



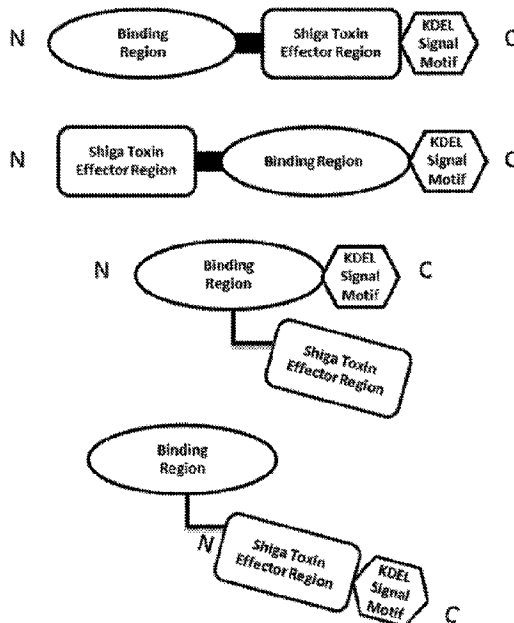
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(54) Titre : PROTEINES COMPORTANT DES REGIONS DE LIAISON, DES REGIONS EFFECTRICES A SOUS-MOTIFS A DE SHIGA-TOXINE, ET DES MOTIFS SIGNAL DE LOCALISATION DU RETICULUM ENDOPLASMIQUE CARBOXY-TERMINAUX

(54) Title: PROTEINS COMPRISING BINDING REGIONS, SHIGA TOXIN A SUBUNIT EFFECTOR REGIONS, AND CARBOXY-TERMINAL, ENDOPLASMIC RETICULUM LOCALIZATION SIGNAL MOTIFS

Schematic Drawing of the General Architecture of Exemplary Cytotoxic Proteins



(57) **Abrégé/Abstract:**

The present invention provides proteins comprising binding regions for cell-type specific targeting, Shiga toxin effector regions derived from A Subunits of members of the Shiga toxin family for providing Shiga toxin effector functions (e.g. cellular internalization

(57) **Abrégé(suite)/Abstract(continued):**

and cytotoxicity), and carboxy-terminal endoplasmic reticulum localization signal motifs. The presently disclosed proteins can comprise additional exogenous materials, such as, e.g., antigens, cytotoxic agents, and detection-promoting agents, and are capable of targeted delivery of these additional exogenous materials into the interiors of target cells. The proteins of the present invention have uses in methods such as, e.g., methods involving targeted killing of target cells, delivering exogenous materials into target cells, labeling subcellular compartments of target cells, and diagnosing and/or treating a variety of conditions including cancers, tumors, other growth abnormalities, immune disorders, and microbial infections.

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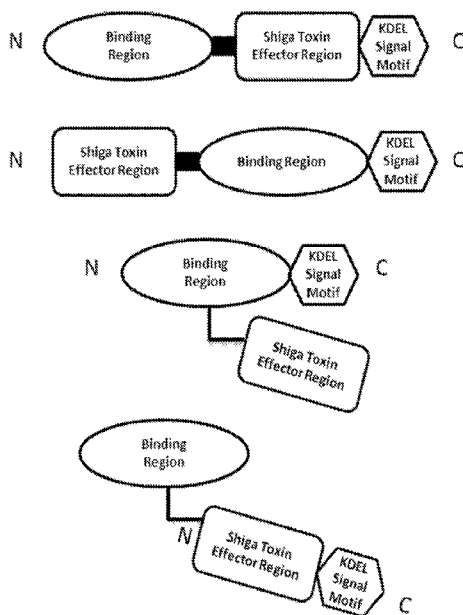
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[Continued on next page]

(54) Title: PROTEINS COMPRISING BINDING REGIONS, SHIGA TOXIN A SUBUNIT EFFECTOR REGIONS, AND CARBOXY-TERMINAL, ENDOPLASMIC RETICULUM LOCALIZATION SIGNAL MOTIFS

Figure 1. Schematic Drawing of the General Architecture of Exemplary Cytotoxic Proteins



(57) Abstract: The present invention provides proteins comprising binding regions for cell-type specific targeting, Shiga toxin effector regions derived from A Subunits of members of the Shiga toxin family for providing Shiga toxin effector functions (e.g. cellular internalization and cytotoxicity), and carboxy-terminal endoplasmic reticulum localization signal motifs. The presently disclosed proteins can comprise additional exogenous materials, such as, e.g., antigens, cytotoxic agents, and detection-promoting agents, and are capable of targeted delivery of these additional exogenous materials into the interiors of target cells. The proteins of the present invention have uses in methods such as, e.g., methods involving targeted killing of target cells, delivering exogenous materials into target cells, labeling subcellular compartments of target cells, and diagnosing and/or treating a variety of conditions including cancers, tumors, other growth abnormalities, immune disorders, and microbial infections.

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**PROTEINS COMPRISING BINDING REGIONS, SHIGA TOXIN A  
SUBUNIT EFFECTOR REGIONS, AND CARBOXY-TERMINAL,  
ENDOPLASMIC RETICULUM LOCALIZATION SIGNAL MOTIFS**

5 **FIELD OF THE INVENTION**

[1] The present invention relates to proteins comprising binding regions for mediating cell targeting, Shiga toxin effector regions, and carboxy-terminal endoplasmic reticulum-localization signal motifs. The proteins of this invention have uses, *e.g.*, for the selective killing of specific cell types, delivering  
10 exogenous materials inside target cells, labeling subcellular compartments of target cells, and as therapeutic molecules for the treatment of a variety of diseases, disorders, and conditions, including cancers, tumors, immune disorders, and microbial infections.

15 **BACKGROUND**

[2] The development of synthetic, fusion proteins from toxins that are effective as therapeutics has challenged scientists for decades (Pastan I et al., *Annu Rev Med* 58: 221-37 (2007)). One hurdle is that the enzymatic toxin moieties of synthetic cytotoxic proteins derived from bacterial and plant toxins  
20 must reach their cytosolic target substrates in order to kill cells. For many recombinant cytotoxic proteins, the potency of cytotoxicity depends on the molecule's efficiency in intracellular routing (Pirie C et al., *J Biol Chem* 286: 4165-72 (2011)); however, understanding how toxins direct their own intracellular transport from endosomes to the cytosol remains a challenge to  
25 scientific inquiry (Antignani A, Fitzgerald D, *Toxins* 5: 1486-502 (2013)).

[3] Naturally occurring toxins or truncated toxin fragments have been linked or fused to immunoglobulin domains or receptor ligands through chemical conjugation or recombinant protein engineering techniques with the hope of creating cell-targeted therapeutic molecules (Moolten F, Cooperband S, *Science*  
30 169: 68-70 (1970); Thorpe P et al., *Nature* 271: 752-5 (1978); Krolick K et al., *Proc Natl Acad Sci USA* 77: 5419-23 (1980); Krolick K et al., *Cancer Immunol Immunother* 12: 39-41 (1981); Blythman H et al., *Nature* 290: 145-46 (1981); Chaudhary V et al., *Nature* 339: 394-7 (1989); Strom T et al., *Semin Immunol* 2: 467-79 (1990); Pastan I et al., *Annu Rev Biochem* 61: 331-54 (1992); Foss F et

al., *Curr Top Microbiol Immunol* 234: 63-81 (1998)). One aim of such molecular engineering is to design chimeric molecules with the dual functionality of: 1) delivering toxins to specific cell types or places within an organism after systemic administration; and 2) effectuating a targeted cytotoxicity to specific cells using potent cytotoxicity mechanisms effective in eukaryotic cells.

[4] The Shiga toxin family of related protein toxins, notably toxins isolated from *S. dysenteriae* and *E. coli*, is composed of various naturally occurring toxins which are structurally and functionally related (Johannes L, Römer W, *Nat Rev Microbiol* 8: 105-16 (2010)). For example, the Shiga toxin family encompasses true Shiga toxin (Stx) isolated from *S. dysenteriae* serotype 1, Shiga-like toxin 1 variants (SLT1 or Stx1 or SLT-1 or Slt-1) 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 residue from Stx, and both have been referred to as Verocytotoxins or Verotoxins (VTs) (O'Brien A et al., *Curr Top Microbiol Immunol* 180: 65-94 (1992)). 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* 35:1431-37 (1997)). 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, *Nat Rev Microbiol* 8: 105-16 (2010)).

[5] Members of the Shiga toxin family are employed by bacteria as virulence factors during infection of a host (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)). In an infected host, Shiga toxins are cytotoxic because of the toxins' potent ability to inhibit protein synthesis and to trigger apoptotic cell death (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)). The potent cytotoxic effects of Shiga toxins on host cells can result in hemorrhagic colitis and hemolytic uremic syndrome (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)).

[6] Members of the Shiga toxin family share a common, multimeric, protein structure characterized by an A(B)<sub>5</sub> arrangement of Shiga protein subunits (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)). Each Shiga toxin is composed of two protein subunits, A and B, that associate in an A(B)<sub>5</sub> arrangement to form a holotoxin protein complex. The Shiga toxin A Subunit is a 32 kilodalton monomer that contains an enzymatic domain, and the Shiga toxin B Subunit is a 7.7 kilodalton subunit that associates with four other Shiga toxin B Subunits to form a pentamer of Shiga toxin B Subunits. The pentamer of B subunits associates with one A subunit to form the Shiga holotoxin, which is about 70 kilodaltons (O'Brien A, Holmes, R, *Microbiol Rev* 51: 206-20 (1987)).

[7] Members of the Shiga toxin family share a common process for the intoxication of a host cell that can be divided into five main phases: cell surface binding, endocytosis, retrograde subcellular movement to the endoplasmic reticulum, translocation from the endoplasmic reticulum to the cytosol, and the enzymatic inactivation of ribosomes in the cytosol. First, Shiga holotoxins are directed to the cellular surfaces of specific host cells by the B subunit's ability to specifically bind the glycosphingolipid globotriaosylceramide Gb<sub>3</sub>, also known as CD77, present on the exoplasmic membrane leaflet (Ling, H et al, *Biochemistry* 37: 1777-88 (1998); Thorpe C et al., *Infect Immun* 67: 5985-93 (1999); Soltyk A et al., *J Biol Chem* 277: 5351-59 (2002)). Second, Shiga holotoxins exploit the host cell's endocytotic machinery to enter into the host cell, where the holotoxins are initially contained within the endosomes (Sandvig K et al., *J Cell Biol* 108: 1331-43 (1989); Sandvig K et al., *Histochem Cell Biol* 117: 131-141 (2002)). Third, Shiga holotoxins exploit the host cell's intracellular-transport machinery to reach the endoplasmic reticulum and gain access to the cytosol (Nichols B et al., *J Cell Biol* 153: 529-41 (2001); Lauvrak S et al., *J Cell Sci* 117: 2321-31 (2004); Saint-Pol A et al., *Dev Cell* 6: 525-38 (2004)). Fourth, enzymatically active fragments of the Shiga holotoxins retrotranslocate from the lumen of the endoplasmic reticulum to the cytosol (Yu M, Haslam D, *Infect Immun* 73: 2524-32 (2005); LaPointe P et al., *J Biol Chem* 280: 23310-18 (2005); Falguieres T, Johannes L, *Biol Cell* 98: 125-34 (2006); Tam P, Lingwood C, *Microbiology* 153: 2700-10 (2007)). Fifth, the cytosolic fraction of Shiga toxin enzymatically active fragments causes cytotoxicity by inactivating host-cell ribosomes (Tam, *Microbiology* 153: 2700-10 (2007)).

[8] During the Shiga toxin intoxication process, the Shiga toxin A Subunit is proteolytically cleaved between a conserved arginine residue and a methionine residue (e.g. Arg251-Met252 in StxA and SLT-1A) by furin, a host cell endoprotease (Garred Ø et al., *J Biol Chem* 270: 10817-21 (1995)). The amino-terminal fragment of the furin-cleaved, Shiga-toxin A Subunit is called the Shiga toxin “A1 fragment” (or Stxn-A1, SLTn-A1, SLT-nA1). The Shiga toxin A1 fragment is a 28 kilodalton protein that contains the catalytic domain of the Shiga toxin (Fraser M et al., *Nat Struct Biol* 1: 59-64 (1994)). The mechanism of Shiga toxin cytotoxicity to host cells is predominantly through the A1 fragment’s potent catalytic inactivation of eukaryotic ribosomes and cell-wide inhibition of protein synthesis (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)).

[9] The Shiga toxin A1 fragment inhibits protein translation by its potent depurination activity towards a universally conserved adenine nucleobase at position 4,324 in the alpha-sarcin-ricin loop of the 28S ribosomal RNA of the eukaryotic ribosome (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)). After a threshold number of ribosomes is inactivated, the host cell is predicted to experience sufficient reduction in protein synthesis to induce cell death via apoptosis (Iordanov M et al., *Mol Cell Biol* 17: 3373-81 (1997); Smith W et al., *Infect Immun* 71: 1497-504 (2003); Lee S et al., *Cell Microbiol* 10: 770-80 (2008); Tesh V, *Future Microbiol* 5: 431-53 (2010)).

[10] The potency of A-B toxins is reported to be extremely high, such that as little as one toxin molecule can kill a cell (Yamaizumi M et al., *Cell* 15: 245-50 (1978); Antignani A, Fitzgerald D, *Toxins* 5: 1486-502 (2013)). A ribosome-inactivating A-B toxin can permanently cripple one ribosome after another within the same cell at a rate of approximately 1,500 ribosomes per minute (Endo Y, Tsurugi K, *Eur J Biochem* 171: 45-50 (1988); Endo Y et al., *J Biol Chem* 263: 8735-9 (1988)). The catalytic efficiency of this enzymatic reaction ( $K_{cat}/K_m$ ) is near the diffusion limit (Jasheway K et al., *Toxins* 3: 1233-48 (2011)). It is believed that a single molecule of A-B toxin can irreversibly inactivate 300 ribosomes in 35 minutes and is sufficient to kill a cancer cell (see Weldon J, Pastan I, *FEBS Journal* 278: 4683-700 (2011)). This level of cytotoxic potency is further predicted for the Shiga toxin A Subunit, for which it has been suggested that one molecule translocated into the cytosol would be sufficient to kill a cell (Tam, *Microbiology* 153: 2700-10 (2007)).

[11] Holotoxins of the Shiga toxin family are predicted to be too toxic for untargeted use as a therapeutic (Jain, R, Tumor physiology and antibody delivery, *Front Radiat Ther Oncol* 24: 32-46 (1990)). However, members of the Shiga toxin family have the potential to be synthetically engineered for therapeutic applications by rational alterations to the toxin's structure, characteristics, and biological activities (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010); Engedal, *Microbial Biotech* 4: 32-46 (2011)). Shiga holotoxins have a bipartite structure composed of two non-covalently attached, modular parts: an A-moiety containing the enzymatically active A1 fragment and a B-moiety containing binding sites to the cell-surface target Gb3. Because the Shiga toxin subunits are modular, it has been hypothesized that therapeutic compositions may be created based on the separate structures and functions of the A and B moieties (U.S. application 20090156417 A1; Johannes, *Nat Rev Microbiol* 8: 105-16 (2010); Engedal, *Microbial Biotech* 4: 32-46 (2011); E.P. application 2402367 A1; U.S. application 20130196928 A1.

[12] The A-moiety of members of the Shiga toxin family is stable, enzymatically active, and cytotoxic independent of any B-moiety (Engedal, *Microbial Biotech* 4: 32-46 (2011)). The Shiga toxin I A Subunit is catalytically active, capable of enzymatically inactivating ribosomes *in vitro*, and cytotoxic even if truncated or fused to other protein domains (Haddad J et al., *J Bacteriol* 175: 4970-8 (1993); Al-Jaufy A et al., *Infect Immun* 62: 956-60 (1994); Al-Jaufy A et al., *Infect Immun* 63: 3073-8 (1995); LaPointe, *J Biol Chem* 280: 23310-18 (2005); Di R et al., *Toxicon* 57: 525-39 (2011)). Shiga-like toxin I A Subunit truncations are catalytically active, capable of enzymatically inactivating ribosomes *in vitro*, and cytotoxic when expressed within a cell (LaPointe, *J Biol Chem* 280: 23310-18 (2005)).

[13] Members of the Shiga toxin family take advantage of eukaryotic cellular machinery to enter and reach the cytosol of intoxicated cells, where their cytotoxic effect is exerted. After cell entry, Shiga toxins utilize retrograde transport mechanisms to move from the endocytotic compartment to the lumen of the endoplasmic reticulum (Sandvig K et al., *J Cell Biol* 126: 53-64 (1994); Johannes L et al., *J Biol Chem* 272: 19554-61 (1997); Rapak A et al., *Proc Natl Acad Sci USA* 94: 3783-8 (1997)). Retrograde transport is the intracellular movement of proteins in the reverse direction of the more standard movement of

proteins during their production and secretion from eukaryotic cells (Pelham H et al., *Trends Cell Biol* 2: 183-5 (1992)). In a seminal study, Shiga toxins were observed in endocytotic compartments, the Golgi complex, the endoplasmic reticulum and the cytosol of intoxicated eukaryotic cells (Sandvig K et al.,  
5 *Nature* 358: 510-12 (1992)).

[14] Shiga holotoxins travel via retrograde transport from endocytotic compartments to the endoplasmic reticulum, where retrotranslocation of the Shiga toxin enzymatic fragment into the cytosol can occur. First, Shiga toxins exploit host cell endocytotic mechanisms to reach the trans-Golgi network  
10 (Sandvig, *J Cell Biol* 108: 1331-43 (1989); Sandvig K et al., *J Cell Biol* 113: 553-62 (1991)). Next, Shiga toxins travel from the Golgi complex to the endoplasmic reticulum (Simpson J et al., *J Biol Chem* 270: 20078-83 (1995)). Finally, Shiga toxins exploit endoplasmic reticulum machinery to penetrate into the cytosolic compartment via retrotranslocation (LaPointe, *J Biol Chem* 280:  
15 23310-18 (2005); Li S et al., *PLoS One* 7: e41119 (2012)). Once in the cytosol, a Shiga toxin A1 fragment can irreversibly cripple one eukaryotic ribosome after another via the A1 fragment's potent enzymatic activity (Tam P, *Microbiology* 153: 2700-10 (2007)).

[15] After endocytosis into a eukaryotic cell, soluble molecules like toxins are  
20 hypothesized to be sorted in the early endosomes to be 1) recycled for secretion, 2) degraded in the lysosomal compartment, or 3) retrogradely transported from endocytotic organelles to the Golgi complex (Bonifacino J, Hurley J, *Curr Opin Cell Biol* 20: 427-36 (2008); Pfeffer S, *FEBS Lett* 583: 3811-6 (2009); Varkouhi et al., *J Control Release* 151: 220-8 (2011)). Molecules can be shuttled from the  
25 cis-Golgi to the endoplasmic reticulum via vesicles, maturation of cisterns, or tubular connections (Jackson C, *J Cell Sci* 122: 443-52 (2009)). The exact mechanisms that traffic Shiga holotoxins to Golgi complex are not clear (Sandvig K et al., *Toxicon* 56: 1181-5 (2010)) but might involve clathrin, retrograde tubules, actin microfilaments, microtubules, and many other factors,  
30 such as annexins, dynamin, small GTPases, and syntaxins.

[16] Numerous Shiga toxin constructs have been shown to be cytotoxic, which shows that their enzymatic domains reach the cytosol of intoxicated cells to effectuate cell-kill (Haddad, *J Bacteriol* 175: 4970-8 (1993); Al-Jaufy, *Infect Immun* 62: 956-60 (1994); Al-Jaufy, *Infect Immun* 63: 3073-8 (1995); Su et al.,

*Protein Expr Purif* 66: 149-57 (2009); U.S. Patent 7,700,557). These Shiga toxin constructs are capable of self-directing their own intracellular routing to eventually delivery their enzymatic toxin fragments to the cytosol after retrotranslocating from the lumen of the endoplasmic reticulum. For example, a

5 Stx A Subunit fusion protein was shown to be cytotoxic when administered to cells, and the cytotoxicity depending on proper function of the Golgi complex, presumably for retrograde transport (Al-Jaufy, *Infect Immun* 63: 3073-8 (1995)).

[17] The mechanism(s) for retrograde transport of Shiga toxins neither rely on nor benefit from the KDEL-endoplasmic-reticulum-retention/retrieval system to

10 exert their cytotoxic effects (Johannes L et al., *J Biol Chem* 272: 19554-61 (1997); Girod A et al., *Nat Cell Biol* 1: 423-30 (1999); Jackson M et al., *J Cell Sci* 112: 467-75 (1999); Luna A et al., *Mol Biol Cell* 13: 866-79 (2002); Kano F et al., *J Cell Sci* 122: 2218-27 (2009)). The prior art teaches that Shiga toxins do not require any KDEL family signal motif to function as a cytotoxin. Unlike

15 other toxins such as *Pseudomonas* exotoxin and cholera toxin, Shiga toxins reach the endoplasmic reticulum via a retrograde transport pathway that does not involve the use of a signal motif of the KDEL family (Kano F et al., *J Cell Sci* 122: 2218-27 (2009)). Further, overexpressing KDEL receptors enhanced ricin cytotoxicity but did not alter Shiga toxin cytotoxicity (Jackson, *J Cell Sci* 112:

20 467-75 (1999)). Finally, treating cells with antibodies against the KDEL receptor to inhibit the KDEL retrieval system results in protection of cells against *Pseudomonas* exotoxin intoxication but has no effect on Shiga toxin intoxication (Kreitman R, Pastan I, *Biochem J* 307: 29-37 (1995)).

[18] Retrograde transport of Shiga toxins from endocytotic compartments to

25 the endoplasmic reticulum is completely independent of KDEL-receptors and not dependent on COPI-coated vesicles. This is in stark contrast to other toxins, such as *Pseudomonas* exotoxin with a REDLK signal motif at its carboxy-terminus, cholera toxin with a KDEL at its carboxy-terminus, and heat-labile toxin with a RDEL signal sequence at its carboxy-terminus (Chaudhary V et al.,

30 *Proc Natl Acad Sci USA* 87: 308-12 (1990); Lencer W et al., *J Cell Biol* 131: 951-62 (1995)). *Pseudomonas* exotoxin requires the KDEL-endoplasmic-reticulum-retention/retrieval system for intoxication of eukaryotic cells (Jackson, *J Cell Sci* 112: 467-75 (1999); Bard F et al., *J Biol Chem* 278, 46601-6 (2003)). *Pseudomonas* exotoxin intoxication requires empty KDEL-receptors in the Golgi

complex for retrograde transport and most likely requires the COPI-coated vesicle system as well. In addition, it was demonstrated that *Pseudomonas* exotoxin's cytotoxicity can be increased by mutating its RDEL to KDEL (Kreitman, *Biochem J* 307: 29-37 (1995)). Similarly, the addition of the KDEL  
5 signal motif to the carboxyl-terminus of ricin increase the cytotoxicity of a ricin construct, likely because the KDEL signal motif increases ricin's rate of entry into the cytosol (Wales R et al., *J Biol Chem* 268: 23986-90 (1993)). However, Shiga toxins differ from toxins that utilize the endoplasmic-reticulum-retention-system via KDEL-type, signal motifs located at their carboxy terminals.

10 [19] The lack of understanding how Shiga toxins direct intracellular routing inside intoxicated cells makes development of effective Shiga toxin-based therapeutics challenging.

[20] The idea of linking a toxin to a targeting domain to make a chimeric molecule that selectively kills cancer cells is not new (Strebhardt K, Ullrich A, *Nat Rev Cancer* 8: 473-80 (2008)). For example, since the 1970s immunotoxins  
15 have been developed using three, primary, toxin candidates: the bacterial diphtheria toxin, the bacterial *Pseudomonas* exotoxin, and plant toxins exemplified by ricin (Antignani, *Toxins* 5: 1486-502 (2013)). By 2013, however, these three toxins remained "among the top choices for immunotoxin  
20 development" (Antignani, *Toxins* 5: 1486-502 (2013)). To date, no one has described a cytotoxic protein comprising an amino acid sequence derived from a Shiga-toxin combined with a cell-targeting, binding region and an endoplasmic reticulum retrieval signal motif that was capable of specifically and selectively killing a targeted cell type.

25 [21] It would be desirable to have cell-targeting cytotoxic proteins comprising Shiga-toxin-Subunit-A derived regions that self-direct their own intracellular routing and display potent cytotoxicity for uses involving the targeted killing of specific cell types and for use as therapeutics in the treatment of a variety of diseases, such as, *e.g.*, cancers, tumors, immune disorders, and microbial  
30 infections. Thus, there remains a need in the art for ways of engineering cytotoxic proteins comprising Shiga toxin regions and cell-targeting binding regions that exhibit effective intracellular-routing to provide potent cytotoxicity.

