymphoma, Non-Hodgkin's lymphoma (NHL), plasma cell leukemia, plasmacytoma, primary effusion lymphoma, pro-lymphocytic leukemia, promyelocytic leukemia, small lymphocytic lymphoma, splenic marginal zone lymphoma, T-cell lymphoma (TCL), heavy chain disease, monoclonal gammopathy, monoclonal immunoglobulin deposition disease, myelodusplastic syndromes (MDS), smoldering multiple myeloma, and Waldenstrom macroglobulinemia.

[659] In certain embodiments, the Shiga toxin effector polypeptides and cell-targeting molecules of the present invention, or pharmaceutical compositions thereof, are used for both diagnosis and treatment, or for diagnosis alone. In some situations, it would be desirable to determine or verify the HLA variant(s) and/or HLA alleles expressed in the subject and/or diseased tissue from the subject, such as, *e.g.*, a

patient in need of treatment, before selecting a cell-targeting molecule of the invention for use in treatment(s).

[660] Any embodiment of the Shiga toxin effector polypeptide of the present invention and cell-targeting molecule of the present invention (*e.g.* embodiments of embodiment Sets #1–3 in the Summary) may be used with each individual embodiment of the methods of the present invention.

[661] The present invention is further illustrated by the following non-limiting examples of 1) Shiga toxin effector polypeptides of the present invention, 2) cell-targeting molecules of the present invention, and 3) cytotoxic, cell-targeting molecules of the present invention comprising the aforementioned polypeptides and capable of specifically targeting certain cell types.

EXAMPLES

[662] The following examples demonstrate certain embodiments of the present invention. However, it is to be understood that these examples are for illustration purposes only and do not intend, nor should any be construed, to be wholly definitive as to conditions and scope of this invention. The experiments in the following examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described.

[663] The following examples describe several, exemplary, cytotoxic, Shiga toxin A Subunit derived polypeptide scaffolds comprising Shiga toxin effector polypeptides of the present invention. The Shiga toxin effector polypeptides in the Examples are de-immunized while retaining potent cytotoxic activities.

[664] The following examples also describe several, cytotoxic, cell-targeting molecules, each molecule comprising a Shiga toxin effector polypeptide linked, either directly or indirectly, to a cell-targeting binding region capable of specifically binding an extracellular part of a HER2 target biomolecule physically associated with a cellular surface of a cell. Exemplary, cytotoxic, cell-targeting molecules described below bound to cell-surface, target biomolecules expressed by targeted, tumor cell-types and entered those targeted cells. The internalized, cell-targeting molecules effectively routed their Shiga toxin effector polypeptides to the cytosols of target cells where the Shiga toxin effector polypeptides inactivated ribosomes and subsequently caused the apoptotic death of the targeted cells.

[665] Additionally, some of the exemplary cell-targeting molecules comprise protease-cleavage resistant, de-immunized, Shiga toxin effector polypeptides that exhibit improved *in vivo* immunogenicity profiles (reductions in antibody responses) as compared to parental cytotoxic molecules comprising a furin-cleavage resistant, Shiga toxin effector polypeptide that has not been further de-immunized by the disruption of additional, endogenous epitope regions. Furthermore, these exemplary, protease-cleavage resistant, de-immunized cell-targeting molecules exhibit improved *in vivo* tolerability as compared to related cell-targeting molecules comprising more protease-cleavage sensitive Shiga toxin effector polypeptide regions.

[666] The Examples below describe certain, cell-targeting molecules of the present invention and their properties. Certain Examples describe cell-targeting molecules of the present invention wherein a Shiga toxin effector polypeptide component (1) is de-immunized; (2) is on or proximal to an amino-terminus of

a polypeptide component of the cell-targeting molecule; (3) is furin-cleavage resistant; and/or (4) comprises an embedded or inserted T-cell epitope.

Example 1. HER2-Targeting Molecules Comprising Furin-Cleavage Resistant, Shiga Toxin A Subunit Derived Polypeptides

[667] Various HER2-targeting molecules, each comprising (1) at least one immunoglobulin-type binding region targeting HER2 and (2) at least one Shiga toxin A Subunit effector polypeptide were constructed and tested for use in killing HER2-positive cancer cells.

A. Construction and Production of HER2-Targeting Molecules

[668] Cytotoxic, cell-targeting molecules were designed to target HER2 using various Shiga toxin A Subunit effector polypeptides (each capable of providing one or more Shiga toxin A Subunit functions) and various immunoglobulin-type binding regions, each capable of binding an extracellular part of human HER2, as cell-targeting binding regions. The immunoglobulin-type binding region of these HER2-targeting molecules was either a single-chain antibody variable fragment or a camelid V_HH that binds with high-affinity, specificity, and selectivity to a cell-surface HER2 target biomolecule physically coupled to the surface of human cancer cells. Polynucleotides were constructed which encode fusion proteins comprising the aforementioned components: (1) at least one anti-HER2 antibody variable fragment and (2) at least one Shiga toxin A Subunit effector polypeptide. These polynucleotides were used to produce cytotoxic, cell-targeting molecules of the present invention, including 114773 (SEQ ID NO:22), 115172 (SEQ ID NO:23), 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), 114791 (SEQ ID NO:26), 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), 115411 (SEQ ID NO:30), 114898 (SEQ ID NO31), 115195 (SEQ ID NO:32), 115194 (SEQ ID NO:33), 115645 (SEQ ID NO:34), and 115845 (SEQ ID NO:35). All of the cell-targeting molecules tested in the experiments of this Example, including reference cell-targeting molecules, were produced in a bacterial system and purified by column chromatography using techniques well-known to the skilled worker. The purification of certain exemplary HER2-targeting molecules of the present invention was facilitated by the use of a fused affinity tag, such as, *e.g.*, a chitin-binding domain (SEQ ID NO:43) or a 6xHis polyhistidine tag (SEQ ID NO:44).

1. Chitin Affinity Based Purification

[669] For certain exemplary HER2-binding proteins of this Example, cloning and purification were done essentially as described in the manufacturer's manual for the IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system (New England Biolabs, Ipswich, MA, U.S.A.). An affinity tag used to purify some of the HER2-targeting molecules of this Example was the intein chitin binding domain (CBD) sequence (SEQ ID NO:43), which was fused to the carboxy-terminals of some of the fusion proteins of this Example using the *E. coli* expression vector pTxb1 (New England Biolabs, Ipswich, MA, U.S.A.). These CBD fusion proteins were expressed in bacteria, extracted from

the soluble fraction, and then allowed to bind to a chitin column. The intein was then cleaved away from the fusion protein by incubation with dithiolthreitol (DTT), and the HER2-binding proteins of interest were eluted away from the chitin column after removal of the CBD affinity tag (SEQ ID NO:43).

[670] Exemplary HER2-targeting fusion proteins of the present invention 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), and 114791 (SEQ ID NO:26) were expressed and samples were analyzed by SDS-PAGE (Figure 2). All three of these protein samples were predominantly comprised by a protein species of about 55 kDa as measured by SDS-PAGE in reducing conditions (Figure 2).

[671] These exemplary HER2-targeting molecules were then tested for cytotoxic activity using the following cytotoxicity assay. Certain human tumor cell-line cells were plated in 20 μL cell culture medium in 384-well plates (typically at 1–2 x 10^3 cells per well for adherent cells, plated the day prior or day of addition of HER2-targeting molecule). A series of dilutions (typically 10-fold) of the molecules to be tested was prepared in an appropriate buffer, and 5 μL of the dilutions or buffer-only control were added to the plated cells. Control wells containing only cell culture medium were used for baseline correction. The cell samples were incubated with the HER2-targeting molecule or just buffer for 3 or 5 days at 37°C and in an atmosphere of 5% carbon dioxide (CO_2). The total cell survival or percent viability was determined using a luminescent readout using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp., Madison, WI, U.S.A.) according to the manufacturer's instructions. The human cells tested included cells from the HCC1954 and NCI/ADR-RES cell lines, where certain samples of NCI/ADR-RES cells were transfected with a HER2 expression vector to make them express HER2 to a cell-surface in sufficient quantities to make them HER2 positive (referred to herein as "NCI/ADR-RES-HER2+").

[672] The Percent Viability of cells in experimental wells was calculated using the following equation: (Test RLU − Average Media RLU) ÷ (Average Cells RLU − Average Media RLU) × 100. The logarithm of the cell-targeting molecule protein concentration versus Percent Viability was plotted in Prism (GraphPad Prism, San Diego, CA, U.S.A.) and log (inhibitor) versus response (3 parameter) analysis or and log (inhibitor) versus normalized response analysis were used to determine the half-maximal cytotoxic concentration (CD_{50}) value for the tested molecule. The CD_{50} value(s) for each molecule tested were calculated, when possible. When CD_{50} values could not be calculated based on the shape of the curve over the concentrations tested, then a CD_{50} value was noted as being beyond the maximum tested concentration. All graphs and non-linear regressions were done with GraphPad Prism and flow cytometry data was analyzed with FloJo software.

[673] Results of the cytotoxicity assay are reported below (*see* Table 1 and Figure 3). The exemplary HER2-targeting molecules 114778 (SEQ ID NO:24), 114795 (SEQ ID NO:25), and 114791 (SEQ ID NO:26) were cytotoxic to HER2 positive cells (Table 1; Figure 3). In some experiments, HER2 negative cells were also treated with the maximum concentration of the HER2-targeting molecule in the dilution series, and, under these conditions, the HER2 negative cells did not show any change in viability as compared to a buffer only control.

Table 1. Cytotoxicities of Exemplary HER2-Targeting Molecules of the Present Invention Purified Using a Chitin-Binding Affinity Tag and Intein-Mediated Cleavage Away from the Tag

Purification Method: Chitin binding via intein tag and tag cleavage with DTT		
HER2-Targeting Molecule	**CD_{50} (ng/mL)** HER2 positive HCC1954 cells	**CD_{50} (ng/mL)** HER2 positive NCI/ADR-RES cells
114778	36.9	26.4
114791	20.9	17.1
114795	14.9	5.5

5 [674] This data demonstrated similar cytotoxic potencies among 114795 (SEQ ID NO:25), 114778 (SEQ ID NO:24), and 114791 (SEQ ID NO:26), all of which were fusion proteins purified using the IMPACT™ CBD intein affinity tag, chitin-binding purification system.

2. Protein L Affinity Based Purification

10 [675] An alternative method of protein purification based on Protein L binding affinity was used and compared to the intein-CBD affinity tag method used above involving the IMPACT™ system. The binding affinity between bacterial Protein L and certain scFv's was used to purify exemplary HER2-targeting molecules of the present invention: 114773 (SEQ ID NO:22) comprising a carboxy-terminal intein-CBD tag (SEQ ID NO:43), 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), and 115411 (SEQ 15 ID NO:30). Figures 4–5 show SDS-PAGE analyses of samples of 114773 (SEQ ID NO:22), 114791 (SEQ ID NO:26), 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), and 115411 (SEQ ID NO:30) after purification using a Protein L binding affinity method.

[676] Figure 4 shows an SDS-PAGE analysis of 114773 (SEQ ID NO:22) (with a carboxy-terminal intein-CBD tag (SEQ ID NO:43)) and 114791 (SEQ ID NO:26) (with a carboxy-terminal intein-CBD tag 20 (SEQ ID NO:43)) samples after purification using a Protein L binding affinity method. Figure 5 shows SDS-PAGE analysis of 114912 (SEQ ID NO:28) (without any intein-CBD tag), 115111 (SEQ ID NO:29) (without any intein-CBD tag), and 115411 (SEQ ID NO:30) (without any intein-CBD tag).

[677] Exemplary HER2-targeting molecules 114912 (SEQ ID NO:28) and 115111 (SEQ ID NO:29) purified using Protein L binding were tested for cytotoxic activity using the assay as described above for 25 the samples purified using the CBD intein system. The results of the cytotoxicity assay are reported below (*see* Table 2 and Figure 6).

Table 2. Cytotoxicities of Exemplary HER2-Targeting Molecules of the Present Invention Purified Using Protein L Binding Affinity

Purification Method: Protein L binding via scFv						
	CD_{50} (ng/mL)					
HER2-targeting molecule	HER2 positive HCC1954 cells	HER2 positive NCI/ADR-RES cells	HER2 positive JIMT-1 cells	HER2 positive SK-OV-3 cells	HER2 positive HCC1419 cells	HER2 negative JIMT-1 cells
114912	9.0	14.0	78.9	43.2	33.3	>2,000
115111	1.6	2.8	5.9	6.4	11.8	>2,000

[678] The exemplary HER2-targeting fusion proteins 114912 (SEQ ID NO:28) and 115111 (SEQ ID NO:29) were cytotoxic to HER2 positive cells (Table 2; Figure 6). This data demonstrated the greater cytotoxic potency of 115111 (SEQ ID NO:29) as compared to 114912 (SEQ ID NO:28), both of which were purified using Protein L affinity. No cytotoxicity toward MCF-7 cells, which express very low levels of HER2, was observed for most of the HER2-targeting molecule concentrations tested (Figure 6).

[679] Additional exemplary HER2-targeting molecules of the present invention that are related to 115111 (SEQ ID NO:29) were tested for cytotoxic activities toward HER2 positive cell lines using the cytotoxicity assay described above. The proteins 115172 (SEQ ID NO:23), 115195 (SEQ ID NO:32), and 115194 (SEQ ID NO:33) are related to 115111 (SEQ ID NO:29) because they each comprise identical heavy and light variable domains.

[680] The skilled worker will appreciate that the length of the linker between variable domains (or "interdomain linker") in a scFv can affect the spontaneous assaembly of non-covalent, multimeric, multivalent molecules. Generally, linkers that are between three amino acid residues and twelve amino acid residues in length (*e.g.* the pentamer G_4S (SEQ ID NO:94)) promote diabody formation via intermolecular variable domain swapping; whereas longer linkers (*e.g.* $(G_4S)_5$ (SEQ ID NO:92)) allow for intramolecular heavy and light chain pairing, resulting in predominantly monomeric molecules (*see e.g.* WO 2018/140427). 115111 (SEQ ID NO:29) comprises a 25-mer interdomain linker and was verified to predominatly form monovalent monomers. 115195 (SEQ ID NO:32) comprises a pentamer interdomain linker and was verified to predominantly form divalent dimers. 115194 (SEQ ID NO:33) comprises an identical scFv to 115195 (SEQ ID NO:32) having the same pentamer interdomain linker and is predicted to form divalent dimers like 115195 (SEQ ID NO:32). 115172 (SEQ ID NO:23) and 115194 (SEQ ID NO:33) differ from 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) in that its Shiga toxin A Subunit effector polypeptide SLTA-FR (SEQ ID NO:37) comprises mostly wild-type sequences, having mutations only in the minimal furin-cleavage site at the carboxy-terminus of the A1 fragment and disrupting Epitope Region #8 (Table B, *supra*). Cytotoxicity data for these molecules are reported below (*see* Table 3 and Figure 7.

Table 3. Cytotoxicity of 115111 and Related HER2-Targeting Molecules

			CD50 (ng/mL)			
Cell Line	**Cancer Type**	**HER2 Expression**	**115111**	**115195**	**115172**	**115194**
HCC1954	breast	high	4.6	5.6	1.5	3.1
NCI/ADR-RES-HER2+	ovarian, transfected with HER2	high	5.1	3.6	1.8	1.8
HCC1569	breast	high	21.1 (45%)	17.2 (55%)	9.1 (25%)	9.9 (35%)
JIMT-1	breast	medium	5.9 (35%)	6.7 (37%)	3.3 (21%)	6.8 (22%)
ST486	lymphoma	negative	~ 5,000	~ 5,000	~ 5,000	~ 5,000

[681] The data from this cytotoxicity experiment indicated that the monomer 115111 (SEQ ID NO:29), the dimer 115195 (SEQ ID NO:32), the predicted monomer 115172 (SEQ ID NO:23), and the predicted dimer 115194 (SEQ ID NO:33) all exhibited similar cytotoxic activites *in vitro* (*see e.g.* Table 3 and Figure 7). No cytotoxicity toward HER2 negative cells was observed for most of the HER2-targeting molecule concentrations tested (*e.g.* at concentrations below 100 ng/mL).

B. Testing *In Vitro* Activities of Exemplary HER2-Targeting Molecules of the Present Invention

1. Ribosome Inhibition Activities

[682] Exemplary HER2-targeting molecules of the present invention 115111 (SEQ ID NO:29) and 115411 (SEQ ID NO:30) were tested for enzymatic activity after purification using Protein L binding as described above. Their catalytic activities regarding ribosome inactivation were compared with the de-immunized SLT-1A1 fragment alone (DI-2 (SEQ ID NO:20)) and the HER2-targeting molecule 115172 (SEQ ID NO:23) comprising a mostly wild-type SLT-1A sequence having alterations only to mutate the furin cleave motif (SLTA-FR) and disrupt Epitope Region #8 (Table B, *supra*) (SEQ ID NO:37).

[683] The ribosome inhibition assay used a cell-free, *in vitro* protein translation assay using the TNT® Quick Coupled Transcription/Translation kit (L1170 Promega Madison, WI, U.S.A.). The kit includes Luciferase T7 Control DNA (L4821 Promega Madison, WI, U.S.A.) and TNT® Quick Master Mix. The ribosome activity reaction was prepared according to manufacturer instructions. A series (typically 10-fold) of dilutions were prepared in appropriate buffer and a series of identical TNT reaction mixture components were created for each dilution. The protein samples were combined with each of the TNT reaction mixtures along with the Luciferase T7 Control DNA. The test samples were incubated for 1.5 hours at 30°C. After the incubation, Luciferase Assay Reagent (E1483 Promega, Madison, WI, U.S.A.) was added to all test samples and the amount of luciferase protein translation was measured by luminescence according to the manufacturer instructions. The level of translational inhibition was determined by non-linear regression analysis of log-transformed concentrations of total protein versus relative luminescence units. Using statistical software (GraphPad Prism, San Diego, CA, U.S.A.), the

half maximal inhibitory concentration (IC_{50}) value was calculated for each sample using the Prism software function of log(inhibitor) vs. response (three parameters) [Y = Bottom + ((Top − Bottom) / (1+10^(X – Log IC_{50})))] under the heading dose-response-inhibition. The results of the ribosome inhibition assay are reported below (*see* Table 4 and Figure 8).

5 **Table 4. Ribosome Inhibition Activities and Cytotoxicities of Exemplary HER2-Targeting Molecules Purified Using Protein L Binding Affinity**

HER2-Targeting Molecule	**Ribosome Inhibition** **IC_{50}** (pM)	**Cytotoxicity** **CD_{50}** HER2 positive NCI/ADR-RES cells
115172	32.01	0.77 ng/mL
115111	24.20	1.15 ng/mL
115411	6.60	4.59 ng/mL
SLTA-DI-2	20.27	> 2,000 ng/mL

[684] The exemplary HER2-targeting fusion proteins of the present invention 115172 (SEQ ID NO:23), 115111 (SEQ ID NO:29), and 115411 (SEQ ID NO:30) inhibited ribosomes with high potency,
10 *e.g.* in the low picomolar (pM) range (*see* Table 4; Figure 8). The IC_{50} value for ribosome inhibition measured for 115111 (SEQ ID NO:29) was 24.2 pM (Table 4). The similar levels of protein ribosome inhibition exhibited by the Shiga toxin effector polypeptide SLTA-DI-2 (SEQ ID NO:20) and the exemplary HER2-targeting molecules 115172 (SEQ ID NO:23), 115111 (SEQ ID NO:29) and 115411 (SEQ ID NO:30) demonstrated these molecules retained the expected mechanism of action (catalytic
15 inhibition of protein synthesis) as fusion proteins wherein the Shiga toxin A Subunit effector polypeptide component of either 115111 (SEQ ID NO:29) or 115411 (SEQ ID NO:30) or 115172 (SEQ ID NO:23),was fused to an immunoglobulin-type HER2-targeting binding region.

[685] The exemplary HER2-targeting molecules 115172 (SEQ ID NO:23), 115111 (SEQ ID NO:29), and 115411 (SEQ ID NO:30) were also tested for cytotoxic activity toward HER2 positive NCI/ADR-
20 RES cells transfected with HER2 as described above and the results are reported here, in Table 4 above, and in Figure 9. The cytotoxic activities of these exemplary HER2-targeting fusion proteins were compared with the de-immunized SLT-1A1 fragment alone (SLTA-DI-2 (SEQ ID NO:20)) and the HER2-targeting molecule 115172 (SEQ ID NO:23) comprising a Shiga-like toxin A1 fragment SLTA-FR (SEQ ID NO:37) having alterations to mutate the furin cleavage motif and disrupt Epitope Region #8
25 (Table B, *supra*). The cytotoxicity data from this experiment demonstrated that the HER2-targeting moiety was necessary to kill target cells, as there was no cytotoxic effect at concentrations up to 2,000 μg/mL with the untargeted SLTA-DI-2 construct (SEQ ID NO:20) (*see* Figure 9). The cytotoxicity CD_{50} value measured for 115111 (SEQ ID NO:29) was 1.2 ng/mL to NCI/ADR-RES-HER2+ cells.

30 **2. HER2 Binding Activities**

[686] To investigate the cell-binding properties of exemplary HER2-targeting molecules of the present invention, the binding activities of HER2-targeting molecules to human HER2 positive HCC1954 cells

were measured using a flow cytometry-based method. A dilution series of each HER2-targeting molecule being tested was added to cells, the cells were washed, and cell-bound HER2-targeting protein was detected using an anti-SLTA-DI-2 monoclonal antibody (mAb) conjugated to FITC, which recognizes de-immunized Shiga toxin effector polypeptides (*e.g.* SEQ ID NO:20). The FITC signal was
5 measured using flow cytometry to generate mean fluorescent intensity (MFI) values for each sample. The MFI values were plotted as a function of log transformed HER2-targeting molecule concentration to determine the Bmax and K_D using a non-linear curve regression analysis (Prism (GraphPad Prism, San Diego, CA, U.S.A., Prism software function sigmoidal 4PL). Results of this study are reported below (*see* Table 5 and Figure 10).

10 **Table 5. Binding of HER2-Targeting Molecules to HER2 Positive HCC1954 Cells**

HER2-Targeting Molecule	K_D (µg/mL)	Bmax (MFI)
114912	1.28	2119
115111	0.42	2747
115845	0.32	2707
115195	0.30	2447
115645	0.25	1327

[687] The exemplary HER2-targeting fusion proteins of the present invention 115111 (SEQ ID NO:29), 115845 (SEQ ID NO:35), and 115195 (SEQ ID NO:32) exhibited similar binding *in vitro* to HCC1954 cells. 115111 (SEQ ID NO:29) bound HER2 positive HCC1954 cells with a K_D of 0.42
15 µg/mL, and 115845 bound HER2 positive HCC1954 cells with a K_D of 0.32 µg/mL. In this binding assay, 114912 (SEQ ID NO:28) exhibited slightly less binding affinity to HCC1954 cells compared to the other HER2-targeting molecules tested. In this assay, 115645 (SEQ ID NO:34) exhibited a similar K_D but had a reduced Bmax compared to the other HER2-targeting molecules tested.

20 a. HER2 Epitope Mapping of Binding by Exemplary HER2-Targeting Molecule 115111

[688] The extracellular epitope of human HER2 bound by 115111 (SEQ ID NO:29) was mapped to identify contact residues using a shotgun mutagenesis method by Integral Molecular (Philadelphia, PA, U.S.A.). To determine the optimal concentration of 115111 (SEQ ID NO:29) for screening HER2 epitope binding, HEK-293T cells were transfected with a vector designed to express a wild-type (WT)
25 construct of a target human HER2 protein (UniProt ID: P04626 (SEQ ID NO:38)) or with vector alone in a 384-well format. Serial dilutions of 115111 (SEQ ID NO:29) were tested via high-throughput flow cytometry for immunoreactivity against cells expressing the WT HER2 target protein (SEQ ID NO:38) or vector alone. The optimal screening concentration for 115111 (SEQ ID NO:29) for this binding assay was determined based on the raw signal values and signal-to-background calculations. The same process
30 was conducted to determine the optimal screening concentration for trastuzumab, a control HER2-binding antibody.

[689] An alanine scanning mutagenesis library based on the human HER2 protein (SEQ ID NO:38) mentioned above was created and screened for 115111 (SEQ ID NO:29) binding in duplicate by high-throughput flow cytometry. For each test point, background fluorescence was subtracted from the raw data, which were then normalized to 115111 (SEQ ID NO:29) binding with WT HER2 protein (SEQ ID NO:38). Critical mutageneis clones were identified related to the binding of 115111 (SEQ ID NO:29) to WT HER2 (SEQ ID NO:38). The primary critical residues for 115111 (SEQ ID NO:29) binding to HER2 (SEQ ID NO:38) were residues whose mutations were reduced for 115111 (SEQ ID NO:29) binding when tested with 115111 (SEQ ID NO:29) but were similar to WT HER2 target protein control when tested for trastuzumab binding (a HER2-binding, positive control mAb). The resulting mean binding reactivity values for selected critical residue positions in WT human HER2 (SEQ ID NO:38) expressed as a percentage of WT HER2 binding with 115111 (SEQ ID NO:29) measured using this epitope mapping assay are listed in Table 6.

Table 6. Certain Crititcal and Secondary Residues in HER2 Involved in Binding 115111

Binding Reactivity (% WT)		
HER2 mutation	**115111**	**trastuzumab**
Y112A	9.2	87.7
Q178A	34.9	119.5
L181A	20.1	95.8
G152A	39.2	89.7

[690] Using the approach above, the HER2 epitope bound by 115111 (SEQ ID NO:29) appeared to comprise Y112, Q178, and L181, which all map to domain I of the HER2 extracellular domain (ECD) (NCBI accessions NP_004439.2, amino acid residues 23 to 653) (SEQ ID NO:39). One residue, G152, was identified in the epitope mapping assay as a secondary residue in that it did not meet the threshold guidelines for being 'critical', but the G152A mutation did result in a large decrease of 115111 (SEQ ID NO:29) binding compared to the WT HER2 (SEQ ID NO:38). Binding to the G152A expressing clone showed a level of binding that was 39% of WT binding as opposed to the 35% threshold level required to categorize G152 a primary critical residue. The G152 position in HER2 is in close proximity to the primary critical residues mapped using the assay, which suggested that it may also be part of the epitope in human HER2 (SEQ ID NO:38) bound by 115111 (SEQ ID NO:29).