## SUMMARY OF THE INVENTION

[22] The present invention provides various proteins comprising 1) binding regions, such as from immunoglobulins; 2) Shiga toxin effector regions, such as from SLT-1A; and 3) carboxy-terminal located, endoplasmic reticulum retention/retrieval signal motifs, such as KDEL (SEQ ID NO:34). The linking of  
5 binding regions with Shiga-toxin-Subunit-A-derived polypeptides enabled the engineering of cell-type specific targeting of Shiga toxin cytotoxicity, and the addition of carboxy-terminal signal motifs increased that cytotoxicity. The proteins of the invention have uses such as, *e.g.*, for targeted cell-killing, delivering exogenous materials, as diagnostic agents, and as therapeutic  
10 molecules for the treatment of a variety of diseases, disorders, and conditions, including cancers, tumors, growth abnormalities, immune disorders, and microbial infections.

[23] A protein of the present invention comprises (a) a binding region capable of specifically binding at least one extracellular target biomolecule, (b) a Shiga  
15 toxin effector region comprising a polypeptide derived from the amino acid sequence of the A Subunit of at least one member of the Shiga toxin family, and (c) a carboxy-terminal endoplasmic reticulum retention/retrieval signal motif of a member of the KDEL family.

[24] In certain embodiments, the protein of the present invention comprises  
20 the binding region comprising a polypeptide selected from the group consisting of: single-domain antibody (sdAb) fragment, nanobody, heavy-chain antibody domain derived from a camelid (V<sub>H</sub>H fragment), heavy-chain antibody domain derived from a cartilaginous fish, immunoglobulin new antigen receptor (IgNAR), V<sub>NAR</sub> fragment, single-chain variable fragment (scFv), antibody  
25 variable fragment (Fv), a complementary determining region 3 (CDR3) fragment, constrained FR3-CDR3-FR4 (FR3-CDR3-FR4) polypeptide, Fd fragment, small modular immunopharmaceutical (SMIP) domain, antigen-binding fragment (Fab), fibronectin-derived 10<sup>th</sup> fibronectin type III domain (10Fn3) (*e.g.* monobody), tenascin type III domain (*e.g.* TNfn3), ankyrin repeat  
30 motif domain(ARD), low-density-lipoprotein-receptor-derived A-domain (A domain of LDLR or LDLR-A), lipocalin (anticalin), Kunitz domain, Protein-A-derived Z domain, gamma-B crystalline-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.

[25] For certain embodiments, whereby administration of the protein of the present invention to a cell physically coupled with the extracellular target biomolecule of the protein's binding region, the protein is capable of causing death of the cell. In certain further embodiments, upon administration of the protein of the invention to two different populations of cell types which differ with respect to the presence or level of an extracellular target biomolecule, the protein is capable of causing cell death of the cell-types physically coupled with an extracellular target biomolecule of the cytotoxic protein's binding region at a  $CD_{50}$  that is at least three times less than the  $CD_{50}$  observed for cell types which are not physically coupled with an extracellular target biomolecule of the protein's binding region. For certain embodiments, whereby administration of the protein of the invention to a first population of cells whose members are physically coupled to extracellular target biomolecules of the protein'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-targeted 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 protein of the invention to a first population of cells whose members are physically coupled to a significant amount of the extracellular target biomolecule of the protein'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-targeted 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 protein of the 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 protein's binding region at a cellular surface, the cytotoxic effect of the protein to members of the first population of cells relative to members of the second population of cells is at least 3-fold greater.

[26] In certain embodiments, the binding region is designed or selected by its ability to bind the extracellular target biomolecule selected from the group consisting of: CD20, CD22, CD40, CD74, CD79, CD25, CD30, HER2/neu/ErbB2, EGFR, EpCAM, EphB2, prostate-specific membrane antigen, Cripto, CDCP1, endoglin, fibroblast activated protein, Lewis-Y, CD19, CD21, CS1/ SLAMF7, CD33, CD52, CD133, EpCAM, CEA, gpA33, mucin, TAG-72, tyrosine-protein kinase transmembrane receptor (ROR1 or NTRKR1), carbonic anhydrase IX, folate binding protein, ganglioside GD2, ganglioside GD3, ganglioside GM2, ganglioside Lewis-Y2, VEGFR, Alpha Vbeta3, Alpha5beta1, ErbB1/EGFR, Erb3, c-MET, IGF1R, EphA3, TRAIL-R1, TRAIL-R2, RANK, FAP, tenascin, CD64, mesothelin, BRCA1, MART-1/MelanA, gp100, tyrosinase, TRP-1, TRP-2, MAGE-1, MAGE-3, GAGE-1/2, BAGE, RAGE, NY-ESO-1, CDK-4, beta-catenin, MUM-1, caspase-8, KIAA0205, HPVE6, SART-1, PRAME, carcinoembryonic antigen, prostate specific antigen, prostate stem cell antigen, human aspartyl (asparaginy) beta-hydroxylase, EphA2, HER3/ErbB-3, MUC1, MART-1/MelanA, gp100, tyrosinase associated antigen, HPV-E7, Epstein-Barr virus antigen, Bcr-Abl, alpha-fetoprotein antigen, 17-A1, bladder tumor antigen, CD38, CD15, CD23, CD53, CD88, CD129, CD183, CD191, CD193, CD244, CD294, CD305; C3AR, FceRIa, galectin-9, mrp-14, programmed death-ligand 1 (PD-L1), siglec-8, siglec-10, CD49d, CD13, CD44, CD54, CD63, CD69, CD123, CD193, TLR4, FceRIa, IgE, CD107a, CD203c, CD14, CD15, CD33, CD64, CD68, CD80, CD86, CD105, CD115, F4/80, ILT-3, galectin-3, CD11a-c, GITRL, MHC Class I molecule (optionally complexed with a peptide), MHC Class II molecule (optionally complexed with a peptide), CD284-TLR4, CD107-Mac3, CD195-CCR5, HLA-DR, CD16/32, CD282-TLR2, CD11c, CD123, and any immunogenic fragment of any of the foregoing.

[27] In certain embodiments, the proteins of the present invention comprise the Shiga toxin effector region derived from amino acids 75 to 251 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3. In certain further embodiments, the protein of the present invention comprises the Shiga toxin effector region derived from amino acids 1 to 241 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3. In certain further embodiments, the Shiga toxin effector region is derived from amino acids 1 to 251 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

In certain further embodiments, the Shiga toxin effector region is derived from amino acids 1 to 261 of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3.

[28] In certain embodiments, the proteins of the present invention comprise the carboxy-terminal endoplasmic reticulum retention/retrieval signal motif

5 selected from the group consisting of: KDEL (SEQ ID NO:34), HDEF (SEQ ID NO:35), HDEL (SEQ ID NO:36), RDEF (SEQ ID NO:37), RDEL (SEQ ID NO:38), WDEL (SEQ ID NO:39), YDEL (SEQ ID NO:40), HEEF (SEQ ID NO:41), HEEL (SEQ ID NO:42), KEEL (SEQ ID NO:43), REEL (SEQ ID NO:44), KAEL (SEQ ID NO:45), KCEL (SEQ ID NO:46), KFEL (SEQ ID NO:47), KGEL (SEQ ID NO:48), KHEL (SEQ ID NO:49), KLEL (SEQ ID NO:50), KNEL (SEQ ID NO:51), KQEL (SEQ ID NO:52), KREL (SEQ ID NO:53), KSEL (SEQ ID NO:54), KVEL (SEQ ID NO:55), KWEL (SEQ ID NO:56), KYEL (SEQ ID NO:57), KEDL (SEQ ID NO:58), KIEL (SEQ ID NO:59), DKEL (SEQ ID NO:60), FDEL (SEQ ID NO:61), KDEF (SEQ ID NO:62), KKEL (SEQ ID NO:63), HADL (SEQ ID NO:64), HAEL (SEQ ID NO:65), HIEL (SEQ ID NO:66), HNEL (SEQ ID NO:67), HTEL (SEQ ID NO:68), KTEL (SEQ ID NO:69), HVEL (SEQ ID NO:70), NDEL (SEQ ID NO:71), QDEL (SEQ ID NO:72), REDL (SEQ ID NO:73), RNEL (SEQ ID NO:74), RTDL (SEQ ID NO:75), RTEL (SEQ ID NO:76), SDEL (SEQ ID NO:77), TDEL (SEQ ID NO:78), SKEL (SEQ ID NO:79), STEL (SEQ ID NO:80), and EDEL (SEQ ID NO:81).

[29] In certain embodiments, the protein of the present invention comprises the binding region comprising or consisting essentially of amino acids 2-241 of any one of SEQ ID NOs: 4-16.

25 [30] In certain embodiments, the protein of the present invention comprises or consists essentially of the polypeptide shown in any one SEQ ID NOs: 4-33.

[31] In certain embodiments, the proteins of the present invention comprise a Shiga toxin effector region which comprises a mutation relative to a naturally occurring A Subunit of a member of the Shiga toxin family that changes the enzymatic activity of the Shiga toxin effector region. In certain further  
30 embodiments, the mutation is selected from at least one amino acid residue deletion, insertion, or substitution that reduces or eliminates cytotoxicity of the Shiga toxin region. In certain further embodiments, the protein comprises a mutation which reduces or eliminates catalytic activity but retains other Shiga

toxin effector functions, such as, *e.g.*, promoting cellular internalization and/or directing intracellular routing. In certain embodiments, the mutation 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  
5 ID NO:2, or SEQ ID NO:3.

[32] The present invention also provides pharmaceutical compositions comprising a protein of the present invention and at least one pharmaceutically acceptable excipient or carrier; and the use of such a protein or a composition comprising it in the methods of the invention as further described herein.  
10 Certain embodiments of the present invention are pharmaceutical compositions comprising any protein of the present invention and at least one pharmaceutically acceptable excipient or carrier.

[33] Among certain embodiments of the present invention is a diagnostic composition comprising a protein of the invention further comprising a detection  
15 promoting agent for the collection of information, such as diagnostically useful information about a cell type, tissue, organ, disease, disorder, condition, and/or patient.

[34] Beyond the proteins of the present invention and compositions thereof, polynucleotides capable of encoding a protein of the invention are within the  
20 scope of the present invention, as well as expression vectors which comprise a polynucleotide of the invention and host cells comprising an expression vector of the invention. Host cells comprising an expression vector may be used, *e.g.*, in methods for producing a protein of the invention or a polypeptide component or fragment thereof by recombinant expression.

[35] The invention further provides a system for conferring improved cytotoxicity to a protein comprising the step of adding a carboxy-terminal endoplasmic reticulum retention/retrieval signal motif of a member of the KDEL family to a carboxy terminus of the protein or polypeptide component of the protein, wherein the protein comprises (a) a binding region capable of  
25 specifically binding at least one extracellular target biomolecule and (b) a Shiga toxin effector region comprising a polypeptide derived from the amino acid sequence of the A Subunit of at least one member of the Shiga toxin family.  
30

[36] Additionally, the present invention provides methods of killing cell(s) comprising the step of contacting a cell(s) with a protein of the invention or a

pharmaceutical composition comprising a protein of the 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 further  
embodiments of the cell killing methods, the method is capable of selectively  
5 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 protein.

[37] The present invention further provides methods of treating diseases,  
10 disorders, and/or conditions in patients comprising the step of administering to a  
patient in need thereof a therapeutically effective amount of a protein or a  
pharmaceutical composition of the invention. In 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  
15 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,  
20 skin cancer, and uterine cancer. In 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 diseases,  
25 lupus erythematosus, multiple sclerosis, polyarteritis nodosa, polyarthritis,  
psoriasis, psoriatic arthritis, rheumatoid arthritis, scleroderma, septic shock,  
Sjorgren's syndrome, ulcerative colitis, and vasculitis.

[38] The use of any composition of the present invention for the treatment or  
prevention of a cancer, tumor, growth abnormality, and/or immune disorder is  
30 within the scope of the present invention. Among certain embodiments of the  
present invention is a protein 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 protein of the 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.

5 [39] Certain embodiments of the proteins of the invention may be utilized for the delivery of additional exogenous material into a cell physically coupled with an extracellular target biomolecule of the protein of the 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 protein, pharmaceutical composition, and/or diagnostic composition of the present invention. The present invention further provides a method for  
10 delivering exogenous material to the inside of a cell(s) in a patient, the method comprising the step of administering to the patient a protein of the present invention (with or without cytotoxic activity), wherein the target cell(s) is physically coupled with an extracellular target biomolecule of the protein.

15 [40] The use of any composition 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. Among certain embodiments of the present invention is a method of using a protein of the invention comprising a detection promoting agent and/or composition of the invention (*e.g.* a diagnostic  
20 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 using a protein and/or diagnostic composition of the invention comprising the steps of contacting a cell with the protein and/or diagnostic composition and detecting  
25 the presence of said cell-targeted 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  
30 embodiments, the method involves the detection of the location of the protein in an organism using one or more imaging procedures after the administration of the protein to said organism. For example, proteins 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 invention. For example, certain compounds (*e.g.* proteins) of the invention, compositions (*e.g.* pharmaceutical compositions and diagnostic compositions) of the invention, and methods of the invention may be used to determine if a patient belongs to a group that responds to a pharmaceutical composition of the invention.

[41] Among certain embodiments of the present invention are kits comprising a composition of matter of the invention, and optionally, instructions for use, additional reagent(s), and/or pharmaceutical delivery device(s).

[42] 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 combination or removal hereinafter.

15

#### **BRIEF DESCRIPTION OF THE FIGURES**

[43] **Figure 1** shows the general arrangement of the proteins of the invention with “N” and “C” denoting an amino-terminus and carboxy-terminus, respectively, of the protein or a polypeptide component of the protein.

[44] **Figure 2** graphically shows  $\alpha\text{HER2scFv}::\text{SLT-1A}::\text{KDEL}$  exhibited improved target cell-type specific cytotoxicity as compared to  $\alpha\text{HER2scFv}::\text{SLT-1A}$  which lacked a KDEL signal motif. The percent viability of cells was plotted over the logarithm to base 10 of the cytotoxic protein concentration.

[45] **Figure 3** shows microscopy images of the subcellular localization of  $\alpha\text{HER2scFv}::\text{SLT-1A}$  and  $\alpha\text{HER2scFv}::\text{SLT-1A}::\text{KDEL}$ . The images showed both cytotoxic proteins entered target cells within one hour of administration.

[46] **Figure 4** graphically shows improved target cell-type specific cytotoxicity of the protein  $\alpha\text{CD38scFv}::\text{SLT-1A}::\text{KDEL}$  compared to the protein  $\alpha\text{CD38scFv}::\text{SLT-1A}$  which lacked a KDEL signal motif. The percent viability of cells was plotted over the logarithm to base 10 of the cytotoxic protein concentration.

[46a] **Figure 5** graphically shows improved target cell-type specific cytotoxicity of the protein  $\alpha\text{CD19scFv}::\text{SLT-1A}::\text{KDEL}$  compared to the protein

$\alpha$ CD19scFv::SLT-1A which lacked a KDEL signal motif. The percent viability of cells was plotted over the logarithm to base 10 of the cytotoxic protein concentration.

[46b] **Figure 6** graphically shows improved target cell-type specific cytotoxicity of the protein  $\alpha$ CD74scFv::SLT-1A::KDEL compared to the protein  $\alpha$ CD74scFv::SLT-1A which lacked a KDEL signal motif. The percent viability of cells was plotted over the logarithm to base 10 of the cytotoxic protein concentration.

## 10 DETAILED DESCRIPTION

[47] 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.

5 Rather, these embodiments are provided so that this disclosure is thorough and conveys the scope of the invention to those skilled in the art.

[48] 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.

10 [49] 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.

[50] 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  
15 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).

[51] Throughout this specification, the word “comprise” or variations such as  
20 “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).

[52] Throughout this specification, the term “including” is used to mean  
25 “including but not limited to.” “Including” and “including but not limited to” are used interchangeably.

[53] 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  
30 term “polypeptide sequence” refers to a series of amino acids or amino acid residues from which a polypeptide is physically composed. A “protein” is a macromolecule comprising one or more polypeptides or polypeptide “chains.” A “peptide” is a small polypeptide of sizes less than a total of 15-20 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.

- 5 [54] The terms “amino acid,” “amino acid residue,” “amino acid sequence,” or polypeptide sequence include naturally occurring amino acids (including L and D isosteromers) 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, 10 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

<b>Name</b>	<b>3-letter</b>	<b>1-letter</b>
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

- 15 [55] The phrase “conservative substitution” with regard to a polypeptide, refers to a change in the amino acid composition of the polypeptide that does not substantially alter the function and structure of the overall polypeptide (*see* Creighton, *Proteins: Structures and Molecular Properties* (W. H. Freeman and Company, New York (2nd ed., 1992))).

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

[57] 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.

10 [58] As used herein, the symbol “ $\alpha$ ” is shorthand for an immunoglobulin-type binding region capable of binding to the biomolecule following the symbol. The symbol “ $\alpha$ ” is used to refer to the functional characteristic of an immunoglobulin-type binding region based on its capability of binding to the biomolecule following the symbol.

15 [59] The symbol “::” means the polypeptide regions before and after it are physically linked together to form a continuous polypeptide.

[60] The term “selective cytotoxicity” with regard to the cytotoxic activity of a cytotoxic protein refers to the relative levels of cytotoxicity between a targeted cell population and a non-targeted bystander cell population, which can be  
20 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 show preferentiality of cell killing of the targeted cell type.

[61] For purposes of the present invention, the term “effector” means providing a biological activity, such as cytotoxicity, biological signaling,  
25 enzymatic catalysis, subcellular routing, and/or intermolecular binding resulting in the recruitment of a factor(s) and/or allosteric effects.

[62] For purposes of the present invention, the phrase “derived from” means that the polypeptide region comprises amino acid sequences originally found in a protein and which may now comprise additions, deletions, truncations, or other  
30 alterations from the original sequence such that overall function and structure are substantially conserved.

[63] 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. Non-limiting examples of Shiga toxin effector functions include

cellular internalization, subcellular routing, catalytic activity, 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. RIPs can

5 depurinate nucleic acids, polynucleosides, polynucleotides, rRNA, ssDNA, dsDNA, mRNA (and polyA), and viral nucleic acids (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.,

10 *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 Biochem* 128: 883-9 (2000); 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

15 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*. Assays for Shiga toxin effector activity can measure various activities,

20 such as, *e.g.*, protein synthesis inhibitory activity, depurination activity, inhibition of cell growth, cytotoxicity, supercoiled DNA relaxation activity, and/or nuclease activity.

[64] As used herein, the retention of Shiga toxin effector function refers to a level of Shiga toxin functional activity, as measured by an appropriate

25 quantitative assay with reproducibility comparable to a wild-type Shiga toxin effector region control. For ribosome inhibition, Shiga toxin effector function is exhibiting an IC<sub>50</sub> of 10,000 picomolar (pM) or less. For cytotoxicity in a target positive cell kill assay, Shiga toxin effector function is exhibiting a CD<sub>50</sub> of 1,000 nanomolar (nM) or less, depending on the cell type and its expression of

30 the appropriate extracellular target biomolecule.

[65] The effectiveness and potency of immunotoxins and ligand-toxin fusions as cytotoxic molecules is influenced by the densities of their target antigen(s) on a target cell surface (*see e.g.* Deckert T et al., *Blood* 103: 2718-26 (2004); Du X et al., *Blood* 111: 338-43 (2008); Baskar S et al., *mAbs* 4: 349-61 (2012)), epitope

location (Press O et al., *J Immunol* 141: 4410-7 (1988); Godal A et al., *In J Cancer* 52: 631-5 (1992); Yazdi P et al., *Cancer Res* 55: 3763-71 (1995)), rate of internalization of the surface bound cytotoxic molecule (*see e.g.* Du X et al., *Cancer Res* 68: 6300-5 (2008)), and the intracellular itinerary (Tortorella L et al., *PLoS One* 7: e47320 (2012)).

[66] The cell surface representation and/or density of a given extracellular target biomolecule may influence the applications for which certain proteins of the invention may be most suitably used. Differences in cell surface representation and/or density of a given target biomolecule between cells may alter the internalization and/or cytotoxicity of a given protein of the invention both quantitatively and qualitatively. 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 on a particular cell or population of cells may be determined using methods known to the skilled worker, such as the fluorescence-activated cell sorting (FACS) flow cytometry methods.

#### Introduction

[67] The present invention solves problems for engineering proteins comprising Shiga-toxin-Subunit-A derived regions linked to heterologous polypeptide regions, such as binding regions for cell targeting, when translocation of the protein to certain intracellular compartment(s) is desired, *e.g.* to the endoplasmic reticulum and/or cytosol. The present invention is based on the unexpected discovery that the addition of KDEL family signal motifs to the carboxyl-terminals of the Shiga-toxin-Subunit-A-derived toxin region within a cytotoxic protein improved cytotoxicity. This discovery contradicted previous research results published in the scientific literature (Jackson M et al., *J Cell Sci* 112: 467-75 (1999)). As described in more detail in the Examples, the addition of endoplasmic reticulum retention signal motifs to the carboxy-terminals of cytotoxic proteins allowed for the engineering of cell-type specific targeting of more potent Shiga toxin cytotoxicity.

[68] Previously, Shiga toxin A Subunit fusion constructs were shown to be cytotoxic and presumably capable of self-directing their own intracellular

routing to deliver an enzymatically active toxin fragment to the cytosol (Al-Jaufy, *Infect Immun* 62: 956-60 (1994); Al-Jaufy, *Infect Immun* 63: 3073-8 (1995); Su, *Protein Expr Purif* 66: 149-57 (2009)). When multiple cytotoxic proteins were created and tested with Shiga toxin derived regions linked to immunoglobulin derived targeting regions, these engineered proteins did not display the expected levels of cytotoxicity (*see*, Examples, *infra*). However, these reduced-cytotoxicity proteins exhibited cell binding and entry, as well as similar *in vitro* enzymatic activities and binding affinities as compared to more cytotoxic variants with the same polypeptide regions linked in a different configuration (*see*, Examples, *infra*; U.S. patent application 61/951,121). This unexpected problem was solved as described below.

[69] As described further in the Examples, the toxicity of Shiga-toxin-Subunit-A-based, cytotoxic proteins was improved by adding KDEL-like signal motifs to their carboxy-terminals. This modification resulted in significantly more potent cell kill results than observed in variants of the cytotoxic proteins lacking KDEL-type signal motifs. However, the addition of the signal motif did not alter either the catalytic activity of the Shiga toxin-derived region or the binding kinetics of the binding region. The addition of endoplasmic reticulum retention and retrieval signal motifs improved the cytotoxicity of these cytotoxic proteins most likely by directing sufficient intracellular routing to effectively deliver the Shiga toxin effector to the cytosol. The present invention provides a specific way of engineering such Shiga toxin derived proteins by adding a carboxy-terminal KDEL signal motif to at least one polypeptide of the protein in order to provide for desired intracellular routing and/or improved cytotoxic.

25

#### I. The General Structure of the Proteins of the Invention

[70] The present invention provides various proteins, the proteins each comprising (a) a binding region for cell targeting, (b) a Shiga toxin effector region for providing one or more Shiga toxin effector functions (*e.g.* cellular internalization, intracellular routing, and/or cell killing), and (c) an endoplasmic reticulum retention/retrieval signal motif for directing subcellular routing. The linking of cell targeting binding regions with Shiga-toxin-Subunit-A-derived regions enables the engineering of cell-type specific targeting of the potent Shiga toxin cytotoxicity. A protein of the invention comprises a binding region which

can bind specifically to at least one extracellular target biomolecule in physical association with a cell, such as a target biomolecule expressed on the surface of a cell. This general structure is modular in that any number of diverse binding regions may be linked to Shiga toxin Subunit A derived effector regions and  
5 KDEL-type signal motifs to produce variations of the same general structure.

#### A. Binding Regions

[71] The binding region of a protein of the present invention comprises a peptide or polypeptide region capable of binding specifically to a target  
10 biomolecule. Binding region 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. In certain embodiments, a protein of the invention comprises a  
15 binding region comprising one or more polypeptides capable of selectively and specifically binding an extracellular target biomolecule.

[72] There are numerous binding regions known in the art that are useful for targeting proteins to specific cell-types via their binding characteristics, such as ligands, monoclonal antibodies, engineered antibody derivatives, and engineered  
20 alternatives to antibodies.

[73] According to one specific but non-limiting aspect, the binding region of the protein of the present invention comprises a naturally occurring ligand or derivative thereof that retains binding functionality to an extracellular target biomolecule, commonly a cell surface receptor. For example, various cytokines,  
25 growth factors, and hormones known in the art may be used to target the protein to the cell-surface of specific cell types expressing a cognate cytokine receptor, growth factor receptor, or hormone receptor. Certain non-limiting examples of ligands include (alternative names are indicated in parentheses) angiogenin, B-cell activating factors (BAFFs, APRIL), colony stimulating factors (CSFs),  
30 epidermal growth factors (EGFs), fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs), insulin-like growth factors (IGFs), interferons, interleukins (such as IL-2, IL-6, and IL-23), nerve growth factors (NGFs), platelet derived growth factors, transforming growth factors (TGFs), and tumor necrosis factors (TNFs).

[74] According to certain other embodiments, the binding region comprises a synthetic ligand capable of binding an extracellular target biomolecule (*see e.g.* Liang S et al., *J Mol Med* 84: 764-73 (2006); Ahmed S et al., *Anal Chem* 82: 7533-41 (2010); Kaur K et al., *Methods Mol Biol* 1248: 239-47 (2015)).

5 [75] 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  
10 target molecules. 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.

[76] Immunoglobulin (Ig) proteins have a structural domain known as an Ig domain. Ig domains range in length from about 70-110 amino acid residues and  
15 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  
20 intermediate (IgI or I-set). Some Ig domains 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  
25 proteins. The HUGO Gene Nomenclature Committee (HGNC) provides a list of members of the Ig-like domain containing family.

[77] 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  
30 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 to obtain desired characteristics, such as smaller size, cell entry, or other therapeutic improvements. 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.

5 [78] 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 immunoglobulin binding region by providing high-affinity binding to an extracellular target biomolecule. This engineered polypeptide may optionally include polypeptide scaffolds comprising or consisting essentially of complementary determining regions from immunoglobulins as described herein.

10 [79] 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 present proteins is selected from the group which includes single-domain antibody domains (sdAbs), nanobodies, heavy-chain antibody domains derived from camelids ( $V_{HH}$  fragments), bivalent nanobodies, heavy-chain antibody domains derived from cartilaginous fishes, immunoglobulin new antigen receptors (IgNARs),  $V_{NAR}$  fragments, single-chain variable (scFv) fragments, multimerizing scFv fragments (diabodies, triabodies, tetrabodies), bispecific tandem scFv fragments, 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, divalent  $F(ab')_2$  fragments, Fd fragments consisting of the heavy chain and  $C_{H1}$  domains, single chain Fv- $C_{H3}$  minibodies, bispecific 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<sub>L</sub>, V<sub>H</sub>, C<sub>L</sub> and C<sub>H1</sub> domains, bivalent nanobodies, bivalent minibodies, bivalent F(ab')<sub>2</sub> fragments (Fab dimers), bispecific tandem V<sub>H</sub>H fragments, bispecific tandem scFv fragments, 5 bispecific nanobodies, bispecific minibodies, and any genetically manipulated counterparts of the foregoing that retain its paratope and binding function (see 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)). There are a variety of binding regions 10 comprising polypeptides derived from the constant regions of immunoglobulins, such as, *e.g.*, engineered dimeric Fc domains, monomeric Fcs (mFcs), scFv-Fcs, V<sub>H</sub>H-Fcs, C<sub>H2</sub> domains, monomeric C<sub>H3</sub>s domains (mC<sub>H3</sub>s), synthetically reprogrammed immunoglobulin domains, and/or hybrid fusions of immunoglobulin domains with ligands (Hofer T et al., *Proc Natl Acad Sci U. S.* 15 *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 Biol Chem* 20 288: 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)).

[80] In accordance with certain other embodiments, the binding region comprises an engineered, alternative scaffold to immunoglobulin domains. 25 Engineered alternative scaffolds are known in the art which exhibit similar functional characteristics to immunoglobulin-derived structures, such as high-affinity and specific binding of target biomolecules, and may provide improved characteristics to immunoglobulin domains, such as, *e.g.*, greater stability or reduced immunogenicity. Generally, alternative scaffolds to immunoglobulins 30 are less than 20 kilodaltons, consist of a single polypeptide chain, lack cysteine residues, and exhibit relatively high thermodynamic stability.

[81] For certain embodiments of the proteins of the present invention, the binding region comprises an alternative scaffold selected from the group which includes engineered, fibronectin-derived, 10<sup>th</sup> fibronectin type III (10Fn3)

domain (monobodies, AdNectins™, or AdNexins™); engineered, tenascin-derived, tenascin type III domain (Centryns™); engineered, ankyrin repeat motif containing polypeptide (DARPin™); engineered, low-density-lipoprotein-receptor-derived, A domain (LDLR-A) (Avimers™); lipocalin (anticalins);  
5 engineered, protease inhibitor-derived, Kunitz domain; engineered, Protein-A-derived, Z domain (Affibodies™); engineered, gamma-B crystalline-derived scaffold or engineered, ubiquitin-derived scaffold (Affilins); Sac7d-derived polypeptides (Nanoffitins® or affitins); engineered, Fyn-derived, SH2 domain (Fynomers®); miniproteins; C-type lectin-like domain scaffolds, engineered  
10 antibody mimic, and any genetically manipulated counterparts of the foregoing that retains its binding functionality (Wörn A, Plückthun A, *J Mol Biol* 305: 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  
15 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)).

[82] For example, numerous alternative scaffolds have been identified which  
20 bind to the extracellular 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;  
25 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).

[83] Any of the above binding regions may be used as a component of the present invention as long as the binding region component has a dissociation  
30 constant of  $10^{-5}$  to  $10^{-12}$  moles/liter, preferably less than 200 nM, towards an extracellular target biomolecule as described herein.

#### Extracellular Target Biomolecules

[84] The binding region of the protein of the invention comprises a polypeptide region capable of binding specifically to an extracellular target biomolecule, preferably which is physically-coupled to the surface of a cell type of interest, such as a cancer cell, tumor cell, plasma cell, infected cell, or host  
5 cell harboring an intracellular pathogen.

[85] The term “target biomolecule” refers to a biological molecule, commonly a protein or a protein modified by post-translational modifications, such as glycosylation, which is capable of being bound by a binding region to target a protein to a specific cell-type or location within an organism. Extracellular  
10 target biomolecules may include various epitopes, including unmodified polypeptides, polypeptides modified by the addition of biochemical functional groups, and glycolipids (*see e.g.* U.S. Patent 5,091,178; EP 2431743). It is desirable that an extracellular target biomolecule be endogenously internalized or be readily forced to internalize upon interaction with the protein of the  
15 invention.

[86] For purposes of the present invention, the term “extracellular” with regard to modifying a target biomolecule refers to a biomolecule that has at least a portion of its structure exposed to the extracellular environment. Extracellular  
20 target biomolecules include cell membrane components, transmembrane spanning proteins, cell membrane-anchored biomolecules, cell-surface-bound biomolecules, and secreted biomolecules.

[87] With regard to the present invention, the phrase “physically coupled” when used to describe a target biomolecule means both covalent and/or non-covalent intermolecular interactions that couple the target biomolecule, or a  
25 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 about 1-5 kiloCalories (*e.g.* electrostatic bonds, hydrogen bonds, Van der Waals interactions, hydrophobic forces, etc.). All integral membrane proteins can be found physically coupled to a cell  
30 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.

[88] Extracellular target biomolecules of the binding region of the proteins of the invention may include biomarkers over-proportionately or exclusively present on cancer cells, immune cells, and cells infected with intracellular pathogens, such as viruses, bacteria, fungi, prions, or protozoans.

5 [89] The binding regions of the proteins of the invention may be designed or selected based on numerous criteria, such as the cell-type specific expression of their target biomolecules and/or the physical localization of their target biomolecules with regard to specific cell types. For example, certain proteins of the present invention comprise binding domains capable of binding cell-surface  
10 targets which are expressed exclusively by only one cell-type to the cell surface.

[90] The general structure of the proteins of the present invention is modular, in that various, diverse binding regions may be used with the same Shiga toxin effector region to provide for diverse targeting of various extracellular target biomolecules and thus targeting of cytotoxicity, cytostasis, diagnostic agents,  
15 and/or exogenous material delivery to various diverse cell types.

#### B. Shiga Toxin Effector Regions Derived from A Subunits of Members of the Shiga Toxin Family

[91] For purposes of the present invention, the phrase “Shiga toxin effector region” refers to a polypeptide region derived from a Shiga toxin A Subunit of a  
20 member of the Shiga toxin family that is capable of exhibiting at least one Shiga toxin function. Shiga toxin functions include, *e.g.*, cell entry, lipid membrane deformation, directing subcellular routing, avoiding degradation, catalytically inactivating ribosomes, effectuating cytotoxicity, and effectuating cytostatic  
25 effects.

[92] A member of the Shiga toxin family refers to any member of a family of naturally occurring protein toxins which are structurally and functionally related, notably toxins isolated from *S. dysenteriae* and *E. coli* (Johannes, *Nat Rev Microbiol* 8: 105-16 (2010)). For example, the Shiga toxin family encompasses  
30 true Shiga toxin (Stx) isolated from *S. dysenteriae* serotype 1, Shiga-like toxin 1 variants (SLT1 or Stx1 or SLT-1 or Slt-1) 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 residue from Stx, and both have been referred to as Verocytotoxins or

Verotoxins (VTs) (O'Brien, *Curr Top Microbiol Immunol* 180: 65-94 (1992)). Although SLT1 and SLT2 variants are only about 53-60% similar to each other at the amino acid sequence level, they share mechanisms of enzymatic activity and cytotoxicity common to the members of the Shiga toxin family (Johannes, *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); 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, *J Clin Microbiol* 50: 2951-63 (2012)).

[93] Shiga toxin effector regions of the invention comprise or consist essentially of a polypeptide derived from a Shiga toxin A Subunit dissociated from any form of its native Shiga toxin B Subunit. In addition, the proteins of the present invention do not comprise any polypeptide comprising or consisting essentially of a functional binding domain of a native Shiga toxin B subunit. Rather, the Shiga toxin A Subunit derived regions are functionally associated with heterologous binding regions to effectuate cell targeting.

[94] In certain embodiments, a Shiga toxin effector region of the invention may comprise or consist 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)), 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. In other embodiments, the Shiga toxin effector region of the invention comprises or consists essentially of a truncated Shiga toxin A Subunit which is shorter than a full-length Shiga toxin A Subunit.

[95] Shiga-like toxin 1 A Subunit truncations are catalytically active, capable of enzymatically inactivating ribosomes *in vitro*, and cytotoxic when expressed within a cell (LaPointe, *J Biol Chem* 280: 23310-18 (2005)). The smallest Shiga toxin A Subunit fragment exhibiting full enzymatic activity is a polypeptide  
5 composed of residues 1-239 of Slt1A (LaPointe, *J Biol Chem* 280: 23310-18 (2005)). Although the smallest fragment of the Shiga toxin A Subunit reported to retain substantial catalytic activity was residues 75-247 of StxA (Al-Jaufy, *Infect Immun* 62: 956-60 (1994)), a StxA truncation expressed *de novo* within a eukaryotic cell requires only up to residue 240 to reach the cytosol and exert  
10 catalytic inactivation of ribosomes (LaPointe, *J Biol Chem* 280: 23310-18 (2005)).

[96] Shiga toxin effector regions may commonly be smaller than the full length A subunit. It is preferred that the Shiga toxin effector region maintain the polypeptide region from amino acid position 77 to 239 (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.* 77 to 238 of (SEQ ID NO:3)). For example, in certain embodiments of the invention, a Shiga toxin effector region derived from SLT-1A may comprise 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  
15 261 of SEQ ID NO:1. Among certain other embodiments, a Shiga toxin effector region derived from StxA may comprise or consist essentially of 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. Among certain other embodiments, a Shiga toxin effector region derived from SLT-2 may comprise or consist  
20 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.

[97] The invention further provides variants of the proteins of the invention, wherein the Shiga toxin effector region differs from a naturally occurring Shiga toxin A Subunit by up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40 or  
25 30 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 polypeptide region 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%, 95%, 99% or more amino acid sequence identity is maintained to a naturally occurring Shiga toxin A Subunit.

[98] Accordingly, in certain embodiments, the Shiga toxin effector region comprises or consists essentially of amino acid sequences having at least 55%,  
5 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, such as SLT-1A (SEQ ID NO:1), StxA (SEQ ID NO:2), and/or SLT-2A (SEQ ID NO:3).

[99] Optionally, either a full length or a truncated version of the Shiga toxin A  
10 Subunit may comprise one or more mutations (*e.g.* substitutions, deletions, insertions or inversions). It is preferred in certain embodiments of the invention that the Shiga toxin effector region has sufficient sequence identity to a naturally occurring Shiga toxin A Subunit to retain cytotoxicity after entry into a cell, either by well-known methods of host cell transformation, transfection, infection  
15 or induction, or by internalization mediated by the cell targeting binding region linked with the Shiga toxin effector region. 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 arginine-176 among others (Di, *Toxicon* 57: 525-39  
20 (2011)). In any one of the embodiments of the invention, the Shiga toxin effector region 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  
25 activity. The capacity of a cytotoxic protein of the invention to cause cell death, *e.g.* its cytotoxicity, may be measured using any one or more of a number of assays well known in the art.

[100] In certain embodiments of the invention, one or more amino acid residues may be mutated, inserted, or deleted in order to increase the enzymatic  
30 activity of the Shiga toxin effector region. For example, mutating residue-position alanine-231 in Stx1A to glutamate increased its enzymatic activity *in vitro* (Suhan M, Hovde C, *Infect Immun* 66: 5252-9 (1998)).

[101] In certain embodiments of the 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 region. The catalytic and/or cytotoxic activity of the A Subunits of members of the Shiga toxin family may be reduced or eliminated by mutation or truncation. The positions labeled tyrosine-77, glutamate-167, arginine-170, tyrosine-114, and tryptophan-203 have  
5 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, *Infect Immun* 66: 5252-9 (1998)).  
10 Mutating both glutamate-167 and arginine-170 eliminated the enzymatic activity of Slt-I A1 in a cell-free ribosome inactivation assay (LaPointe, *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 or truncating it to residues 1-239 eliminated Slt-I A1 fragment cytotoxicity at  
15 that expression level (LaPointe, *J Biol Chem* 280: 23310-18 (2005)).

[102] 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. For *in vitro* ribosome inhibition,  
20 significant Shiga toxin effector function is exhibiting an IC<sub>50</sub> of 300 pM or less depending on the source of the ribosomes (*e.g.* bacteria, archaea, or eukaryote (algae, fungi, plants, or animals)). This is significantly greater inhibition as compared to the approximate IC<sub>50</sub> of 100,000 pM for the catalytically inactive SLT-1A 1-251 double mutant (Y77S/E167D). For cytotoxicity in a target  
25 positive cell kill assay in laboratory cell culture, significant Shiga toxin effector function is exhibiting a CD<sub>50</sub> of 100, 50, or 30 nM or less, depending on the cell line and its expression of the appropriate extracellular target biomolecule. This is significantly greater cytotoxicity to the appropriate target cell line as compared to the SLT-1A component alone, without a cell targeting binding region, which  
30 has a CD<sub>50</sub> of 100-10,000 nM, depending on the cell line.

[103] For some samples, accurate values for either IC<sub>50</sub> or CD<sub>50</sub> might be unobtainable due to the inability to collect the required data points for an accurate curve fit. Inaccurate IC<sub>50</sub> and/or CD<sub>50</sub> values should not be considered when determining significant Shiga toxin effector function activity. 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, should not be considered as representative of actual Shiga toxin effector function. For example, theoretically, neither an  $IC_{50}$  or  $CD_{50}$  can be  
5 determined if greater than 50% ribosome inhibition or cell death, respectively, does not occur in a concentration series for a given sample.

[104] The failure to detect activity in Shiga toxin effector function may be due to improper expression, polypeptide folding, and/or polypeptide stability rather than a lack of cell entry, subcellular routing, and/or enzymatic activity. Assays  
10 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  
15 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; improper polypeptide folding and/or stability may benefit from stabilizing terminal sequences, or compensatory mutations in non-  
20 effector regions which stabilize the three-dimensional structure of the protein, etc. When new assays for individual Shiga toxin functions become available, Shiga toxin effector regions or polypeptides may be analyzed for any level of those Shiga toxin effector functions, such as for being within a certain-fold activity of a wild-type Shiga toxin effector polypeptide. Examples of  
25 meaningful activity differences are, *e.g.*, Shiga toxin effector regions that have 1000-fold or 100-fold or less the activity of a wild-type Shiga toxin effector polypeptide; or that have 3-fold to 30-fold or more activity compared to a functional knock-down or knockout Shiga toxin effector polypeptide.

[105] Certain Shiga toxin effector functions are not easily measurable, *e.g.*  
30 subcellular routing functions. Currently there is no routine, quantitative assay to distinguish whether the failure of a Shiga toxin effector polypeptide to be cytotoxic 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 region.

[106] It should be noted that even if the cytotoxicity of a Shiga toxin effector polypeptide is reduced relative to wild-type, in practice, applications using attenuated Shiga toxin effector polypeptides may be equally or more effective than those using wild-type Shiga toxin effector polypeptides because the highest potency variants might exhibit undesirable effects which are minimized in reduced potency variants. Wild-type Shiga toxin effector polypeptides are very potent, being able to kill with only one molecule reaching the cytosol or perhaps 40 molecules being internalized. 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 involving targeted cell killing and/or specific cell detection.

#### C. Endoplasmic Reticulum Retention/Retrieval Signal Motif of a Member of the KDEL Family

[107] For purposes of the present invention, the phrase “endoplasmic reticulum retention/retrieval signal motif,” KDEL-type signal motif, or signal motif refers to any member of the KDEL family capable of functioning within a eukaryotic cell to promote subcellular localization of a protein to the endoplasmic reticulum via KDEL receptors.

[108] The carboxy-terminal lysine-asparagine-glutamate-leucine (KDEL) sequence (SEQ ID NO:34) is a canonical, endoplasmic reticulum retention and retrieval signal motif for soluble proteins in eukaryotic cells and is recognized by KDEL receptors (*see*, Capitani M, Sallese M, *FEBS Lett* 583: 3863-71 (2009), *for review*). The KDEL family of signal motifs includes many KDEL-like motifs, such as HDEL (SEQ ID NO:36), RDEL (SEQ ID NO:38), WDEL (SEQ ID NO:39), YDEL (SEQ ID NO:40), HEEL (SEQ ID NO:42), KEEL (SEQ ID NO:43), REEL (SEQ ID NO:44), KFEL (SEQ ID NO:47), KIEL (SEQ ID NO:59), DKEL (SEQ ID NO:60), KKEL (SEQ ID NO:63), HNEL (SEQ ID NO:67), HTEL (SEQ ID NO:68), KTEL (SEQ ID NO:69), and HVEL (SEQ ID NO:70), all of which are found at the carboxy-terminals of proteins which are known to be residents of the lumen of the endoplasmic reticulum of organisms

throughout multiple phylogenetic kingdoms (Munro S, Pelham H, *Cell* 48: 899-907 (1987); Raykhel I et al., *J Cell Biol* 179: 1193-204 (2007)). The KDEL signal motif family includes at least 46 polypeptide variants shown using synthetic constructs (Raykhel, *J Cell Biol* 179: 1193-204 (2007)). Additional  
5 KDEL signal motifs include ALEDEL (SEQ ID NO: 82), HAEDEL (SEQ ID NO: 83), HLEDEL (SEQ ID NO: 84), KLEDEL (SEQ ID NO: 85), IRSDEL (SEQ ID NO: 86), ERSTEL (SEQ ID NO: 87), and RPSTEL (SEQ ID NO: 88) (Alanen H et al., *J Mol Biol* 409: 291-7 (2011)). A generalized consensus motif representing the majority of KDEL signal motifs has been described as  
10 [KRHQSA]-[DENQ]-E-L (Hulo N et al., *Nucleic Acids Res* 34: D227-30 (2006)).

[109] Proteins containing KDEL family signal motifs are bound by KDEL receptors distributed throughout the Golgi complex and transported to the endoplasmic reticulum by a microtubule-dependent mechanism for release into  
15 the lumen of the endoplasmic reticulum (Griffiths G et al., *J Cell Biol* 127: 1557-74 (1994); Miesenböck G, Rothman J, *J Cell Biol* 129: 309-19 (1995)). KDEL receptors dynamically cycle between the Golgi complex and endoplasmic reticulum (Jackson M et al., *EMBO J* 9: 3153-62 (1990); Schutze M et al., *EMBO J*. 13: 1696-1705 (1994)).

[110] For purposes of the present invention, the members of the KDEL family include synthetic signal motifs able to function within a eukaryotic cell to promote subcellular localization of a protein to the endoplasmic reticulum via KDEL receptors. In other words, some members of the KDEL family might not occur in nature or have yet to be observed in nature but have or may be  
25 constructed and empirically verified using methods known in the art; see *e.g.*, Raykhel I et al., *J Cell Biol* 179: 1193-204 (2007).

[111] As a component of the proteins of the invention, the KDEL-type signal motif is physically located, oriented, or arranged within the protein such that it is on a carboxy-terminal of a polypeptide.

[112] For the purposes of the present invention, the specific order or orientation is not fixed for the Shiga toxin effector region and the binding region in relation to each other or the entire protein (*see e.g.* Figure 1). The components of the proteins of the invention may be arranged in any order provided that the desired activities of the binding region and the Shiga toxin effector region are not

eliminated. Desired activities include providing the protein with the ability, *e.g.*, to bind target expressing cells, induce cellular internalization, cause cytoxicity, cause cytotoxicity, and/or deliver exogenous materials to the interiors of cells.

[113] In the above embodiments of proteins of the invention, the binding  
5 regions, Shiga toxin effector regions (which may be cytotoxic and/or harbor one or more mutations reducing or eliminating catalytic activity and/or cytotoxicity), and endoplasmic reticulum retention/retrieval signal motif may be directly linked to each other and/or suitably linked to each other via one or more intervening polypeptide sequences, such as with one or more linkers well known in the art  
10 and/or described herein.

#### D. Linkages Connecting Polypeptide Components of the Invention and/or Their Subcomponents

[114] Individual polypeptide and/or protein components of the invention, *e.g.*  
15 the binding regions and Shiga toxin effector regions (which may be cytotoxic and/or harbor one or more mutations altering, reducing, or eliminating catalytic activity and/or cytotoxicity), 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.* CDR and/or ABR  
20 regions, may be suitably linked to each other via one or more linkers well known in the art and/or described herein (*see e.g.* Weisser N, Hall J, *Biotechnol Adv* 27: 502-20 (2009); Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)). Polypeptide components of the invention, *e.g.*, multi-chain binding regions, may be suitably linked to each other or other polypeptide components of the  
25 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.

[115] Suitable linkers are generally those which allow each polypeptide  
30 component of the 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, *e.g.*,

various non-proteinaceous carbon chains, whether branched or cyclic (*see e.g.* Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)).

[116] 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 (*see e.g.* Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)).

[117] Suitable linkers may be non-proteinaceous, such as, *e.g.* chemical linkers (*see e.g.* Dosio F et al., *Toxins* 3: 848-83 (2011); Feld J et al., *Oncotarget* 4: 397-412 (2013)). Various non-proteinaceous linkers known in the art may be used to link binding regions to the Shiga toxin effector regions, such as linkers commonly used to conjugate immunoglobulin-derived polypeptides to heterologous polypeptides. For example, polypeptide regions of the proteins of the present invention may be linked using the functional side chains of their amino acid residues 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 (*see e.g.* Fitzgerald D et al., *Bioconjugate Chem* 1: 264-8 (1990); Pasqualucci L et al., *Haematologica* 80: 546-56 (1995)). In addition, non-natural amino acid residues may be used with other functional side chains, such as ketone groups (*see e.g.* Sun S et al., *Chembiochem* Jul 18 (2014); Tian F et al., *Proc Natl Acad Sci USA* 111: 1766-71 (2014)). Examples of non-proteinaceous chemical linkers include but are not limited to N-succinimidyl (4-iodoacetyl)-aminobenzoate, *S*-(*N*-succinimidyl) thioacetate (SATA), N-succinimidyl-oxycarbonyl- $\alpha$ -methyl- $\alpha$ -(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- $\alpha$ -(2-pyridyldithio) toluene, sulfosuccinimidyl-6-( $\alpha$ -methyl- $\alpha$ -(pyridyldithiol)-toluamido) hexanoate, N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), succinimidyl 6(3-(2-pyridyldithio)-propionamido) 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-(2-thiopyridyl)-L-cysteine (*see e.g.* Thorpe P et al., *Eur J Biochem* 147: 197-206 (1985); Thorpe P et al., *Cancer Res* 47: 5924-31 (1987); Thorpe P et al., *Cancer Res* 48: 6396-403 (1988); Grossbard M et al., *Blood* 79: 576-85 (1992); Lui C et al., *Proc Natl Acad Sci USA* 93: 8618-23 (1996); Doronina S et al., *Nat Biotechnol* 21: 778-84 (2003); Feld J et al., *Oncotarget* 4: 397-412 (2013)).