[691] Figure 11 shows a diagram of human HER2 with markings to highlight the critical and secondary residues for binding by 115111 (SEQ ID NO:29) as measured by the approach described above. In Figure 11 on the left, primary residues critical for 115111 (SEQ ID NO:29) binding to HER2 (SEQ ID NO:38) are visualized as red spheres based on a crystal structure of the HER2 (PDB 1S78, Franklin M et al., *Cancer Cell* 5: 317-28 (2004)) along with a secondary residue visualized as a blue sphere. In Figure 11 on the right, the critical HER2 binding residues for 115111 (SEQ ID NO:29) binding are shown as blue spheres, for pertuzumab binding are shown as magenta spheres, and for trastuzumab binding are shown as purple spheres based on the data above and information in Cho H et al., *Nature* 421: 756-60

(2003); Franklin M et al., *Cancer Cell* 5: 317-28 (2004). The HER2 epitope bound by 115111 (SEQ ID NO:29) was mapped within the HER2 extracellular domain (ECD) (SEQ ID NO:39) to domain I; in contrast, pertzumab binds to an epitope mapped to domain II of the ECD of HER2 and trastuzumab binds to an epitope mapped to domain IV of the ECD of HER2 (SEQ ID NO:39) (*see* Figure 11). Thus, 115111 (SEQ ID NO:29) binds an epitope in HER2 that is different from the epitopes bound by trastuzumab and pertuzumab.

b. HER2 Binding Specificity of Exemplary HER2-Targeting Molecule 115111

[692] The binding specificity, affinity, and selectivity of the exemplary HER2-targeting fusion protein of the present invention 115111 (SEQ ID NO:29) were tested by analyzing binding of this purified fusion protein to a membrane proteome array, which comprised 5,300 different proteins transfected to be expressed on the cell surface of HEK-293T cells (Integral Molecular, Inc., Philadelphia, PA, U.S.A.). The results shown in Figure 12 show that only HER2 was identified and validated among the 5,300 proteins as a selective binding target of 115111 (SEQ ID NO:29).

3. Cytotoxic Activities

1. Cytotoxic Activities *In Vitro* Compared to Other Drugs

[693] The cytotoxicity of 115111 (SEQ ID NO:29) to HER2 positive cells was evaluated in comparison to and in combination with reference HER2-targeted therapeutics, including T-DM1 (trastuzumab emtansine), trastuzumab, and pertuzumab. As all of these therapeutic molecules specifically bind to an extracellular part of human HER2, these molecules might compete for HER2 binding with each other or otherwise interact so as to alter their functional activities when combined. For example, when different, cytotoxic, HER2-targeted molecules are administered in combination, either a reduction or increase in cytotoxic activities toward HER2 positive cells might be observed. In a further example, when different, cytotoxic, HER2-targeted molecules are administered in combination, an increase in the death of HER2-expressing cells might be observed as result of additive or synergistic effects on cytotoxicity.

[694] To investigate the number of possible HER2 receptors on the cell surfaces of cells of different cell types, an experiment was performed to quantify the number of HER2-targeted antibody molecules bound per cell. This experiment involved the incubation of cells with an anti-HER2 antibody conjugated to phycoerythrin (PE) (anti-HER2-PE, clone 24D2, Biolegend, San Diego, CA, U.S.A.). Then the samples were analyzed using flow cytometry to quantify binding. Standard curves generated with BD Quantibrite™ PE beads having known PE loads were used to convert MFI signal values to the number of antibodies bound per cell for each sample. These HER2 positive cells were then used in cytotoxicity assays performed essentially as described above to compare the cytotoxic activities of 115111 (SEQ ID NO:29) to T-DM1 and lapatinib. Results from these cytotoxicity assays are reported below and in Table 7, and a representative data set for T-DM1 is shown in Figure 13.

Table 7. HER2 Positive Cell Binding Sites per Cell and *In Vitro* Cytotoxic Activities of 115111 as Compared to T-DM1 and Lapatinib

HER2 Positive Cell Binding			***In Vitro* Cytotoxicity CD_{50}**		
Cell Line	cancer type	antibodies bound per cell	115111	T-DM1	lapatinib
HCC1954	breast	2,250,000	1.39 ng/mL	8.98 ng/mL	786 nM
NCI-N87	gastric	2,270,000	3.76 ng/mL	18.48 ng/mL	112 nM
NCI/ADR-RES-HER2+	ovarian, transfected with HER2	1,340, 000	1.17 ng/mL	no viability change	2,704 nM
HCC1569	breast	DNT	2.17 ng/mL	13.66 ng/mL	885 nM
HCC1419	breast	3,070,000	6.58* ng/mL	74.56* ng/mL	DNT
AU565	breast	DNT	7.04 ng/mL	0.63 ng/mL	DNT
JIMT-1	breast	307,000	6.55* ng/mL	248.7* ng/mL	5,650 nM

'*' Indicates that cell viability plateaus above 20% in this assay; DNT = did not test

[695] 115111 (SEQ ID NO:29) exhibited potent cytotoxicity to all HER2+ cells tested in this experiment: HCC1954, NCI-N87, HCC1569, HCC1419, AU565, and JIMT-1 cells (Table 7; *see also* Tables 2–3, 8–10; Figures 6–7, and 13–19).

[696] Both 115111 (SEQ ID NO:29) and T-DM1 were potently cytotoxic to HCCC1954, NCI-N87, HCC1569, HCC1419, and AU565 cells (Table 7), with 115111 exhibiting higher cytotoxic potency than T-DM1 to HCCC1954, NCI-N87, HCC1569, and HCC1419 cells (Table 7; *see e.g.* Figure 13). 115111 (SEQ ID NO:29) exhibited more potent cytotoxicity than T-DM1 toward NCI/ADR-RES-HER2+ and JIMT-1 cells (Table 7; *see* Figure 13).

[697] NCI/ADR-RES cells are considered resistant to T-DM1 due to MDR1 expression. Because 115111 (SEQ ID NO:29) killed HER2 positive NCI/ADR-RES cells (Tables 2–3 and 7; Figures 6–7, 9, and 13), 115111 (SEQ ID NO:29) is likely to be cytotoxic to other HER2 positive cells expressing p-glycoprotein 1 (Pgp) type multidrug resistance efflux pumps.

[698] JIMT-1 cells are considered resistant to trastuzumab due to epitope masking via MUC-4 and/or CD44 expression (*see e.g.* Wilken J, Maihle N, *Ann N Y Acad Sci* 1210: 53-65 (2010)). Because 115111 (SEQ ID NO:29) killed JIMT-1 cells (Tables 1–2 and 7; Figures 6–7), the presence of MUC-4 and/or CD44 does not prevent 115111 (SEQ ID NO:29) cytotoxicity, such as, *e.g.*, via epitope masking (*see e.g.* Figures 6–7 and Table 7).

[699] As 115111 (SEQ ID NO:29) binds to an extracellular part of HER2 and internalizes into target cells to seeking out their ribosomes for inactivation, the cytotoxicity activity of 115111 (SEQ ID NO:29) is not dependent on the binding to or inactivation of a kinase domain of HER2. In contrast, lapatinib is a

dual tyrosine kinase inhibitor which targets HER2 and EGFR by binding to their kinase domains and inhibiting kinase activity. Cancer therapies relying on drugs like lapatinib may be rendered less effective due to the development of resistance mechanisms, such as protective mutations in the kinase catalytic domain of HER2 or *de novo* aberrations resulting in constitutive/unregulated HER2-signaling-pathway activation or overactivation of the HER2 signaling pathway at a point downstream of HER2 thereby bypassing the receptor, such as in the absence of HER2 kinase activity or even HER2 expression. Thus, certain HER2-targeting molecules of the present invention (*e.g.* 115111 (SEQ ID NO:29)) may be effective in refractory or non-responding treatment resistance settings, *e.g.*, tumors resistant to therapies involving tyrosine kinase inhibitors, such as, *e.g.*, lapatinib, and/or neratinib.

2. Cytotoxic Activities in the Presence of Other Drugs

a. Lapatinib and T-DM1

[700] The cytotoxicity of 115111 (SEQ ID NO:29) to HER2 positive cells was evaluated in combination with lapatinib or T-DM1 using a cytoxicity assay performed essential as described above. The cytotoxicities of 115111 (SEQ ID NO:29) over range of concentrations combined with either 1 μM lapatinib or a range of T-DM1 concentrations are shown in Figures 14 and 15. Neither combination appeared deleterious to the cytotoxicity caused by 115111 (SEQ ID NO:29) to HER2 positive cells observed in this *in vitro* cell-kill assay. Furthermore, the results of these expreiments show the potential of combinations of 115111 (SEQ ID NO:29) with other agents to achieve even more potent cytotoxicity to HER2-expressing cells.

[701] Figure 14 shows the results of cytotoxicity assays using HER2 positive HCC1419 or HCC1954 cells where 115111 (SEQ ID NO:29), lapatinib, or a combination of both were administered to the cells. Concentrations for 115111 (SEQ ID NO:29) and lapatinib were selected such that the single agent resulted in about 50% viability for each cell line. The results of this experiment showed that 115111 (SEQ ID NO:29) may be administered with lapatinib to achieve more cytotoxicity than either administrated individually. The data in Figure 14 showed that a higher percentage of cells were killed by the combination 115111 (SEQ ID NO:29) and lapatinib than by treatment with either lapatinib or 115111 (SEQ ID NO:29) alone. These results showed that 115111 (SEQ ID NO:29) may be combined with laptinib and/or may be administered in the presence of laptinib without significant loss in 115111 (SEQ ID NO:29) cell-kill activity to HER2 positive cells. Furthermore, these results suggested that the administration of the combination of 115111 (SEQ ID NO:29) (or a similar HER2-targeting molecule of the present invention) and lapatinib may kill more HER2 positive cells than using either one alone at the same respective dose.

[702] Figure 15 shows the results of a cytotoxicity assay using HER2 positive HCC1954 cells where 115111 (SEQ ID NO:29), T-DM1, or a combination of both were administered to the cells. The data in Figure 15 showed that a higher percentage of cells were killed by the combination 115111 (SEQ ID NO:29) and T-DM1 than by treatment with either T-DM1 or 115111 (SEQ ID NO:29) alone. These

results showed that 115111 (SEQ ID NO:29) may be combined with T-DM1 and/or may be administered in the presence of T-DM1 without significant loss in 115111 (SEQ ID NO:29) cell-kill activity to HER2 positive cells. Furthermore, these results suggested that the administration of the combination of 115111 (SEQ ID NO:29) (or a similar HER2-targeting molecule of the present invention) and T-DM1 may kill more HER2 positive cells than using either one alone at the same respective dose.

b. T-DM1 and Trastuzumab

[703] The cytotoxic activities of 114912 (SEQ ID NO:28) and 115111 (SEQ ID NO:29) to HER2 positive HCC1954 cells were evaluated in the presence of excess trastuzumab using a cytotoxicity assay performed essentially as described above, except that the cells were pre-treated with trastuzumab (20 μg/mL) for one hour prior to addition of the other HER2-targeting molecules. Figure 16 shows the results of cytotoxicity assays using HER2 positive HCC1954 cells where 114912 (SEQ ID NO:28) and 115111 (SEQ ID NO:29) cytotoxic activities were evaluated in comparison to T-DM1, both in the absence of trastuzumab and in the presence of excess trastuzumab. The pretreatment of HER2 positive cancer cells with excess trastuzumab did not alter the cytotoxic activity of 115111 (SEQ ID NO:29) (*see* Figure 16, middle). This was in contrast to the combination of excess trastuzumab with T-DM1 or the exemplary HER2-targeting molecule 114912 (SEQ ID NO:28), both of which comprise the heavy and light variable domains of the trastuzumab antigen binding regions. These data suggest that HER2-targeting molecules of the present invention may kill cells in the presence of other HER2-binding therapeutics which bind non-overlapping epitopes and do not compete or interfere with the HER2-targeting molecule's mechanism of action (*see* Figure 11, trastuzumab binds to an epitope mapped to domain IV of the ECD whereas 115111 (SEQ ID NO:29) interacts with domain I of the ECD). The monoclonal antibody trastuzumab exhibited no cytotoxicity in this *in vitro* cytotoxic assay (involving just HER2 positive cancer cells and culture medium).

c. Trastuzumab and Pertuzumab

[704] The cytotoxic activities of 115111 (SEQ ID NO:29) to HER2 positive cells over a range of concentrations were evaluated in the presence of excess trastuzumab, excess pertuzumab, or an excess of both trastuzumab and pertuzumab using a cytotoxicity assay performed essentially as described above, except that the cells were pre-treated with trastuzumab (100 μg/mL), pertuzumab (100 μg/mL) or both trastuzumab (100 μg/ml) and pertuzumab (100 μg/mL) (for a total of 200 μg/mL antibody) for one hour prior to addition of the other HER2-targeting molecules. Figure 17 and Table 8 show the results of the cytotoxicity assay using HER2 positive HCC1954 or NCI-N87 cells where 115111 (SEQ ID NO:29) activity was evaluated in the presence of excess trastuzumab, pertuzumab, or both. The HER2-targeting fusion protein 115111 (SEQ ID NO:29) killed HCC1954 cells in the presence of excess trastuzumab (Figures 16–17). 115111 (SEQ ID NO:29) killed HCC1954 cells and NCI-N87 cells in the presence of both excess trastuzumab and excess pertuzumab or in the excess of both (Figure 17). The monoclonal

antibodies trastuzumab or pertuzumab exhibited no cell-killing activity in this *in vitro* cytotoxic assay (involving just the cancer cells and culture medium).

Table 8. Cytotoxic Activities of 115111 in the Presence of Excess Trastuzumab, Pertuzumab, or both Trastuzumab and Pertuzumab

Test Condition	CD_{50} (ng/mL)	
	HCC-1954	NCI-N87
115111 alone	3.9	19.2
115111 + trastuzumab	5.4	44.0
115111 + pertuzumab	18.0	140.0
115111 + trastuzumab and pertuzumab	16.4	89.7

5

[705] The data in Figure 17 and Table 8 showed that the HER2-targeting molecule 115111 (SEQ ID NO:29), tested at concentrations up to 20 µg/mL, was cytotoxic to HER2-expressing cells in the presence of excess trastuzumab or pertuzumab (either 100 µg/mL individually or 100 µg/mL of each, for a total of 200 µg/mL concurrent exposure) thereby suggesting a potential combination therapy involving
10 administering 115111 (SEQ ID NO:29) and another HER-targeting therapeutic, such as a monoclonal antibody like trastuzumab or pertuzumab having a non-overlapping HER2 binding epitope (*see e.g.* Figure 11). Thus, the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) has the potential to be effective in killing HER2 expressing cancer cells in combination with other HER2-targeted therapies as long as their HER2 binding epitopes are different.
15 [706] The mechanism of action of the HER2-targeting molecules of the present invention — internalization and targeted ribosome inactivation— is different than that of monoclonal antibodies targeting HER2 (trastuzumab binding HER2 induces ADCC and inhibits HER2 signaling) (pertuzumab binding HER2 inhibits dimerization) and is different than antibody drug conjugates like T-DM1 (targeted mictrotubule inhibition). Cancer therapies relying on drugs using mechanisms of action other than the
20 one(s) used by HER2-targeting molecules may be rendered less effective due to the development of resistance mechanisms specific to one or more of these other mechanisms of action. For example, tumors may become resistant to therapies involving monoclonal antibodies which bind specific HER2 epitopes, such as, *e.g.*, T-DM1, trastuzumab, and/or pertuzumab, and/or involving tyrosine kinase inhibitors, such as, *e.g.*, lapatinib and/or neratinib. Certain HER2-targeting molecules of the present invention (*e.g.*
25 115111 (SEQ ID NO:29)) may be effective in refractory or non-responding treatment resistance settings, *e.g.*, tumors resistant to therapies involving T-DM1, trastuzumab, pertuzumab, lapatinib, and/or neratinib. Furthermore, the unique mechanism of action of HER2-targeting molecules of the present invention may offer new oppurtunites for monotherapies for benefit-treatment resistant/non-responder patients as well as for combination therapies combining different and/or complementary mechanisms of action, such as
30 combinations with T-DM1, trastuzumab, pertuzumab, lapatinib, and/or neratinib.

3. Cytotoxicies of Exemplary HER2-Targeting Molecules Based on Exposure Duration

[707] To study the cytotoxic effects of exemplary HER2-targeting molecules of the present invention, kinetic cell-kill experiments were performed. The cell-kill assay described above was used here but with

different durations of exposure of the HER2-expressing cells to HER2-targeting molecules. In this study, HER2 positive SKBR3 or HCC1954 were exposed to a HER2-targeting molecule for a specific and short duration of time (*e.g.* 1 or 4 hours), then the cells were washed and the media replaced ("washout"). Control samples were incubated with the HER2-targeting molecule continuously throughout the
5 experiment (with "no washing"). All data was nomalized by comparing results to samples treated with vehicle alone under the same conditions. Results from this study are shown in Tables 9–10 and Figures 18–19.

Table 9. Cytotoxicity of Exemplary HER2-Targeting Molecules to HER2 Positive SKBR3 Cells after Different Durations of Exposure

	SKBR3	
Test Condition	**CD_{50} (ng/mL)**	**Fold change from continuous**
114912 continuous	44.6	N/A
114912 4-hour exposure	430.1	9.6
114912 1-hour exposure	3736.0	83.7
115111 continuous	11.1	N/A
115111 4-hour exposure	25.0	2.2
115111 1-hour exposure	118.3	10.6

10 * 'N/A' denotes not applicable

Table 10. Cytotoxicity of HER2 Positive HCC1954 Cells Under Different Duration of Exposure

	HCC-1954		
Test Condition	**CD_{50} (ng/mL)**	**Fold change from continuous**	**Cell viability at 2 µg/mL (%)**
Experiment 1			
115111 continuous	37.3		4.5%
115111 4-hour exposure	137.3	3.7	10.0%
114898 continuous	110.1		9.6%
114898 4-hour exposure	487.4	4.4	44.9%
Experiment 2			
115111 continuous	5.6		2.6%
115111 4-hour exposure	14.9	2.7	3.1%
115195 continuous	3.9		3.5%
115195 4-hour exposure	14.3	3.7	3.5%
115645 continuous	41.5		16.4%
115645 4-hour exposure	422.3	10.2	55%
115845 continuous	25.3		16.2%
115845 4-hour exposure	179.4	7.1	45.2%

15 [708] Under continuous exposure, 115111 (SEQ ID NO:29) exhibited a CD_{50} value of 5.6 ng/mL toward HER2 positive HCC1954 cells, which is similar to the CD_{50} value of 3.9 ng/mL measured for 115195 (SEQ ID NO:32). Under continuous exposure, 115111 (SEQ ID NO:29) exhibited CD_{50} values of 5.6 and 37.3 ng/mL toward HCC1954 cells, which is similar to the CD_{50} value 25.3 ng/mL measured

for 115845 (SEQ ID NO:35) and 41.5 ng/mL measured for 115645 (SEQ ID NO:34). However, the shorter exposure condition revealed some surprising differences between 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) and the other HER2-targeting molecules tested.

[709] The data from these experiments demonstrated that 115111 (SEQ ID NO:29) has potent cytotoxic activity when exposed to HER2-expressing cells for just a short duration (1 to 4 hours) (*see* Tables 9–10 and Figures 18–19). The dimeric/divalent 115195 (SEQ ID NO:32) is related to monomeric/monovalent 115111 (SEQ ID NO:29), differing only in a linker (which affects multimerization), exhibited similar activity to 115111 (SEQ ID NO:29) under both continuous and washout conditions (Table 10, Figure 19). In contrast, other HER2-targeting molecules like 114912 (SEQ ID NO:28), 114898 (SEQ ID NO:31), 115645 (SEQ ID NO:34), and 115845 (SEQ ID NO:35) exhibited greater reductions in cytotoxic activity at shorter exposure durations as compared to continuous exposure (*see e.g.* Tables 9–10 and Figures 18–19).

[710] The HER2 binding and catalytic activity data shown above did not provide a clear indication that the cytotoxic potency under relatively short durations of exposure would be highest for 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) as opposed to other HER2-targeting molecules tested, such as the trastuzumab based 114912 (SEQ ID NO:28). Furthermore, the cytotoxicity assay data above gathered under conditions of continuous exposure (*e.g.* 3 to 5 days) did not provide any indication as to which HER2-targeting molecules would be more potent under conditions of shorter exposure durations (*e.g.* 4 hours or less).

[711] Comparing different HER2-targeting molecules, it is apparent that the binding affinity does not always correlate with the cytotoxic activity. For example, 115645 (SEQ ID NO:34) and 115845 (SEQ ID NO:35) have similar cytotoxic activities toward HER2 positive HCC 1954 (Table 10; Figure 19) but differ in HER2 binding as measured by Bmax (Table 5 and Figure 10). Conversely, 115111 (SEQ ID NO:29) is more potently cytotoxic to HCC1954 cells than 115845 (SEQ ID NO:35) (*see* Tables 7–9; Figures 6–7 and 18–19), but these two HER2-targeting molecules demonstrated similar Bmax and K_D binding characteristics to those cells (*see* Tables 5 and 10; Figure 10). The shorter duration exposures reveal quite large differences between molecules, and this finding could not be predicted by the HER2 binding data, catalytic data, and the continuous exposure data.

4. Species Cross-Reactivity of HER2 Binding

[712] In preparation for animal studies, *in vitro* binding assays were used to investigate the binding of 115111 (SEQ ID NO:29) to recombinant HER2 proteins from different species. The wells of enzyme-linked immunosorbent assay (ELISA) plates were coated with recombinant HER2 extracellular domain (ECD) proteins derived from human (SEQ ID NO:38), cynomolgus monkey species (GenBank EHH58073.1 and NCBI reference XP_001090430.1 (SEQ ID NOs: 40–41)), and mouse (UnitProt P70424 (SEQ ID NO:42)) sources. The wells were blocked, washed, and then incubated with one of the exemplary HER2-targeting molecules: 115111 (SEQ ID NO:29), 115195 (SEQ ID NO:32), or 114912 (SEQ ID NO:28). Unbound protein was removed by washing, and HER2 bound HER2-targeting

molecules were detected using a horse radish peroxidase (HRP)-conjugated mAb anti-SLTA-DI-2 that recognizes the Shiga toxin effector polypeptide component in these molecules. The results of these binding assays are reported below (*see* Table 11 and Figure 20).

Table 11. Binding to Recombinant HER2 Proteins from Different Species

HER2 ECD recombinant protein	115111 binding		115195 binding		114912 binding	
	K_D (ng/mL)	Bmax (Abs 450 nM)	K_D (ng/mL)	Bmax (Abs 450 nM)	K_D (ng/mL)	Bmax (Abs 450 nM)
human	19.11	4.00	12.30	3.96	44.80	3.57
cynomolgus monkey	19.34	3.99	12.70	3.99	61.86	3.92

[713] The data in Table 11 and Figure 20 show that 115111 (SEQ ID NO:29), the related molecule 115195 (SEQ ID NO:32) (which is a divalent dimer variant of the monovalent 115111 (SEQ ID NO:29) differing only in a linker), and the trastuzumab binding domain-derived molecule 114912 (SEQ ID NO:28) all bound to both human and cynomolgus monkey recombinant HER2 ECD proteins (Sino Biological Inc., Beijing, CN, catalog nos. 10004-H02H and 90295-C02H, respectively). In this assay, 114912 (SEQ ID NO:28) did not bind the mouse HER2 ECD protein (Sino Biological Inc., Beijing, CN, catalog no. 50714-M02H). 115111 (SEQ ID NO:29) did not bind to the mouse HER2 ECD protein in this assay (Figure 20). As shown in Table 11 and Figure 20, the monomeric/monovalent 115111 (SEQ ID NO:29) and the related dimeric/divalent 115195 (SEQ ID NO:32) exhibited similar binding to human and cynomolgus monkey recombinant HER2 ECD proteins. Although 114912 (SEQ ID NO:28) bound with a similar affinity to human and cynomolgus monkey HER2 ECD proteins as 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) regarding Bmax (*see* Table 11; Figure 20), the trastuzumab based 114912 (SEQ ID NO:28) exhibited a slightly higher K_D (2 to 5 -fold) as compared to both 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) (*see* Table 11).

Summary of *In Vitro* Data:

[714] Based on the *in vitro* data above, the most promising HER2-targeting molecules tested were 114912 (SEQ ID NO:28), 115111 (SEQ ID NO:29), 115195 (SEQ ID NO:32), 115172 (SEQ ID NO:23), 115194 (SEQ ID NO:33), 115411 (SEQ ID NO:30), 115645 (SEQ ID NO:34), and 115845 (SEQ ID NO:35) (*see* Tables 1–3 and 9–10; Figures 3, 6–7, and 18–19). These HER2-targeting molecules exhibited potent cytotoxic activities *in vitro* under conditions of continuous exposure. In the *in vitro* ribosome inhibition assay, no significant differences were observed between the HER2-targeting molecules tested. In the *in vitro* cell-binding assay, some differences were observed between the HER2-targeting molecules tested; however, binding characterstics do not necessarily coorelate with cytotoxic potency (see above discussion regarding 115645 (SEQ ID NO:34) and 115845 (SEQ ID NO:35)). The *in vitro* cytotoxicity data above gave no indication of which molecule(s) from among 115111 (SEQ ID NO:29), 115195 (SEQ ID NO:32), 115172 (SEQ ID NO:23), 115194 (SEQ ID NO:33), and 115411 (SEQ ID NO:30) would be the most effective and safe *in vivo*. However, the Shiga toxin A Subunit

effector polypeptide components in 115172 (SEQ ID NO:23) and 115194 (SEQ ID NO:33) were less de-immunized than the Shiga toxin A Subunit effector polypetide components of the other promising HER2-targeting molecules 115111 (SEQ ID NO:29), 115411 (SEQ ID NO:30), and 115195 (SEQ ID NO:32). Thus, administration of 115111 (SEQ ID NO:29), 115411 (SEQ ID NO:30), or 115195 (SEQ ID NO:32) to animals was expected to be better tolerated than administration of either 115172 (SEQ ID NO:23) or 115194 (SEQ ID NO:33). Animal studies involving the administration of different exemplary HER2-targeting molecules at different dosages were undertaken, particularly to discern any differences in their safety profiles and/or efficacy for treating cancer and to pick the most promising candidate to take into human clinical trials. Neither the *in vitro* data above nor the fact that 115111 (SEQ ID NO:29) was monovalent where as 115195 (SEQ ID NO:32) forms a dimeric divalent molecule suggested which one of these two exemplary HER2-targeting molecules might perform better *in vivo*.