[118] 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 (*see e.g.* Dosio F et al., *Toxins* 3: 848-83 (2011); Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013); Feld J et al., *Oncotarget* 4: 397-412 (2013)).

[119] Proteinaceous linkers may be chosen for incorporation into recombinant fusion proteins of the present invention. For example, the component polypeptides of the proteins of the present invention or their subcomponents may be joined by one or more linkers comprising one or more amino acids, peptides, and/or polypeptides. For recombinant fusion proteins of the invention, linkers typically comprise about 2 to 50 amino acid residues, preferably about 5 to 30 amino acid residues (Argos P, *J Mol Biol* 211: 943-58 (1990); Williamson M, *Biochem J* 297: 240-60 (1994); George R, Heringa J, *Protein Eng* 15: 871-9 (2002); Kreitman R, *AAPS J* 8: E532-51 (2006)). Commonly, proteinaceous linkers comprise a majority of amino acid residues with polar, uncharged, and/or charged residues, such as, *e.g.*, threonines, prolines, glutamines, glycines, and alanines (*see e.g.* Huston J et al. *Proc Natl Acad Sci USA* 85: 5879-83 (1988); Pastan I et al., *Annu Rev Med* 58: 221-37 (2007); Li J et al., *Cell Immunol* 118: 85-99 (1989); Cumber A et al. *Bioconj Chem* 3: 397-401 (1992); Friedman P et al., *Cancer Res* 53: 334-9 (1993); Whitlow M et al., *Protein Engineering* 6: 989-95 (1993); Siegall C et al., *J Immunol* 152: 2377-84 (1994); Newton et al. *Biochemistry* 35: 545-53 (1996); Ladurner et al. *J Mol Biol* 273: 330-7 (1997); Kreitman R et al., *Leuk Lymphoma* 52: 82-6 (2011); U.S. 4,894,443). Non-

limiting examples of proteinaceous linkers include alanine-serine-glycine-glycine-proline-glutamate (ASGGPE) (SEQ ID NO: 89), valine-methionine (VM), alanine-methionine (AM), AM(G<sub>2 to 4</sub>S)<sub>x</sub>AM (SEQ ID NO: 90) where G is glycine, S is serine, and x is an integer from 1 to 10.

5 [120] 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  
10 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 (*see e.g.* Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)). The skilled worker may use databases and linker design software tools when choosing linkers. Certain linkers may be chosen to optimize expression (*see e.g.*  
15 Turner D et al., *J Immunol Methods* 205: 43-54 (1997)). 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  
20 proteins of the invention, such as, *e.g.*, interactions related to the formation dimers and other higher order multimers (*see e.g.* U.S. 4,946,778).

[121] 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,  
25 and threonines (*see e.g.* Bird R et al., *Science* 242: 423-6 (1988); Friedman P et al., *Cancer Res* 53: 334-9 (1993); Siegall C et al., *J Immunol* 152: 2377-84 (1994)). 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  
30 skilled worker and are composed of multiple glycines and/or one or more serines, sometimes in repeating units, such as, *e.g.*, (G<sub>x</sub>S)<sub>n</sub> (SEQ ID NO: 91), (S<sub>x</sub>G)<sub>n</sub> (SEQ ID NO: 92), (GGGS)<sub>n</sub> (SEQ ID NO: 93), and (G)<sub>n</sub> (SEQ ID NO: 94). in which x is 1 to 6 and n is 1 to 30 (*see e.g.* WO 96/06641). Non-limiting examples of flexible proteinaceous linkers include GKSSGSGSESKS (SEQ ID

NO: 95), GSTSGSGKSSEGKG (SEQ ID NO: 96), GSTSGSGKSSEGSGSTKG (SEQ ID NO: 97), , GSTSGSGKPGSGEGSTKG (SEQ ID NO: 99), EGKSSGSGSESKEF (SEQ ID NO: 100), SRSSG (SEQ ID NO: 101), and SGSSC (SEQ ID NO: 102).

5 [122] Rigid proteinaceous linkers are often stiff alpha-helical structures and rich in proline residues and/or one or more strategically placed prolines (*see* Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)). Rigid linkers may be chosen to prevent intramolecular interactions between components.

[123] Suitable linkers may be chosen to allow for *in vivo* separation of  
10 components, such as, *e.g.*, due to cleavage and/or environment-specific instability (*see* Dosio F et al., *Toxins* 3: 848-83 (2011); Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)). *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 (*see e.g.*

15 Doronina S et al., *Bioconjug Chem* 17: 144-24 (2006); Erickson H et al., *Cancer Res* 66: 4426-33 (2006)). *In vivo* cleavable proteinaceous linkers often comprise protease sensitive motifs and/or disulfide bonds formed by one or more cysteine pairs (*see e.g.* Pietersz G et al., *Cancer Res* 48: 4469-76 (1998); The J et al., *J Immunol Methods* 110: 101-9 (1998); *see* Chen X et al., *Adv Drug Deliv Rev* 65:

20 1357-69 (2013)). *In vivo* cleavable proteinaceous linkers can 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.*, proteases with abnormally high levels, proteases overexpressed at certain disease sites, and  
25 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

30 AMGRSGGCAGNRVGSLSLSCGGLNLQAM (SEQ ID NO: 103).

[124] In certain embodiments of the proteins 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 proteins of the invention, a linker may be used which is not cleavable to

reduce unwanted toxicity after administration to a vertebrate organism (*see e.g.* Polson A et al., *Cancer Res* 69: 2358-64 (2009)).

[125] Suitable linkers may include, *e.g.*, protease sensitive, environmental redox potential sensitive, pH sensitive, acid cleavable, photocleavable, and/or  
5 heat sensitive linkers, whether proteinaceous or non-proteinaceous (*see* Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013)).

[126] Suitable cleavable linkers may include linkers comprising cleavable groups which are known in the art such as, *e.g.*, linkers noted by Zarling D et al.,  
10 *J Immunol* 124: 913-20 (1980); Jung S, Moroi M, *Biochem Biophys Acta* 761: 152-62 (1983); Bouizar Z et al., *Eur J Biochem* 155: 141-7 (1986); Park L et al., *J Biol Chem* 261: 205-10 (1986); Browning J, Ribolini A, *J Immunol* 143: 1859-67 (1989); Joshi S, Burrows R, *J Biol Chem* 265: 14518-25 (1990).

[127] Suitable linkers may include pH sensitive linkers. For example, certain suitable linkers may be chosen for their instability in lower pH environments to  
15 provide for dissociation inside a subcellular compartment of a target cell. For example, linkers that comprise one or more trityl groups, derivatized trityl groups, bismaleimideoxy propane groups, adipic acid dihydrazide groups, and/or acid labile transferrin groups, may provide for release of components of the invention, *e.g.* a polypeptide component, in environments with specific pH  
20 ranges (*see e.g.* Welhöner H et al., *J Biol Chem* 266: 4309-14 (1991); Fattom A et al., *Infect Immun* 60: 584-9 (1992)). Certain linkers may be chosen which are cleaved in pH ranges corresponding to physiological pH differences between tissues, such as, *e.g.*, the pH of tumor tissue is lower than in healthy tissues (*see e.g.* U.S. 5,612,474).

[128] Photocleavable linkers are linkers that are cleaved upon exposure to  
25 electromagnetic radiation of certain wavelength ranges, such as light in the visible range (*see e.g.* Goldmacher V et al., *Bioconj Chem* 3: 104-7 (1992)). Photocleavable linkers may be used to release a component of a protein of the invention, *e.g.* a polypeptide component, upon exposure to light of certain  
30 wavelengths. Non-limiting examples of photocleavable linkers include a nitrobenzyl group as a photocleavable protective group for cysteine, nitrobenzyloxycarbonyl chloride cross-linkers, hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer, and methylrhodamine copolymer (Hazum E et al., *Pept Proc Eur Pept Symp*, 16th, Brunfeldt K, ed.,

105-110 (1981); Senter et al., *Photochem Photobiol* 42: 231-7 (1985); Yen et al.,  
*Makromol Chem* 190: 69-82 (1989); Goldmacher V et al., *Bioconj Chem* 3: 104-  
7 (1992)). Photocleavable linkers may have particular uses in linking  
components to form proteins of the invention designed for treating diseases,  
5 disorders, and conditions that can be exposed to light using fiber optics. In  
certain embodiments of the proteins of the invention, a cell-targeting binding  
region is linked to a Shiga toxin effector region using any number of means  
known to the skilled worker, including both covalent and noncovalent linkages  
(see e.g. Chen X et al., *Adv Drug Deliv Rev* 65: 1357-69 (2013); Behrens C, Liu  
10 B, *MAbs* 6: 46-53 (2014).

[129] In certain embodiments of the proteins of the present invention, the  
protein comprises a binding region which is a scFv with a linker connecting a  
heavy chain variable (V<sub>H</sub>) domain and a light chain variable (V<sub>L</sub>) domain. There  
are numerous linkers known in the art suitable for this purpose, such as, e.g., the  
15 15-residue (Gly4Ser)<sub>3</sub> peptide (SEQ ID NO: 104). Suitable scFv linkers which  
may be used in forming non-covalent multivalent structures include GGS,  
GGGS (Gly3Ser or G3S) (SEQ ID NO: 105), GGGGS (Gly4Ser or G4S) (SEQ  
ID NO: 106), GGGGSGGG (SEQ ID NO: 107), GGSGGGG (SEQ ID NO:  
108), GSTSGGGSGGGSGGGSS (SEQ ID NO: 109), and  
20 GSTSGSGKPGSSEGSTKG (SEQ ID NO: 110) (Plückthun A, Pack P,  
*Immunotechnology* 3: 83-105 (1997); Atwell J et al., *Protein Eng* 12: 597-604  
(1999); Wu A et al., *Protein Eng* 14: 1025-33 (2001); Yazaki P et al., *J Immunol*  
*Methods* 253: 195-208 (2001); Carmichael J et al., *J Mol Biol* 326: 341-51  
(2003); Arndt M et al., *FEBS Lett* 578: 257-61 (2004); Bie C et al., *World J*  
25 *Hepatol* 2: 185-91 (2010)).

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

## II. Examples of Specific Structural Variations of the Proteins of the Invention

[131] Among certain embodiments of the present invention, the proteins of the invention comprise a binding region derived from an immunoglobulin-type polypeptide selected for specific and high-affinity binding to a surface antigen on the cell surface of a cancer cell, where the antigen is restricted in expression to cancer 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 a surface antigen on the cell surface of a cancer cell, where the antigen is over-expressed or preferentially expressed by cancer cells as compared to non-cancer cells. Some representative target biomolecules include, but are not limited to, the following enumerated targets associated with cancers and/or specific immune cell types.

[132] Many immunoglobulin-type binding regions that recognize epitopes associated with cancer cells exist in the prior art, such as binding regions that target (alternative names are indicated in parentheses) annexin AI, B3 melanoma antigen, B4 melanoma antigen, CD2, CD3, CD4, CD20 (B-lymphocyte antigen protein CD20), CD22, CD25 (interleukin-2 receptor IL2R), CD30 (TNFRSF8), CD38 (cyclic ADP ribose hydrolase), CD40, CD44 (hyaluronan receptor), ITGAV (CD51), CD66, CD71 (transferrin receptor), CD73, CD74 (HLA-DR antigens-associated invariant chain), CD79, CD98, endoglin (END or CD105), CD106 (VCAM-1), chemokine receptor type 4 (CD4, fusin, CD184), CD200, insulin-like growth factor 1 receptor (CD221), mucin1 (MUC1, CD227), basal cell adhesion molecule (B-CAM or CD239), CD248 (endosialin or TEM1), tumor necrosis factor receptor 10b (TNFRSF10B, CD262), tumor necrosis factor receptor 13B (TNFRSF13B, TACI, CD276), vascular endothelial growth factor receptor 2 (KDR, CD309), epithelial cell adhesion molecule (EpCAM, CD326), human epidermal growth factor receptor 2 (HER2/Neu/ErbB2/CD340), cancer antigen 15-3 (CA15-3), cancer antigen 19-9 (CA 19-9), cancer antigen 125 (CA125, MUC16), CA242, carcinoembryonic antigen-related cell adhesion molecules (*e.g.* CEACAM3 (CD66d) and CEACAM5), carcinoembryonic antigen protein (CEA), chondroitin sulfate proteoglycan 4 (CSP4, MCSP, NG2), CTLA4, DLL4, epidermal growth factor receptor (EGFR/ErbB1), folate receptor (FOLR), G-28, ganglioside GD2, ganglioside GD3, HLA-Dr10, HLA-DRB, human epidermal growth factor receptor 1 (HER1), Ephrin type-B receptor 2 (EphB2), epithelial cell adhesion

molecule (EpCAM), fibroblast activation protein (FAP/seprase), insulin-like growth factor 1 receptor (IGF1R), interleukin 2 receptor (IL-2R), interleukin 6 receptor (IL-6R), integrins alpha-V beta-3 ( $\alpha_v\beta_3$ ), integrins alpha-V beta-5 ( $\alpha_v\beta_5$ ), integrins alpha-5 beta-1 ( $\alpha_5\beta_1$ ), L6, MPG, melanoma-associated antigen 1 protein (MAGE-1), melanoma-associated antigen 3 (MAGE-3), mesothelin (MSLN), MPG, MS4A, p21, p97, polio virus receptor-like 4 (PVRL4), protease-activated-receptors (such as PAR1), prostate-specific membrane antigen protein (PSMA), trophoblast glycoprotein (TPGB), and tumor-associated calcium signal transducers (TACSTDs) (*see e.g.* Lui B et al., *Cancer Res* 64: 704-10 (2004); 5 Novellino L et al., *Cancer Immunol Immunother* 54: 187-207 (2005); Bagley R et al., *Int J Oncol* 34: 619-27 (2009); Gerber H et al., *mAbs* 1: 247-53 (2009); Beck A et al., *Nat Rev Immunol* 10: 345-52 (2010); Andersen J et al., *J Biol Chem* 287: 22927-37 (2012); Nolan-Stevaux O et al., *PLoS One* 7: e50920 (2012); Rust S et al., *Mol Cancer* 12: 11 (2013)). This list of target 10 biomolecules is intended to be non-limiting. It will be appreciated by the skilled worker that any desired target biomolecule associated with a cancer cell or other desired cell type may be used to design or select a binding region to be coupled with the Shiga toxin effector region to produce a protein of the invention.

[133] Examples of other target biomolecules which are strongly associated with 20 cancer cells and immunoglobulin-type binding regions known to bind them include BAGE proteins (B melanoma antigens), basal cell adhesion molecules (BCAMs or Lutheran blood group glycoproteins), bladder tumor antigen (BTA), cancer-testis antigen NY-ESO-1, cancer-testis antigen LAGE proteins, CD19 (B-lymphocyte antigen protein CD19), CD21 (complement receptor-2 or 25 complement 3d receptor), CD26 (dipeptidyl peptidase-4, DPP4, or adenosine deaminase complexing protein 2), CD33 (sialic acid-binding immunoglobulin-type lectin-3), CD52 (CAMPATH-1 antigen), CD56 (neural cell adhesion molecule or NCAM), CD133 (prominin-1), CS1 (SLAM family number 7 or SLAMF7), cell surface A33 antigen protein (gpA33), Epstein-Barr virus antigen 30 proteins, GAGE/PAGE proteins (melanoma associated cancer/testis antigens), hepatocyte growth factor receptor (HGFR or c-Met), MAGE proteins, melanoma antigen recognized by T-cells 1 protein (MART-1/MelanA, MARTI), mucins, Preferentially Expressed Antigen of Melanoma (PRAME) proteins, prostate specific antigen protein (PSA), prostate stem cell antigen protein (PSCA),

Receptor for Advanced Glycation Endproducts (RAGE), tumor-associated glycoprotein 72 (TAG-72), tyrosine-protein kinase transmembrane receptor (ROR1 or NTRKR1), vascular endothelial growth factor receptors (VEGFRs), and Wilms' tumor antigen.

5 [134] Examples of other target biomolecules which are strongly associated with cancer cells are carbonic anhydrase IX (CA9/CAIX), claudin proteins (CLDN3, CLDN4), ephrin type-A receptor 3 (EphA3), folate binding proteins (FBP), ganglioside GM2, insulin-like growth factor receptors, integrins (such as CD11a–c), receptor activator of nuclear factor kappa B (RANK), receptor  
10 tyrosine-protein kinase erB-3, tumor necrosis factor receptor 10A (TRAIL-R1/DR4), tumor necrosis factor receptor 10B (TRAIL-R2), tenascin C, and CD64 (FcγRI) (*see* Hough C et al., *Cancer Res* 60: 6281-7 (2000); Thepen T et al., *Nat Biotechnol* 18: 48-51 (2000); Pastan I et al., *Nat Rev Cancer* 6: 559-65 (2006); Pastan, *Annu Rev Med* 58: 221-37 (2007); Fitzgerald D et al., *Cancer Res* 71: 6300-9 (2011); Scott A et al., *Cancer Immun* 12: 14-22 (2012)). This list of target biomolecules is intended to be non-limiting.

[135] In addition, there are numerous other examples of contemplated, target biomolecules such as ADAM metalloproteinases (*e.g.* ADAM-9, ADAM-10, ADAM-12, ADAM-15, ADAM-17), ADP-ribosyltransferases (ART1, ART4),  
20 antigen F4/80, bone marrow stroma antigens (BST1, BST2), break point cluster region-c-abl oncogene (BCR-ABL) proteins, C3aR (complement component 3a receptors), CD7, CD13, CD14, CD15 (Lewis X or stage-specific embryonic antigen 1), CD23 (FC epsilon RII), CD49d, CD53, CD54 (intercellular adhesion molecule 1), CD63 (tetraspanin), CD69, CD80, CD86, CD88 (complement  
25 component 5a receptor 1), CD115 (colony stimulating factor 1 receptor), CD123 (interleukin-3 receptor), CD129 (interleukin 9 receptor), CD183 (chemokine receptor CXCR3), CD191 (CCR1), CD193 (CCR3), CD195 (chemokine receptor CCR5), CD203c, CD225 (interferon-induced transmembrane protein 1), CD244 (Natural Killer Cell Receptor 2B4), CD282 (toll-like receptor 2), CD284  
30 (Toll-like receptor 4), CD294 (GPR44), CD305 (leukocyte-associated immunoglobulin-like receptor 1), ephrin type-A receptor 2 (EphA2), FcεRIa, galectin-9, alpha-fetoprotein antigen 17-A1 protein, human aspartyl (asparaginyl) beta-hydroxylase (HAAH), immunoglobulin-like transcript ILT-3, lysophosphatidlycerol acyltransferase 1 (LPGAT1/IAA0205), lysosome-

associated membrane proteins (LAMPs, such as CD107), melanocyte protein PMEL (gp100), myeloid-related protein-14 (mrp-14), programmed death-ligand 1 (PD-L1), receptor tyrosine-protein kinase erbB-3, SART proteins, scavenger receptors (such as CD64 and CD68), Siglecs (sialic acid-binding immunoglobulin-type lectins), syndecans (such as SDC1 or CD138), tyrosinase, tyrosinase-related protein 1 (TRP-1), tyrosinase-related protein 2 (TRP-2), tyrosinase associated antigen (TAA), APO-3, BCMA, CD2, CD3, CD4, CD8, CD18, CD27, CD28, CD29, CD41, CD49, CD90, CD95 (Fas), CD103, CD104, CD134 (OX40), CD137 (4-1BB), CD152 (CTLA-4), chemokine receptors, complement proteins, cytokine receptors, histocompatibility proteins, ICOS, interferon-alpha, interferon-beta, c-myc, osteoprotegerin, PD-1, RANK, TACI, TNF receptor superfamily member (TNF-R1, TNFR-2), Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 (*see*, Cheever M et al., *Clin Cancer Res* 15: 5323-37 (2009); Scott A et al., *Cancer Immun* 12: 14 (2012)), for target biomolecules and note the target molecules described therein are non-limiting examples). It will be appreciated by the skilled worker that any desired target biomolecule may be used to design or select a binding region to be coupled with the Shiga toxin effector region to produce a protein of the invention.

[136] In certain embodiments, the binding region comprises or consists essentially of an immunoglobulin-type polypeptide selected for specific and high-affinity binding to the cellular surface of a cell type of the immune system. For example, immunoglobulin-type binding domains are known that bind to CD1, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD9, CD10, CD11, CD12, CD13, CD14, CD15, CD16, CD17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD33, CD34, CD35, CD36, CD37, CD38, CD40, CD41, CD56, CD61, CD62, CD66, CD95, CD117, CD123, CD235, CD146, CD326, interleukin-2 receptor (IL-2R), receptor activator of nuclear factor kappa B ligand (RANKL, TNFSF11, TRANCE, OPG, ODF), SLAM-associated protein (SAP), and TNFSF18 (tumor necrosis factor ligand 18 or GITRL).

[137] In certain embodiments, the binding region binds with high-affinity to the target biomolecule which is a chemokine receptor selected from the following CXCR- 1, CXCR-2, CXCR-3 A, CXCR3B, CXCR-4, CXCR-5, CCR-I, CCR-2A, CCR-2B, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9,

CCR-10, CX3CR-1, XCR1, CXCR-6, CXCR-7, chemokine binding protein-2 (CCBP2, D6 receptor), and Duffy antigen/chemokine receptor (DARC, Fy glycoprotein, FY, CD234). For more non-limiting target biomolecules, see Table 11 in the Examples below.

5 [138] In certain embodiments, the binding region comprises or consists essentially of ligand selected for targeting an extracellular receptor. Some representative ligands include, but are not limited to, the following: bone morphogenetic proteins and activin membrane-bound inhibitor BAMBI (also known as TGFBR), chemokines, B cell-attracting chemokine 1 (BCA-1), breast  
10 and kidney-expressed chemokine (BRAK), C10, chemokine C-C motif ligand 2 (CCL2), CD137L (also known as 4-1BB), cutaneous T-cell attracting chemokine (CTACK), decoy receptor 3 DcR3 (also known as TR6 and TNFRSF6B), epithelial-derived neutrophil-activating protein 78 (ENA-78), eosinophils chemotactic proteins (*e.g.* Eotaxin-1, Eotaxin-2, and Eotaxin-3), exodus-2 (SLC),  
15 fractalkine, granulocyte chemotactic protein 2 (GCP-2), growth regulated protein  $\alpha$  (GRO- $\alpha$ ), hemo filtrate CC chemokine 1 (HCC-1), interleukin-8 (IL-8), interferon-inducible protein 10 (IP-10), interferon-inducible T-cell alpha chemoattractant (I-TAC), liver-expressed chemokine (LEC), lungkine, lymphotactin, macrophage-derived chemokine (MDC), mucosae-associated  
20 epithelial chemokine (MEC), gamma interferon-induced monokine (MIG), macrophage inhibitory proteins (*e.g.* MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-1 $\gamma$ , MIP-2, MIP-2 $\alpha$ , MIP-2 $\beta$ , MIP-3, MIP-3 $\beta$ , MIP-3 $\alpha$ , MIP-4, and MIP-5), monocytes chemotactic proteins (*e.g.* MCP-1 or MCP-1, MCP-2, MCP-3, MCP-4, and MCP-5), platelet basic protein (PBP), platelet factor 4 (PF-4), Regulated on Activation, Normal T-  
25 cell Expressed and Secreted (RANTES) protein, stromal derived factors (*e.g.* SDF-1 and SDF-2), single C motif 1- $\beta$  (SCM-1 $\beta$ ), thymus and activation-regulated chemokine (TARC), thymus expressed chemokine (TECK), the tumor necrosis factor TWEAK (also known as TNFSF12 and APO3L), and allelic or species variants thereof. For more non-limiting exemplary ligands, see Table 11  
30 in the Examples below.

[139] Among certain embodiments, the proteins of the present invention comprise the Shiga toxin effector region comprising 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). Further embodiments are proteins in which the Shiga

toxin effector region comprises or consists 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). Further embodiments are proteins in which the Shiga toxin effector region comprises or consists 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). Further embodiments are proteins in which the Shiga toxin effector region comprises or consists essentially of 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).

[140] In certain embodiments, the proteins of the present invention comprise the immunoglobulin-type binding region comprising or consisting essentially of amino acids 2-245 of SEQ ID NO:4, which exhibits high affinity binding specifically to human HER2. Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 4-7.

[141] In certain embodiments, the proteins of the present invention comprise the immunoglobulin-type binding region comprising or consisting essentially of amino acids 2-241 of SEQ ID NO:8, which exhibits high affinity binding specifically to human CD38. Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 8-11.

[142] In certain embodiments, the proteins of the present invention comprise the immunoglobulin-type binding region comprising or consisting essentially of amino acids 2-250 of SEQ ID NO:12, which exhibits high affinity binding specifically to human CD19. Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 12-15.

[143] In certain embodiments, the proteins of the present invention comprise the immunoglobulin-type binding region comprising or consisting essentially of amino acids 2-251 of SEQ ID NO:16, which exhibits high affinity binding specifically to human CD74. Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 16-19.

[144] Among certain embodiments of the present invention, the binding region is a single domain immunoglobulin-derived region V<sub>H</sub>H which exhibits high

affinity binding specifically to HER2, such as derived from a single-domain variable region of the camelid antibody (V<sub>H</sub>H) protein 5F7, as described in U.S. Patent Application Publication 2011/0059090. In certain further embodiments, the proteins of the present invention comprise the immunoglobulin-type binding region comprising or consisting essentially of amino acids 2-119 of SEQ ID NO:20, which exhibits high affinity binding specifically to human HER2. Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 20-27.

[145] In certain embodiments, the proteins of the present invention comprise the binding region comprising or consisting essentially of amino acids 2-99 of SEQ ID NO:28, which exhibits high affinity binding specifically to human CD20. Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 28-29.

[146] In certain embodiments, the proteins of the present invention comprise the binding region comprising or consisting essentially of amino acids 2-134 of SEQ ID NO:30, which exhibits high affinity binding specifically to human interleukin-2 receptor (IL-2R). Further embodiments are the proteins comprising or consisting essentially of any one of the polypeptides shown in SEQ ID NOs: 30-33.

[147] As used herein, the term “heavy chain variable (V<sub>H</sub>) domain” or “light chain variable (V<sub>L</sub>) domain” respectively refer to any antibody V<sub>H</sub> or V<sub>L</sub> domain (*e.g.* a human V<sub>H</sub> or V<sub>L</sub> domain) as well as any derivative thereof retaining at least qualitative antigen binding ability of the corresponding native antibody (*e.g.* a humanized V<sub>H</sub> or V<sub>L</sub> domain derived from a native murine V<sub>H</sub> or V<sub>L</sub> domain). A V<sub>H</sub> or V<sub>L</sub> domain consists of a “framework” region interrupted by the three CDRs or ABRs. The framework regions serve to align the CDRs for specific binding to an epitope of an antigen. From amino-terminus to carboxyl-terminus, both V<sub>H</sub> and V<sub>L</sub> domains comprise the following framework (FR) and CDR regions: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. For camlied V<sub>H</sub>H fragments, IgNARs of cartilaginous fish, V<sub>NAR</sub> fragments, and derivatives thereof, there is a single heavy chain variable domain comprising the same basic arrangement: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4.

[148] It is within the scope of the present invention to use fragments, variants, and/or derivatives of the polypeptides of the proteins of the invention which

contain a functional extracellular target biomolecule binding site, and even more preferably capable of binding the target biomolecule with high affinity (*e.g.* as shown by  $K_D$ ). For example, while the invention provides polypeptide sequences that can specifically bind to CD19, CD20, CD38, CD74, and HER2, any binding region that binds to extracellular target biomolecule physically coupled to a cell, expressed on a cell surface, with a dissociation constant of  $10^{-5}$  to  $10^{-12}$  moles per liter, preferably less than 200 nM, may be substituted for use in making proteins of the invention and methods of the invention.

[149] Among certain embodiments of the present invention, the immunoglobulin-type binding region is derived from a nanobody or single domain immunoglobulin-derived region  $V_{HH}$ . Generally, nanobodies are constructed from fragments of naturally occurring single, monomeric variable domain antibodies (sdAbs) of the sort found in camelids and cartilaginous fishes (Chondrichthyes). Nanobodies are engineered from these naturally occurring antibodies by truncating the single, monomeric variable domain to create a smaller and more stable molecule, such as, *e.g.* IgNAR,  $V_{HH}$ , and  $V_{NAR}$  constructs. Due to their small size, nanobodies are able to bind to antigens that are not accessible to whole antibodies. Among certain embodiments of the present invention, the immunoglobulin-type binding region is derived from a nanobody or single domain immunoglobulin-derived region  $V_{HH}$  which exhibits high affinity binding specifically to human HER2 proteins.

### III. General Functions of the Proteins of the Invention

[150] The present invention provides various proteins, each comprising 1) a binding region for cell targeting and 2) a cytotoxic Shiga toxin effector region. The linking of cell targeting binding regions with Shiga-toxin-Subunit-A-derived regions enables the engineering of cell-type specific targeting of the potent Shiga toxin cytotoxicity. In certain embodiments, the proteins of the 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 proteins of the invention are capable of routing a cytotoxic Shiga toxin effector polypeptide fragment into the cytosol of the target cell. Once in the cytosol of a targeted cell type, certain embodiments of the cytotoxic proteins of the invention are capable of

enzymatically inactivating ribosomes, interfering with cell homeostasis, and eventually killing the cell. This system is modular, in that any number of diverse binding regions can be used to target this potent cytotoxicity or cytostasis to various, diverse cell types. Alternatively, nontoxic variants of the proteins of the invention may be used to deliver additional exogenous materials into target cells, such as or detection promoting agents to label the interiors of target cells for diagnostic information collection functions.

#### A. Cell Kill via Targeted Shiga Toxin Cytotoxicity

[151] Because members of the Shiga toxin family are adapted to killing eukaryotic cells, proteins designed using Shiga toxin effector regions can show potent cell-kill activity. The A Subunits of members of the Shiga toxin family comprise enzymatic domains capable of killing a eukaryotic cell once in the cell's cytosol. Certain embodiments of the cytotoxic proteins of the invention take advantage of this cytotoxic mechanism but must be capable of getting the Shiga toxin effector region to the cytosol of a targeted cell type. The addition of a KDEL type signal motif improves cytotoxicity of the cytotoxic proteins of the invention most likely by providing sufficient intracellular routing to promote delivery of a Shiga toxin effector polypeptide to the cytosol.

[152] In certain embodiments of the cytotoxic proteins of the present invention, upon contacting a cell physically coupled with an extracellular target biomolecule of the binding region of a cytotoxic protein of the invention, the cytotoxic protein is capable of causing death of the cell. Cell kill may be accomplished using a cytotoxic protein of the invention under varied conditions of target cells, such as 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 vivo*.

[153] The expression of the target biomolecule need not be native in order for targeted cell killing by a cytotoxic protein of the invention. 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. An example of inducing expression of a target biomolecule is the upregulation of

CD38 expression of cells exposed to retinoids, like all-*trans* retinoic acid and various synthetic retinoids, or any retinoic acid receptor (RAR) agonist (Drach J et al., *Cancer Res* 54: 1746-52 (1994); Uruno A et al., *J Leukoc Biol* 90: 235-47 (2011)). In another example, CD20, HER2, and EGFR expression may be  
5 induced by exposing a cell to ionizing radiation (Wattenberg M et al., *Br J Cancer* 110: 1472-80 (2014)).

#### B. Selective Cytotoxicity among Cell Types

[154] By targeting the delivery of enzymatically active Shiga toxin regions  
10 using high-affinity binding regions to specific cell types, this potent cell-kill activity can be restricted to preferentially killing selected cell types. The present invention provides various cytotoxic proteins with this functional ability.

[155] In certain embodiments, administration of the cytotoxic protein of the present invention to a mixture of cell types, the cytotoxic protein is capable of  
15 selectively killing those cells which are physically coupled with a certain extracellular target biomolecule compared to cell types not physically coupled with any extracellular target biomolecule specifically bound by the binding region of that cytotoxic protein. Because members of the Shiga toxin family are adapted for killing eukaryotic cells, cytotoxic proteins designed using Shiga  
20 toxin effector regions can show potent cytotoxic activity. By targeting the delivery of enzymatically active Shiga toxin regions to specific cell types using high-affinity binding regions, this potent cell kill activity can be restricted to preferentially killing only those cell types desired to be targeted by their physical association with a target biomolecule specifically bound by chosen binding  
25 regions.

[156] In certain embodiments, the cytotoxic protein 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 the targeting of cytotoxic activity to specific cell types with a high preferentiality, such as a 3-  
30 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.

5 [157] Levels of extracellular target biomolecules on the surface of cells may be determined using various methods known to the skilled worker, such as, *e.g.*, FACS methods. As used herein, a significant amount of an extracellular target biomolecule expressed at a cellular surface is greater than 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, or 70,000 mean fluorescence intensity (MFI) by  
10 FACS analysis depending on the cell type.

[158] In certain further embodiments, administration of the cytotoxic protein of the invention to two populations of cell types which differ with respect to the presence and/or polypeptide sequence of an extracellular target biomolecule, the cytotoxic protein is capable of causing cell death as defined by the half-maximal  
15 cytotoxic concentration ( $CD_{50}$ ) on a population of target cells, whose members express an extracellular target biomolecule of the binding region of the cytotoxic protein, *e.g.*, at a dose at least three-times lower than the  $CD_{50}$  dose of the same cytotoxic protein to a population of cells whose members do not express an extracellular target biomolecule of the binding region of the cytotoxic protein.

20 [159] In certain embodiments, the cytotoxic activity of a protein 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 bound specifically by that protein of the  
25 invention. 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  
30 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, 1000-fold, or 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.

[160] This preferential cell-killing function allows a target cell to be killed by certain cytotoxic proteins of the 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).

[161] In certain embodiments, the proteins of the present invention are 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 the targeted 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 extracellular target bound specifically by the binding region of that cytotoxic protein of the invention. Alternatively, the expression of an extracellular target biomolecule may be non-exclusive to one cell type if the extracellular target biomolecule is expressed in low enough amounts by cell types that are not to be targeted. This enables the preferential cell-killing of only those cell type(s) expressing the highest amounts of extracellular target biomolecules, such as a 3-fold cytotoxic effect, over “bystander” cell types that do not express significant amounts of the target biomolecule bound specifically by that cytotoxic protein and/or are not physically coupled to significant amounts of extracellularly exposed target biomolecule bound specifically by that cytotoxic protein.

[162] The cytotoxic proteins of the present invention are useful for the elimination of populations of specific cell types. For example, the cytotoxic proteins of the invention are useful for the treatment of certain tumors, cancers, and/or other growth abnormalities by eliminating “target biomolecule+” cells that express elevated levels of target biomolecule at one or more cellular surfaces.

[163] In certain embodiments, the cytotoxic activity of a protein of the present invention toward populations of cell types physically coupled with a certain extracellular target biomolecule is at least 3-fold higher than the cytotoxic activity toward populations of cell types not physically coupled with significant amounts of an extracellular target biomolecule bound specifically by the binding

region of that particular protein of the invention. 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 physically coupled with a significant amount of an extracellular target biomolecule bound by the binding region of the cytotoxic protein of the invention to (b) cytotoxicity towards a population of cells of a cell type not physically coupled with a significant amount of any extracellular target biomolecule bound specifically by the binding region of that particular cytotoxic protein of the invention. 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, 1000-fold or higher for populations of cells or cell types expressing an extracellular target biomolecule or physically coupled with an extracellular target biomolecule bound by the binding region of the cytotoxic protein of the invention compared to populations of cells or cell types which do not express an extracellular target biomolecule or that are not physically coupled with significant amounts of an extracellular target biomolecule bound specifically the binding region of that particular cytotoxic protein of the invention. For example, upon administration of certain cytotoxic proteins of the present invention to two different populations of cells which differ with respect to the presence and/or polypeptide sequence of an extracellular target biomolecule, the cytotoxic protein of the invention is capable of causing cell death of the cell-types physically coupled with an extracellular target biomolecule bound by the cytotoxic protein's binding region, *e.g.*, at a  $CD_{50}$  that is at least three times less than the  $CD_{50}$  observed for cell types that are not physically coupled with an extracellular target biomolecule bound by the cytotoxic protein's binding region or for cell types that are physically coupled only with forms of that extracellular target biomolecule which comprise sequence variations or mutations which disrupt binding specificity by the binding region of that cytotoxic protein.

[164] In certain embodiments of the cytotoxic proteins of the present invention, administration of the cytotoxic protein to two different populations of cell types, the cytotoxic protein is capable of causing cell death as defined by the half-maximal cytotoxic concentration ( $CD_{50}$ ) on a first cell population, whose members express a certain target biomolecule at a cellular surface, at a dose at

least three-times lower than the CD<sub>50</sub> dose of the same cytotoxic protein to a second population of cells whose members do not express that target biomolecule, do not express a significant amount of that target biomolecule, or are not exposing a significant amount of that target biomolecule bound by the cytotoxic protein's binding region.

### C. Delivery of Additional Exogenous Material into the Interior of a Target Cell

[165] In addition to direct cell killing, proteins of the invention optionally may be used for delivery of additional exogenous materials into the interiors of target cells. The delivery of additional exogenous materials may be used, *e.g.*, for cytotoxic, cytostatic, information gathering, and/or diagnostic functions. Non-toxic variants of the cytotoxic proteins of the invention, or optionally toxic 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 cytotoxic protein. Various types of cells and/or cell populations which express target biomolecules to at least one cellular surface may be targeted by the proteins of the invention for receiving exogenous materials. The functional components of the present invention are modular, in that various Shiga toxin effector regions and additional exogenous materials may be linked to various binding regions to provide diverse applications, such as non-invasive *in vivo* imaging of tumor cells.

[166] Because the proteins of the present invention, whether toxic or nontoxic, and catalytically inactive forms thereof, are capable of entering cells physically coupled with an extracellular target biomolecule recognized by its binding region, certain embodiments of the proteins of the invention may be used to deliver additional exogenous materials into the interior of targeted cell types. In one sense, the entire protein is an exogenous material which will enter the cell; thus, the "additional" exogenous materials are heterologous materials linked to but other than the core protein itself.

[167] "Additional exogenous material" as used herein refers to one or more molecules, often not generally present within a native target cell, where the proteins of the present invention may be used to specifically transport such material to the interior of a cell. Non-limiting examples of additional exogenous

materials are cytotoxic agents, peptides, polypeptides, proteins, polynucleotides, detection promoting agents, and small molecule chemotherapeutic agents.

[168] In certain embodiments of the proteins of the present invention for delivery of additional exogenous material, the additional exogenous material is a  
5 cytotoxic agent, such as, *e.g.*, a small molecule chemotherapeutic agent, cytotoxic antibiotic, alkylating agent, antimetabolite, topoisomerase inhibitor, and/or tubulin inhibitor. Non-limiting examples of cytotoxic agents include aziridines, cisplatins, tetrazines, procarbazine, hexamethylmelamine, vinca  
10 alkaloids, taxanes, camptothecins, etoposide, doxorubicin, mitoxantrone, teniposide, novobiocin, aclarubicin, anthracyclines, actinomycin, bleomycin, plicamycin, mitomycin, daunorubicin, epirubicin, idarubicin, dolastatins, maytansines, docetaxel, adriamycin, calicheamicin, auristatins, pyrrolbenzodiazepine, carboplatin, 5-fluorouracil (5-FU), capecitabine, mitomycin C, paclitaxel, 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU),  
15 rifampicin, cisplatin, methotrexate, and gemcitabine.

[169] 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  
20 certain embodiments, the additional exogenous material is an antigen, such as antigens derived from 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  
25 complementary determining regions capable of functioning as exogenous antigens.

#### D. Information Gathering for Diagnostic Functions

[170] Certain proteins of the invention have uses in the *in vitro* and/or *in vivo*  
30 detection of specific cells, cell types, and/or cell populations. In certain embodiments, the cytotoxic proteins described herein are used for both diagnosis and treatment, or for diagnosis alone. When the same cytotoxic protein is used for both diagnosis and treatment, the cytotoxic protein variant which incorporates a detection promoting agent for diagnosis may be rendered non-

toxic by catalytic inactivation of a Shiga toxin effector region via one or more amino acid substitutions, including exemplary substitutions described herein. Catalytically inactive forms of the cytotoxic proteins 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.

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[171] The ability to conjugate detection promoting agents known in the art to various proteins of the invention provides useful compositions for the detection of cancer, tumor, immune, and infected cells. These diagnostic embodiments of the proteins of the present invention may be used for information gathering via various imaging techniques and assays known in the art. For example, diagnostic embodiments of the proteins 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, or infected cells in a patient or biopsy sample.

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[172] Various types of information may be gathered using the diagnostic embodiments of the proteins 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 antineoplastic 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.

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[173] For example, subpopulations of patients might be ascertained using information gathered using the diagnostic variants of the proteins of the present invention, and then individual patients could be 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 one type of criterion used to define a patient subpopulation. For example, a non-toxic diagnostic variant of a particular cytotoxic protein 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

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the same cytotoxic protein of the invention. Accordingly, associated methods for patient identification, patient stratification and diagnosis using cytotoxic proteins of the invention and/or their non-toxic variants are considered to be within the scope of the present invention.

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IV. Variations in the Polypeptide Sequences of the Proteins of the Invention which Maintain Overall Structure and Function

[174] The skilled worker will recognize that variations may be made to the proteins of the present invention (and polynucleotides encoding them) without diminishing their biological activities, *e.g.* by maintaining the overall structure and function of the protein of the invention. For example, some modifications may facilitate expression, purification, pharmacokinetic properties, and/or 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.

[175] Also contemplated herein is the inclusion of additional amino acid residues at the amino and/or carboxy termini, such as sequences for epitope tags or other moieties. The additional amino acid residues may be used for various purposes including, *e.g.*, to facilitate cloning, expression, post-translational modification, synthesis, purification, detection, and/or 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.

[176] In certain of the above embodiments, the protein of the present invention is a variant in which there are one or more conservative amino acid substitutions introduced into the polypeptide region(s). 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 B below). 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)).

[177] In the conservative substitution scheme in Table B below, 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.

TABLE B. 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	

[178] In certain embodiments, a protein of the present invention may comprise functional fragments or variants of a polypeptide region of the invention that have, at most, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitution(s)

compared to a polypeptide sequence recited herein, as long as the substituted polypeptide region retains measurable biological activity alone or as a component of a protein of the invention. Variants of proteins of the invention are within the scope of the invention as a result of changing a polypeptide of the protein of the invention by altering one or more amino acids or deleting or  
5 inserting one or more amino acids, such as within the binding region or the Shiga toxin effector region, in order to achieve desired properties, such as changed cytotoxicity, changed cytostatic effects, changed immunogenicity, and/or changed serum half-life. A polypeptide of a protein of the invention may  
10 further be with or without a signal sequence.

[179] In certain embodiments, a protein of the present invention shares at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or more amino acid sequence identity to any one of the amino acid sequences of a protein recited herein, as long as it retains measurable biological activity, such as cytotoxicity, extracellular target  
15 biomolecule binding, enzymatic catalysis, or subcellular routing. The immunoglobulin-type binding region may differ from the amino acid sequences of a protein recited herein, as long as it retains binding functionality to its extracellular target biomolecule. Binding functionality will most likely be retained if the amino acid sequences of the CDRs or ABRs are identical. For  
20 example, a protein is within the claim scope that comprises or consists essentially of 85% amino acid identity to a protein recited herein which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDRs or ABRs are disregarded. Binding functionality can be determined by the skilled worker using standard techniques.

[180] In certain embodiments, the Shiga toxin effector region may be altered to change its enzymatic activity and/or cytotoxicity as long as the Shiga toxin effector region retains one or more other Shiga toxin effector functions. This change may or may not result in a change in the cytotoxicity of a protein of which the altered Shiga toxin effector region is a component. Possible  
25 alterations include mutations to the Shiga toxin effector region selected from the group consisting of: a truncation, deletion, inversion, insertion, rearrangement, and substitution.  
30

[181] The cytotoxicity of the A Subunits of members of the Shiga toxin family may be altered, reduced, or eliminated by mutation 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, *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, *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 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, *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, *J Biol Chem* 280: 23310-18 (2005)).

[182] 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 arginine-176 among others (Di, *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.

[183] 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)).

[184] Shiga-like toxin 1 A Subunit truncations are catalytically active, capable of enzymatically inactivating ribosomes *in vitro*, and cytotoxic when expressed within a cell (LaPointe, *J Biol Chem* 280: 23310-18 (2005)). The smallest Shiga toxin A Subunit fragment exhibiting full enzymatic activity is a polypeptide  
5 composed of residues 1-239 of Sl1A (LaPointe, *J Biol Chem* 280: 23310-18 (2005)). Although the smallest fragment of the Shiga toxin A Subunit reported to retain substantial catalytic activity was residues 75-247 of StxA (Al-Jaufy, *Infect Immun* 62: 956-60 (1994)), a StxA truncation expressed *de novo* within a eukaryotic cell requires only up to residue 240 to reach the cytosol and exert  
10 catalytic inactivation of ribosomes (LaPointe, *J Biol Chem* 280: 23310-18 (2005)).

[185] In certain embodiments derived from SLT-1A (SEQ ID NO:1) or StxA (SEQ ID NO:2), these changes include substitution of the asparagine at position 75, tyrosine at position 77, tyrosine at position 114, glutamate at position 167,  
15 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 at position 167 to aspartate,  
20 substitution of the arginine at position 170 to alanine, substitution of the arginine at position 176 to lysine, and/or substitution of the tryptophan at position 203 to alanine.

[186] Proteins of the present invention may optionally be conjugated to one or more additional agents, such as therapeutic and/or diagnostic agents known in  
25 the art, including such agents as described herein.

#### V. Production, Manufacture, and Purification of a Protein of the Invention

[187] The proteins of the present invention may be produced using biochemical engineering techniques well known to those of skill in the art. For example,  
30 proteins of the invention may be manufactured by standard synthetic methods, by use of recombinant expression systems, or by any other suitable method. The proteins of the invention may be produced as fusion proteins, chemically coupled conjugates, and/or combinations thereof, such as, *e.g.*, a fusion protein component covalently coupled to one or more components. Thus, the proteins 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 protein of the invention using standard solid-phase or liquid-phase methodology, either stepwise or by fragment assembly, and isolating and purifying the final peptide compound product; (2) expressing a polynucleotide that encodes a polypeptide or polypeptide component of a protein 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 protein 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 peptide component, subsequently joining (*e.g.* ligating) the fragments to obtain the peptide component, and recovering the peptide component.

[188] It may be preferable to synthesize a polypeptide or polypeptide component of a protein of the present invention by means of solid-phase or liquid-phase peptide synthesis. Proteins of the 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 1998/11125 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.

[189] Proteins of the present invention may be prepared (produced and purified) using recombinant techniques well known in the art. In general, methods for preparing polypeptides by culturing host cells transformed or transfected with a vector comprising the encoding polynucleotide and recovering the polypeptide 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 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 protein of the invention may be produced by modifying the polynucleotide encoding the 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

5 cytostatic effects, changed immunogenicity, and/or changed serum half-life. [190] There are a wide variety of expression systems which may be chosen to produce a protein of the present invention. For example, host organisms for expression of proteins of the invention include prokaryotes, such as *E. coli* and 10 *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*).

15 [191] Accordingly, the present invention also provides methods for producing a protein of the present invention according to above recited methods and using (i) a polynucleotide encoding part or all of a protein of the invention or a polypeptide component thereof, (ii) an expression vector comprising at least one polynucleotide of the invention capable of encoding part or all of a protein of the 20 invention or a polypeptide component thereof when introduced into a suitable host cell or cell-free expression system, and/or (iii) a host cell comprising a polynucleotide or expression vector of the invention.

[192] When a polypeptide or protein is expressed using recombinant techniques in a host cell or cell-free system, it is advantageous to separate (or purify) the 25 desired polypeptide or 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 30 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 protein of the invention. In certain embodiments, the proteins of the invention may optionally be purified in homo-multimeric forms (*i.e.* a protein complex of two or more identical proteins) or in hetero-multimeric forms (*i.e.* a protein complex of two or more non-identical proteins).

[193] In the Examples below are descriptions of non-limiting examples of methods for producing a protein of the present invention, as well as specific but non-limiting aspects of protein production for the disclosed, exemplary, cytotoxic proteins.

10

#### VI. Pharmaceutical and Diagnostic Compositions Comprising a Protein of the Invention

[194] The present invention provides proteins 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). The present invention further provides pharmaceutical compositions comprising a protein of the 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 pharmaceutical composition of the invention may comprise homo-multimeric and/or hetero-multimeric forms of the proteins of the invention. The pharmaceutical compositions will be useful in methods of treating, ameliorating, or preventing a disease, condition, disorder, or symptom described in further detail below. 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.

30

[195] 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.*).

[196] As used herein, “treat,” “treating,” or “treatment” and grammatical  
5 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  
10 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”  
15 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  
20 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 reductions in overall tumor burden and/or individual tumor size.