C. *In Vivo* Studies of Exemplary HER-Targeting Molecules of the Present Invention

[715] Animal models were used to investigate the *in vivo* effects of exemplary HER-targeting molecules of the present invention. Different mouse strains were used to test the effect(s) of 115111 (SEQ ID NO:29) after administration on both healthy, immunocompetent mice and on xenograft tumors in immunocompromised mice resulting from the injection into those mice of human neoplastic cells which express HER2 on their cell surfaces. Non-human primates were used to investigate the effect(s) of 115111 (SEQ ID NO:29) after intravenous administration to healthy non-human primates. The exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was observed to be better tolerated than other HER2-targeting molecules tested in animals. Suprisingly, 115111 (SEQ ID NO:29) administration was observed to be better tolerated than administration of the closely related candidate HER2-targeting molecule 115195 (SEQ ID NO:32).

1. Serum Exposure

[716] Immunocompetent C57BL/6 mice were used to investigate the serum exposure (pharmacokinetics) of 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) after intravenous (IV) administration of a single dose at 1 milligram per kilogram (mg/kg) of body weight. The HER2-targeting molecules 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) were each diluted in phosphate buffered saline (PBS) and administered by IV to mice, with three mice were per treatment group. At each timepoint, blood sera were collected from each mouse, and the concentration of HER2-targeting molecule was measured by means of a mesoscale discovery assay (Cambridge Biomedical Inc., Boston, MA, U.S.A.) using recombinant, human HER2 protein to capture any HER2-binding molecules and using the anti-SLTA-DI-2 mAb for quantitation of HER2-targeting molecule present, both quantified using standard curves. Results from this study are shown in Table 12.

Table 12. Serum Exposure to Exemplary HER2-Targeting Molecules in C57BL/6 Mice after Repeat Dosing

	Serum HER2-Targeting Molecule (ng/mL)	
Time (hr)	115111	115195
0.083	22,344	30,280
0.5	3,603	29,899
1	1,354	23,334
2	355	13,518
4	182	6,742
8	84	3,428
12	21	602
24	-	111

[717] Data in Table 12 indicate that the serum exposure of 115195 (SEQ ID NO:32) was significantly
5 longer than that of 115111 (SEQ ID NO:29) in mice.

2. Tolerability

a. Tolerability in Immunocompetent Mice

10 [718] Immunocompetent BALB/c mice were used to investigate the toxicity and immunogenicity of exemplary HER2-targeting molecules 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115195 (SEQ ID NO:32), and 115194 (SEQ ID NO:33) after repeated administrations over time to the same mice (6 doses total per mouse). 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115195(SEQ ID NO:32), and 115194 (SEQ ID NO:33) were diluted in PBS and administered to mice (n = 6 per group at
15 intravenous (IV) at doses 1 mg/kg of body weight with dosing on days 1, 3, 5, 8, 10, and 12). As a control, vehicle-only samples were administered to a negative control treatment group. The body weights and health of the mice were monitored during the study. Treatment tolerability results from this study are shown in Table 13 and Figure 21, with changes in body weight and deaths as indicators of tolerability. The percentage change in the mean body weight of the treatment group compared to Day 0
20 is shown along with the number of animal deaths per total animals in the treatment group.

Table 13. Effects of Exemplary HER2-Targeting Molecules in BALB/c Mice after Repeat Dosing

Sample	Days dosed	Amount per Dose	Percentage Body Weight Nadir (Day)	Deaths / Number of Mice in Group
Vehicle Control	1, 3, 5, 8, 10, 12	N/A	−2% (14)	0/6
115111	1, 3, 5, 8, 10, 12	1	−1% (12)	0/6
115195	1, 3, 5, 8, 10, 12	1	−24% (14)	5/6
115172	1, 3, 5, 8, 10, 12	1	−9.5% (12)	0/6
115194	1, 3, 5, 8 (dosing halted)	1	−28% (11)	6/6

* 'N/A' denotes not applicable

5 [719] The data from this study demonstrated that 115111 (SEQ ID NO:29) was the most well-tolerated molecule among the exemplary HER-targeting molecules tested (*see* Table 13 and Figure 21). The group of mice that received 115111 (SEQ ID NO:29) exhibit no loss in body weight compared to the control group and an absence of animal deaths. By contrast, the group of mice administered 115195 (SEQ ID NO:32) lost an average of over 20% body weight per mouse and 5 of 6 mice (83%) died during the first 10 two weeks of the study. This indicates that the monomeric/monovalent 115111 (SEQ ID NO:29) was better tolerated than the related dimeric/divalent molecule 115195 (SEQ ID NO:32). In addition, the 115111 (SEQ ID NO:29) molecule, comprising SLTA-DI-2 (SEQ ID NO:20), was better tolerated as compared to 115172 (SEQ ID NO:23), which comprises the SLTA-FR (SEQ ID NO:37) component. This might be expected as 115111 (SEQ ID NO:29) comprises a more de-immunized Shiga toxin effector 15 polypeptide component compared to 115172 (SEQ ID NO:23). The group of mice that received 115172 (SEQ ID NO:23) had an average of 9.5% body weight loss and no animal deaths with all the mice recovering body weight after dosing was complete. The least tolerated molecule in this study was 115194 (SEQ ID NO:33), which was dimeric/divalent and comprised the SLTA-FR Shiga toxin effector polypeptide component (SEQ ID NO:37). The group of mice that received 115194 (SEQ ID NO:33) had 20 the most body weight loss, resulting in a ceasation of dosing after the 4th dose; however, all the mice (6 of 6 (100%)) in this dosing group died by Day 12 of the study.

[720] Together, these data indicated that 115111 (SEQ ID NO:29) was the most well-tolerated HER2-targeting molecule in the study. Based on the serum exposure data for 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32), one might expect administration of 115195 (SEQ ID NO:32) to result in less 25 free Shiga toxin in the serum and thus better tolerability. However, 115195 (SEQ ID NO:32) resulted in more toxicity for reasons not yet fully elucidated.

[721] Immunocompetent C57BL/6 mice were used to investigate the tolerability of 115111 (SEQ ID NO:29) after repeated administrations over time to the same mice (2 cycles, 12 doses total per mouse). A 115111 (SEQ ID NO:29) sample was diluted in PBS and administered intravenously (IV) to mice (n = 6– 30 10 mice per group) at doses of 0.1 mg/kg of body weight. As a control, the *in vivo* effects of the less de-

immunized 115172 (SEQ ID NO:23) (*see e.g.* WO 2016/196344) and 115411 (SEQ ID NO:30) were studied in parallel in the same study. As a control, vehicle-only samples were administered to a negative control treatment group. The body weights and health of the mice were monitored during the study and the results for mice dosed at 1 mg/kg per dose are shown in Table 14 and Figure 22.

5 **Table 14. Effects of Exemplary HER2-Targeting Molecules in C57BL/6 Mice after Repeat Dosing**

Sample	Days dosed	Amount per Dose	Body Weight Nadir % (Day)	Deaths / Number of Mice in Group
Vehicle Control	1, 3, 5, 8, 10, 12; 22, 24, 26, 29, 31, 33	N/A	--	0/6
115111	1, 3, 5, 8, 10, 12; 22, 24, 26, 29, 31, 33	1	−6.2% (11)	0/6
115172	1, 3, 5, 8, 10, 12, 22 (dosing halted)	1	−14.5% (11)	6/6
115411	1, 3, 5, 8, 10, 12; 22, 24, 26, 29, 31, 33	1	−10.2% (13)	0/6

* 'N/A' denotes not applicable

[722] The body weight and animal death results in Table 14 showed that 115111 (SEQ ID NO:29) and 10 115411 (SEQ ID NO:30) were better tolerated than 115172 (SEQ ID NO:23). None of the mice in the 115111 (SEQ ID NO:29) or 115411 (SEQ ID NO:30) treatment groups died, whereas 6 of 6 mice (100%) died in the 115172 (SEQ ID NO:23) treatment group during the first twenty-two days of the study. The body weight data in Figure 22 showed that 115111 (SEQ ID NO:29) was better tolerated than 115172 (SEQ ID NO:23). These results indicate the 115111 (SEQ ID NO:29) and 115411 (SEQ ID NO:30) 15 molecules, comprising SLTA-DI-2 (SEQ ID NO:20), were better tolerated as compared to 115172 (SEQ ID NO:23), which comprised the less de-immunized SLTA-FR Shiga toxin effector polypeptide component (SEQ ID NO:37).

b. Immunogenicity

20 [723] Immunocompetent BALB/c mice were used to investigate the immunogenicities of 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115195 (SEQ ID NO:32), and 115194 (SEQ ID NO:33) after repeated administrations over time to the same mice (6 doses total per mouse). In one study, 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), 115195 (SEQ ID NO:32), and 115194 (SEQ ID NO:33) were diluted in PBS and administered to mice (n = 6 per group) at intravenous (IV) doses of 1 mg/kg of 25 body weight. Immune response results measured in this study are reported below and in Figure 23. In another study, 115111 (SEQ ID NO:29), 115172 (SEQ ID NO:23), or a vehicle-only control sample were administered to mice (n = 6 per group) via intraperitoneal (IP) injection at doses of 0.25 mg/kg of body weight.

[724] The quantities of anti-drug antibodies (ADA) to HER2-targeting molecule in the mice were 30 monitored during these studies. The anti-drug antibody levels were monitored by taking serum samples from the mice, diluting the samples, and incubating the diluted samples with the HER2-targeting

molecule being tested (115111 (SEQ ID NO:29) or 115172 (SEQ ID NO:23)) overnight to form immune complexes. Then, molecules comprising a human HER2 binding domain (*e.g.* a HER2-targeting molecule of the present invention) were captured using an ELISA method involving a plate coated with recombinant human HER2 protein. The quantity of immune complexes present in each sample was then estimated using an anti-mouse immunoglobulin G conjugated to horseradish peroxidase (IgG-HRP) to quantify the number of anti-mouse IgG's captured by the human HER2 ELISA method. Results from this anti-drug antibody (ADA) assay are shown in Figure 23.

The top of Figure 23 showed the more de-immunized 115111 (SEQ ID NO:29) resulted in a lower IgG anti-drug antibody (ADA) ELISA signal than 115172 (SEQ ID NO:23) during the first 36 days of a study involving intraperitoneal (IP) dosing at 0.25 mg/kg body weight. The bottom of Figure 23 showed administration of the more de-immunized 115111 (SEQ ID NO:29) resulted in a lower IgG ADA ELISA signal than administration of 115172 (SEQ ID NO:23) at Day 22 of the study involving IV dosing at 1 mg/kg body weight. These results are not unexpected as 115111 (SEQ ID NO:29) comprised a more de-immunized Shiga toxin effector polypeptide component compared to 115172 (SEQ ID NO:23).

c. Non-Human Primates

[725] Two toxicology studies were performed in cynomolgus monkeys involving the administration of 115111 (SEQ ID NO:29) in six intravenous (IV) bolus doses, three times per week for 2 weeks, to evaluate the potential reversibility, progression or delayed progression of any findings following a 3-week (or 18 days for the low dose study) post-dose observation period.

[726] A "high dose" good laboratory practice (GLP) study tested doses of 50, 150, and 300 µg/kg of body weight. These "high" doses were not well tolerated.

[727] A "low dose" GLP study tested doses of 1, 5, and 25 µg/kg of body weight. These "low" doses showed effects that were not considered to have adversely impacted the health of the animals and so the No-Observed-Adverse-Effect Level (NOAEL) and Highest Non-Severely Toxic Dose (HNSTD) for these two studies was 25 µg/kg body weight (i.e. the highest dose tested in the "low-dose" study).

3. HER2 Expressing Tumor Xenograft Efficacy

[728] A mouse model of human breast cancer was used to test the effect(s) of 115111 (SEQ ID NO:29) after intraperitoneal (IP) administration. First, human neoplastic cells which express HER2 on their cell surfaces were injected into the mice and then the mice were treated with 115111 (SEQ ID NO:29).

[729] The activity of 115111 (SEQ ID NO:29) was studied in a subcutaneous tumor model using SCID Beige mice bearing subcutaneous, human HER2 positive tumors. HCC1954 cells from a human breast cancer derived cell line were implanted subcutaneously, then treatment was initiated when tumors reached an average of 100–150 mm^3, thereby defining study Day 0. The mice were intravenously administered two cycles of 115111 (SEQ ID NO:29) at doses of 0.1, 0.5 or 2 mg/kg body weight on study days 1, 3, 5, 8, 10, 12, 22, 24, 26, 29, 31 (and 33 for the groups administered doses of 0.1 or 0.5 mg/kg). Tumor volume was monitored during the treatment period and results are shown from Day 0 to

Day 60 (latest point where greater then fifty percent of the animals in all groups survived) are shown in Figure 24. In addition, survival results from the study are shown from Day 0 to Day 84 (end of study) in Figure 24.

[730] This study showed that repeat administration of 115111 (SEQ ID NO:29) at 0.1, 0.5, and 2 mg/kg body weight resulted in delays in tumor growth and provided survival benefits through study Day 84 (*see e.g.* Figure 24). Weight losses observed in the 115111 (SEQ ID NO:29) treatment group that was administered doses of 2 mg/kg of body weight resulted in the last scheduled dose (Day 33) to be withheld, and two mice in the 2 mg/kg 115111 (SEQ ID NO:29) treatment group died during the study.

SUMMARY

[731] Surprisingly, the exemplary HER2-targeting molecule 115111 (SEQ ID NO:29) was much safer to administer to animals at doses ranging between 1 to 2 mg/kg body weight than any other exemplary HER2-targeting molecules tested in this Example. This safety result could not be predicted from the *in vitro* data above. It was particularly surprising that the monomer 115111 (SEQ ID NO:29) was better tolerated than the dimer 115195 (SEQ ID NO:32) because these two molecules share identical components: the Shiga toxin effector polypeptide, heavy variable chain, light variable chain, and linker between the Shiga toxin effector polypeptide and the HER2-binding region are the same in these molecules. Furthermore, the *in vitro* data revealed no significant difference between these molecules except that 115195 (SEQ ID NO:32) was dimeric and divalent whereas 115111 (SEQ ID NO:29) was monomeric and monovalent. For example, both 115195 (SEQ ID NO:32) and 115111 (SEQ ID NO:29) exhibited similar cytotoxicity *in vitro* (*see* Tables 3 and 10; Figure 19) and similar binding characterstics to HER2 positive cells (*see* Tables 5 and 11; Figure 10). Both 115111 (SEQ ID NO:29) and 115195 (SEQ ID NO:32) maintained cytotoxic potency at shorter durations of exposure compared to continuous exposure (*see* Tables 9–10 and Figures 18–19). 115111 (SEQ ID NO:29) exhibited more cytotoxic potency than 114912 (SEQ ID NO:28) (*see* Tables 2 and 9; and Figures 6 and 18), under conditions involving continuous or shorter exposures. The HER2-targeting molecules 114912 (SEQ ID NO:28) and 114778 (SEQ ID NO:24), both of which are partly based on the widely used medicine trastuzumab (Herceptin®), were not among the most potent HER2-targeting molecules tested herein.

Example 2. Subject Response Prediction and Appropriate Subject Identification for a Therapeutic Use of a HER2-Targeting Molecule of the Present Invention

[732] Exemplary HER2-targeting molecules, such as one described above in Example 1, are administered to patient derived cell-lines *in vitro* in order to identify the most sensitive or resistant histologies and/or histological subtypes for different diseases, disorders, and/or conditions. Then, patients are categorized into different subpopulations using methods known to the skilled worker. For example, panels of breast cancer derived cell-lines (or alternatively gastric, ovary, lung, colorectal, melanoma, or pancreas) are tested *in vitro* using the cytotoxicity assay described in Example 1 or other methods known to the skilled worker. In addition, these cytoxicity assays are performed in combination

with an additional HER2-targeted therapeutic, such as, *e.g.*, including T-DM1 (trastuzumab emtansine), trastuzumab, and pertuzumab, and/or an additional drug which impinges on HER2 function, such as, *e.g.*, lapatinib and/or neratinib. The cytotoxicity results reveal certain associations useful to identify or predict which indications, cell-types, histologies, histological subtypes, and/or subjects may respond or be resistant to a particular HER2-targeting molecule of the present invention and/or a particular combination of a HER2-targeting molecule of the present invention and an additional therapeutic, such as another HER2-targeted therapeutic described herein, based on information regarding each cell line, such as, *e.g.*, molecular markers (including biomarker expression, gene copy numbers, mutations and polymorphisms, pro-proliferation pathway activation or growth-inhibitor pathway inhibition, metabolic activities, genotyping); responses to standard chemotherapeutic/cytotoxic agents, hormonal agents (*e.g.* estrogen, estradiol, etc.), and biologic agents (*e.g.* immune checkpoint inhibitor); and abilities to grow on plastic and in soft agar, as well as to grow *in vivo* subcutaneously and orthotopically. Further, malignant tissue specimens of cancers from individual patients or circulating tumor cells are tested for the presence of biomarkers of sensitivity/resistance using routine methods known to the skilled worker. Based on this biomarker associations, histologic associations, and cell characteristics *in vitro*, patients are selected or categorized as appropriate candidates (or inappropriate) to receive HER2-targeting molecule therapies (including combination therapies) as part of the treatment regimen for their malignancy.

[733] While some embodiments of the invention have been described by way of illustration, it will be apparent that the invention may be put into practice with many modifications, variations and adaptations, and with the use of numerous equivalents or alternative solutions that are within the scope of persons skilled in the art, without departing from the spirit of the invention or exceeding the scope of the claims.

[734] All publications, patents, and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety. The international patent application publications WO 2014/164680, WO 2014/164693, WO 2015/138435, WO 2015/138452, WO 2015/113005, WO 2015/113007, WO 2015/191764, WO 2016/196344, WO 2017/019623, and WO 2018/140427 are each incorporated herein by reference in its entirety. The disclosures of U.S. patent applications US2015/259428, US2016/17784, US2017/143814, and US 62/659,116 are each incorporated herein by reference in its entirety. The complete disclosures of all electronically available biological sequence information from GenBank (National Center for Biotechnology Information, U.S.) for amino acid and nucleotide sequences cited herein are each incorporated herein by reference in their entirety.

Sequence Listing		
ID Number	Text Description	Biological Sequence
SEQ ID NO:1	Shiga-like toxin 1 Subunit A (SLT-1A)	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDSGSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHASRVARMASDEFPSMCPADGRVRGITHNKILWDSSTLGAILMRRTISS
SEQ ID NO:2	Shiga toxin Subunit A (StxA)	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDSGTGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHASRVARMASDEFPSMCPADGRVRGITHNKILWDSSTLGAILMRRTISS
SEQ ID NO:3	Shiga-like toxin 2 Subunit A (SLT-2A)	DEFTVDFSSQKSYVDSLNSIRSAISTPLGNISQGGVSVSVINHVLGGNYISLNVRGLDPYSERFNHLRLIMERNNLYVAGFINTETNIFYRFSDFSHISVPDVITVSMTTDSSYSSLQRIADLERTGMQIGRHSLVGSYLDLMEFRGRSMTRASSRAMLRFVTVIAEALRFRQIQRGFRPALSEASPLYTMTAQDVDLTLNWGRISNVLPEYRGEEGVRIGRISFNSLSAILGSVAVILNCHSTGSYSVRSVSQKQKTECQIVGDRAAIKVNNVLWEANTIAALLNRKPQDLTEPNQ
SEQ ID NO:4	Shiga toxin subtype c Subunit A (Stx1cA)	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDSGTGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGRLSSVLPDYHGQDSVRVGRISFGSVNAILGSVALILNCHHHASRVAR
SEQ ID NO:5	Shiga toxin subtype d Subunit A (Stx1dA)	KEFTLDFSTAKKYVDSLNVIRSAIGTPLQTISSGGTSLLMIDSGTGDNLFAVDIMGLEPEEERFNNLRLIVERNNLYVTGFVNRTNNVFYRFADFSHVTFPGTRAVTLSGDSSYTTLQRVAGISRTGMQINRHSLTTSYLDLMSYSGTSLTQSVARAMLRFVTVTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGRLSSILPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHASRVAR
SEQ ID NO:6	Shiga toxin subtype e Subunit A (Stx1eA)	QDFTVDFSTAKKYVDSLNAIRSAIGTPLHSISSGGTSLLMIDNGTGDNLFAVDIRGLDPEEERFDNLRLIIERNNLYVTGFVNRTSNIFYRFADFSHVTFPGTRAVTLSGDSSYTTLQRVAGIGRTGMQINRHSLTTSYLDLMSYSGSSLTQPVARAMLRFVTVTAEALRFRQIQRGFRTTLDDVSGHSYTMTVEDVDLTLNWGRLSSVLPDYHGQDSVRVGRISFGGVNAILGSVALILNCHHHTSRVSR
SEQ ID NO:7	Shiga toxin subtype 2c Subunit A (Stx2cA) variant 1	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVR

SEQ ID NO:8	Shiga toxin subtype 2c Subunit A (Stx2cA) variant 2	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFAHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVR
SEQ ID NO:9	Shiga toxin subtype 2c Subunit A (Stx2cA) variant 3	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDIYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVR
SEQ ID NO:10	Shiga toxin subtype 2c Subunit A (Stx2cA) variant 4	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPSVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVR
SEQ ID NO:11	Shiga toxin subtype 2c Subunit A (Stx2cA) variant 5	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYMAGFVNTATNTFYRFSDFTHISVPSVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVR
SEQ ID NO:12	Shiga toxin subtype 2c Subunit A (Stx2cA) variant 6	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQVLSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGVRVGRISFNNISAILSTVAVILNCHHQGARSVR
SEQ ID NO:13	Shiga toxin subtype 2d Subunit A (Stx2dA) variant 1	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFAHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVIPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVR
SEQ ID NO:14	Shiga toxin subtype 2d Subunit A (Stx2dA) variant 2	REFMIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPEEVDLTLNWGRISNVLPEFRGEGGVRVGRISFNNISAILGTVAVILNCHHQGARSVR
SEQ ID NO:15	Shiga toxin subtype 2d Subunit A (Stx2dA) variant 3	REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISN

		VIPEYRGEDGVRVGRISFNNISAILSTVAVILNCHHQGARSV R
SEQ ID NO:16	Shiga toxin subtype 2e Subunit A (Stx2eA) variant 1	QEFTIDFSTQQSYVSSLNSIRTAISTPLEHISQGATSVSVINHT PPGSYISVGIRGLDVYQERFDHLRLIIERNNLYVAGFVNTTT NTFYRFSDFAHISLPGVTTISMTTDSSYTTLQRVAALERSG MQISRHSLVSSYLALMEFSGNTMTRDASRAVLRFVTVTAE ALRFRQIQREFRLALSETAPVYTMTPEDVDLTLNWGRISNV LPEYRGEAGVRVGRISFNNISAILGTVAVILNCHHQGARSV R
SEQ ID NO:17	Shiga toxin subtype 2e Subunit A (Stx2eA) variant 2	QEFTIDFSTQQSYVSSLNSIRTAISTPLEHISQGATSVSVINHT PPGSYISVGIRGLDVYQAHFDHLRLIIEQNNLYVAGFVNTA TNTFYRFSDFAHISLPGVTTISMTTDSSYTTLQRVAALERSG MQISRHSLVSSYLALMEFSGNTMTREASRAVLRFVTVTAE ALRFRQIQREFRQALSETAPVYTMTPEDVDLTLNWGRISNV LPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSV R
SEQ ID NO:18	Shiga toxin subtype 2f Subunit A (Stx2fA)	DEFTVDFSSQKSYVDSLNSIRSAISTPLGNISQGGVSVSVINH VPGGNYISLNVRGLDPYSERFNHLRLIMERNNLYVAGFINT ETNTFYRFSDFSHISVPDVITVSMTTDSSYSSLQRIADLERTG MQIGRHSLVGSYLDLMEFRGRSMTRASSRAMLRFVTVIAE ALRFRQIQRGFRPALSEASPLYTMTAQDVDLTLNWGRISNV LPEYRGEEGVRIGRISFNSLSAILGSVAVILNCHSTGSYSVR
SEQ ID NO:19	SLTA-DI-1	AEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAA
SEQ ID NO:20	SLTA-DI-2	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAA
SEQ ID NO:21	SLTA-DI-3	REFTLDFSTARTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLALMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAA
SEQ ID NO:22	114773 (SLTA-FR::scFv1)	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHH ASAVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRV TITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGT KVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKD TYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTIS

		ADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY WGQGTLVTVSSA
SEQ ID NO:23	115172 (SLTA-FR::scFv-6)	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHH ASAVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVK ISCKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGES TFADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARW EVYHGYVPYWGQGTTVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSDIQMTQSPSSLSASVGDRVTITCKASQDVYNAV AWYQQKPGQSPKLLIYSASSRYTGVPSRFTGSGSGPDFTFTI SSVQAEDLAVYFCQQHFRTPFTFGSGTKLEIK
SEQ ID NO:24	114778 (SLTA-DI-1::scFv-1)	MAEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRV TITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGT KVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKD TYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY WGQGTLVTVSSA
SEQ ID NO:25	114795 (SLTA-DI-1::scFv2)	MAEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPQVQLLQSGAELKKPGESLK ISCKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDTK YSPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHD VGYCSSSNCAKWPEYFQHWGQGTLVTVSSGGGGSQSVLT QPPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPK LLIYGHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYY CAAWDDSLSGWVFGGGTKLTVLA
SEQ ID NO:26	114791 (SLTA-DI-1::scFv3)	MAEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVK ISCKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGES TFADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARW EVYHGYVPYWGQGTTVTVSSGGGGSDIQLTQSHKFLSTSV GDRVSITCKASQDVYNAVAWYQQKPGQSPKLLIYSASSRY TGVPSRFTGSGSGPDFTFTISSVQAEDLAVYFCQQHFRTPFT FGSGTKLEIK

SEQ ID NO:27	SLTA-DI-1::scFv4	MAEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRV TITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRYTGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGT KVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFTD YTMDWVRQAPGKGLEWVADVNPNSGGSIYNQRFKGRFTL SVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYW GQGTLVTVSSA
SEQ ID NO:28	114912 (SLTA-DI-2::scFv-5)	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRV TITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGT KVEIKGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGG GLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAE DTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
SEQ ID NO:29	115111 (SLTA-DI-2::scFv-6)	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVK ISCKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGES TFADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARW EVYHGYVPYWGQGTTVTVSSGGGGSGGGGSGGGGSGGG GSGGGGSDIQMTQSPSSLSASVGDRVTITCKASQDVYNAV AWYQQKPGQSPKLLIYSASSRYTGVPSRFTGSGSGPDFTFTI SSVQAEDLAVYFCQQHFRTPFTFGSGTKLEIK
SEQ ID NO:30	115411 (SLTA-DI-3::scFv-7)	MREFTLDFSTARTYVDSLNVIRSAIGTPLQTISSGGTSLLMID SGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVN RTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGIS RTGMQINRHSLTTSYLALMSHSGTSLTQSVARAMLRFVTV TAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWG RLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHA SAVAASPSTPPTPSPSTPPASQVQLVQSGPEVKKPGASVKVS CKASGYPFTNYGMNWVRQAPGQGLEWMGWINTSTGESTF ADDFKGRVTMTTDTSTSTTYMELRSLRPDDTAVYFCARWE VYHGYVPYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG SGGGGSDIQMTQSPSSLSASIGDRVTITCKASQDVYNAVAW YQQKPGEAPKLLVYSASSRYTGVPSRFSGSGSGTDFTFTISS LQPEDIATYFCQQHFRTPFTFAPGTKLEIK
SEQ ID NO:31	114898	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI

		SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAAHHSEDPSSKAPKAPEVQLVESGGGLVQAGGSLR LSCAASGITFSINTMGWYRQAPGKQRELVALISSIGDTYYA DSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCKRFRT AAQGTDYWGQGTQVTVSSA
SEQ ID NO:32	115195	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVK ISCKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGES TFADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARW EVYHGYVPYWGQGTTVTVSSGGGGSDIQMTQSPSSLSASV GDRVTITCKASQDVYNAVAWYQQKPGQSPKLLIYSASSRY TGVPSRFTGSGSGPDFTFTISSVQAEDLAVYFCQQHFRTPFT FGSGTKLEIK
SEQ ID NO:33	115194	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHH ASAVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVK ISCKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGES TFADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARW EVYHGYVPYWGQGTTVTVSSGGGGSDIQMTQSPSSLSASV GDRVTITCKASQDVYNAVAWYQQKPGQSPKLLIYSASSRY TGVPSRFTGSGSGPDFTFTISSVQAEDLAVYFCQQHFRTPFT FGSGTKLEIK
SEQ ID NO:34	115645	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEVQLVESGGGLVQAGGSLRLSCAASGITFSINTMG WYRQAPGKQRELVALISSIGDTYYADSVKGRFTISRDNAK NTVYLQMNSLKPEDTAVYYCKRFRTAAQGTDYWGQGTQ VTVSSA
SEQ ID NO:35	115845	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLALMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAQVQLQESGGGSVQAGGSLKLTCAASGYIFNSCGM GWYRQSPGREREL VSRISGDGDTWHKESVKGRFTISQDNV KKTLYLQMNSLKPEDTAVYFCAVCYNLETYWGQGTQVTV SSHHHHHH
SEQ ID NO:36	SLTA-DI-2::scFv-8	MKEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMI DSGIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFV

		NRTNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVT VTAEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNW GRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHH ASAVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVK ISCKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGES TFADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARW EVYHGYVPYWGQGTTVTVSSGGGGSGGGGSGGGGSGGG GSDIQMTQSPSSLSASVGDRVTITCKASQDVYNAVAWYQQ KPGQSPKLLIYSASSRYTGVPSRFTGSGSGPDFTFTISSVQAE DLAVYFCQQHFRTPFTFGSGTKLEIK
SEQ ID NO:37	SLTA-FR	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAS AVAA
SEQ ID NO:38	HER2 from *Homo sapiens*	MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPE THLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEV QGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNG DPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLC YQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSR CWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAG CTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPN PEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTA EDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEF AGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEI TGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQG LGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRN PHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGPTQ CVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPEC QPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPD LSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQR ASPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRR LLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSG AFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEA YVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVR ENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARN VLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMA LESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREI PDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFREL VSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDD MGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSST RSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMG AAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCS PQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSP GKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAFSP AFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
SEQ ID NO:39	predicted extracellular domain of HER2 from *Homo sapiens*	TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLE LTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIV RGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQL RSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLI DTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGC ARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGIC

		ELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYL STDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYG LGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDP ASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQN LQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIH HNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGL ACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQ GLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACA HYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPIN CTHSCVDLDDKGCPAEQRASPLT
SEQ ID NO:40	HER2 from *Macaca fascicularis*	MELAAWYRWGLLLALLPPGAAGTQVCTGTDMKLRLPASP ETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQE VQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDN GNPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQL CYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPVCKGS RCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAA GCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMP NPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVT AEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQE FAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLRVFETLEE ITGYLYISAWPDSLPDLSVLQNLQVIRGRILHNGAYSLTLQG LGISWLGLRSLRELGSGLALIHHNTRLCFVHTVPWDQLFRN PHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGPTQ CVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPEC QPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPD LSYMPIWKFPDEEGTCQSCPINCTHSCVDLDDKGCPAEQRA SPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRRLL QETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAF GTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAY VMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRE NRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARN VLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMA LESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREI PDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFREL VSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDD MGDLVDAEEYLVPQQGFFCPDPAPGTGGMVHHRHRSSST RSGGGDLTLGLEPSEEEAPRSPRAPSEGTGSDVFDGDLGMG AAKGLQSLPAHDPSPLQRYSEDPTVPLPSETDGYVAPLTCS PQPEYVNQPDVRPQPPLPQEGPLSPARPTGATLERPKTLSPG KNGVVKDVFAFGGAVENPEYLAPRGGAAPQPHLPPAFSPA FDNLYYWDQDPSERGAPPSTFKGTPTAENPEYLGLDVPV
SEQ ID NO:41	HER2 from *Macaca mulatta*	MELAAWYRWGLLLALLPPGAAGTQVCTGTDMKLRLPASP ETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQE VQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDN GDLLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQ LCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPVCKG SRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCA AGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESM PNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEV TAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQ EFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLRVFETLE EITGYLYISAWPDSLPDLSVLQNLQVIRGRILHNGAYSLTLQ GLGISWLGLRSLRELGSGLALIHHNTRLCFVHTVPWDQLFR NPHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGPT QCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPE

		CQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKP DLSYMPIWKFPDEEGTCQSCPINCTHSCVDLDDKGCPAEQR ASPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRR LLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSG AFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEA YVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVR ENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARN VLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMA LESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREI PDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFREL VSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDD MGDLVDAEEYLVPQQGFFCPDPAPGTGGMVHHRHRSSST RSGGGDLTLGLEPSEEEAPRSPRAPSEGTGSDVFDGDLGMG AAKGLQSLPAHDPSPLQRYSEDPTVPLPSETDGYVAPLTCS PQPEYVNQPDVRPQPPSPQEGPLSPARPTGATLERPKTLSPG KNGVVKDVFAFGGAVENPEYLAPRGGAAPQPHLPPAFSPA FDNLYYWDQDPSERGAPPSTFKGTPTAENPEYLGLDVPV
SEQ ID NO:42	HER2 from *Mus musculus*	MELAAWCRWGFLLALLSPGAAGTQVCTGTDMKLRLPASP ETHLDMLRHLYQGCQVVQGNLELTYLPANASLSFLQDIQE VQGYMLIAHNRVKHVPLQRLRIVRGTQLFEDKYALAVLDN RDPLDNVTTAAPGRTPEGLRELQLRSLTEILKGGVLIRGNP QLCYQDMVLWKDVLRKNNQLAPVDMDTNRSRACPPCAP TCKDNHCWGESPEDCQILTGTICTSGCARCKGRLPTDCCHE QCAAGCTGPKHSDCLACLHFNHSGICELHCPALITYNTDTF ESMLNPEGRYTFGASCVTTCPYNYLSTEVGSCTLVCPPNNQ EVTAEDGTQRCEKCSKPCAGVCYGLGMEHLRGARAITSDN IQEFAGCKKIFGSLAFLPESFDGNPSSGVAPLKPEHLQVFET LEEITGYLYISAWPESFQDLSVFQNLRVIRGRILHDGAYSLT LQGLGIHSLGLRSLRELGSGLALIHRNTHLCFVNTVPWDQL FRNPHQALLHSGNRPEEACGLEGLVCNSLCARGHCWGPGP TQCVNCSQFLRGQECVEECRVWKGLPREYVRGKHCLPCHP ECQPQNSSETCYGSEADQCEACAHYKDSSSCVARCPSGVK PDLSYMPIWKYPDEEGICQPCPINCTHSCVDLDERGCPAEQ RASPVTFIIATVVGVLLFLIIVVVIGILIKRRRQKIRKYTMRR LLQETELVEPLTPSGAVPNQAQMRILKETELRKLKVLGSGA FGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAY VMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRE HRGRLGSQDLLNWCVQIAKGMSYLEEVRLVHRDLAARNV LVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMAL ESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIP DLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELV SEFSRMARDPQRFVVIQNEDLGPSSPMDSTFYRSLLEDDDM GELVDAEEYLVPQQGFFSPDPALGTGSTAHRRHRSSSARSG GGELTLGLEPSEEEPPRSPLAPSEGAGSDVFDGDLAVGVTK GLQSLSPHDLSPLQRYSEDPTLPLPPETDGYVAPLACSPQPE YVNQPEVRPQSPLTPEGPPPPIRPAGATLERPKTLSPGKNGV VKDVFAFGGAVENPEYLAPRAGTASQPHPSPAFSPAFDNL YYWDQNSSEQGPPPSTFEGTPTAENPEYLGLDVPV
SEQ ID NO:43	intein and chitin binding domain	CITGDALVALPEGESVRIADIVPGARPNSDNAIDLKVLDRH GNPVLADRLFHSGEHPVYTVRTVEGLRVTGTANHPLLCLV DVAGVPTLLWKLIDEIKPGDYAVIQRSAFSVDCAGFARGKP EFAPTTYTVGVPGLVRFLEAHHRDPDAQAIADELTDGRFY YAKVASVTDAGVQPVYSLRVDTADHAFITNGFVSHATGLT GLNSGLTTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQP HTSLAGWEPSNVPALWQLQ

SEQ ID NO:44	polyhistidine tag (6xHis)	HHHHHH
SEQ ID NO:45	vhCDR1	DTYIH
SEQ ID NO:46	vhCDR2	RIYPTNGYTRYADSVKG
SEQ ID NO:47	vhCDR3	WGGDGFYAMDY
SEQ ID NO:48	vlCDR1	RASQDVNTAVA
SEQ ID NO:49	vlCDR2	SASFLYS
SEQ ID NO:50	vlCDR3	QQHYTTPPT
SEQ ID NO:51	vhCDR1	SYWIA
SEQ ID NO:52	vhCDR2	LIYPGDSDTKYSPSFQG
SEQ ID NO:53	vhCDR3	HDVGYCSSSNCAKWPEYFQH
SEQ ID NO:54	vlCDR1	SGSSSNIGNNYVS
SEQ ID NO:55	vlCDR2	SASYRYT
SEQ ID NO:56	vlCDR3	QQYYIYPYT
SEQ ID NO:57	vhCDR1	NYGMN
SEQ ID NO:58	vhCDR2	WINTSTGESTFADDFKG
SEQ ID NO:59	vhCDR3	WEVYHGYVPY
SEQ ID NO:60	vlCDR1	KASQDVYNAVA
SEQ ID NO:61	vlCDR2	SASSRYT
SEQ ID NO:62	vlCDR3	QQHFRTPFT
SEQ ID NO:63	vhCDR1	DYTMD
SEQ ID NO:64	vhCDR2	DVNPNSGGSIYNQRFKG
SEQ ID NO:65	vhCDR3	NLGPSFYFDY
SEQ ID NO:66	vlCDR1	KASQDVSIGVA
SEQ ID NO:67	vlCDR2	SASYRYT
SEQ ID NO:68	vlCDR3	QQYYIYPYT
SEQ ID NO:69	vhhCDR1	INTMG
SEQ ID NO:70	vhhCDR2	LISSIGDTYYADSVKG

SEQ ID NO:71	vhhCDR3	FRTAAQGTDY
SEQ ID NO:72	vhhCDR1	SCGMG
SEQ ID NO:73	vhhCDR2	RISGDGDTWHKESVKG
SEQ ID NO:74	vhhCDR3	CYNLETY
SEQ ID NO:75	SLT-1A-combo variant 1	KEFILRFSVAHKYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAS AVAA
SEQ ID NO:76	SLT-1A-combo variant 2	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDN LVPMVATVVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVN RTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGIS RTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTV TAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWG RLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHA SAVAA
SEQ ID NO:77	SLT-1A-combo variant 3	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS NLVPMVATVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVN RTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGIS RTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTV TAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWG RLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHA SAVAA
SEQ ID NO:78	SLT-1A-combo variant 4	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GILGFVFTLDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRT NNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVTA EALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGRL SSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHASA VAA
SEQ ID NO:79	SLT-1A-combo variant 5	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVGILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAS AVAA
SEQ ID NO:80	SLT-1A-combo variant 6	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDILGFVFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAS AVAA
SEQ ID NO:81	SLT-1A-combo variant 7	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR

		LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAS AVAA
SEQ ID NO:82	SLT-1A-combo variant 8	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVGILGFVFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAS AVAA
SEQ ID NO:83	SLT-1A-combo variant 9	KEFILDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDVRGIAPIEARFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLAALSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAA
SEQ ID NO:84	SLT-1A-combo variant 10	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVGILGFVFTLEGRFNNLRLIVERNNLYVTGFVN RTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGIS RTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTV TAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWG RLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHA SAVAA
SEQ ID NO:85	SLT-1A-combo variant 11	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVNLVPMVATVGRFNNLRLIVERNNLYVTGFVN RTNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGIS RTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTV TAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWG RLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHA SAVAA
SEQ ID NO:86	SLT-1A-combo variant 12	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTNLVPMVATVSYTTLQRVAGIS RTGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTV TAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWG RLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHA SAVAA
SEQ ID NO:87	SLT-1A-combo variant 13	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRGILGDVFTLSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAA
SEQ ID NO:88	SLT-1A-combo variant 14	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHIL RFSVAHKASAVAA
SEQ ID NO:89	SLT-1A-combo variant 15	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT

		AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHAR NLVPMVATVASAVAA
SEQ ID NO:90	linker 1	EFPKPSTPPGSSGGAP
SEQ ID NO:91	linker 2	GGGGSGG
SEQ ID NO:92	linker 3	GGGGSGGGGSGGGGSGGGGSGGGGS
SEQ ID NO:93	linker 4	GSTSGSGKPGSGEGS
SEQ ID NO:94	linker 5	GGGGS
SEQ ID NO:95	linker 6	AHHSEDPSSKAPKAP
SEQ ID NO:96	linker 7	GSTSGSGKPGSGEGSTKG
SEQ ID NO:97	exemplary HER2-targeting molecule #1	AEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRVTIT CRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKV EIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTY IHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISAD TSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWG QGTLVTVSSA
SEQ ID NO:98	exemplary HER2-targeting molecule #2	AEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPQVQLLQSGAELKKPGESLKIS CKGSGYSFTSYWIAWVRQMPGKGLEYMGLIYPGDSDTKY SPSFQGQVTISVDKSVSTAYLQWSSLKPSDSAVYFCARHDV GYCSSSNCAKWPEYFQHWGQGTLVTVSSGGGGSQSVLTQ PPSVSAAPGQKVTISCSGSSSNIGNNYVSWYQQLPGTAPKL LIYGHTNRPAGVPDRFSGSKSGTSASLAISGFRSEDEADYY CAAWDDSLSGWVFGGGTKLTVLA
SEQ ID NO:99	exemplary HER2-targeting molecule #3	AEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVKIS CKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGEST FADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARWE VYHGYVPYWGQGTTVTVSSGGGGSDIQLTQSHKFLSTSVG DRVSITCKASQDVYNAVAWYQQKPGQSPKLLIYSASSRYT

		GVPSRFTGSGSGPDFTFTISSVQAEDLAVYFCQQHFRTPFTF GSGTKLEIK
SEQ ID NO:100	exemplary HER2-targeting molecule #4	AEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSATSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRVTIT CKASQDVSIGVAWYQQKPGKAPKLLIYSASYRYTGVPSRF SGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKV EIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFTDYT MDWVRQAPGKGLEWVADVNPNSGGSIYNQRFKGRFTLSV DRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYWGQ GTLVTVSSA
SEQ ID NO:101	exemplary HER2-targeting molecule #5	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPDIQMTQSPSSLSASVGDRVTIT CRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKV EIKGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGL VQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARI YPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDT AVYYCSRWGGDGFYAMDYWGQGTLVTVSS
SEQ ID NO:102	exemplary HER2-targeting molecule #6	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVKIS CKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGEST FADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARWE VYHGYVPYWGQGTTVTVSSGGGGSGGGGSGGGGSGGGG SGGGGSDIQMTQSPSSLSASVGDRVTITCKASQDVYNAVA WYQQKPGQSPKLLIYSASSRYTGVPSRFTGSGSGPDFTFTIS SVQAEDLAVYFCQQHFRTPFTFGSGTKLEIK
SEQ ID NO:103	exemplary HER2-targeting molecule #7	REFTLDFSTARTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLALMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAASPSTPPTPSPSTPPASQVQLVQSGPEVKKPGASVKVS CKASGYPFTNYGMNWVRQAPGQGLEWMGWINTSTGESTF ADDFKGRVTMTTDTSTSTTYMELRSLRPDDTAVYFCARWE VYHGYVPYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGG SGGGGSDIQMTQSPSSLSASIGDRVTITCKASQDVYNAVAW YQQKPGEAPKLLVYSASSRYTGVPSRFSGSGSGTDFTFTISS LQPEDIATYFCQQHFRTPFTFAPGTKLEIK

SEQ ID NO:104	exemplary HER2-targeting molecule #8	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAAHHSEDPSSKAPKAPEVQLVESGGGLVQAGGSLRLS CAASGITFSINTMGWYRQAPGKQRELVALISSIGDTYYADS VKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCKRFRTAA QGTDYWGQGTQVTVSSA
SEQ ID NO:105	exemplary HER2-targeting molecule #9	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVKIS CKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGEST FADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARWE VYHGYVPYWGQGTTVTVSSGGGGSDIQMTQSPSSLSASVG DRVTITCKASQDVYNAVAWYQQKPGQSPKLLIYSASSRYT GVPSRFTGSGSGPDFTFTISSVQAEDLAVYFCQQHFRTPFTF GSGTKLEIK
SEQ ID NO:106	exemplary HER2-targeting molecule #10	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEVQLVESGGGLVQAGGSLRLSCAASGITFSINTMGW YRQAPGKQRELVALISSIGDTYYADSVKGRFTISRDNAKNT VYLQMNSLKPEDTAVYYCKRFRTAAQGTDYWGQGTQVT VSSA
SEQ ID NO:107	exemplary HER2-targeting molecule #11	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLALMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAQVQLQESGGGSVQAGGSLKLTCAASGYIFNSCGMG WYRQSPGRERELVSRISGDGDTWHKESVKGRFTISQDNVK KTLYLQMNSLKPEDTAVYFCAVCYNLETYWGQGTQVTVS S
SEQ ID NO:108	exemplary HER2-targeting molecule #12	KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDS GIGDNLFAVDILGFDFTLGRFNNLRLIVERNNLYVTGFVNR TNNVFYRFADFSHVTFPGTTAVTLSADSSYTTLQRVAGISR TGMQINRHSLTTSYLDLMSHSGTSLTQSVARAMLRFVTVT AEALRFRQIQRGFRTTLDDLSGASYVMTAEDVDLTLNWGR LSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNSHHHAS AVAAEFPKPSTPPGSSGGAPQVQLQQSGPELKKPGETVKIS CKASGYPFTNYGMNWVKQAPGQGLKWMGWINTSTGEST FADDFKGRFDFSLETSANTAYLQINNLKSEDSATYFCARWE VYHGYVPYWGQGTTVTVSSGGGGSGGGGSGGGGSGGGG SDIQMTQSPSSLSASVGDRVTITCKASQDVYNAVAWYQQK

		PGQSPKLLIYSASSRYTGVPSRFTGSGSGPDFTFTISSVQAED LAVYFCQQHFRTPFTFGSGTKLEIK
SEQ ID NO:179	(G4S)7	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
SEQ ID NO:180	(G4S)3	GGGGSGGGGSGGGGS
SEQ ID NO:181	linker 8	SPSTPPTPSPSTPPA

CLAIMS

The invention is claimed as follows:

1. A HER2-targeting molecule comprising

   i) an immunoglobulin binding region capable of specifically binding an extracellular part of HER2/neu/ErbB2, and comprising one or more of:

      an antibody variable fragment, a single-domain antibody fragment, a single-chain variable fragment, a Fd fragment, an antigen-binding fragment, an autonomous V_H domain, a V_HH fragment derived from a camelid antibody, a heavy-chain antibody domain derived from a cartilaginous fish antibody, a V_{NAR} fragment, and an immunoglobulin new antigen receptor;

   and

   ii) a Shiga toxin A subunit effector polypeptide comprising a Shiga toxin A1 fragment region and a carboxy-terminus, wherein the Shiga toxin A subunit effector polypeptide comprises:

      (a) an embedded or inserted, heterologous, CD8+ T-cell epitope which disrupts an endogenous, B-cell and/or CD4+ T-cell epitope region;

      (b) a disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions which do not overlap with the embedded or inserted, heterologous, CD8+ T-cell epitope; and

      (c) a disrupted furin-cleavage motif at the carboxy-terminus of the Shiga toxin A1 fragment region; and

   wherein the Shiga toxin A subunit effector polypeptide is truncated at its carboxy-terminus, relative to a wild-type Shiga toxin A subunit, resulting in the elimination of one or more endogenous, B-cell and/or CD4+ T-cell epitope regions; and

   wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a Shiga toxin effector function.

2. The HER2-targeting molecule of claim 1, wherein the Shiga toxin A subunit effector polypeptide comprises an amino acid sequence that is at least 85% identical to a wild-type Shiga toxin A Subunit amino acid sequence selected from:

   (i) amino acids 75 to 251 of any one of SEQ ID NOs: 1–6;

   (ii) amino acids 1 to 241 of any one of SEQ ID NOs: 1–18;

   (iii) amino acids 1 to 251 of any one of SEQ ID NOs: 1–6; and

   (iv) amino acids 1 to 261 of any one of SEQ ID NOs: 1–3.

3. The HER2-targeting molecule of claim 1 or claim 2, wherein the disrupted furin-cleavage motif further comprises one or more mutations, relative to a wild-type Shiga toxin A Subunit, the mutation altering at least one amino acid residue in a region natively positioned

at 248–251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 variant sequence (SEQ ID NOs: 4–6), or
at 247–250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant sequence (SEQ ID NOs: 7–18).

4. The HER2-targeting molecule of any one of claims 1–3, wherein the disrupted furin-cleavage motif comprises an amino acid residue substitution in the furin-cleavage motif relative to a wild-type Shiga toxin A Subunit.

5. The HER2-targeting molecule of claim 4, wherein the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with a non-positively charged, amino acid residue selected from the group consisting of:
alanine, glycine, proline, serine, threonine, aspartate, asparagine, glutamate, glutamine, cysteine, isoleucine, leucine, methionine, valine, phenylalanine, tryptophan, and tyrosine.

6. The HER2-targeting molecule of claim 5, wherein the disrupted furin-cleavage motif comprises an amino acid substitution in the furin-cleavage motif relative to a wild-type Shiga toxin A Subunit, at the natively positioned amino acid residues 248 and 251 of the A Subunit of Shiga-like toxin 1 (SEQ ID NO:1), Shiga toxin (SEQ ID NO:2), or another Shiga toxin 1 variant (SEQ ID NOs: 4–6); or at the natively positioned amino acid residues 247 and 250 of the A Subunit of Shiga-like toxin 2 (SEQ ID NO:3) or a Shiga-like toxin 2 variant (SEQ ID NOs: 7–18); optionally wherein the substitution of the amino acid residue in the furin-cleavage motif is of an arginine residue with an alanine residue.

7. The HER2-targeting molecule of any one of claims 1–6, wherein the disruption of at least four, endogenous, B-cell and/or CD4+ T-cell epitope regions comprises a mutation, relative to a wild-type Shiga toxin A Subunit, in the B-cell epitope regions selected from the group of natively positioned Shiga toxin A Subunit regions consisting of:
1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2;
and/or in the CD4+ T-cell epitope regions selected from the group of natively positioned Shiga toxin A Subunit regions consisting of:

4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2.

8. The HER2-targeting molecule of any one of claims 1–7, wherein the embedded or inserted, heterologous, CD8+ T-cell epitope disrupts
an endogenous, B-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of:
1–15 of SEQ ID NO:1 or SEQ ID NO:2; 3–14 of SEQ ID NO:3; 26–37 of SEQ ID NO:3; 27–37 of SEQ ID NO:1 or SEQ ID NO:2; 39–48 of SEQ ID NO:1 or SEQ ID NO:2; 42–48 of SEQ ID NO:3; 53–66 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94–115 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141–153 of SEQ ID NO:1 or SEQ ID NO:2; 140–156 of SEQ ID NO:3; 179–190 of SEQ ID NO:1 or SEQ ID NO:2; 179–191 of SEQ ID NO:3; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 210–218 of SEQ ID NO:3; 240–260 of SEQ ID NO:3; 243–257 of SEQ ID NO:1 or SEQ ID NO:2; 254–268 of SEQ ID NO:1 or SEQ ID NO:2;
and/or an endogenous CD4+ T-cell epitope region selected from the group of natively positioned Shiga toxin A Subunit regions consisting of:
4–33 of SEQ ID NO:1 or SEQ ID NO:2; 34–78 of SEQ ID NO:1 or SEQ ID NO:2; 77–103 of SEQ ID NO:1 or SEQ ID NO:2; 128–168 of SEQ ID NO:1 or SEQ ID NO:2; 160–183 of SEQ ID NO:1 or SEQ ID NO:2; and 236–258 of SEQ ID NO:1 or SEQ ID NO:2.

9. The HER2-targeting molecule of any one of claims 1–8, wherein the HER2-targeting molecule has reduced B-cell antigenicity or immunogenicity and/or reduced CD4+ T-cell antigenicity or immunogenicity.

10. The HER2-targeting molecule of any one of claims 1–9, wherein the binding region and the Shiga toxin effector polypeptide are fused forming a continuous polypeptide such that the binding region is associated, either directly or indirectly, with the carboxy-terminus of the Shiga toxin A subunit effector polypeptide.

11. The HER2-targeting molecule of any one of claims 1–10, which comprises disruptions of at least five, six, seven, eight, or more B-cell and/or CD4+ T-cell epitope regions endogenous to a wild-type Shiga toxin A Subunit.

12. The HER2-targeting molecule of any one of claims 1–11, wherein at least three of the four B-cell and/or CD4+ T-cell epitope region disruptions comprise an amino acid residue substitution relative to a wild-type Shiga toxin A Subunit.

13. The HER2-targeting molecule of any one of claims 1–12, wherein at least one substitution occurs at the natively positioned Shiga toxin A Subunit amino acid residue selected from the group consisting of:

    1 of SEQ ID NO:1 or SEQ ID NO:2; 4 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 6 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 8 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 9 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 11 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 12 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 33 of SEQ ID NO:1 or SEQ ID NO:2; 43 of SEQ ID NO:1 or SEQ ID NO:2; 44 of SEQ ID NO:1 or SEQ ID NO:2; 45 of SEQ ID NO:1 or SEQ ID NO:2; 46 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 47 of SEQ ID NO:1 or SEQ ID NO:2; 48 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 49 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 50 of SEQ ID NO:1 or SEQ ID NO:2; 51 of SEQ ID NO:1 or SEQ ID NO:2; 53 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 55 of SEQ ID NO:1 or SEQ ID NO:2; 56 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 57 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 58 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 59 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 62 of SEQ ID NO:1 or SEQ ID NO:2; 84 of SEQ ID NO:1 or SEQ ID NO:2; 88 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 94 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 96 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 104 of SEQ ID NO:1 or SEQ ID NO:2; 105 of SEQ ID NO:1 or SEQ ID NO:2; 107 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 108 of SEQ ID NO:1 or SEQ ID NO:2; 109 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 110 of SEQ ID NO:1 or SEQ ID NO:2; 111 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 112 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 141 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 147 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 154 of SEQ ID NO:1 or SEQ ID NO:2; 179 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 180 of SEQ ID NO:1 or SEQ ID NO:2; 181 of SEQ ID NO:1 or SEQ ID NO:2; 183 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 184 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 185 of SEQ ID NO:1 or SEQ ID NO:2; 186 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3; 187 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 189 of SEQ ID NO:1 or SEQ ID NO:2; 197 of SEQ ID NO:3; 198 of SEQ ID NO:1 or SEQ ID NO:2; 204 of SEQ ID NO:3; 205 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO: 1 or SEQ ID NO:2; 247 of SEQ ID NO:1 or SEQ ID NO:2; 247 of SEQ ID NO:3; 248 of SEQ ID NO:1 or SEQ ID NO:2; 250 of SEQ ID NO:3; 251 of SEQ ID NO:1 or SEQ ID NO:2.

14. The HER2-targeting molecule of claim 13, wherein the at least one substitution occurs at the natively positioned Shiga toxin A Subunit amino acid residues:

45 of SEQ ID NO:1 or SEQ ID NO:2; 54 of SEQ ID NO:1 or SEQ ID NO:2; 55 of SEQ ID NO:1 or SEQ ID NO:2; 57 of SEQ ID NO:1 or SEQ ID NO:2; 59 of SEQ ID NO:1 or SEQ ID NO:2; 60 of SEQ ID NO:1 or SEQ ID NO:2; 61 of SEQ ID NO:1 or SEQ ID NO:2; 110 of SEQ ID NO:1 or SEQ ID NO:2; 141 of SEQ ID NO:1 or SEQ ID NO:2; 188 of SEQ ID NO:1 or SEQ ID NO:2; 242 of SEQ ID NO:1 or SEQ ID NO:2; 248 of SEQ ID NO:1 or SEQ ID NO:2; and 251 of SEQ ID NO:1 or SEQ ID NO:2.

15. The HER2-targeting molecule of claim 13 or claim 14, wherein at least one of the substitutions is selected from the group consisting of:

D to A, D to G, D to V, D to L, D to I, D to F, D to S, D to Q, D to M, D to R, E to A, E to G, E to V, E to L, E to I, E to F, E to S, E to Q, E to N, E to D, E to M, E to R, F to A, F to G, F to V, F to L, F to I, G to A, G to P, H to A, H to G, H to V, H to L, H to I, H to F, H to M, I to A, I to V, I to G, I to C, K to A, K to G, K to V, K to L, K to I, K to M, K to H, L to A, L to V, L to G, L to C, N to A, N to G, N to V, N to L, N to I, N to F, P to A, P to G, P to F, R to A, R to G, R to V, R to L, R to I, R to F, R to M, R to Q, R to S, R to K, R to H, S to A, S to G, S to V, S to L, S to I, S to F, S to M, T to A, T to G, T to V, T to L, T to I, T to F, T to M, T to S, V to A, V to G, Y to A, Y to G, Y to V, Y to L, Y to I, Y to F, Y to M, and Y to T.

16. The HER2-targeting molecule of claim any one of claims 13–15, wherein one or more substitutions are selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of:

K1 to A, G, V, L, I, F, M and H; T4 to A, G, V, L, I, F, M, and S; D6 to A, G, V, L, I, F, S, Q and R; T8 to A, G, V, I, L, F, and M; S8 to A, G, V, I, L, F, and M; T9 to A, G, V, I, L, F, M, and S; S9 to A, G, V, L, I, F, and M; K11 to A, G, V, L, I, F, M and H; T12 to A, G, V, I, L, F, M, S, and K; S12 to A, G, V, I, L, F, and M; S33 to A, G, V, L, I, F, M, and C; S43 to A, G, V, L, I, F, and M; G44 to A or L; S45 to A, G, V, L, I, F, and M; T45 to A, G, V, L, I, F, and M; G46 to A and P; D47 to A, G, V, L, I, F, S, M, and Q; N48 to A, G, V, L, M and F; L49 to A, V, C, and G; Y49 to A, G, V, L, I, F, M, and T; F50 to A, G, V, L, I, and T; D53 to A, G, V, L, I, F, S, and Q; V54 to A, G, I, and L; R55 to A, G, V, L, I, F, M, Q, S, K, and H; G56 to A and P; I57 to A, G, V, and M; L57 to A, V, C, G, M, and F; D58 to A, G, V, L, I, F, S, and Q; P59 to A, G, and F; E60 to A, G, V, L, I, F, S, Q, N, D, M, T, and R; E61 to A, G, V, L, I, F, S, Q, N, D, M, and R; G62 to A; R84 to A, G, V, L, I, F, M, Q, S, K, and H; V88 to A and G; I88 to A, V, C, and G; D94 to A, G, V, L, I, F, S, and Q; S96 to A, G, V, I, L, F, and M; T104 to A, G, V, L, I, F, M; and N; A105 to L; T107 to A, G, V, L, I, F, M, and P; S107 to A, G, V, L, I, F, M, and P; L108 to A, V, C, and G; S109 to A, G, V, I, L, F, and M; T109 to A, G, V, I, L, F, M, and S; G110 to A; S112 to A, G, V, L, I, F, and M; D111 to A, G, V, L, I, F, S, Q, and T; S112 to A, G, V, L, I, F, and M; D141 to A, G, V, L, I, F, S, and Q; G147 to A; V154 to A and G; R179 to A, G, V, L, I, F, M, Q, S, K, and H; T180 to A, G, V, L, I, F, M, and S; T181 to A, G, V, L, I, F, M, and S;

D183 to A, G, V, L, I, F, S, and Q; D184 to A, G, V, L, I, F, S, and Q; L185 to A, G, V and C; S186 to A, G, V, I, L, F, and M; G187 to A; R188 to A, G, V, L, I, F, M, Q, S, K, and H; S189 to A, G, V, I, L, F, and M; D197 to A, G, V, L, I, F, S, and Q; D198 to A, G, V, L, I, F, S, and Q; R204 to A, G, V, L, I, F, M, Q, S, K, and H; R205 to A, G, V, L, I, F, M, Q, S, K and H; C242 to A, G and V; S247 to A, G, V, I, L, F, and M; Y247 to A, G, V, L, I, F, and M; R247 to A, G, V, L, I, F, M, Q, S, K, and H; R248 to A, G, V, L, I, F, M, Q, S, K, and H; R250 to A, G, V, L, I, F, M, Q, S, K, and H; R251 to A, G, V, L, I, F, M, Q, S, K, and H.

17. The HER2-targeting molecule of any one of claims 13–16, wherein the one or more substitutions are selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of:
    K1A, K1M, T4I, D6R, S8I, T8V, T9I, S9I, K11A, K11H, T12K, S33I, S33C, S43N, G44L, S45V, S45I, T45V, T45I, G46P, D47M, D47G, N48V, N48F, L49A, F50T, D53A, D53N, D53G, V54L, V54I, R55A, R55V, R55L, G56P, I57F, I57M, D58A, D58V, D58F, P59A, P59F, E60I, E60T, E60R, E61A, E61V, E61L, G62A, R84A, V88A, D94A, S96I, T104N, A105L, T107P, L108M, S109V, T109V, G110A, D111T, S112V, D141A, G147A, V154A, R179A, T180G, T181I, D183A, D183G, D184A, D184A, D184F, L185V, L185D, S186A, S186F, G187A, G187T, R188A, R188L, S189A, D197A, D198A, R204A, R205A, C242A, S247I, Y247A, R247A, R248A, R250A, and R251A.

18. The HER2-targeting molecule of claim 17, wherein the one or more substitutions are selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of: S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, C242S, R248A, and R251A.

19. The HER2-targeting molecule of claim 18, wherein the one or more substitutions are selected from the group of substitutions at native positions in a Shiga toxin A Subunit consisting of: K1A, S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, G147A, R188A, C242S, R248A, and R251A.

20. The HER2-targeting molecule of any one of claims 13–18, wherein the one or more substitutions comprise the substitutions at native positions in a Shiga toxin A Subunit: S45I, V54I, R55L, I57F, P59F, E60T, E61L, G110A, D141A, R188A, C242S, R248A, and R251A.

21. The HER2-targeting molecule of any one of claims 1–20, wherein the carboxy-terminal truncation of the Shiga toxin effector polypeptide disrupts the furin-cleavage motif at the carboxy-terminus of the Shiga toxin A2 fragment region

22. The HER2-targeting molecule of any one of claims 1–21, wherein the Shiga toxin A subunit effector polypeptide comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical to an amino acid sequence selected from any one of SEQ ID NOs: 19–21 and 75–89.

23. The HER2-targeting molecule of claim 22, wherein the Shiga toxin A subunit effector polypeptide comprises, consists essentially of, or consists of the amino acid sequence of any one of SEQ ID NOs: 19–21.

24. The HER2-targeting molecule of any one of claims 1–23, wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting one or more Shiga toxin effector function selected from: increasing cellular internalization, directing subcellular routing from an endosomal compartment to the cytosol, avoiding intracellular degradation, catalytically inactivating ribosomes, effectuating cytostatic effects and effectuating cytotoxicity.

25. The HER2-targeting molecule of any one of claims 1–24, wherein the Shiga toxin A subunit effector polypeptide is capable of exhibiting a ribosome inhibition activity with a half-maximal inhibitory concentration (IC_{50}) value of 10,000 picomolar or less.

26. The HER2-targeting molecule of any one of claims 1–23, wherein the Shiga toxin A subunit effector polypeptide comprises one or more mutations relative to a naturally occurring A Subunit of a member of the Shiga toxin family which changes an enzymatic activity of the Shiga toxin A subunit effector polypeptide, the mutation selected from at least one amino acid residue deletion, insertion, or substitution.

27. The HER2-targeting molecule of claim 26, wherein the mutation, relative to the naturally occurring A Subunit which changes an enzymatic activity of the Shiga toxin A subunit effector polypeptide, reduces or eliminates cytotoxicity of the Shiga toxin A subunit effector polypeptide.

28. The HER2-targeting molecule of any one of claims 1–27, wherein the immunoglobulin binding region comprises a single-chain variable fragment and/or a V_HH fragment derived from a camelid antibody, optionally wherein the V_HH fragment comprises the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 69, 70, and 71, respectively; or the HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 72, 73, and 74, respectively.

29. The HER2-targeting molecule of any one of claims 1–27, wherein the immunoglobulin binding region comprises a single-chain variable fragment comprising at least one heavy-chain variable domain polypeptide comprising:

(i) HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 51, 52, and 53, respectively; and

(ii) HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 57, 58, and 59, respectively; or

(iii) HCDR1, HCDR2, and HCDR3 amino acid sequences shown in SEQ ID NOs: 63, 64, and 65, respectively.

30. The HER2-targeting molecule of claim 29, wherein the immunoglobulin binding region comprises at least one light-chain variable domain polypeptide comprising:

    (i) LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NOs: 54, 55, and 56, respectively; and

    (ii) LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NOs: 60, 61, and 62, respectively; or

    (iii) LCDR1, LCDR2, and LCDR3 amino acid sequences shown in SEQ ID NOs: 66, 67, and 68, respectively.

31. The HER2-targeting molecule of claim 29 or claim 30, wherein the immunoglobulin binding region comprises at least one heavy-chain variable domain polypeptide linked to at least one light-chain variable domain polypeptide by a linker comprising at least twelve amino acid residues, optionally wherein the linker comprises an amino acid sequence selected from: $(G_4S)_3$ (SEQ ID NO:180), $(G_4S)_4$ (SEQ ID NO:177), $(G_4S)_5$ (SEQ ID NO:92), $(G_4S)_6$ (SEQ ID NO:178), or $(G_4S)_7$ (SEQ ID NO:179).

32. The HER2-targeting molecule of claim 31, wherein the heavy-chain variable domain polypeptide comprises

    amino acids 269 to 387 of SEQ ID NO: 26, 29–30, or 36;

    amino acids 269 to 397 of SEQ ID NO:25; or

    amino acids 381 to 500 of SEQ ID NO: 24 or 27.

33. The HER2-targeting molecule of claim 31 or claim 32, wherein the light-chain variable domain polypeptide comprises

    amino acids 269 to 375 of SEQ ID NO: 24 or 27;

    amino acids 393 to 499 of SEQ ID NO:26;

    amino acids 403 to 513 of SEQ ID NO:25;

    amino acids 408 to 514 of SEQ ID NO:36; or

    amino acids 413 to 519 of SEQ ID NO: 29 or 30.

34. The HER2-targeting molecule of any one of claims 1–33, wherein the immunoglobulin binding region comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical to the amino acid sequence of:

    amino acids 269 to 501 of SEQ ID NO:24;

    amino acids 269 to 513 of SEQ ID NO:25;

amino acids 269 to 499 of SEQ ID NO:26;
amino acids 269 to 499 of SEQ ID NO:27;
amino acids 269 to 519 of SEQ ID NO:29;
amino acids 269 to 519 of SEQ ID NO:30;
amino acids 268 to 386 of SEQ ID NO:31;
amino acids 253 to 370 of SEQ ID NO:34;
amino acids 253 to 367 of SEQ ID NO:35; or
amino acids 269 to 514 of SEQ ID NO:36.

35. The HER2-targeting molecule of any one of claims 1–34, wherein the immunoglobulin binding region and Shiga toxin A subunit effector polypeptide are fused by a proteinaceous linker comprising one or more amino acid residue, optionally wherein the linker comprises or consists of:
    $(G_4S)_3$ (SEQ ID NO:180), $(G_4S)_4$ (SEQ ID NO:177), $(G_4S)_5$ (SEQ ID NO:92), $(G_4S)_6$ (SEQ ID NO:178), $(G_4S)_7$ (SEQ ID NO:179), GSTSGSGKPGSGEGS (SEQ ID NO:93), AHHSEDPSSKAPKAP (SEQ ID NO:95), SPSTPPTPSPSTPPA (SEQ ID NO:181), EFPKPSTPPGSSGGAP (SEQ ID NO:90), and GSTSGSGKPGSGEGSTKG (SEQ ID NO:96).

36. The HER2-targeting molecule of any one of claims 1–35, whereby upon administration of the HER2-targeting molecule to a first population of cells whose members express the HER2 bound by the binding region, and a second population of cells whose members do not express the HER2 bound by the binding region, a cytotoxic effect of the HER2-targeting molecule to members of said first population of cells relative to members of said second population of cells is at least 3-fold greater.

37. The HER2-targeting molecule of any one of claims 1–36, wherein the HER2-targeting molecule is capable of exhibiting improved, *in vivo* tolerability compared to a reference HER2-targeting molecule consisting of the HER2-targeting molecule except for the Shiga toxin A subunit effector polypeptide consists of a wild-type Shiga toxin A1 polypeptide.

38. The HER2-targeting molecule of any one of claims 1–38, which comprises, consists essentially of, or consists of an amino acid sequence that is at least 85% identical to the amino acid sequence shown in any one of SEQ ID NOs: 24–36 and 97–108.

39. The HER2-targeting molecule of any one of claims 1–25 and 28–38, which comprises, consists essentially of, or consists of the amino acid sequence shown in any one of SEQ ID NOs: 24–27, 29–31, 34–36, 97–104 and 106–108.

40. The HER2-targeting molecule of any one of claims 1–39, in the form of a pharmaceutically acceptable salt or solvate.

41. A HER2-targeting molecule which comprises or consists of the amino acid sequence shown in SEQ ID NO: 29 or 102.

42. A pharmaceutical composition comprising
    i) a HER2-targeting molecule according to any one of claims 1–41, and
    ii) at least one pharmaceutically acceptable excipient or carrier.

43. A pharmaceutical composition comprising a HER2-targeting molecule which comprises or consists of the amino acid sequence shown in SEQ ID NO: 29 or 102; and at least one pharmaceutically acceptable excipient or carrier.

44. The pharmaceutical composition of claim 42 or 43, wherein the at least one pharmaceutically acceptable carrier includes a solvent, a dispersion medium, a coating, an antimicrobial agent, an isotonic agent, or an absorption delaying agent; and/or wherein the pharmaceutical composition further comprises an aqueous or non-aqueous carrier; a surfactant; a stabilizer, a preservative, a buffer, an antioxidant, a wetting agent, an emulsifying agent, a dispersing agent; an isotonic agent; and/or an antibacterial or antifungal agent.

45. The pharmaceutical composition of claim 43 or 44, which further comprises one or more of:
    citrate, sorbitol, polysorbate 20, chloride, or sodium.

46. A diagnostic composition comprising
    i) a HER2-targeting molecule according to any one of claims 1–41 and
    ii) a detection promoting agent.

47. A polynucleotide capable of encoding a HER2-targeting molecule according to any one of claims 1–41, or a complement thereof.

48. An expression vector comprising a polynucleotide according to claim 47.

49. A host cell comprising a polynucleotide according to claim 47 or an expression vector according to claim 48.

50. A method of killing a HER2-expressing cell, the method comprising the step of
    contacting the cell with a HER2-targeting molecule according to any one of claims 1–41 or a pharmaceutical composition according to any one of claims 42–45.

51. A method according to claim 50, wherein the contacting occurs *in vivo*.

52. A method of treating a disease, disorder, or condition in a patient, the method comprising the step of administering to a patient in need thereof a therapeutically effective amount of a HER2-targeting molecule according to any one of claims 1–41, or a pharmaceutical composition according to any one of claims 42–45, wherein the disease, disorder, or condition is characterized by cells that are physically coupled with HER2/neu/ErbB2.

53. The method of claim 52, wherein when the patient in need thereof is administered with a therapeutically effective amount of the HER2-targeting molecule, the method further comprises administering to the patient in need thereof a therapeutically effective amount of an additional HER2-targeting therapeutic agent.

54. The method of claim 53, wherein the additional HER2-targeting therapeutic agent comprises a dual tyrosine kinase inhibitor; optionally wherein the inhibitor comprises lapatinib and/or neratinib.

55. The method of claim 52 or claim 53, wherein the additional HER2-targeting therapeutic agent comprises an anti-HER2 antibody that binds an antigenic determinant in HER2 that does not overlap with the antigenic determinant in HER2 bound by the HER2-targeting molecule.

56. The method of claim 55, wherein the additional HER2-targeting therapeutic agent comprises: T-DM1, trastuzumab, and/ or pertuzumab.

57. The method of any one of claims 52–56, wherein the patient has been previously treated with at least one other HER2-targeting therapeutic agent; optionally wherein the at least one other HER2-targeting therapeutic agent is selected from: lapatinib, neratinib, T-DM1, trastuzumab, and pertuzumab.

58. The method of any one of claims 52–57, wherein the patient does not respond to, or does not benefit from, treatment with at least one other HER2-targeting therapeutic agent; optionally wherein the at least one other HER2-targeting therapeutic agent is selected from: lapatinib, neratinib, T-DM1, trastuzumab, and pertuzumab.

59. The method of any one of claims 52–58, wherein the disease, disorder, or condition is a cancer or tumor; optionally wherein the disease, disorder, or condition is selected from the group consisting of: breast cancer, gastrointestinal cancer, germ cell cancer, glandular cancer, gynecologic cancer, head-neck cancer, kidney-urinary tract cancer, and lung/pleura cancer, such as, *e.g.*, endometrial cancer, esophageal cancer, ovarian cancer, pancreatic cancer, prostate cancer, and testicular cancer.

60. A composition for use in the treatment or prevention of a cancer, tumor, or growth abnormality comprising a HER2-targeting molecule according to any one of claims 1–41, or a pharmaceutical composition according to to any one of claims 42–45, wherein the cancer, tumor, or growth abnormality is characterized by cells that are physically coupled with HER2/neu/ErbB2.

61. Use of a composition of matter according to any one of claims 1–49 in the manufacture of a medicament for the treatment or prevention of a cancer, tumor, or growth abnormality.

62. Use of a composition of matter according to any one of claims 1–49 in the diagnosis, prognosis, or characterization of a disease, disorder, or condition.

63. A kit comprising a composition of matter according to any one of claims 1–49 and an additional reagent and/or pharmaceutical delivery device.