[197] As used herein, the terms “prevent,” “preventing,” “prevention” and  
25 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  
30 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.

[198] As used herein, an “effective amount” or “therapeutically effective amount” is an amount or dose of a composition (*e.g.* a therapeutic composition 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 compound (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 compound 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)).

[199] Diagnostic compositions of the present invention comprise a protein of the invention and one or more detection promoting agents. Various detection promoting agents are known in the art, such as isotopes, dyes, colorimetric agents, contrast enhancing agents, fluorescent agents, bioluminescent agents, and magnetic agents. These agents may be incorporated into the protein of the invention at any position. The incorporation of the agent may be via an amino acid residue(s) of the protein of the 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.

[200] When producing or manufacturing a diagnostic composition of the present invention, a protein of the invention may be directly or indirectly linked to one or more detection promoting agents. There are numerous detection promoting agents known to the skilled worker which can be operably linked to the proteins of the present 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)). For example, detection promoting agents include image enhancing contrast agents, such as fluorescent dyes (*e.g.* Alexa680, indocyanine green, and Cy5.5), isotopes and radionuclides, such as <sup>11</sup>C, <sup>13</sup>N, <sup>15</sup>O, <sup>18</sup>F, <sup>32</sup>P, <sup>51</sup>Mn, <sup>52m</sup>Mn, <sup>52</sup>Fe, <sup>55</sup>Co, <sup>62</sup>Cu, <sup>64</sup>Cu, <sup>67</sup>Cu, <sup>67</sup>Ga, <sup>68</sup>Ga, <sup>72</sup>As, <sup>73</sup>Se, <sup>75</sup>Br, <sup>76</sup>Br, <sup>82m</sup>Rb, <sup>83</sup>Sr, <sup>86</sup>Y, <sup>90</sup>Y, <sup>89</sup>Zr, <sup>94m</sup>Tc, <sup>94</sup>Tc, <sup>99m</sup>Tc, <sup>110</sup>In, <sup>111</sup>In, <sup>120</sup>I, <sup>123</sup>I, <sup>124</sup>I, <sup>125</sup>I, <sup>131</sup>I, <sup>154</sup>Gd, <sup>155</sup>Gd, <sup>156</sup>Gd, <sup>157</sup>Gd, <sup>158</sup>Gd, <sup>177</sup>Lu, <sup>186</sup>Re, <sup>188</sup>Re, and <sup>223</sup>Rn, paramagnetic ions, such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III), metals, such as lanthanum (III), gold (III), lead (II), and bismuth (III), ultrasound contrast enhancing agents, such as liposomes, radiopaque agents, such as barium, gallium, and thallium compounds. Detection promoting agents may be incorporated directly or indirectly by using an intermediary functional group, such as chelators like 2-benzyl DTPA, PAMAM, NOTA, DOTA, TETA, analogs thereof, and functional equivalents of any of the foregoing (*see* Leyton J et al., *Clin Cancer Res* 14: 7488-96 (2008)). There are numerous standard techniques known to the skilled worker for incorporating, affixing, and/or conjugating various detection promoting agents to proteins, especially to immunoglobulins and immunoglobulin-derived domains (Wu A, *Methods* 65: 139-47 (2014)). 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 (*see* Kaur S et al., *Cancer Lett* 315: 97-111 (2012), *for review*).

Production or Manufacture of a Pharmaceutical and/or Diagnostic Composition

5 Comprising a Protein of the Invention

[201] Pharmaceutically acceptable salts or solvates of any of the proteins of the present invention are likewise within the scope of the present invention.

[202] The term “solvate” in the context of the present invention refers to a complex of defined stoichiometry formed between a solute (*in casu*, a  
10 polypeptide 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.

[203] 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 compound of the invention, or a salt thereof, in a pharmaceutically acceptable carrier. The term  
15 “pharmaceutically acceptable carrier” includes any of the standard pharmaceutical carriers. Pharmaceutically acceptable carriers for therapeutic 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  
20 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  
25 subcutaneous, intramuscular, intravenous, intradermal, and transdermal) administration. Exemplary pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous  
30 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

5       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 of the invention or other pharmaceutical component may be coated in a material intended to protect the compound from the action of low pH and other natural

10       inactivating conditions to which the active protein of the invention may encounter when administered to a patient by a particular route of administration.

[204] The formulations of the pharmaceutical compositions of the present 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

15       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

20       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.

25       [205] The pharmaceutical compositions of the present invention may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of 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

30       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.

[206] 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.

5  
10 [207] In another aspect, the present invention provides pharmaceutical compositions comprising one or a combination of different proteins of the invention, or an ester, salt or amide of any of the foregoing, and at least one pharmaceutically acceptable carrier.

[208] 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, polyalcohols such as mannitol, sorbitol, or sodium chloride may be desirable in the composition.

15  
20  
25 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.

[209] 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.*,

30

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.

5 [210] Sterile injectable solutions may be prepared by incorporating a protein 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  
10 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.

15 [211] When a therapeutically effective amount of a protein 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,  
20 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  
25 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.

[212] As described elsewhere herein, a protein of the present invention or composition thereof (*e.g.* pharmaceutical or diagnostic composition) may be  
30 prepared with carriers that will protect the composition 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)).

[213] In certain embodiments, the composition of the present invention (*e.g.* pharmaceutical or diagnostic composition) may be formulated to ensure a desired distribution *in vivo*. For example, the blood-brain barrier excludes many large and/or hydrophilic compounds. To target a therapeutic protein or composition of the invention to a particular *in vivo* location, it 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.

[214] 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 dendrimers, liposomes, microspheres, microparticles, nanocapsules, nanoparticles, nanorods, nanospheres, polymeric micelles, and nanotubes (*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. Particulate and polymer formulations may comprise a plasma membrane permeability altering agent(s), such as, *e.g.*, various peptides and proteins like cytolytins, toxin-derived agents, virus derived agents, synthetic biomimetic peptides, and chemical agents (*see e.g. Varkouhi et al., J Control Release* 151: 220-8 (2011); J Pirie C et al., *Mol Cancer Ther* 12: 1774-82 (2013)).

## 30 VII. Polynucleotides, Expression Vectors, and Host Cells

[215] Beyond the proteins of the present invention, the polynucleotides which encode such proteins, or functional portions thereof, are within the scope of the present invention. The term “polynucleotide” is equivalent to the term “nucleic acids” both of which include 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 invention may be single-, double-, or triple-stranded. Disclosed polynucleotides are specifically disclosed to include all  
5 polynucleotides capable of encoding an exemplary protein of the invention, 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)).

[216] In one aspect, the invention provides polynucleotides which encode a  
10 protein of the invention, or a fragment or derivative thereof. The polynucleotides may include, *e.g.*, 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 the protein of the invention. The invention also includes polynucleotides  
15 comprising nucleotide sequences that hybridize under stringent conditions to a polynucleotide which encodes a protein of the invention, or a fragment or derivative thereof, or the antisense or complement of any such sequence.

[217] Derivatives or analogs of the polynucleotides (or proteins) of the invention include, *inter alia*, polynucleotide (or polypeptide) molecules having  
20 regions that are substantially homologous to the polynucleotides or proteins of the invention, *e.g.* by at least about 45%, 50%, 70%, 80%, 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  
25 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  
30 encoding the 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 in *Current Protocols in Molecular Biology* (John Wiley & Sons, NY, U.S., Ch. Sec. 6.3.1-6.3.6 (1989)).

[218] The present invention further provides expression vectors that comprise the polynucleotides within the scope of the invention. The polynucleotides capable of encoding the proteins 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 proteins of the invention within any host cell of choice or cell-free expression systems (*e.g.* pTxb1 and pIVEX2.3 described in the Examples below). 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, or may be purchased.

[219] 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 protein of the invention comprising a single polypeptide chain (*e.g.* a scFv genetically recombined with a Shiga toxin effector region) includes at least an expression unit for the single polypeptide chain, whereas a protein of the invention comprising, *e.g.* two or more polypeptide chains (*e.g.* one chain comprising a V<sub>L</sub> domain and a second chain comprising a V<sub>H</sub> domain linked to a toxin effector region) includes at least two expression units, one for each of the two polypeptide chains of the protein. For expression of multi-chain 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).

[220] 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  
5 known in the art.

[221] 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  
10 the invention or capable of producing a protein of the invention can be accomplished using standard techniques known in the art.

[222] Proteins within the scope of the present invention may be variants or derivatives of the proteins described herein that are produced by modifying the polynucleotide encoding a disclosed protein of the invention by altering one or  
15 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.

#### VIII. Delivery Devices and Kits

[223] In certain embodiments, the invention relates to a device comprising one or more compositions of matter of the invention, such as a pharmaceutical composition, for delivery to a subject. Thus, a delivery devices comprising one or more compounds of the invention may be used to administer to a patient a composition of matter of the invention by various delivery methods, including:  
25 intravenous, subcutaneous, intramuscular or 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.

[224] Also within the scope of the invention are kits comprising at least one  
30 composition of matter of the 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 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. 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 invention as described  
5 herein.

#### IX. Methods for Using a Protein of the Invention or a Composition Thereof

[225] Generally, it is an object of the invention to provide pharmacologically active agents, as well as compositions comprising the same, that can be used in  
10 the prevention and/or treatment of diseases, disorders, and conditions, such as certain cancers, tumors, immune disorders, microbial infections, or further pathological conditions mentioned herein. Accordingly, the present invention provides methods of using the proteins and pharmaceutical compositions of the invention for the targeted killing of cells, for delivering additional exogenous  
15 materials into target cells, for labeling of the interiors of target cells, for collecting diagnostic information, and for treating diseases, disorders, and conditions as described herein.

[226] In particular, it is an object of the invention to provide such pharmacologically active agents, compositions, and/or methods that have certain  
20 advantages compared to the agents, compositions, and/or methods that are currently known in the art. Accordingly, the present invention provides methods of using proteins of the invention characterized by specified polypeptide sequences and pharmaceutical compositions thereof. For example, any of the polypeptide sequences in SEQ ID NOs: 1-34 may be specifically utilized as a  
25 component of the protein used in the following methods.

[227] The present invention provides methods of killing a cell comprising the step of contacting the cell, either *in vitro* or *in vivo*, with a protein or pharmaceutical composition of the present invention. The proteins and pharmaceutical compositions of the invention can be used to kill a specific cell  
30 type upon contacting a cell or cells with one of the claimed compositions of matter. In certain embodiments, a protein 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. In certain embodiments, a protein or pharmaceutical

composition of the present invention can be used to kill cancer cells in a mixture of different cell types. In certain embodiments, a protein 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. In certain  
5       embodiments, a protein 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. In certain embodiments, a protein or pharmaceutical composition of the present invention can be used to selectively kill cells infected by viruses or microorganisms, or otherwise  
10       selectively kill cells expressing a particular extracellular target biomolecule, such as a cell surface biomolecule. The proteins and pharmaceutical compositions of the invention have varied applications, including, *e.g.*, uses in depleting unwanted cell types from tissues either *in vitro* or *in vivo*, uses in modulating immune responses to treat graft-versus-host disease, uses as antiviral  
15       agents, uses as anti-parasitic agents, and uses in purging transplantation tissues of unwanted cell types.

[228] In certain embodiments, a protein or pharmaceutical composition of the present invention, alone or in combination with other compounds or pharmaceutical compositions, can show potent cell-kill activity when  
20       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 regions using high-affinity binding regions to cancer cell types, this potent cell-kill activity can be restricted to specifically and selectively kill  
25       certain cell types within an organism, such as certain cancer cells, neoplastic cells, malignant cells, non-malignant tumor cells, or infected cells.

[229] 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 protein of the present invention or a pharmaceutical composition thereof.

[230] Certain embodiments of the protein of the invention or pharmaceutical compositions thereof can be used to kill a cancer and/or tumor cell in a patient by targeting an extracellular biomolecule found physically coupled with a cancer and/or tumor cell. The terms “cancer cell” or “cancerous cell” refers to various neoplastic cells which grow and divide in an abnormally accelerated fashion and

will be clear to the skilled person. The term “tumor cell” includes both malignant and non-malignant cells (*e.g.* non-cancerous, benign tumor cells, non-cancerous “cancer” stem cells, tumor stem cells, pre-malignant cancer-initiating cells, tumor-initiating cells, or tumorigenic cells all of which can give rise to daughter cells which become malignant tumor and/or cancer cells but are unable to metastasize on their own (*see e.g.* Martinez-Climent J et al., *Haematologica* 95: 293-302 (2010)). 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.

15 [231] Certain embodiments of the proteins of the 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.

[232] Certain embodiments of the proteins of the 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.

[233] It is within the scope of the present invention to utilize the protein of the invention or pharmaceutical composition thereof for the purposes of purging patient cell populations (*e.g.* bone marrow) of infected, malignant, neoplastic, or otherwise unwanted B-cells and/or T-cells and then reinfusing the B-cell and/or T-cell depleted material into the patient (*see e.g.* van Heeckeren W et al., *Br J Haematol* 132: 42-55 (2006); Alpdogan O, van den Brink M, *Semin Oncol* 39: 629-42 (2012)).

[234] It is within the scope of the present invention to utilize the protein of the invention or pharmaceutical composition thereof for the purposes of *ex vivo* depletion of B-cells and/or T-cells from isolated cell populations removed from a patient. In one non-limiting example, the protein of the invention may be used in a method for prophylaxis of organ and/or tissue transplant rejection wherein the donor organ or tissue is perfused prior to transplant with a cytotoxic protein

of the invention or a pharmaceutical composition thereof in order to purge the organ of unwanted donor B-cells and/or T-cells (*see e.g.* Alpdogan O, van den Brink M, *Semin Oncol* 39: 629-42 (2012)).

5 [235] It is also within the scope of the present invention to utilize the protein of the invention or pharmaceutical composition thereof for the purposes of depleting B-cells and/or T-cells from a donor cell population as a prophylaxis against graft-versus-host disease, and induction of tolerance, in a patient to undergo a bone marrow and or stem cell transplant.

10 [236] Certain embodiments of the protein 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.

15 [237] Certain embodiments of the protein of the present invention or pharmaceutical compositions thereof can be used to kill a cell(s) of a multicellular parasite. In certain further embodiments, the cell killing occurs while the multicellular parasite is present in a host organism or subject. In certain further embodiments, the protein of the invention may be used to kill a helminth, such as, *e.g.*, a plathelminth, nemathelminth, cestode, mongenean, nematode, and/or trematode.

20 [238] Additionally, the present invention provides a method of treating a disease, disorder, or condition in a patient comprising the step of administering to a patient in need thereof a therapeutically effective amount of at least one of the proteins of the present invention or a pharmaceutical composition thereof. Contemplated diseases, disorders, and conditions that can be treated using this  
25 method include cancers, malignant tumors, non-malignant tumors, growth abnormalities, immune disorders, and microbial infections. Administration of a “therapeutically effective dosage” of a compound of the invention may 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  
30 disability due to the disease affliction.

[239] The therapeutically effective amount of a compound of the present invention will depend on the route of administration, the type of mammal 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.

[240] 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.

[241] For administration of a pharmaceutical composition of the invention, the dosage range will generally be from about 0.0001 to 100 milligrams per kilogram (mg/kg), and more usually 0.01 to 5 mg/kg, of the host body weight. Exemplary dosages may be 0.25 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 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.

[242] Pharmaceutical compositions of the invention will typically be administered to the same patient on multiple occasions. Intervals between single dosages can be, for example, 2-5 days, weekly, monthly, every two or three months, every six months, or yearly. Intervals between administrations can also  
5 be irregular, based on regulating blood levels or other markers in the subject or patient. Dosage regimens for a compound of the invention include intravenous administration of 1 mg/kg body weight or 3 mg/kg body weight with the compound administered every two to four weeks for six dosages, then every three months at 3 mg/kg body weight or 1 mg/kg body weight.

10 [243] 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 proteins of the present invention or  
15 compositions thereof include, *e.g.* intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal, or other parenteral routes of administration, for example by injection or infusion. In other embodiments, a protein or pharmaceutical composition of the invention may be administered by a non-parenteral route, such as a topical, epidermal or mucosal route of  
20 administration, for example, intranasally, orally, vaginally, rectally, sublingually, or topically.

[244] Proteins or pharmaceutical compositions of the 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  
25 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  
30 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.

[245] A protein 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 protein of the 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-  
5 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  
10 therapeutics which may complement or otherwise be beneficial in a therapeutic or prophylactic treatment regimen.

[246] Treatment of a patient with a protein or pharmaceutical composition of the invention preferably leads to cell death of targeted cells and/or the inhibition of growth of targeted cells. As such, proteins of the invention, and  
15 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*, cancers, tumors, other growth abnormalities, immune disorders, and infected cells. The present invention provides methods for suppressing cell proliferation, and treating cell disorders,  
20 including neoplasia, overactive B-cells, and overactive T-cells.

[247] In certain embodiments, proteins and pharmaceutical compositions of the invention may be used to treat or prevent cancers, tumors (malignant and non-malignant), growth abnormalities, immune disorders, and microbial infections. In a further aspect, the above *ex vivo* method can be combined with the above *in*  
25 *vivo* method to provide methods of treating or preventing rejection in bone marrow transplant recipients, and for achieving immunological tolerance.

[248] 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  
30 administering to a subject in need thereof a therapeutically effective amount of a protein or pharmaceutical composition of the invention.

[249] The proteins and pharmaceutical compositions of the invention have varied applications, including, *e.g.*, uses in removing unwanted B-cells and/or T-cells, uses in modulating immune responses to treat graft-versus-host diseases,

uses as antiviral agents, uses as antimicrobial agents, and uses in purging transplantation tissues of unwanted cell types. The proteins 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.

[250] In certain embodiments, a protein or pharmaceutical composition of the present invention is used to treat a B-cell-, plasma cell-, T-cell-, or antibody-mediated disease or disorder, such as for example leukemia, lymphoma, myeloma, Human Immunodeficiency Virus-related diseases, amyloidosis, hemolytic uremic syndrome, polyarteritis nodosa, polyarthritis, septic shock, Crohn's Disease, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, ulcerative colitis, psoriasis, asthma, Sjorgren's syndrome, graft-versus-host disease, graft rejection, diabetes, vasculitis, scleroderma, and systemic lupus erythematosus.

[251] In another aspect, certain embodiments of the proteins and pharmaceutical compositions of the present invention are antimicrobial agents – meaning they are capable of treating and/or preventing the acquisition, development, or consequences of microbiological pathogenic infections, such as caused by viruses, bacteria, fungi, prions, or protozoans.

[252] It is within the scope of the present invention to provide a prophylaxis or treatment for diseases or conditions mediated by B-cells and/or T-cells, the prophylaxis or treatment involving administering the protein of the invention, or a pharmaceutical composition thereof, to a patient in need thereof for the purpose of killing B-cells and/or T-cells in the patient. This usage is compatible with preparing or conditioning a patient for bone marrow transplantation, stem cell transplantation, tissue transplantation, or organ transplantation, regardless of the source of the transplanted material, *e.g.* human or non-human sources.

[253] It is within the scope of the present invention to provide a bone marrow recipient for prophylaxis or treatment of host-versus-graft disease via the targeted cell-killing of host B-cells, NK cells, and/or T-cells using a protein or pharmaceutical composition of the present invention (*see e.g.* Sarantopoulos S et al., *Biol Blood Marrow Transplant* 21: 16-23 (2015)).

[254] The proteins and pharmaceutical compositions of the present invention may be utilized in a method of treating cancer comprising administering to a patient, in need thereof, a therapeutically effective amount of the protein or a pharmaceutical composition of the present invention. In 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.

[255] The proteins and pharmaceutical compositions of the present invention may be utilized in a method of treating an immune disorder comprising administering to a patient, in need thereof, a therapeutically effective amount of the protein or a pharmaceutical composition of the present invention. In certain embodiments of the methods of the present invention, the immune disorder is related to an inflammation 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 diseases, lupus erythematosus, multiple sclerosis, polyarteritis nodosa, polyarthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, scleroderma, septic shock, Sjorgren's syndrome, ulcerative colitis, and vasculitis.

[256] Among certain embodiments of the present invention is using the protein of the invention as a component of a pharmaceutical composition or medicament for the treatment or prevention of a cancer, tumor, growth abnormality, immune

disorder, and/or microbial infection. For example, immune disorders presenting on the skin of a patient may be treated with such a medicament in efforts to reduce inflammation. In another example, skin tumors may be treated with such a medicament in efforts to reduce tumor size or eliminate the tumor completely.

5 [257] Certain proteins of the present invention may be used in molecular neurosurgery applications such as immunolesioning and neuronal tracing (*see*, Wiley R, Lappi D, *Adv Drug Deliv Rev* 55: 1043-54 (2003), *for review*). For example, the targeting domain may be selected or derived from various ligands, such as neurotransmitters and neuropeptides, which target specific neuronal cell  
10 types by binding neuronal surface receptors, such as a neuronal circuit specific G-protein coupled receptor. Similarly, the targeting domain may be selected from or derived from antibodies that bind neuronal surface receptors. Because Shiga toxins robustly direct their own retrograde axonal transport, certain cytotoxic proteins of the invention may be used to kill a neuron(s) which  
15 expresses the extracellular target at a site of cytotoxic protein injection distant from the cell body (*see* Llewellyn-Smith I et al., *J Neurosci Methods* 103: 83-90 (2000)). These neuronal cell type specific targeting cytotoxic proteins have uses in neuroscience research, such as for elucidating mechanisms of sensations (*see e.g.* Mishra S, Hoon M, *Science* 340: 968-71 (2013)), and creating model  
20 systems of neurodegenerative diseases, such as Parkinson's and Alzheimer's (*see e.g.* Hamlin A et al., *PLoS One* e53472 (2013)).

[258] Among certain embodiments of the present invention is a method of using a protein, pharmaceutical composition, and/or diagnostic composition of the invention to detect the presence of a cell type for the purpose of information  
25 gathering regarding diseases, conditions and/or disorders. The method comprises contacting a cell with a diagnostically sufficient amount of a protein of the invention to detect the protein by an assay or diagnostic technique. The term "diagnostically sufficient amount" refers to an amount that provides adequate detection and accurate measurement for information gathering  
30 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 0.1 mg to 100 mg of the detection promoting agent linked protein per kg of subject per subject. Typically, the amount of protein 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 protein of the invention administered to a subject will be as low as feasibly possible.

5 [259] The cell-type specific targeting of proteins of the 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 proteins of the invention. Imaging of cells using the proteins of the invention may be performed *in vitro* or *in vivo* by any suitable technique known  
10 in the art. For example, the method of using a protein, pharmaceutical composition, or diagnostic composition of the invention to detect the presence of a cell type for the purpose of information gathering may be performed on cells *in vivo* within a patient, including on cells *in situ*, *e.g.* at a disease locus, on cells *in vitro*, and/or in an *ex vivo* setting on cells and tissues removed from an organism,  
15 *e.g.* a biopsy material.

[260] 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,  
20 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  
25 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*).

[261] The method of using a protein, pharmaceutical composition, or diagnostic composition of the invention to detect the presence of a target  
30 biomolecule positive cell type for the purpose of information gathering may be performed on cells *in vivo* within a patient, on cells *in situ*, *e.g.* at a disease locus, on cells *in vitro*, and/or in an *ex vivo* setting on cells and tissues removed from an organism, *e.g.* a biopsy material. The detection of specific cells, cell types, and cell populations using a composition of the invention may be used for

diagnosis and imaging of cells, such as, *e.g.*, tumor, cancer, immune, and infected cells. For example, proteins and diagnostic compositions of the invention may be employed to image or visualize a site of possible accumulation of target biomolecule expressing cells in an organism. These methods may be used to identify sites of tumor development or residual tumor cells after a therapeutic intervention.

[262] 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, AIDS, amyloidosis, ankylosing spondylitis, asthma, autism, cardiogenesis, Crohn's disease, diabetes, erythematosus, gastritis, graft rejection, graft-versus-host disease, Grave's disease, Hashimoto's thyroiditis, hemolytic uremic syndrome, HIV-related diseases, lupus erythematosus, lymphoproliferative disorders, multiple sclerosis, myasthenia gravis, neuroinflammation, polyarteritis nodosa, polyarthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, scleroderma, septic shock, Sjorgren's syndrome, systemic lupus erythematosus, ulcerative colitis, vasculitis, cell proliferation, inflammation, leukocyte activation, leukocyte adhesion, leukocyte chemotaxis, leukocyte maturation, leukocyte migration, neuronal differentiation, 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, myelodysplastic syndromes (MDS), smoldering multiple myeloma, and Waldenstrom macroglobulinemia.

[263] Among certain embodiment of the present invention is a method of using a protein, pharmaceutical composition, and/or diagnostic composition to label or detect the interiors of neoplastic cells and/or immune cell types (*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 the proteins and pharmaceutical compositions 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.