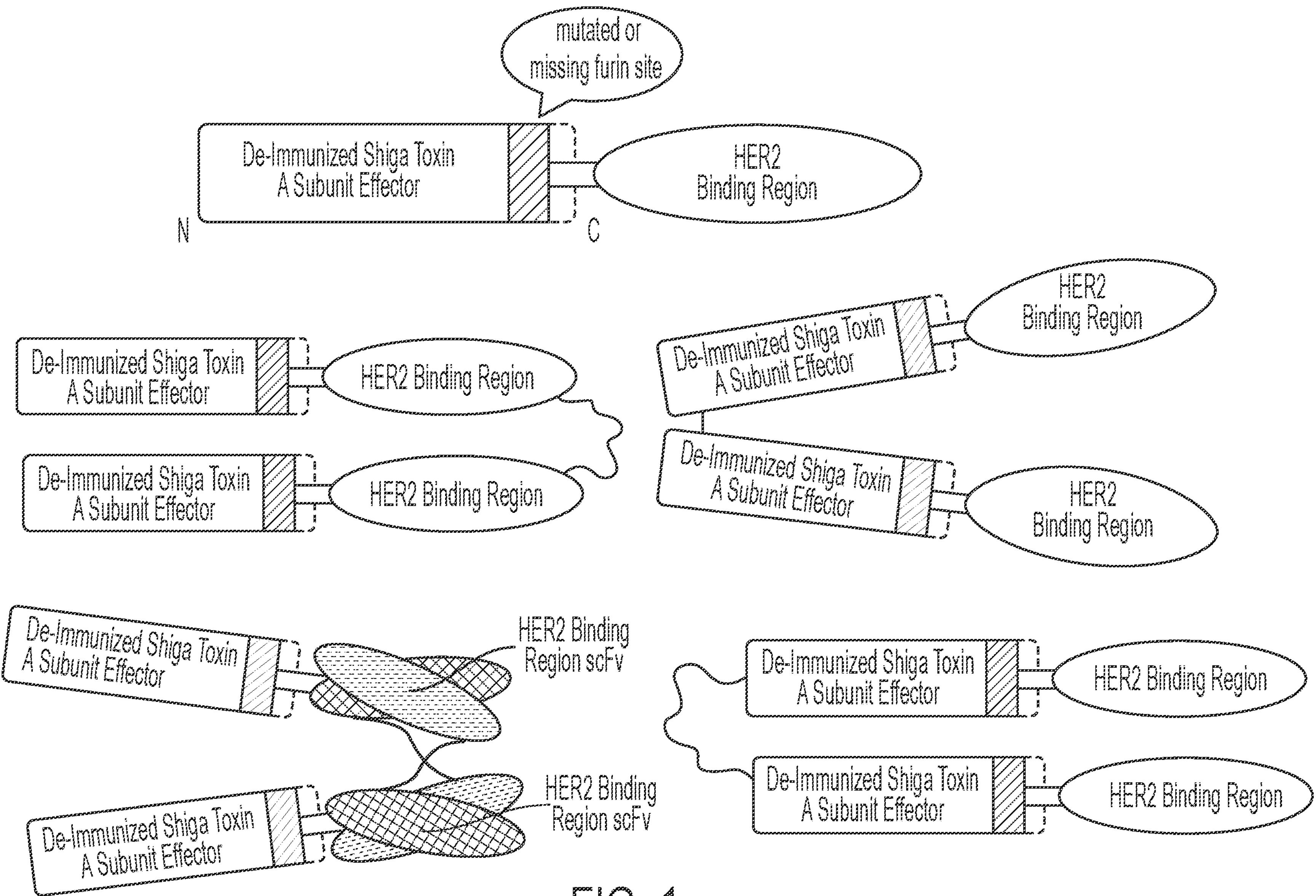
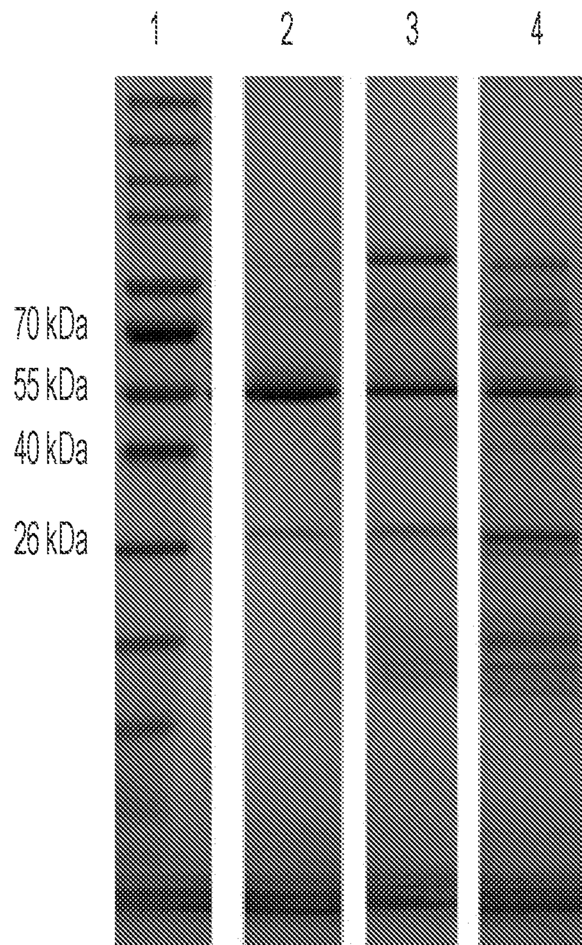


FIG. 1

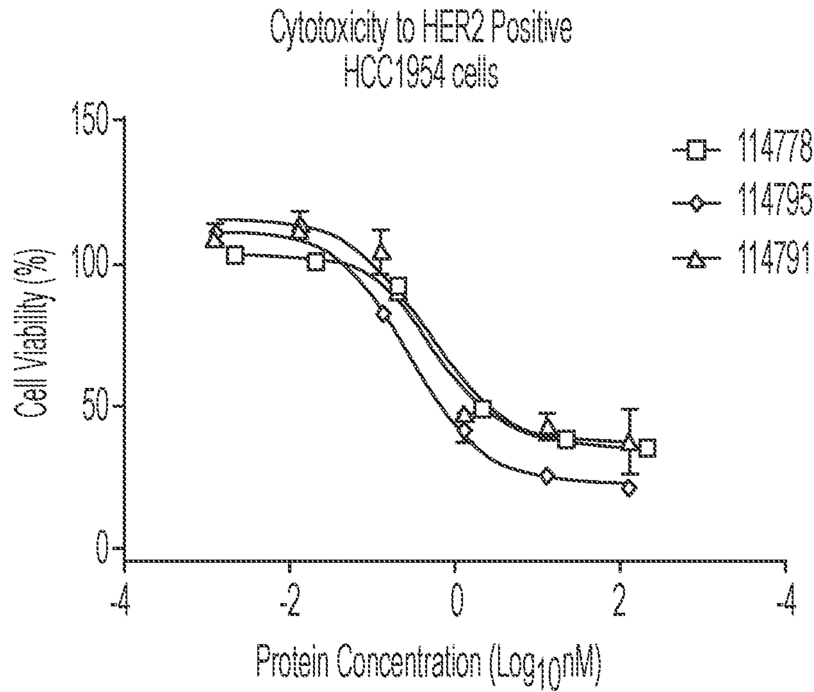
SDS-PAGE Analyses (Reducing Conditions) of Exemplary HER2-Targeting Molecules of the Present Invention Purified Using an Intein CBD Affinity Tag and Intein-Mediated Cleavage from a Chitin Resin Chromatography Column



Lane Descriptions:
1. Molecular Weight Marker (approximate size indicated)
2. 114778
3. 114795
4. 114791

FIG. 2

Cytotoxicities of Exemplary HER2-Targeting Molecules of the Present Invention Purified Using a CBD Affinity Tag and Intein-Mediated Cleavage from a Chitin Column



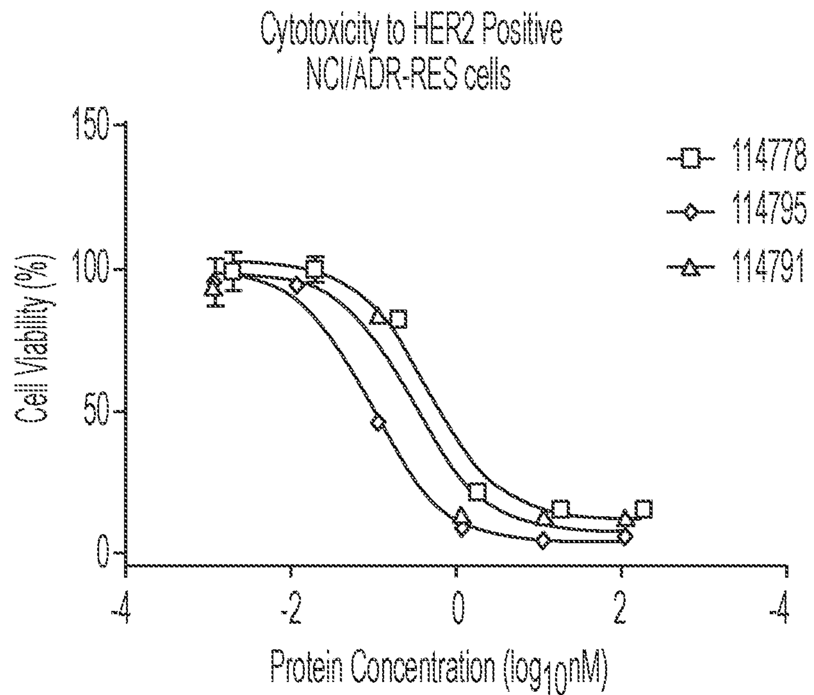
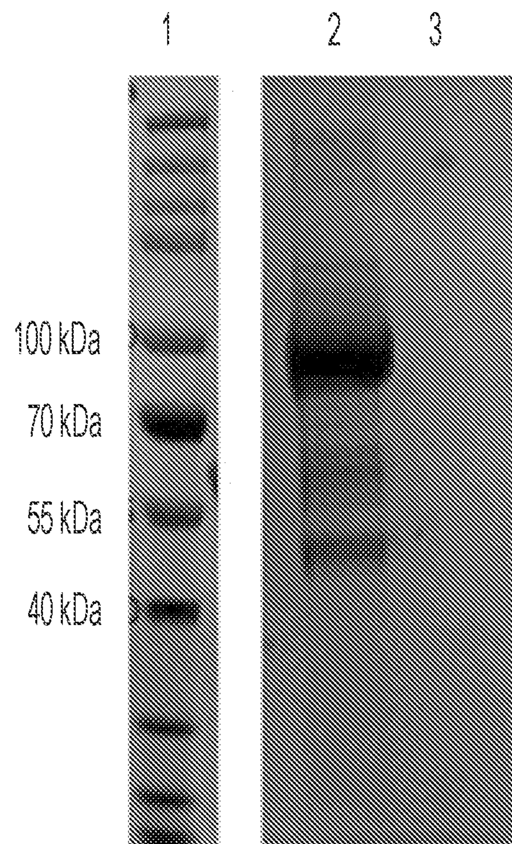


FIG. 3

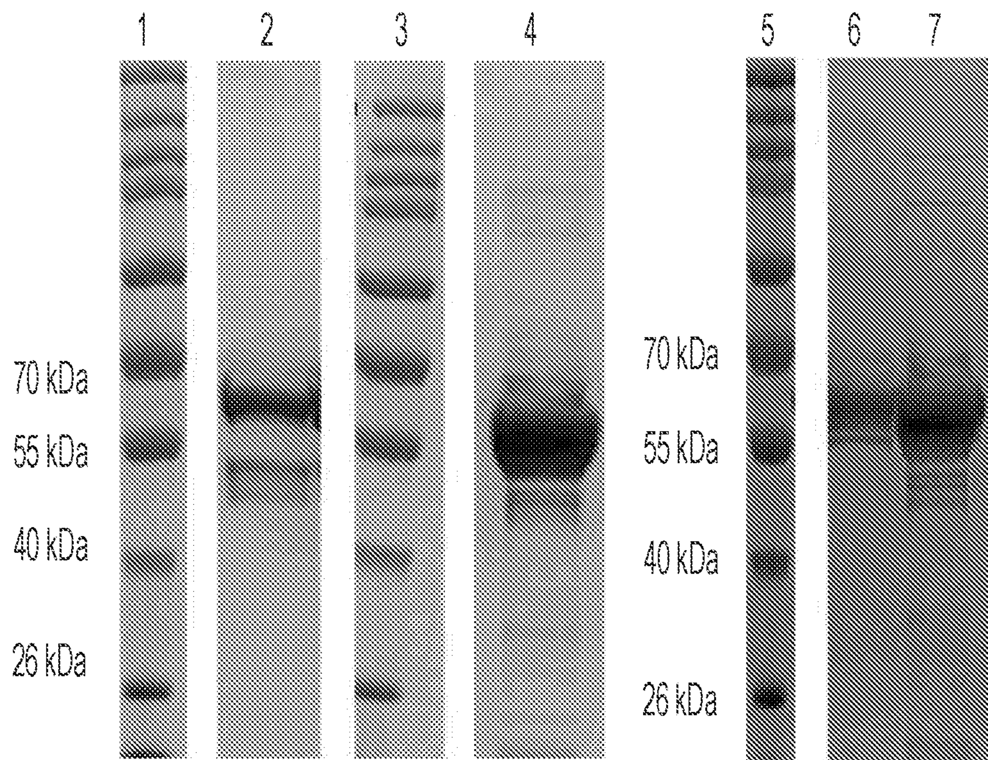
SDS-PAGE Analysis (Non-Reducing Conditions) of Exemplary HER2-Targeting Molecules of the Present Invention Produced with an Intein CBD Affinity Tag and Purified Using a Protein-L Affinity Resin



Lane Descriptions:
1. Molecular Weight Marker (approximate size indicated)
2. 114773
3. 114791

FIG. 4

SDS-PAGE Analyses (Non-Reducing Conditions) of Exemplary HER2-Targeting Molecules of the Present Invention Produced without a CBD Affinity Tag and Purified Using a Protein-L Affinity Resin



Lane Descriptions:
1. Molecular Weight Marker (approximate size indicated)
2. 114912
3. Molecular Weight Marker
4. 115111

Lane Descriptions:
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6. 115411
7. 115111

FIG. 5

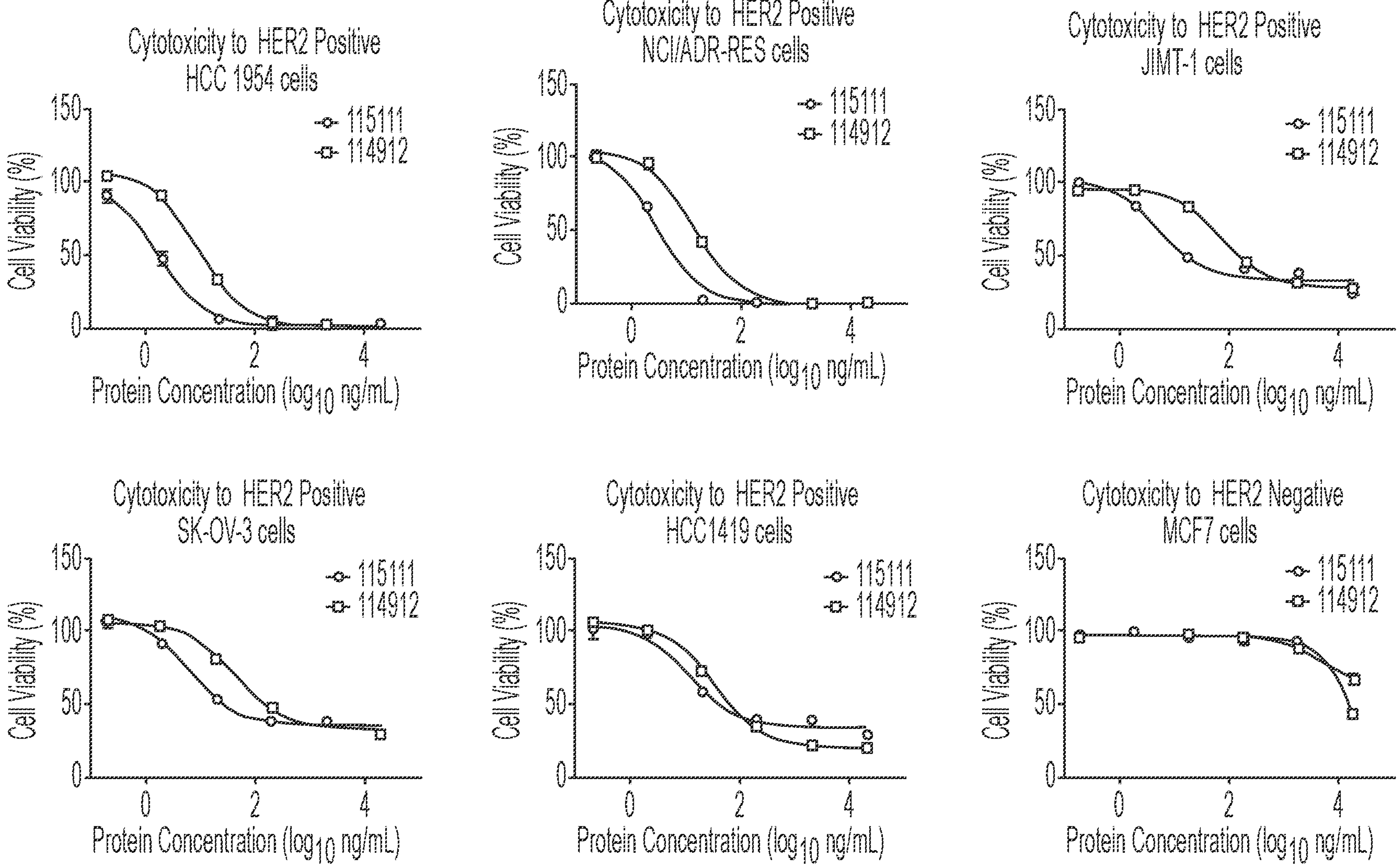
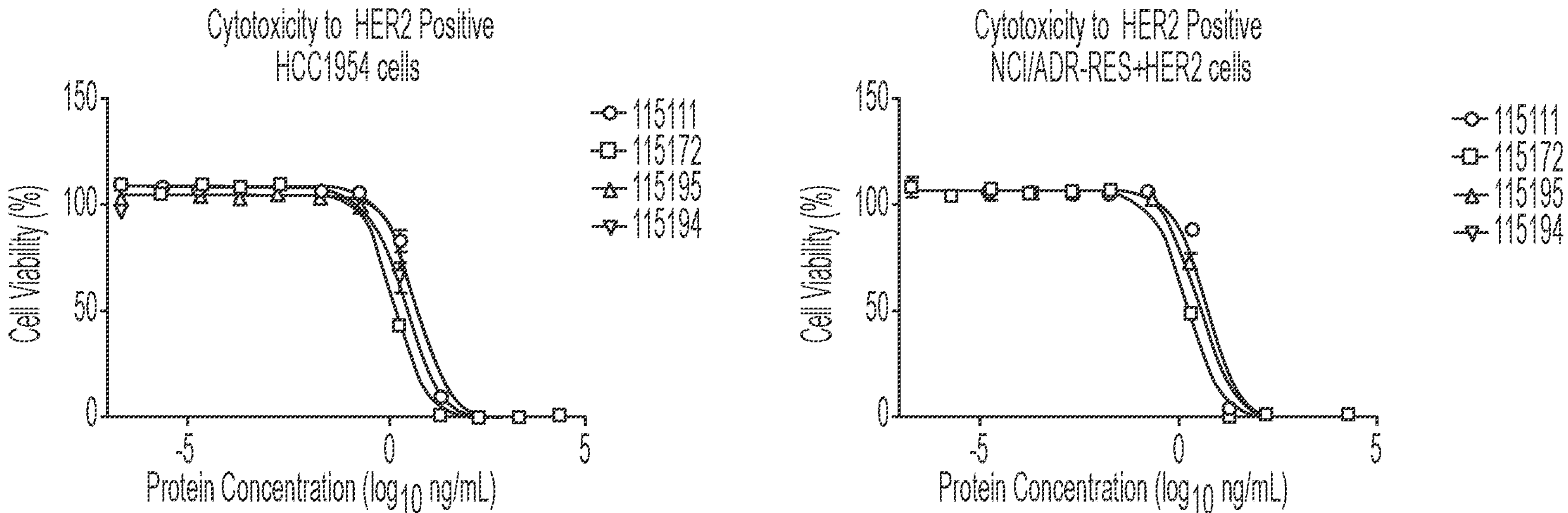


FIG. 6



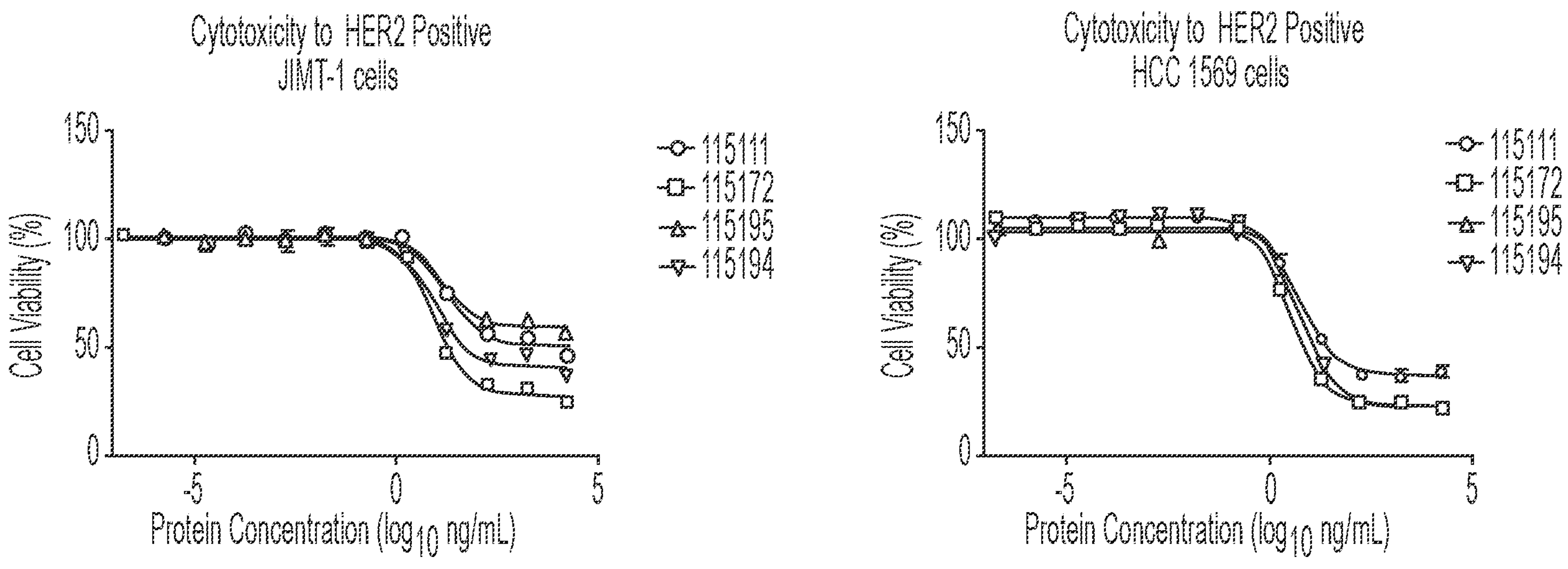


FIG. 7

Ribosome Inhibition by Exemplary HER2-Targeting Molecules of the Present Invention Representing Catalytic Activity

Protein Synthesis Inhibition Assay
Catalytic Activity

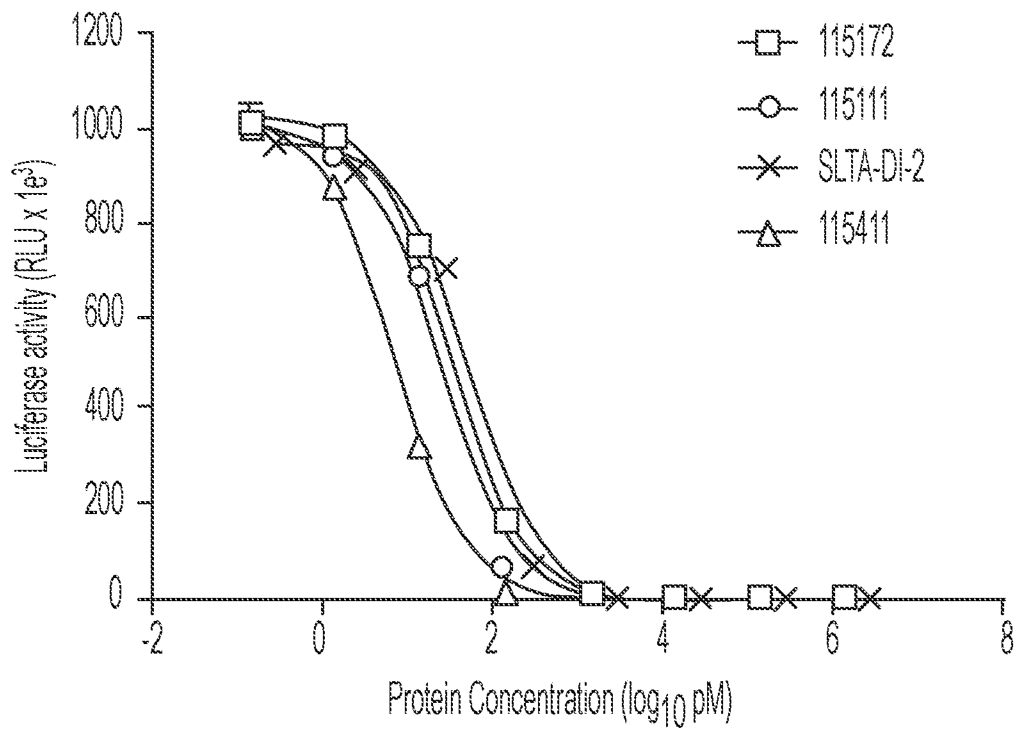


FIG. 8

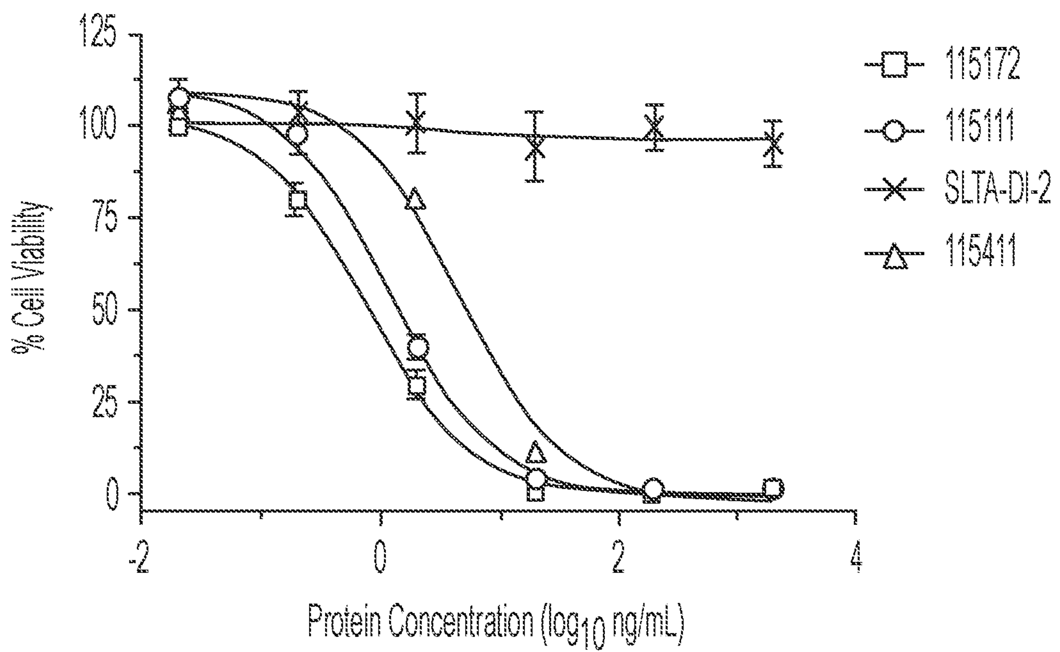


FIG. 9

Binding of Exemplary HER2-Targeting Molecules to HER2-Expressing Human Tumor Cells