[264] Diagnostic compositions of the invention may be used to characterize a disease, disorder, or condition as potentially treatable by a related pharmaceutical composition of the invention. Certain compositions of matter of the 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 invention as described herein or is well suited for using a delivery device of the invention.

[265] Diagnostic compositions of the 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.

[266] Diagnostic compositions of the invention may be used to assess responses to therapeutic(s) regardless of the type of therapeutic, *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, and/or monitor a different marker than the antigen targeted by a therapy already being administered to a patient (*see e.g.* Smith-Jones P et al., *Nat Biotechnol* 22: 701-6 (2004); Evans M et al., *Proc Natl Acad Sci USA* 108: 9578-82 (2011)).

[267] In certain embodiments, the proteins of the invention or pharmaceutical and/or diagnostic compositions thereof are used for both diagnosis and treatment, or for diagnosis alone.

[268] The present invention is further illustrated by the following non-limiting examples of selectively cytotoxic proteins comprising Shiga toxin effector regions derived from A Subunits of members of the Shiga toxin family and binding regions capable of binding extracellular target biomolecules physically coupled to specific cell types.

## EXAMPLES

[269] 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 examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail.

[270] The following examples of cytotoxic proteins demonstrate the improved ability of exemplary cytotoxic proteins to selectively kill cells physically coupled with an extracellular target biomolecule of the immunoglobulin-type binding region as compared to their ancestral variants which lacked signal motifs. The exemplary cytotoxic proteins bound to target biomolecules expressed by targeted cell types and entered the targeted cells. The internalized cytotoxic proteins effectively routed their Shiga toxin effector regions to the

cytosol to inactivate ribosomes and subsequently caused the apoptotic death of the targeted cells.

[271] First, it was observed that the addition of KDEL family signal motifs to multiple Shiga-toxin-based fusion proteins reduces their cytotoxicity. This is  
5 consistent with the scientific literature which never described any example of a Shiga toxin construct that benefits from the presence of a KDEL family signal motif. Then, it was unexpectedly discovered that the addition of a KDEL family signal motif can improve Shiga-toxin-based cytotoxicity of certain constructs. Surprisingly, both the KDEL bearing and non-KDEL bearing variants exhibited  
10 similar *in vitro* enzymatic activities, similar cell binding characteristics, and similar cell internalization; however, the KDEL bearing variants of the cytotoxic proteins of the invention exhibited improved cytotoxicity. These results contradicted previous findings (*see e.g.* Jackson M et al., *J Cell Sci* 112: 467-75 (1999)).

[272] One exemplary cytotoxic protein of the invention comprises a single-chain, variable fragment, binding region capable of binding HER2 with high affinity combined with a Shiga toxin A Subunit fragment and a carboxy-terminal KDEL motif. This exemplary cytotoxic protein is capable of selectively killing cells that express HER2 on their surface. A second exemplary cytotoxic protein  
20 of the invention comprises a single-chain, variable fragment, binding region capable of binding CD38 with high affinity combined with a Shiga toxin A Subunit fragment and a carboxy-terminal KDEL motif. This second exemplary cytotoxic protein is capable of selectively killing cells that express CD38 on their surface. A third exemplary cytotoxic protein of the invention comprises a  
25 single-chain, variable fragment, binding region capable of binding CD19 with high affinity combined with a Shiga toxin A Subunit fragment and a carboxy-terminal KDEL motif. This third exemplary cytotoxic protein is capable of selectively killing cells that express CD19 on their surface. A fourth exemplary cytotoxic protein of the invention comprises a single-chain, variable fragment,  
30 binding region capable of binding CD74 with high affinity combined with a Shiga toxin A Subunit fragment and a carboxy-terminal KDEL motif. This fourth exemplary cytotoxic protein is capable of selectively killing cells that express CD74 on their surface. Other exemplary cytotoxic proteins include those with binding regions targeting Epstein-Barr viral antigens, Leishmania

antigens, neurotensin receptors, epidermal growth factor receptors, the immune cell receptor CCR5, and the viral protein targets Env and UL18.

**Example 1. A HER2-targeted, cytotoxic protein derived from Shiga-like toxin 1 A Subunit with an endoplasmic reticulum signal motif ( $\alpha$ HER2scFv::SLT-1A::KDEL)**

5 [273] The cytotoxic protein of this example  $\alpha$ HER2scFv::SLT-1A::KDEL comprises a single-chain, variable fragment, binding region capable of binding HER2 with high affinity combined with a Shiga toxin A Subunit fragment and a  
10 carboxy-terminal KDEL motif.

Construction, Production, and Purification of the Cytotoxic Protein

$\alpha$ HER2scFv::SLT-1A::KDEL

15 [274] First, a Shiga toxin effector region and a binding region were designed or selected. In this example, the Shiga toxin effector region was derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). A polynucleotide was obtained that encoded amino acids 1-251 of SLT-1A (Cheung M et al., *Mol Cancer* 9: 28 (2010)). An immunoglobulin-type binding region  $\alpha$ HER2scFv was derived from  
20 trastuzumab (marketed as Herceptin®, Genentech, Inc., South San Francisco, CA, U.S.) and the 4D5 monoclonal antibody (Zhao et al., *J Immunol* 183: 5563-74 (2009)) such that a single-chain variable fragment (scFv) is created with the two immunoglobulin variable regions ( $V_L$  and  $V_H$ ) separated by a linker known in the art.

25 [275] Second, the binding region and Shiga toxin effector region were linked together to form a fusion protein. In this example, a polynucleotide encoding the binding region  $\alpha$ HER2scFv comprising amino acids 1-245 of SEQ ID NO:4 was cloned in frame with a polynucleotide encoding a “murine hinge” derived from a murine IgG3 molecule (or other linkers known to the skilled person) and in frame with a polynucleotide encoding Shiga toxin effector region SLT-1A  
30 comprising amino acids 1-251 of SEQ ID NO:1. In certain experiments, the full-length coding sequence of the cytotoxic protein of this example began with a polynucleotide encoding a Strep-tag® to facilitate detection and purification. The polynucleotide sequence encoding the cytotoxic protein  $\alpha$ HER2scFv::SLT-

1A::KDEL of this example was codon optimized for efficient expression in *E. coli* using services from DNA 2.0, Inc. (Menlo Park, CA, U.S.).

[276] Third, a fusion protein was produced by expressing the polynucleotide encoding the cytotoxic protein  $\alpha$ HER2scFv::SLT-1A::KDEL (SEQ ID NO:4).

5 Expression of the  $\alpha$ HER2scFv::SLT-1A::KDEL cytotoxic protein was accomplished using both bacterial and cell-free, protein translation systems.

[277] In this example of  $\alpha$ HER2scFv::SLT-1A::KDEL production by an *E. coli* expression system, the polynucleotide “insert” sequence encoding  $\alpha$ HER2scFv::SLT-1A::KDEL was cloned into the pTxb1 vector (New England Biolabs, Ipswich, MA, U.S.) using standard procedures to produce a polynucleotide sequence encoding the cytotoxic protein  $\alpha$ HER2scFv::SLT-1A::KDEL ligated in frame to polynucleotide sequences encoding the amino-terminal intein of the vector. The plasmid insert polynucleotide sequence was verified by Sanger sequencing (Functional Biosciences, Madison, WI, U.S.) and transformed into T7 Shuffle® cells (New England Biolabs, Ipswich, MA, U.S.). The  $\alpha$ HER2scFv::SLT-1A::KDEL protein was produced and purified according to the IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system manual (New England Biolabs, Ipswich, MA, U.S.). Purification was accomplished using standard techniques known in the art, such as using immobilized targets of the Strep-tag® or the binding region.

[278] In this example of  $\alpha$ HER2scFv::SLT-1A::KDEL production by a cell-free, protein translation system, the polynucleotide “insert” sequence encoding  $\alpha$ HER2scFv::SLT-1A::KDEL was cloned into the pIVEX2.3 vector with a stop codon directly after the coding region using the In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, U.S.) according to the manufacturer’s instructions. The plasmid insert polynucleotide sequence was verified by Sanger sequencing (Functional Biosciences, Madison, WI, U.S.).  $\alpha$ HER2scFv::SLT-1A::KDEL protein was produced using the rapid translation system 5 Prime™ RTS 100 *E. coli* Disulfide Kit (5 Prime, Gaithersburg, MD, U.S.) according to the manufacturer’s instructions. Purification was accomplished using standard techniques known in the art, such as using immobilized targets of the Strep-tag® or the binding region.

Determining the Maximum Specific Binding ( $B_{max}$ ) and Equilibrium Binding Constant ( $K_D$ ) of  $\alpha$ HER2scFv::SLT-1A::KDEL Binding Target Cell Types

[279] The binding characteristics of the  $\alpha$ HER2scFv::SLT-1A::KDEL protein produced as described above was determined by a fluorescence-based, flow-cytometry assay. Samples containing HER2 positive (+) cells and HER2 negative (-) cells were suspended in phosphate buffered saline (1X PBS) (Hyclone Brand, Fisher Scientific, Waltham, MA, U.S.) containing 1 percent bovine serum albumin (BSA) (Calbiochem, San Diego, CA, U.S.), hereinafter referred to as "1X PBS+1%BSA" and incubated for 1 hour at 4 degrees Celsius (°C) with 100  $\mu$ L of various dilutions of the  $\alpha$ HER2scFv::SLT-1A::KDEL protein to be assayed. The highest concentrations of  $\alpha$ HER2scFv::SLT-1A::KDEL protein was selected to lead to saturation of the binding reaction. After the one hour incubation, cell samples were washed twice with 1X PBS+1%BSA. The cell samples were incubated for 1 hour at 4°C with 100  $\mu$ L of 1X PBS+1%BSA containing 0.3  $\mu$ g of anti-Strep-tag® mAb-FITC (# A01736-100, Genscript, Piscataway, NJ, U.S.).

[280] The cell samples were next washed twice with 1X PBS+1%BSA, resuspended in 200  $\mu$ L of 1X PBS and subjected to fluorescence-based, flow cytometry. The mean fluorescence intensity (MFI) data for all the samples was obtained by gating the data using a FITC-only sample as a negative control. Graphs were plotted of MFI versus "concentration of cells" using Prism software (GraphPad Software, San Diego, CA, U.S.). Using the Prism software function of one-site binding [ $Y = B_{max} * X / (K_D + X)$ ] under the heading binding-saturation, the  $B_{max}$  and  $K_D$  were calculated using baseline corrected data. Abs values were corrected for background by subtracting the Abs values measured for wells containing only PBS.  $B_{max}$  is the maximum specific binding reported in MFI.  $K_D$  is the equilibrium binding constant, reported in nanomolar (nM).

[281] The  $B_{max}$  for  $\alpha$ HER2scFv::SLT-1A::KDEL binding to HER2+ cells was measured to be about 110,000 MFI with a  $K_D$  of about 160 nM (Table 1). This result was relatively similar to the  $B_{max}$  for the  $\alpha$ HER2scFv::SLT-1A protein, which lacked a KDEL signal motif, binding to HER2+ cells which was measured to be about 140,000 MFI with a  $K_D$  of about 180 nM (Table 1). Neither protein was observed to have measurable binding to HER2- negative cells in this assay. This shows that the improved cytotoxicity of the KDEL

variant is probably not related to a change in the immunoglobulin-derived domain's target cell binding properties.

**Table 1. Addition of KDEL Had No Significant Effect on Binding Characteristics:** Representative values for  $B_{max}$  and  $K_D$  for  $\alpha$ HER2scFv::SLT-1A::KDEL as compared to its ancestor which lacked a KDEL signal motif

Cytotoxic Protein	target biomolecule	Target Positive Cells	
		$B_{max}$ (MFI)	$K_D$ (nM)
$\alpha$ HER2scFv::SLT-1A::KDEL	HER2	111,000	158
$\alpha$ HER2scFv::SLT-1A	HER2	141,000	182

Determining the Half-Maximal Inhibitory Concentration ( $IC_{50}$ ) of  $\alpha$ HER2scFv::SLT-1A::KDEL to Eukaryotic Ribosomes *In Vitro*

[282] The ribosome inactivation capabilities of  $\alpha$ HER2scFv::SLT-1A::KDEL was determined in a cell-free, *in vitro* protein translation assay using the TNT® Quick Coupled Transcription/Translation Kit (L1170 Promega, Madison, WI, U.S.). The kit includes Luciferase T7 Control DNA and TNT® Quick Master Mix. The ribosome activity reaction was prepared according to the manufacturer's instructions to create "TNT" reaction mixtures.

[283] A series of 10-fold dilutions of  $\alpha$ HER2scFv::SLT-1A::KDEL to be tested was prepared in appropriate buffer and a series of identical TNT reaction mixture components was created for each dilution of  $\alpha$ HER2scFv::SLT-1A::KDEL. Each sample in the dilution series of  $\alpha$ HER2scFv::SLT-1A::KDEL protein was 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.) 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.), the half maximal inhibitory concentration ( $IC_{50}$ ) value was calculated for each sample. Then, the data were normalized by calculating the "percent of SLT-1A-only control protein" using the Prism software function of log(inhibitor) vs. response (three parameters) [ $Y = Bottom + ((Top-Bottom) / (1+10^{(X-LogIC50)}))$ ] under the heading dose-response-inhibition. The  $IC_{50}$  for

experimental proteins and SLT-1A-only control protein were calculated. The percent of SLT-1A-only control protein was calculated by [(IC<sub>50</sub> of SLT-1A control protein / IC<sub>50</sub> of experimental protein) x 100].

[284] The inhibitory effect of αHER2scFv::SLT-1A::KDEL on cell-free  
 5 protein synthesis was strong. Dose dependence experiments determined that the IC<sub>50</sub> of αHER2scFv::SLT-1A::KDEL on protein synthesis in this cell-free assay was about 70 picomolar (pM) or within 6% of the SLT-1A-only positive control (Table 2). This result was not substantially different from the IC<sub>50</sub> for the αHER2scFv::SLT-1A protein, which lacked a KDEL signal motif, which was  
 10 measured to be within 8% of the SLT-1A-only positive control (Table 2). This shows that the improved cytotoxicity of the KDEL variant is probably not related to a change in the Shiga toxin A Subunit enzymatic activity.

**Table 2. Addition of KDEL Had No Significant Effect on Ribosome Inactivation:** Representative half-maximal inhibitory concentration (IC<sub>50</sub>) for αHER2scFv::SLT-1A::KDEL as compared to its ancestor which lacked a KDEL  
 15 signal motif

Cytotoxic Protein	IC <sub>50</sub> (pM)	IC <sub>50</sub> of SLT-1A only positive control (pM)	Percent of IC <sub>50</sub> of SLT-1A control protein
αHER2scFv::SLT-1A::KDEL	69.0	73.0	106%
αHER2scFv::SLT-1A	28.1	30.4	108%

Determining the Selective Cytotoxicity and Half-Maximal Cytotoxic  
 20 Concentration (CD<sub>50</sub>) of αHER2scFv::SLT-1A::KDEL Using a Cell-Kill Assay

[285] The cytotoxicity characteristics of αHER2scFv::SLT-1A::KDEL was determined by the following cell-kill assay. This assay determines the capacity of a cytotoxic protein to kill cells expressing the target biomolecule of cytotoxic protein's binding region as compared to cells that do not express the target  
 25 biomolecule. Cells were plated (2 x 10<sup>3</sup> cells per well) in 20 μL cell culture medium in 384-well plates. The αHER2scFv::SLT-1A::KDEL protein was diluted either 5-fold or 10-fold in a 1X PBS and 5 μL of the dilutions were added to the cells. Control wells containing only cell culture medium were used for baseline correction. The cell samples were incubated with αHER2scFv::SLT-  
 30 1A::KDEL, or just buffer, for 3 days at 37°C and in an atmosphere of 5% carbon dioxide (CO<sub>2</sub>). The total cell survival or percent viability was determined using

a luminescent readout using the CellTiter-Glo® Luminescent Cell Viability Assay (G7573 Promega Madison, WI, U.S.) according to the manufacturer's instructions. The Percent Viability of experimental wells was calculated using the following equation: (Test RLU - Average Media RLU) / (Average Cells RLU - Average Media RLU) \* 100. Log polypeptide concentration versus Percent Viability was plotted in Prism (GraphPad Prism, San Diego, CA, U.S.) and log (inhibitor) vs. normalized response (variable slope) analysis was used to determine the half-maximal cytotoxic concentration (CD<sub>50</sub>) value for αHER2scFv::SLT-1A::KDEL.

[286] Dose dependence experiments determined that the CD<sub>50</sub> of the αHER2scFv::SLT-1A::KDEL protein was measured to be about 0.001 to 0.13 nM for HER2 + cells as compared to 14-37 nM for HER2- cells, depending on the cell line (Table 3; Figure 2). The CD<sub>50</sub> of αHER2scFv::SLT-1A::KDEL was over 100 times greater for cells not physically coupled with the extracellular target biomolecule HER2 as compared to cells that were physically coupled with the extracellular target biomolecule HER2, e.g. expressed HER2 on their cell surface (Table 3; Figure 2).

**Table 3. Addition of KDEL Improved Cytotoxicity:** Representative half-maximal cytotoxic concentrations (CD<sub>50</sub>) for αHER2scFv::SLT-1A::KDEL as compared to its ancestor which lacked a KDEL signal motif

Cell Line	HER2 status	CD <sub>50</sub> (nM)		
		αHER2scFv::SLT-1A::KDEL	αHER2scFv::SLT-1A	SLT-1A only negative control
HCC-1954	positive	0.1100	2.1	320
SKBR3	positive	0.1300	1.3	220
BT474	positive	0.0010	1.6	14
MDA-MB-231	negative	37.0000	1500.0	130
MDA-MB-468	negative	14.0000	546.0	230

[287] The CD<sub>50</sub> of the αHER2scFv::SLT-1A variant lacking a carboxy-terminal KDEL signal motif was measured to be about 1.3-2.1 nM for HER2+ cells (Table 3; Figure 2). Thus, the addition of a KDEL signal motif reduced the CD<sub>50</sub> to HER2+ cells by 10-20 fold. Additionally, αHER2scFv::SLT-1A::KDEL killed more total cells than αHER2scFv::SLT-1A as seen in the decreased cell viability at the highest tested concentration (Figure 2). These results exemplify the effect of adding a KDEL signal motif on both cytotoxicity and selective

cytotoxicity. The differences in cell-kill for these cytotoxic proteins were not predictable based on the *in vitro* results for either protein synthesis inhibition or target cell-binding characteristics.

5 Determining Cell Internalization of  $\alpha$ HER2scFv::SLT-1A with and without a KDEL Signal Motif Using Immunofluorescence

[288] The ability of cytotoxic proteins to enter target cells was investigated using standard immunocytochemical techniques known in the art. Briefly,  $0.8 \times 10^6$  cells of each cell type (SKBR3 (HER2+) and MDA-MB-231 (HER2-)) were  
10 harvested and suspended in 50  $\mu$ L of cell culture medium containing a cocktail of protease inhibitors (*e.g.* P1860 Sigma-Aldrich Co., St. Louis, MO, U.S.) and human Fc receptor protein to reduce non-specific immunofluorescent staining. Next, 100 nM of the cytotoxic protein to be analyzed was added to the cells, and the cells were incubated at 37°C for 1 hour to allow for intoxication to progress.  
15 Then, cells were “fixed” and “permeabilized” using the Cytotfix/Cytoperm™ Kit (BD Biosciences San Diego, CA, U.S.) according to the manufacturer’s instructions. The Shiga toxin effector region was “stained” using a mouse monoclonal antibody (mouse IgG anti-Shiga toxin 1 Subunit A, BEI NR-867 BEI Resources, Manassas, VA, U.S.). The mouse monoclonal antibody  
20 localization was then detected with the Alexa Fluor® 555 Monoclonal Antibody Labeling Kit (Life Technologies, Carlsbad, CA, U.S.) according to manufacturer's instructions.

[289] In this assay, cell internalization was observed in HER2+ cells for both  $\alpha$ HER2scFv::SLT-1A::KDEL and the variant  $\alpha$ HER2scFv::SLT-1A which  
25 lacked a KDEL signal motif (Figure 3). No cell internalization was observed for either protein to HER2- cells. This shows the differences in cytotoxicity (Table 3; Figure 2) between the KDEL variant and the ancestral variant between these two variants is probably not related to any significant change in target cell binding and/or cell entry.  
30

Determining the *In Vivo* Effects of the Cytotoxic Protein  $\alpha$ HER2scFv::SLT-1A Using Animal Models

[290] Animal models are used to determine the *in vivo* effects of the cytotoxic protein  $\alpha$ HER2scFv::SLT-1A on HER2+ neoplastic cells. Various mice strains

are used to test the effect of the cytotoxic protein after intravenous administration on xenograft tumors in mice resulting from the injection into those mice of human neoplastic cells which express HER2 on their cell surfaces.

5 **Example 2. A CD38-targeted, cytotoxic protein derived from Shiga-like toxin 1 A Subunit with a carboxy-terminal endoplasmic reticulum signal motif ( $\alpha$ CD38scFv::SLT-1A::KDEL)**

[291] The cytotoxic protein of this example  $\alpha$ CD38scFv::SLT-1A::KDEL comprises a single-chain, variable fragment, binding region capable of binding  
10 CD38 with high affinity combined with a Shiga toxin A Subunit fragment and a carboxy-terminal KDEL motif.

Construction, Production, and Purification of the Cytotoxic Protein

$\alpha$ CD38scFv::SLT-1A::KDEL

15 [292] In this example, the Shiga toxin effector region was derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). A polynucleotide was obtained that encoded amino acids 1-251 of SLT-1A (Cheung M et al., *Mol Cancer* 9: 28 (2010)). An immunoglobulin-type binding region  $\alpha$ CD38scFv was derived from the monoclonal antibody anti-CD38 HB7 (Peng et al., *Blood* 101: 2557-62  
20 (2003); see also GenBank Accession BD376144, National Center for Biotechnology Information, U.S.) such that a single-chain variable fragment (scFv) is created with the two immunoglobulin variable regions ( $V_L$  and  $V_H$ ) separated by a linker known in the art.

[293] In this example, the binding region and Shiga toxin effector region were  
25 linked together to form a fusion protein. In this example, a polynucleotide encoding the binding region  $\alpha$ CD38scFv comprising amino acids 1-241 of SEQ ID NO:8 was cloned in frame with a polynucleotide encoding a "murine hinge" derived from a murine IgG3 molecule (or other linkers known to the skilled person) and in frame with a polynucleotide encoding Shiga toxin effector region  
30 SLT-1A comprising amino acids 1-251 of SEQ ID NO:1. In certain experiments, the full-length coding sequence of the cytotoxic protein of this example began with a polynucleotide encoding a Strep-tag® to facilitate detection and purification. The polynucleotide sequence encoding the cytotoxic protein  $\alpha$ CD38scFv::SLT-1A::KDEL of this example was codon optimized for

efficient expression in *E. coli* using services from DNA 2.0, Inc. (Menlo Park, CA, U.S.).

[294] A fusion protein was produced by expressing the polynucleotide encoding the cytotoxic protein  $\alpha$ CD38scFv::SLT-1A::KDEL (SEQ ID NO:8).

5 Expression of the  $\alpha$ CD38scFv::SLT-1A::KDEL cytotoxic protein was accomplished using both bacterial and cell-free, protein translation systems.

[295] In this example of  $\alpha$ CD38scFv::SLT-1A::KDEL production by an *E. coli* expression system, the polynucleotide “insert” sequence encoding  $\alpha$ CD38scFv::SLT-1A::KDEL was cloned into the pTxb1 vector (New England Biolabs, Ipswich, MA, U.S.) using standard procedures to produce a  
10 polynucleotide sequence encoding the cytotoxic protein  $\alpha$ CD38scFv::SLT-1A::KDEL ligated in frame to polynucleotide sequences encoding the amino-terminal intein of the vector. The plasmid insert polynucleotide sequence was verified by Sanger sequencing (Functional Biosciences, Madison, WI, U.S.) and  
15 transformed into T7 Shuffle® cells (New England Biolabs, Ipswich, MA, U.S.). The  $\alpha$ CD38scFv::SLT-1A::KDEL protein was produced and purified according to the IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system manual (New England Biolabs, Ipswich, MA, U.S.). Purification was accomplished using standard techniques known in the art, such as using  
20 immobilized targets of the Strep-tag® or the binding region.