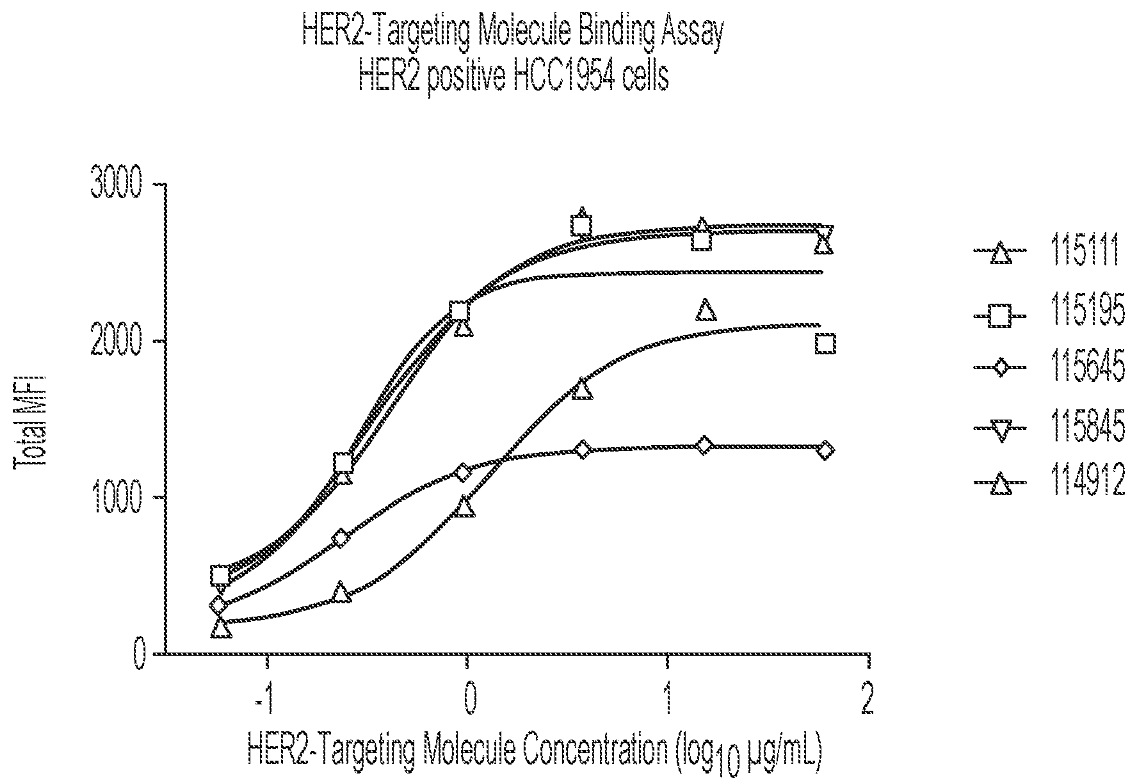


FIG. 10

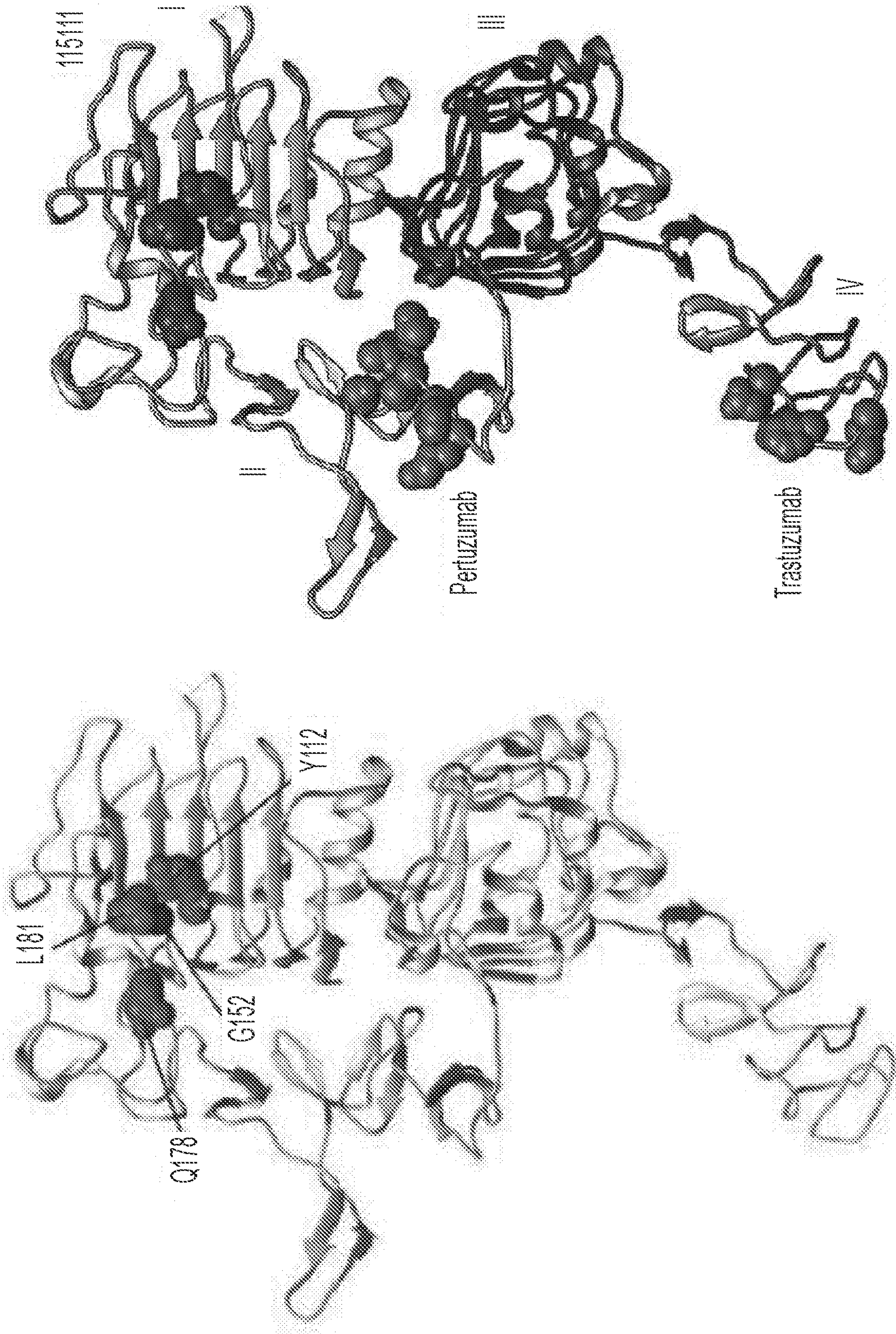


FIG. 11

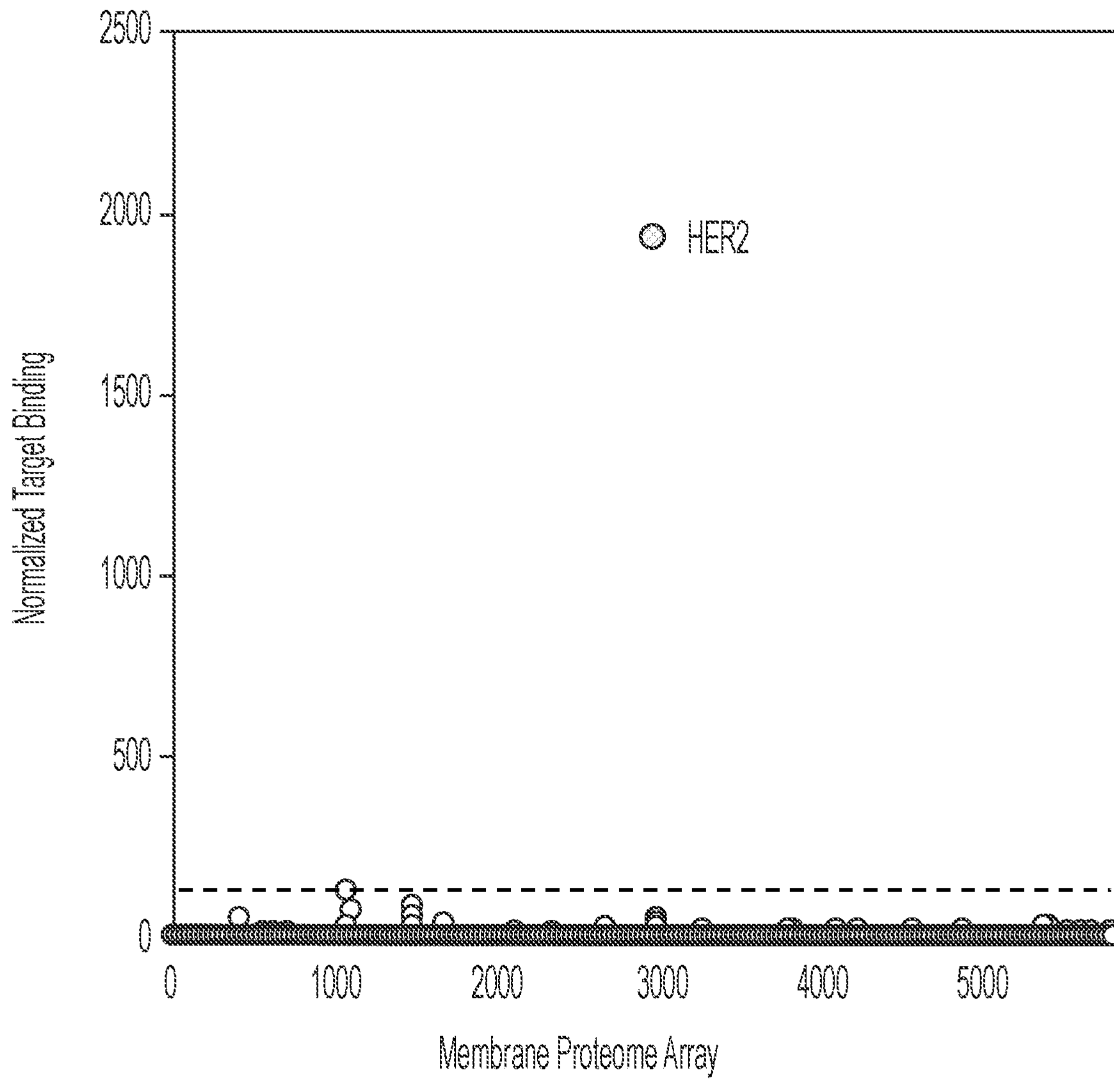


FIG. 12

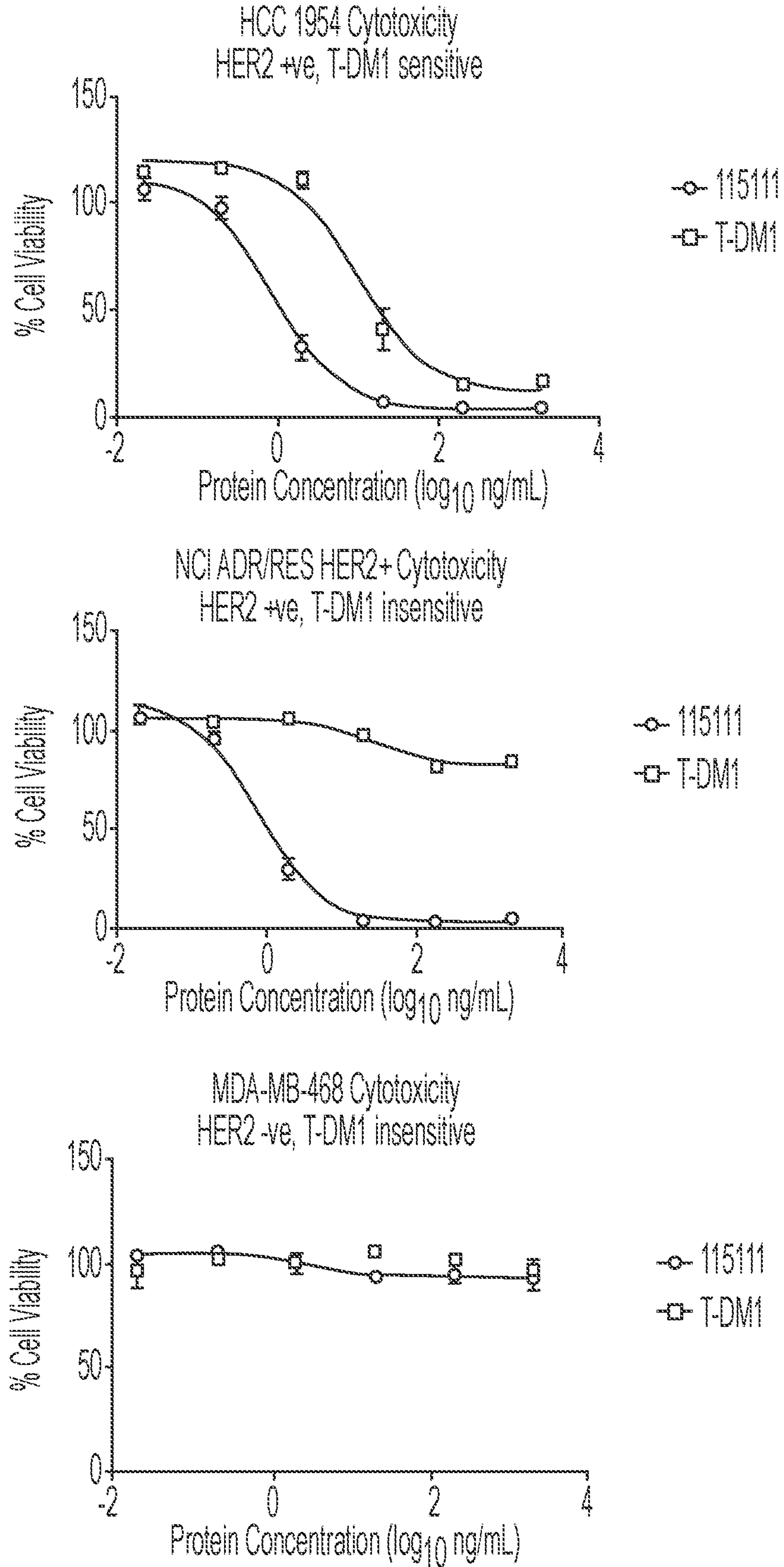


FIG. 13

Cytotoxicity of 115111 in Combination with Lapatinib

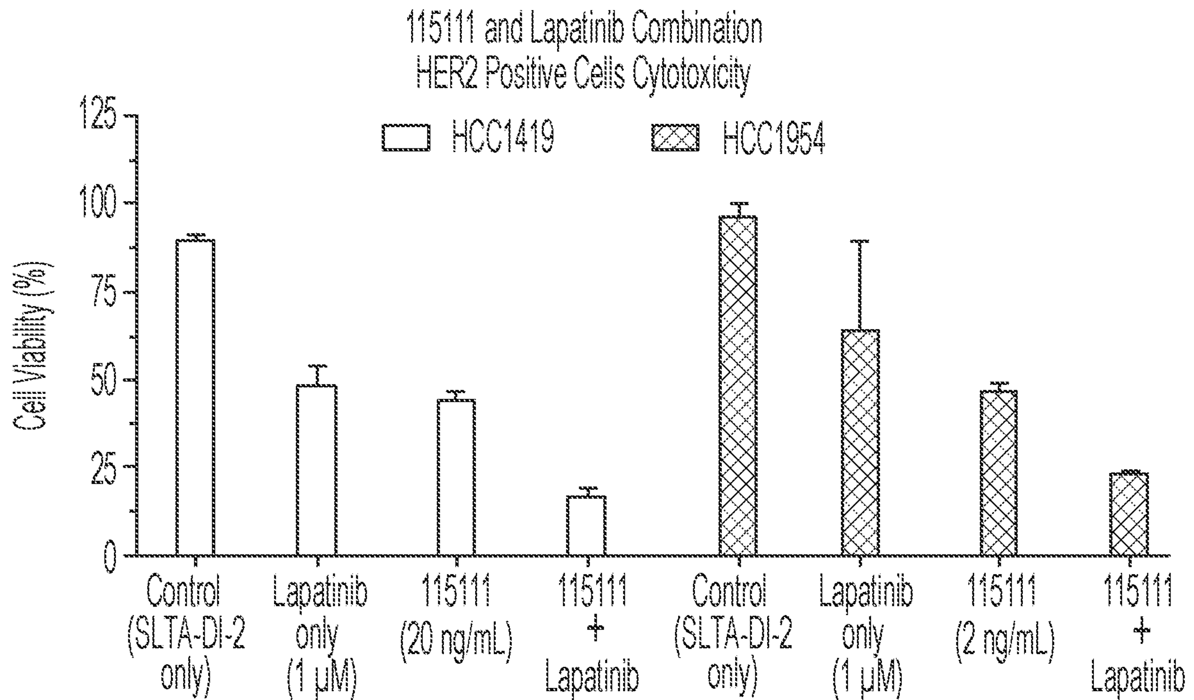


FIG. 14

Cytotoxicity of 115111 in Combination with T-DM1

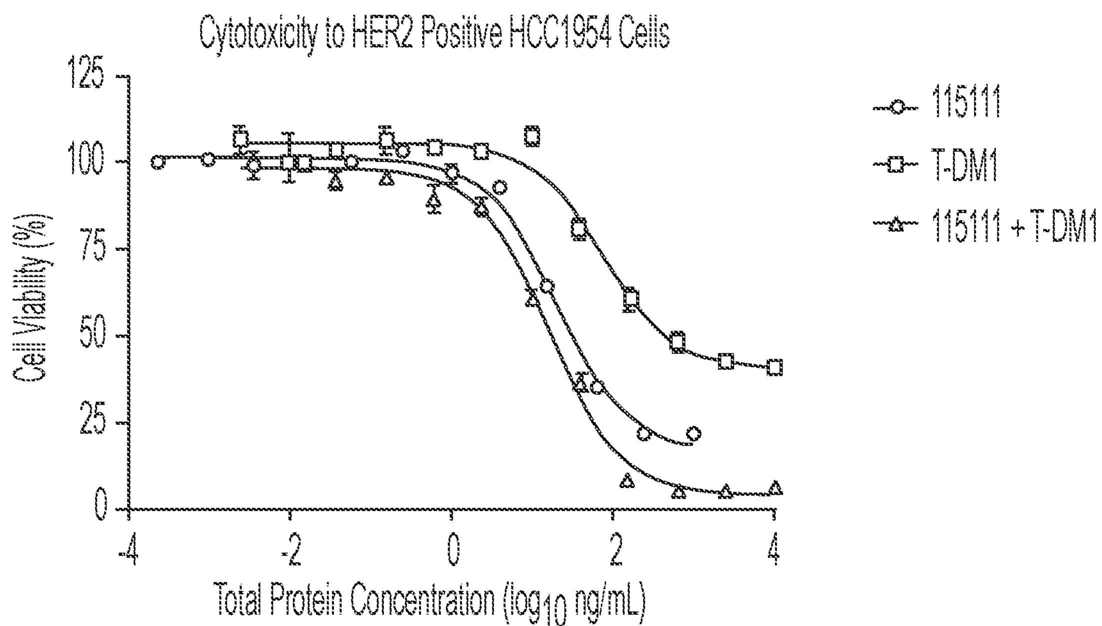


FIG. 15

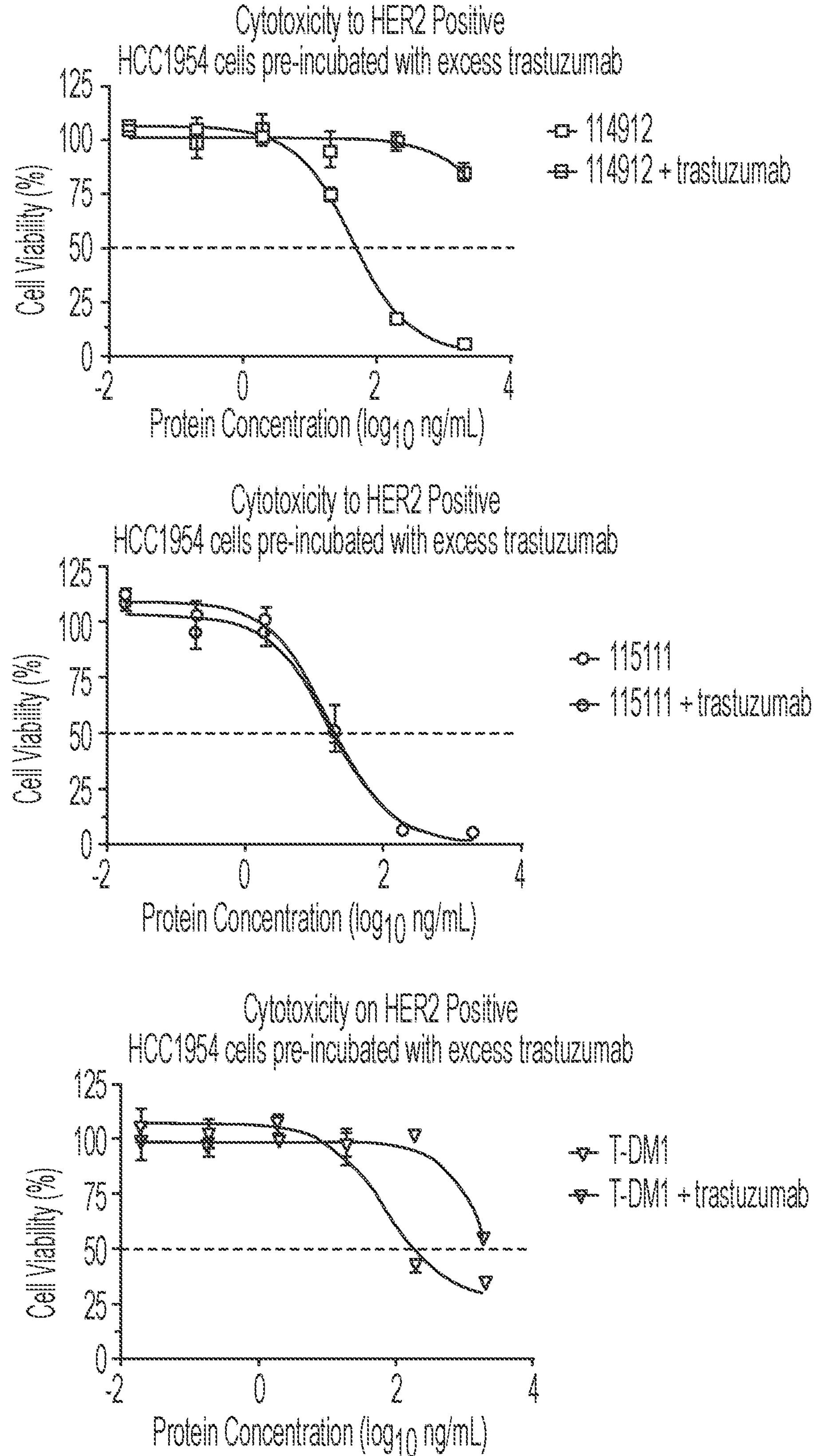
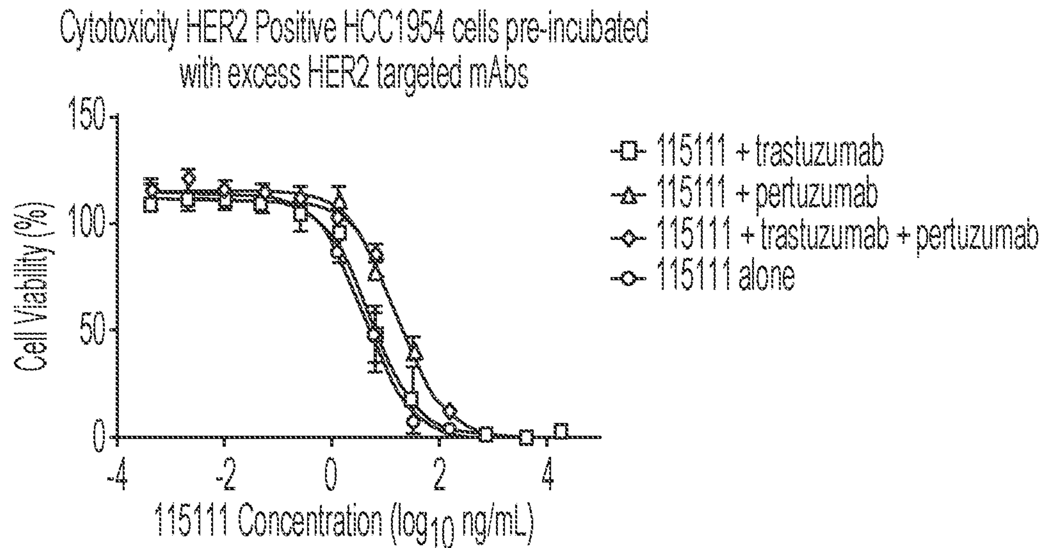


FIG. 16

Cytotoxicities of 115111 in the Presence of Excess Trastuzumab, Pertuzumab, or Excesses of Both Trastuzumab and Pertuzumab



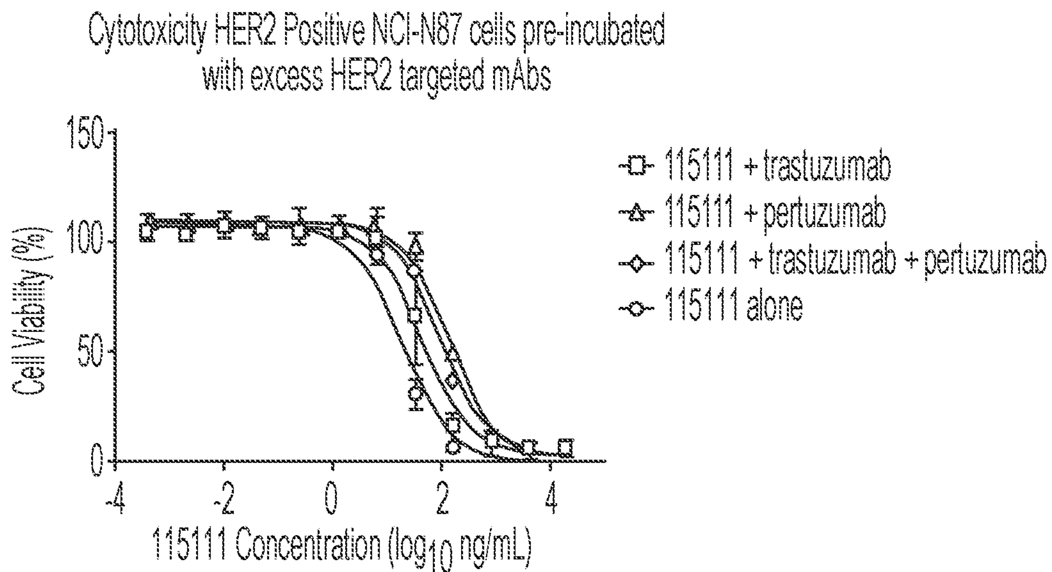


FIG. 17

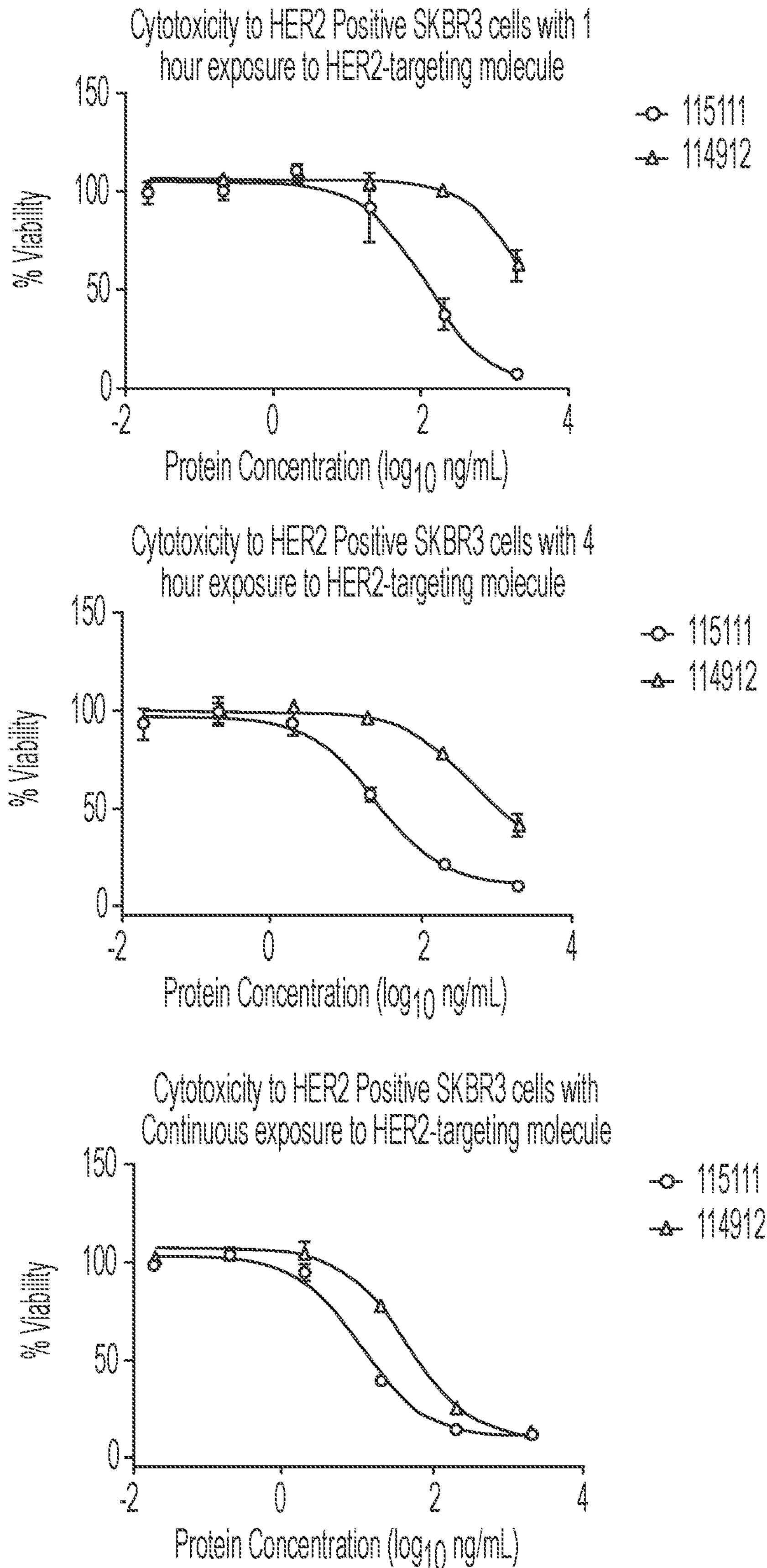
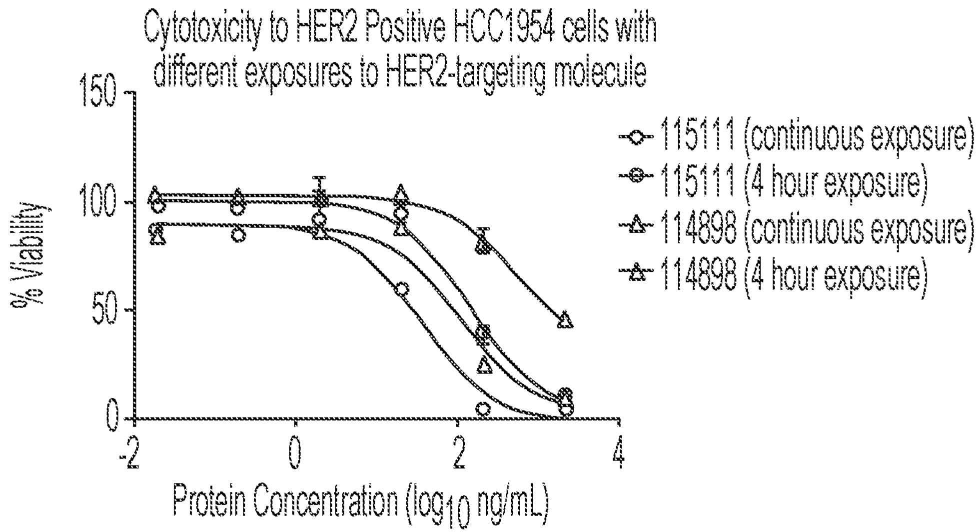
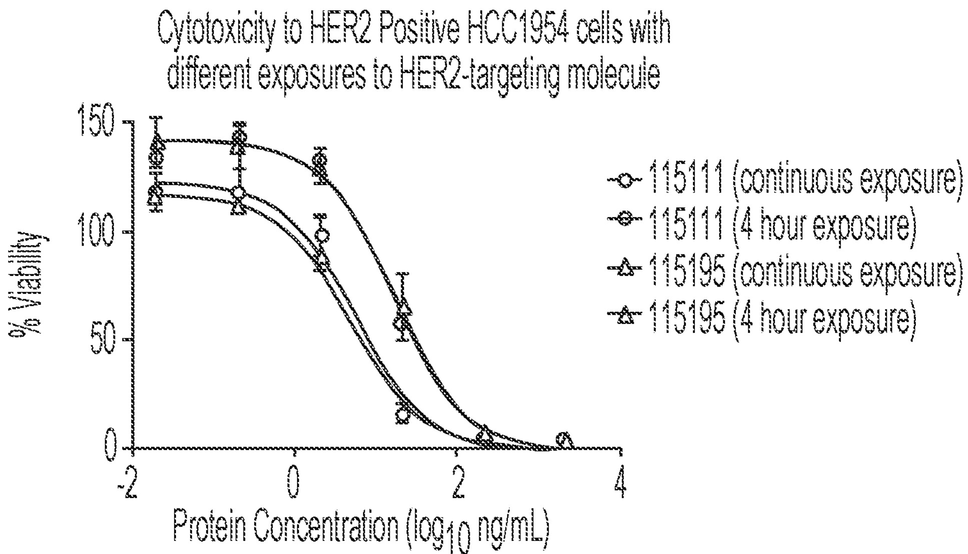


FIG. 18

Cytotoxicities of Exemplary HER2-Targeting Molecules of the Present Invention to HER2 Positive Cells after Different Durations of Exposure





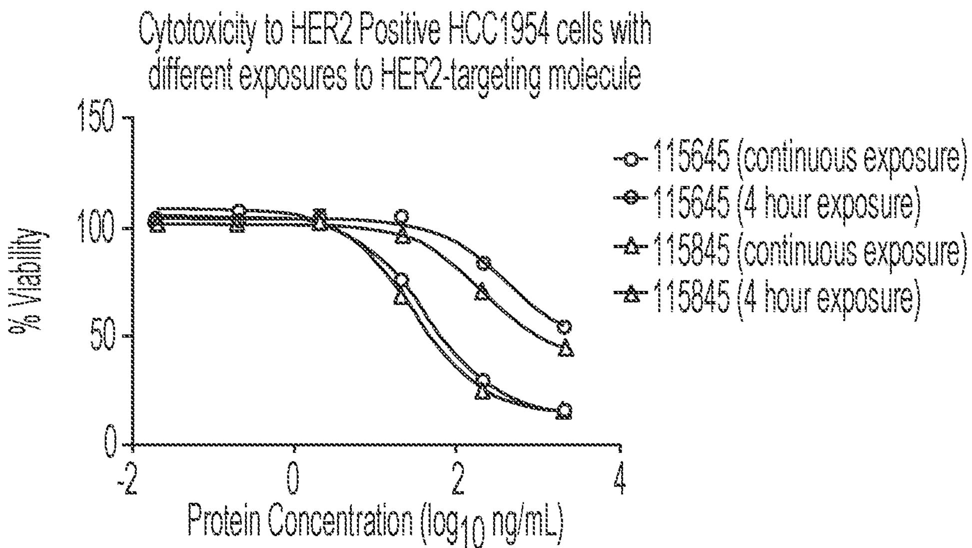
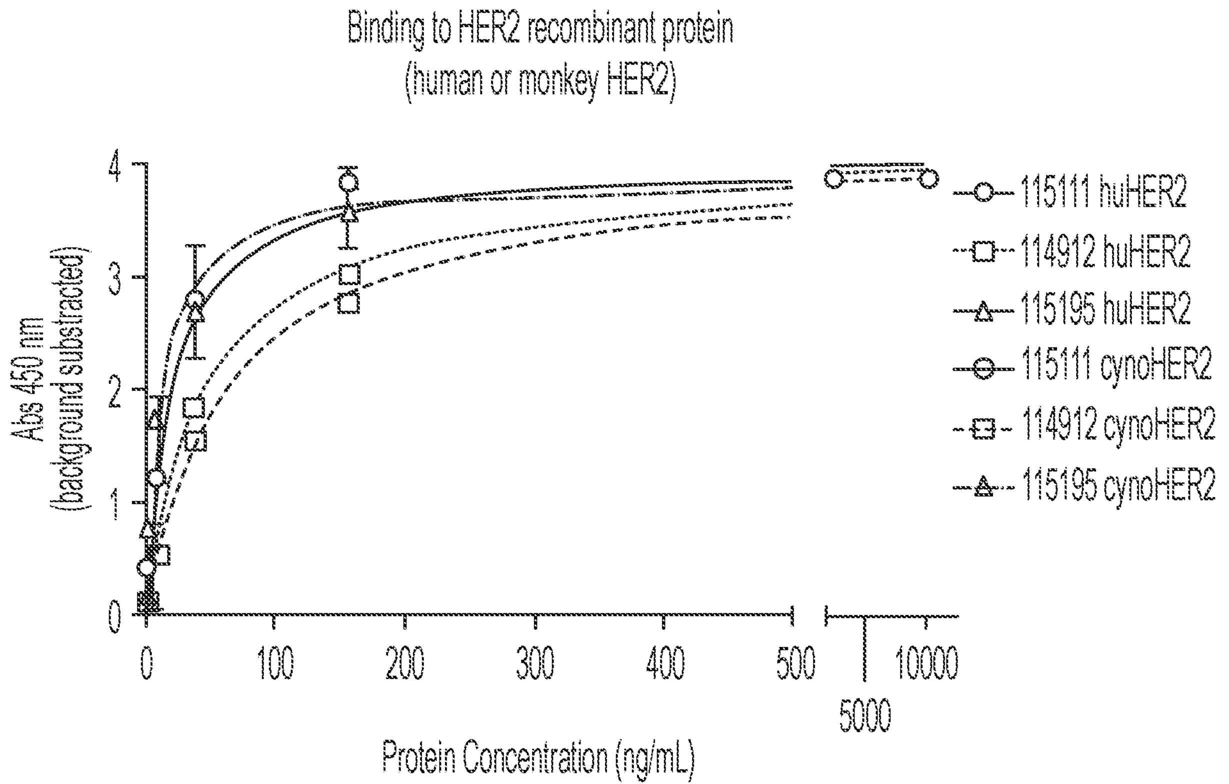


FIG. 19

In Vitro Binding of Exemplary HER2-Targeting Molecules of the Present Invention to Recombinant HER2 Proteins from Different Species



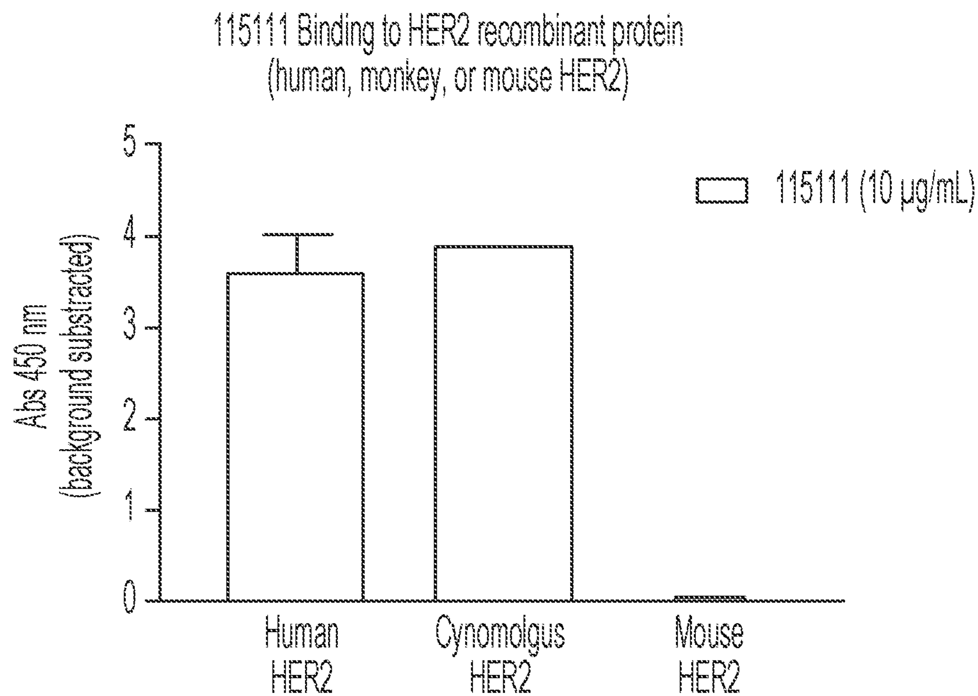


FIG. 20

115111 Is Tolerated by Immunocompetent Mice Better than other Exemplary HER2-Targeting Molecules after Repeated Dosing

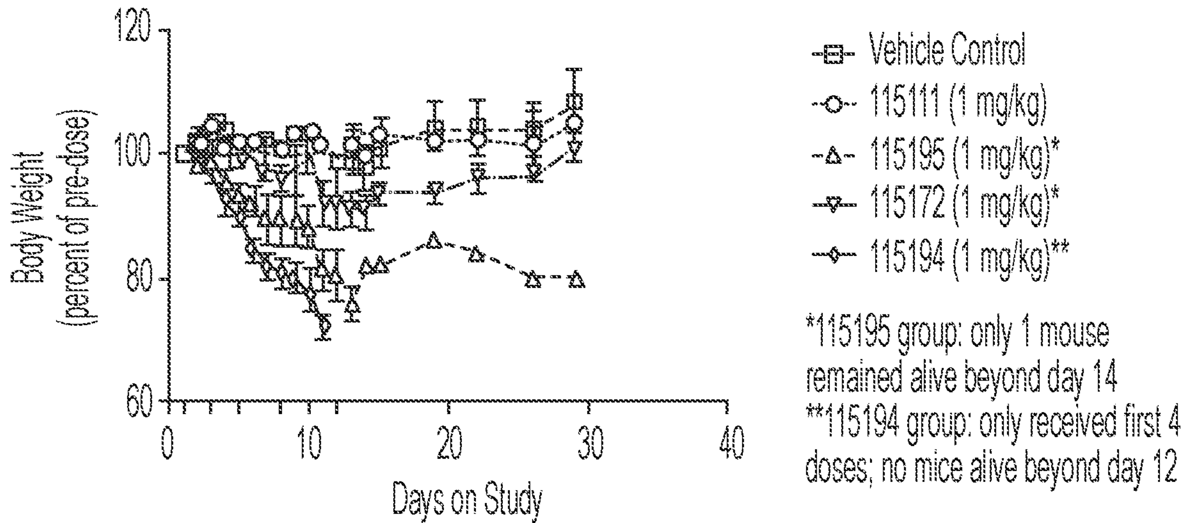


FIG. 21

115111 Is Tolerated by Immunocompetent Mice Better than 115172 after Repeated Dosing

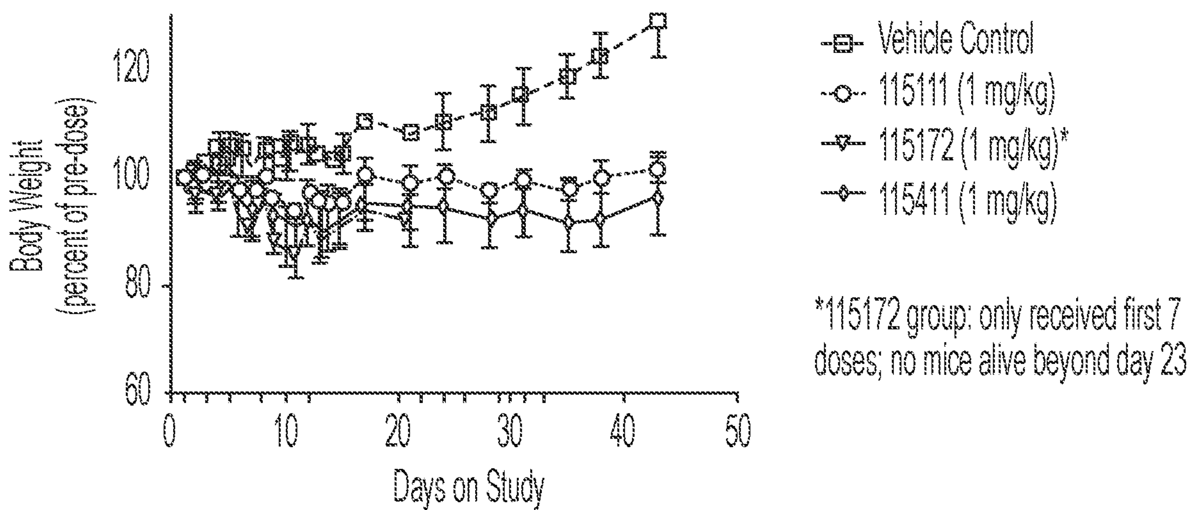


FIG. 22

Administration of 115111 with Repeated Dosing of Immunocompetent Mice Resulted in Lower Anti-Drug Antibody Levels than Administration of 115172

IgG ADA Comparison
BALB/C mice 0.25 mg/kg IP dosing

Percent of Average signal of WT control group
125
100
75
50
25
0
15 20 25 30 35
Day of study

-□- 115172 (0.25 mg/kg)
-○- 115111 (0.25 mg/kg)

Serum dilution increased over time. Signal normalized by setting the average signal for the 115172 treatment group to 100%

IgG ADA Comparison
BALB/C mice 1 mg/kg IV dosing

ELISA Signal (Abs 450 nm)
2
1
0
Day 22 Serum Collection

× Group 1: Vehicle control
□ Group 2: 115172 (1 mg/kg)
○ Group 3: 115111 (1 mg/kg)

FIG. 23

115111 Administration Exhibits Efficacy in an HCC 1954 Xenograft Mouse Model of Human Breast Cancer

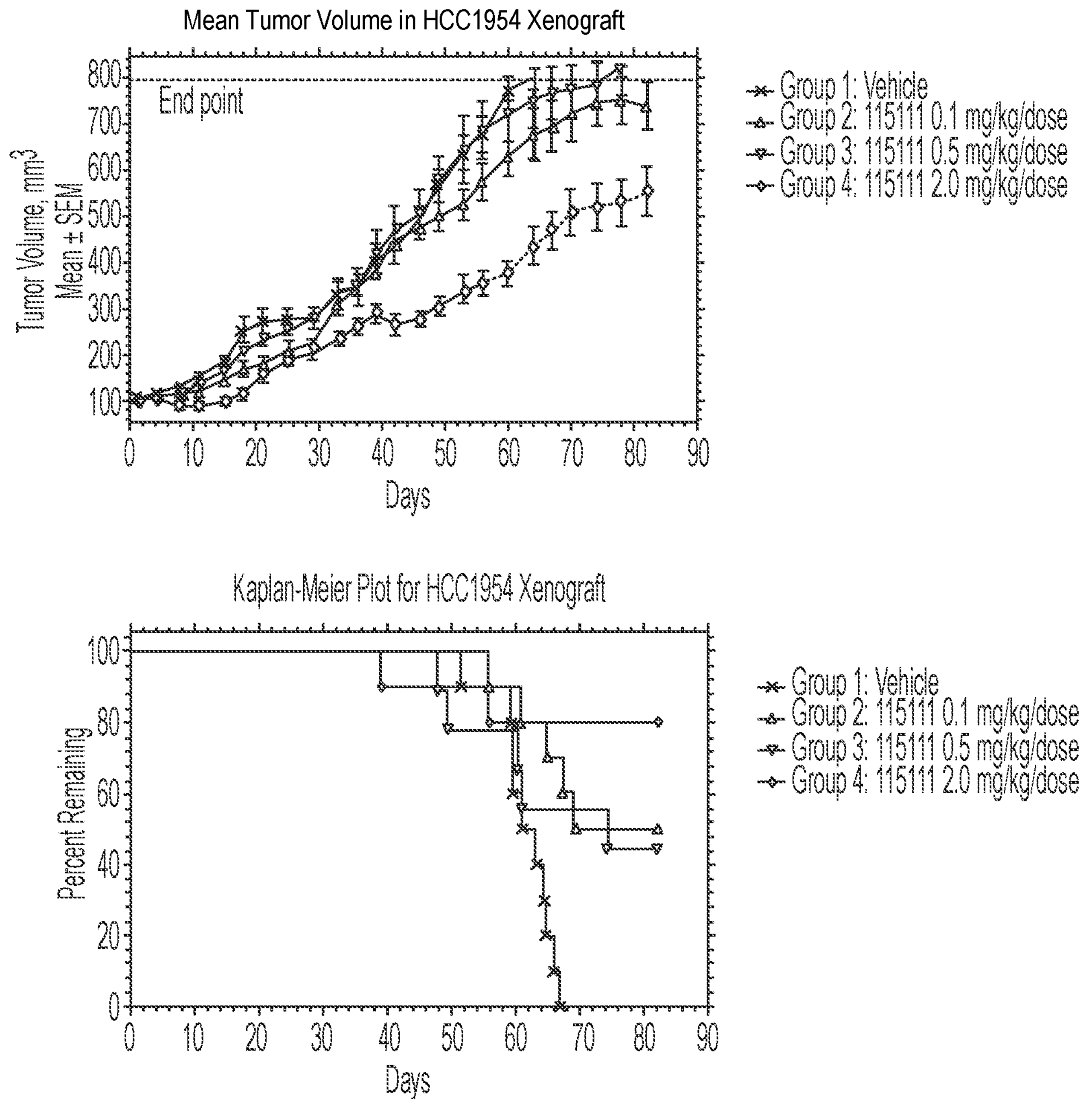


FIG. 24

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/027627

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 C07K16/32 C07K14/245 C07K14/25 C12N9/10
C12N9/24 C12N15/62 A61K47/68 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N C12R C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/113005 A1 (MOLECULAR TEMPLATES INC [US]) 30 July 2015 (2015-07-30) paragraph [0344] - paragraph [0446]; example 1 paragraph [0455] - paragraph [0461] paragraph [0473] - paragraph [0480]; example 2 paragraph [0522] - paragraph [0527]; example 7 -----	1-63
X	WO 2016/196344 A1 (MOLECULAR TEMPLATES INC [US]) 8 December 2016 (2016-12-08) paragraph [0547] - paragraph [0557] paragraph [0881] - paragraph [0892]; example 5 claims 1, 17-20 sequences 479, 511, 66, 454, 72, 38 ----- -/--	1-63

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2019	30/07/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Irion, Andrea

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/019623 A2 (MOLECULAR TEMPLATES INC [US]) 2 February 2017 (2017-02-02) sequences 57, 66, 36, 67 claims 1,8 paragraph [0450] -----	1-63
X,P	WO 2018/140427 A1 (MOLECULAR TEMPLATES INC [US]) 2 August 2018 (2018-08-02) the whole document -----	1-63
X,P	WO 2018/106895 A1 (MOLECULAR TEMPLATES INC [US]) 14 June 2018 (2018-06-14) the whole document -----	1-63

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/027627

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WO 2018106895 A1	14-06-2018	AU 2017373962 A1	30-05-2019
		CA 3043333 A1	14-06-2018
		WO 2018106895 A1	14-06-2018