[296] In this example of  $\alpha$ CD38scFv::SLT-1A::KDEL production by a cell-free, protein translation system, the polynucleotide “insert” sequence encoding  $\alpha$ CD38scFv::SLT-1A::KDEL was cloned into the pIVEX2.3 vector with a stop codon directly after the coding region using the In-Fusion® HD Cloning Kit  
25 (Clontech, Mountain View, CA, U.S.) according to the manufacturer’s instructions. The plasmid insert polynucleotide sequence was verified by Sanger sequencing (Functional Biosciences, Madison, WI, U.S.).  $\alpha$ CD38scFv::SLT-1A::KDEL protein was produced using the rapid translation system 5 Prime™ RTS 100 *E. coli* Disulfide Kit (5 Prime, Gaithersburg, MD, U.S.) according to  
30 the manufacturer’s instructions. Purification was accomplished using standard techniques known in the art, such as using immobilized targets of the Strep-tag® or the binding region.

Determining the Maximum Specific Binding ( $B_{max}$ ) and Equilibrium Binding Constant ( $K_D$ ) of  $\alpha$ CD38scFv::SLT-1A::KDEL Binding Target Cell Types

[297] The binding characteristics of the  $\alpha$ CD38scFv::SLT-1A::KDEL protein produced as described above were determined by a fluorescence-based, flow-cytometry assay. Samples containing CD38 positive (+) cells and CD38 negative (-) cells were suspended in 1X PBS+1%BSA and incubated for 1 hour at 4°C with 100  $\mu$ L of various dilutions of the  $\alpha$ CD38scFv::SLT-1A::KDEL protein to be assayed. The highest concentrations of  $\alpha$ CD38scFv::SLT-1A::KDEL protein was selected to lead to saturation of the binding reaction.

After the one hour incubation, cell samples were washed twice with 1X PBS+1%BSA. The cell samples were incubated for 1 hour at 4°C with 100  $\mu$ L of 1X PBS+1%BSA containing 0.3  $\mu$ g of anti-Strep-tag® mAb-FITC (# A01736-100, Genscript, Piscataway, NJ, U.S.).

[298] The cell samples were next washed twice with 1X PBS+1%BSA, resuspended in 200  $\mu$ L of 1X PBS and subjected to fluorescence-based, flow cytometry. The mean fluorescence intensity (MFI) data for all the samples was obtained by gating the data using a FITC-only sample as a negative control. Graphs were plotted of MFI versus “concentration of cells” using Prism software (GraphPad Software, San Diego, CA, U.S.). Using the Prism software function of one-site binding [ $Y = B_{max} * X / (K_D + X)$ ] under the heading binding-saturation, the  $B_{max}$  and  $K_D$  were calculated using baseline corrected data. Abs values were corrected for background by subtracting the Abs values measured for wells containing only PBS.  $B_{max}$  is the maximum specific binding reported in MFI.  $K_D$  is the equilibrium binding constant, reported in nM.

[299] The  $B_{max}$  for  $\alpha$ CD38scFv::SLT-1A::KDEL binding to CD38+ cells was measured to be about 100,000 MFI with a  $K_D$  of about 13 nM (Table 4). This result was similar to the  $B_{max}$  for the  $\alpha$ CD38scFv::SLT-1A protein, which lacked a KDEL signal motif, binding to CD38+ cells which was measured to be about 110,000 MFI with a  $K_D$  of about 17 nM (Table 4). Neither protein bound to CD38- cells. This shows that the improved cytotoxicity of the KDEL variant is probably not related to a change in the immunoglobulin-derived domain’s target cell binding properties.

**Table 4. Addition of KDEL Had No Effect on Binding Characteristics:**  
Representative values for  $B_{max}$  and  $K_D$  for  $\alpha$ CD38scFv::SLT-1A::KDEL as compared to its ancestor which lacked a KDEL signal motif

Cytotoxic Protein	target biomolecule	Target Positive Cells	
		B <sub>max</sub> (MFI)	K <sub>D</sub> (nM)
$\alpha$ CD38scFv::SLT-1A::KDEL	CD38	102,000	12.5
$\alpha$ CD38scFv::SLT-1A	CD38	108,000	17.0

Determining the Half-Maximal Inhibitory Concentration (IC<sub>50</sub>) of  $\alpha$ CD38scFv::SLT-1A::KDEL to Eukaryotic Ribosomes *In Vitro*

- 5 [300] The ribosome inactivation capabilities of  $\alpha$ CD38scFv::SLT-1A::KDEL was determined in a cell-free, *in vitro* protein translation assay using the TNT® Quick Coupled Transcription/Translation Kit (L1170 Promega, Madison, WI, U.S.). The kit includes Luciferase T7 Control DNA and TNT® Quick Master Mix. The ribosome activity reaction was prepared according to the
- 10 manufacturer's instructions to create "TNT" reaction mixtures.
- [301] A series of 10-fold dilutions of  $\alpha$ CD38scFv::SLT-1A::KDEL to be tested were prepared in appropriate buffer and a series of identical TNT reaction mixture components were created for each dilution of  $\alpha$ CD38scFv::SLT-
- 15 1A::KDEL. Each sample in the dilution series of  $\alpha$ CD38scFv::SLT-1A::KDEL protein was 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.) was added to all test samples and the amount of luciferase protein translation was measured by luminescence according to the manufacturer
- 20 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.), the half maximal inhibitory concentration (IC<sub>50</sub>) value was calculated for each sample. Then, the data were normalized by calculating the
- 25 "percent of SLT-1A-only control protein" using the Prism software function of log(inhibitor) vs. response (three parameters) [ $Y = \text{Bottom} + ((\text{Top}-\text{Bottom}) / (1+10^{-(X-\text{LogIC}_{50})}))$ ] under the heading dose-response-inhibition. The IC<sub>50</sub> for experimental proteins and SLT-1A-only control protein were calculated. The percent of SLT-1A-only control protein was calculated by [(IC<sub>50</sub> of SLT-1A
- 30 control protein / IC<sub>50</sub> of experimental protein) x 100].

[302] The inhibitory effect of  $\alpha$ CD38scFv::SLT-1A::KDEL on cell-free protein synthesis was strong. Dose dependence experiments determined that the IC<sub>50</sub> of  $\alpha$ CD38scFv::SLT-1A::KDEL on protein synthesis in this cell-free assay was about 15 pM or 99% of the SLT-1A-only positive control (Table 5). This result was similar to the IC<sub>50</sub> for the  $\alpha$ CD38scFv::SLT-1A protein, which lacked a KDEL signal motif, which was measured to be about 15 pM or 101% of the SLT-1A-only positive control (Table 5). This showed the improved cytotoxicity of the KDEL comprising variant is probably not related to a significant change in the Shiga toxin A Subunit enzymatic activity.

10 **Table 5. Addition of KDEL Had No Effect on Ribosome Inactivation:**  
Representative half-maximal inhibitory concentration (IC<sub>50</sub>) for  $\alpha$ CD38scFv::SLT-1A::KDEL as compared to its ancestor which lacked a KDEL signal motif

Cytotoxic Protein	IC <sub>50</sub> (pM)	IC <sub>50</sub> of SLT-1A only positive control (pM)	Percent of IC <sub>50</sub> of SLT-1A control protein
$\alpha$ CD38scFv::SLT-1A::KDEL	15.1	15.0	99%
$\alpha$ CD38scFv::SLT-1A	14.8	15.0	101%

15 Determining the Selective Cytotoxicity and Half-Maximal Cytotoxic Concentration (CD<sub>50</sub>) of  $\alpha$ CD38scFv::SLT-1A::KDEL Using a Cell-Kill Assay

[303] The cytotoxicity characteristics of  $\alpha$ CD38scFv::SLT-1A::KDEL was determined by the following cell-kill assay. This assay determines the capacity of a cytotoxic protein to kill cells expressing the target biomolecule of cytotoxic protein's binding region as compared to cells that do not express the target biomolecule. Cells were plated (2 x 10<sup>3</sup> cells per well) in 20  $\mu$ L cell culture medium in 384-well plates. The  $\alpha$ CD38scFv::SLT-1A::KDEL protein was diluted either 5-fold or 10-fold in a 1X PBS and 5  $\mu$ L of the dilutions were added to the cells. Control wells containing only cell culture medium were used for baseline correction. The cell samples were incubated with  $\alpha$ CD38scFv::SLT-1A::KDEL, or just buffer, for 3 days at 37°C and in an atmosphere of 5% CO<sub>2</sub>. The total cell survival or percent viability was determined using a luminescent readout using the CellTiter-Glo® Luminescent Cell Viability Assay (G7573 Promega Madison, WI, U.S.) according to the manufacturer's instructions. The Percent Viability of experimental wells was calculated using the following equation: (Test RLU - Average Media RLU) / (Average Cells RLU - Average

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Media RLU) \* 100. Log polypeptide concentration versus Percent Viability was plotted in Prism (GraphPad Prism, San Diego, CA, U.S.) and log (inhibitor) vs. normalized response (variable slope) analysis was used to determine the half-maximal cytotoxic concentration (CD<sub>50</sub>) value for αCD38scFv::SLT-1A::KDEL.

5 [304] The CD<sub>50</sub> of the αCD38scFv::SLT-1A::KDEL protein was measured to be about 0.2-1 nM for CD38 + cells, depending on the cell line, as compared to 300 nM for a CD38- cell line, which was relatively similar to the CD<sub>50</sub> for the SLT-1A-only negative control (Table 6; Figure 4). The CD<sub>50</sub> of the αCD38scFv::SLT-1A::KDEL was about 300-2000 fold greater for cells not  
10 physically coupled with the extracellular target biomolecule CD38 as compared to cells that were physically coupled with the extracellular target biomolecule CD38, e.g. expressed CD38 on their cell surface.

[305] Dose dependence experiments determined that the CD<sub>50</sub> for the αCD38scFv::SLT-1A, which lacked a KDEL signal motif, was about 0.8-3.2 nM  
15 (Table 6; Figure 4), which was 2-4 fold greater (less cytotoxic) than the same protein with a carboxy-terminal KDEL signal motif. This exemplifies the effect of the addition of a KDEL signal motif on both cytotoxicity and selective cytotoxicity. The differences in cell-kill for these cytotoxic proteins were not predictable based on the *in vitro* results for either ribosome inactivation or target  
20 cell-binding characteristics.

**Table 6. Addition of KDEL Improved Cytotoxicity:** Representative half-maximal cytotoxic concentrations (CD<sub>50</sub>) for αCD38scFv::SLT-1A::KDEL compared to its ancestor which lacked a KDEL signal motif

Cell Line	CD38 status	CD <sub>50</sub> (nM)		
		αCD38scFv::SLT-1A::KDEL	αCD38scFv::SLT-1A	SLT-1A only negative control
Daudi	positive	0.32	1.10	750
Raji	positive	0.98	3.20	1,100
ST486	positive	0.18	0.75	940
BC-1	positive	0.59	1.10	510
U226	negative	300.00	670.00	490

25 Determining Cell Internalization of SLT-1A::αCD38scFv Using Immunofluorescence

[306] The ability of αCD38scFv::SLT-1, the ancestor of αCD38scFv::SLT-1::KDEL ancestor which lacked a KDEL signal motif, to enter target cells was investigated using standard immunocytochemical techniques known in the art.

Briefly,  $0.8 \times 10^6$  cells of each cell type (Raji (CD38+), Daudi (CD38+), and U266 (CD38-)) were harvested and suspended in 50  $\mu$ L of cell culture medium containing a cocktail of protease inhibitors (e.g. P1860 Sigma-Aldrich Co., St. Louis, MO, U.S.) and human Fc receptor protein to reduce non-specific immunofluorescent staining. Next, 100 nM of the cytotoxic protein to be analyzed was added to the cells, and the cells were incubated at 37°C for 1 hour to allow for intoxication to progress. Then, cells were “fixed” and “permeabilized” using the Cytofix/Cytoperm™ Kit (BD Biosciences San Diego, CA, U.S.) according to the manufacturer’s instructions. The Shiga toxin effector region was “stained” using a mouse monoclonal antibody (mouse IgG anti-Shiga toxin 1 Subunit A, BEI NR-867 BEI Resources, Manassas, VA, U.S.). The mouse monoclonal antibody localization was then detected with the Alexa Fluor® 555 Monoclonal Antibody Labeling Kit (Life Technologies, Carlsbad, CA, U.S.) according to manufacturer's instructions.

[307] In this assay, cell surface binding and cell internalization was observed in CD38+ cells for  $\alpha$ CD38scFv::SLT-1. No cell internalization was observed for either protein to CD38- cells. This showed the differences in cytotoxicity (Table 6; Figure 4) between the cytotoxic protein KDEL variant and its ancestor was probably not caused by significant changes to the ability of  $\alpha$ CD38scFv::SLT-1 to bind target cells and become internalized.

#### Determining the *In Vivo* Effects of the Cytotoxic Protein $\alpha$ CD38scFv::SLT-1::KDEL Using Animal Models

[308] Animal models are used to determine the *in vivo* effects of the cytotoxic protein  $\alpha$ CD38scFv::SLT-1::KDEL on CD38+ neoplastic cells and/or immune cells. Various mice strains are used to test the effect of the cytotoxic protein after intravenous administration on xenograft tumors in mice resulting from the injection into those mice of human neoplastic and/or human immune cells which express CD38 on their cell surfaces.

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#### **Example 3. A CD19-targeted, cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 ( $\alpha$ CD19scFv::SLT-1A::KDEL)**

[309] The cytotoxic protein of this example  $\alpha$ CD19scFv::SLT-1A::KDEL comprises a single-chain, variable fragment, binding region capable of binding

CD19 with high affinity combined with a Shiga toxin A Subunit fragment and a carboxy-terminal KDEL motif.

Construction, Production, and Purification of the Cytotoxic Protein

5  $\alpha$ CD19scFv::SLT-1A::KDEL

[310] First, a Shiga toxin effector region and an immunoglobulin-type binding region were designed or selected. In this example, the Shiga toxin effector region was derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). A polynucleotide was obtained that encoded amino acids 1-251 of SLT-1A  
10 (Cheung M et al., *Mol Cancer* 9: 28 (2010)). An immunoglobulin-type binding region  $\alpha$ CD19scFv was derived from the humanized monoclonal antibody anti-CD19 4G7 (Peipp M et al., *J Immunol Methods* 285: 265-80 (2004) and references therein) such that a single-chain variable fragment (scFv) is created with the two immunoglobulin variable regions ( $V_L$  and  $V_H$ ) separated by a linker  
15 known in the art.

[311] Second, the binding region and Shiga toxin effector region were linked together to form a fusion protein. In this example, a polynucleotide encoding the immunoglobulin-type binding region  $\alpha$ CD19scFv comprising amino acids 1-250 of SEQ ID NO:12 was cloned in frame with a polynucleotide encoding a linker,  
20 such as a “murine hinge” derived from a murine IgG3 molecule (or other linkers known to the skilled person) and in frame with a polynucleotide encoding the Shiga toxin effector region from SLT-1A (amino acids 1-251 of SEQ ID NO:1) fused to a carboxy-terminal KDEL motif. The polynucleotide sequence encoding the cytotoxic protein  $\alpha$ CD19scFv::SLT-1A::KDEL of this example  
25 was codon optimized for efficient expression in *E. coli* using services from DNA 2.0, Inc. (Menlo Park, CA, U.S.).

[312] A fusion protein was produced by expressing the polynucleotide encoding the cytotoxic protein  $\alpha$ CD19scFv::SLT-1A::KDEL (SEQ ID NO:12). Expression of the  $\alpha$ CD19scFv::SLT-1A::KDEL cytotoxic protein was  
30 accomplished using a bacterial system known in the art.

[313] In this example of  $\alpha$ CD19scFv::SLT-1A::KDEL production by an *E. coli* expression system, the polynucleotide “insert” sequence encoding  $\alpha$ CD19scFv::SLT-1A::KDEL was cloned into the pTxb1 vector (New England Biolabs, Ipswich, MA, U.S.) using standard procedures to produce a

polynucleotide sequence encoding the cytotoxic protein  $\alpha$ CD19scFv::SLT-1A::KDEL ligated in frame to polynucleotide sequences encoding the amino-terminal intein of the vector. The plasmid insert polynucleotide sequence was verified by Sanger sequencing (Functional Biosciences, Madison, WI, U.S.) and transformed into T7 Shuffle® cells (New England Biolabs, Ipswich, MA, U.S.). The  $\alpha$ CD19scFv::SLT-1A::KDEL protein was produced and purified according to the IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system manual (New England Biolabs, Ipswich, MA, U.S.). Purification was accomplished using standard techniques known in the art, such as affinity chromatography.

Determining the Half-Maximal Inhibitory Concentration (IC<sub>50</sub>) of  $\alpha$ CD19scFv::SLT-1A::KDEL to Eukaryotic Ribosomes *in vitro*

[314] The ribosome inactivation capabilities of  $\alpha$ CD19scFv::SLT-1A::KDEL was determined in a cell-free, *in vitro* protein translation assay using the TNT® Quick Coupled Transcription/Translation Kit (L1170 Promega, Madison, WI, U.S.). The kit includes Luciferase T7 Control DNA and TNT® Quick Master Mix. The ribosome activity reaction was prepared according to the manufacturer's instructions to create "TNT" reaction mixtures.

[315] A series of 10-fold dilutions of  $\alpha$ CD19scFv::SLT-1A::KDEL to be tested was prepared in appropriate buffer and a series of identical TNT reaction mixture components was created for each dilution of  $\alpha$ CD19scFv::SLT-1A::KDEL. Each sample in the dilution series of  $\alpha$ CD19scFv::SLT-1A::KDEL protein was 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.) 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.), the half maximal inhibitory concentration (IC<sub>50</sub>) value was calculated for each sample.

[316] The inhibitory effect of  $\alpha$ CD19scFv::SLT-1A::KDEL on cell-free protein synthesis was strong. Dose dependence experiments determined that the  $IC_{50}$  of  $\alpha$ CD19scFv::SLT-1A::KDEL on protein synthesis in this cell-free assay was about 2.7 pM (Table 7). This result was not substantially different from the  $IC_{50}$  for the protein  $\alpha$ CD19scFv::SLT-1A, which lacked a KDEL signal motif, that was measured to be about 3.2 pM or equivalent of the SLT-1A-only positive control (Table 7). This shows that the improved cytotoxicity of the KDEL variant is probably not related to any significant perturbation of Shiga toxin A Subunit enzymatic activity.

10 **Table 7. Addition of KDEL Had No Significant Effect on Ribosome Inactivation:** Representative half-maximal inhibitory concentrations ( $IC_{50}$ ) for  $\alpha$ CD19scFv::SLT-1A::KDEL as compared to  $\alpha$ CD19scFv::SLT-1A

Cytotoxic Protein	$IC_{50}$ (pM)	$IC_{50}$ of SLT-1A only positive control (pM)
$\alpha$ CD19scFv::SLT-1A::KDEL	2.7	7.9
$\alpha$ CD19scFv::SLT-1A	3.2	7.9

15 Determining the Selective Cytotoxicity and Half-Maximal Cytotoxic Concentration ( $CD_{50}$ ) of  $\alpha$ CD19scFv::SLT-1A::KDEL Using a Cell-Kill Assay  
 [317] The cytotoxicity characteristics of  $\alpha$ CD19scFv::SLT-1A::KDEL was determined by the following cell-kill assay. This assay determines the capacity of a cytotoxic protein to kill cells expressing the target biomolecule of its immunoglobulin-type binding region as compared to the SLT-1A protein that does not possess the binding region. Cells were plated ( $2 \times 10^3$  cells per well) in 20  $\mu$ L of cell culture medium in 384-well plates. The  $\alpha$ CD19scFv::SLT-1A::KDEL protein was diluted 10-fold in buffer and 5  $\mu$ L of the dilutions were added to the cells. Control wells containing only cell culture medium were used for baseline correction. The cell samples were incubated with  $\alpha$ CD19scFv::SLT-1A::KDEL, or just buffer, for 3 days at 37°C and in an atmosphere of 5% CO<sub>2</sub>. The total cell survival or percent viability was determined using a luminescent readout using the CellTiter-Glo® Luminescent Cell Viability Assay (G7573 Promega Madison, WI, U.S.) according to the manufacturer's instructions. The Percent Viability of experimental wells was calculated using the following equation: (Test RLU - Average Media RLU) / (Average Cells RLU - Average Media RLU) \* 100. Log polypeptide

concentration versus Percent Viability was plotted in Prism (GraphPad Prism, San Diego, CA, U.S.) and log (inhibitor) vs. normalized response (variable slope) analysis was used to determine the half-maximal cytotoxic concentration (CD<sub>50</sub>) value for  $\alpha$ CD19scFv::SLT-1A::KDEL.

5 [318] Dose dependence experiments determined that the CD<sub>50</sub> of the  $\alpha$ CD19scFv::SLT-1A::KDEL protein was about 0.15 nM for CD19 + Daudi cells (Table 8, Figure 5). The CD<sub>50</sub> for the SLT-1A-only negative control and the same protein lacking the KDEL motif,  $\alpha$ CD19scFv::SLT-1A, could not be accurately measured based on the shape of the curve. These results exemplify  
 10 the effect of adding a KDEL signal motif on both cytotoxicity and selective cytotoxicity (Table 8, Figure 5). The differences in cell-kill for these cytotoxic proteins were not predictable based on the *in vitro* results for protein synthesis inhibition and are not expected to be predictable based on target cell-binding characteristics.

15 **Table 8. KDEL Motif Affected Cytotoxicity:** Representative half-maximal cytotoxic concentrations (CD<sub>50</sub>) of  $\alpha$ CD19scFv::SLT-1A::KDEL as compared to  $\alpha$ CD19scFv::SLT-1A

Cell Line	CD19 status	CD <sub>50</sub> (nM)		
		$\alpha$ CD19scFv::SLT-1A::KDEL	$\alpha$ CD19scFv::SLT-1A	SLT-1A only negative control
Daudi	positive	0.15	NC	NC

\* "NC" denotes not calculable.

20 Determining the *In Vivo* Effects of the Cytotoxic Protein  $\alpha$ CD19scFv::SLT-1A::KDEL Using Animal Models

[319] Animal models are used to determine the *in vivo* effects of the cytotoxic protein  $\alpha$ CD19scFv::SLT-1A::KDEL on neoplastic and/or immune cells. Various mice strains are used to test the effect of the cytotoxic protein after  
 25 intravenous administration on xenograft tumors in mice resulting from the injection into those mice of human neoplastic and/or human immune cells which express CD19 on their cell surfaces.

30 **Example 4. A CD74-targeted, cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 ( $\alpha$ CD74scFv::SLT-1A::KDEL)**

[320] A fourth exemplary cytotoxic protein comprises a Shiga toxin A Subunit fragment recombined with a single-chain, variable fragment, binding region capable of binding CD74 with high affinity. This fourth exemplary cytotoxic protein is capable of selectively killing cells that express CD74 on their surface.

5

Construction, Production, and Purification of the Cytotoxic Protein  
 $\alpha$ CD74scFv::SLT-1A::KDEL

[321] First, a Shiga toxin effector region and an immunoglobulin-type binding region were designed or selected. In this example, the Shiga toxin effector region was derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). A polynucleotide was obtained that encoded amino acids 1-251 of SLT-1A (Cheung M et al., *Mol Cancer* 9: 28 (2010)). An immunoglobulin-type binding region  $\alpha$ CD74scFv was derived from the humanized monoclonal antibody anti-CD74, Milatuzumab (Sapra P et al., *Clin Cancer Res* 11: 5257-64 (2005) and references therein) such that a single-chain variable fragment (scFv) is created with the two immunoglobulin variable regions ( $V_L$  and  $V_H$ ) separated by a linker known in the art.

[322] Second, the binding region and Shiga toxin effector region were linked together to form a fusion protein. In this example, a polynucleotide encoding the immunoglobulin-type binding region  $\alpha$ CD74scFv comprising amino acids 1-251 of SEQ ID NO:16 was cloned in frame with a polynucleotide encoding a linker, such as a “murine hinge” derived from a murine IgG3 molecule (or other linkers known to the skilled person) and in frame with a polynucleotide encoding the Shiga toxin effector region from SLT-1A (amino acids 1-251 of SEQ ID NO:1) fused to a carboxy-terminal KDEL motif. The polynucleotide sequence encoding the cytotoxic protein  $\alpha$ CD74scFv::SLT-1A::KDEL of this example was codon optimized for efficient expression in *E. coli* using services from DNA 2.0, Inc. (Menlo Park, CA, U.S.).

[323] A fusion protein was produced by expressing the polynucleotide encoding the cytotoxic protein  $\alpha$ CD74scFv::SLT-1A::KDEL (SEQ ID NO:16). Expression of the  $\alpha$ CD74scFv::SLT-1A::KDEL cytotoxic protein was accomplished using a bacterial system known in the art.

[324] In this example of  $\alpha$ CD74scFv::SLT-1A::KDEL production by an *E. coli* expression system, the polynucleotide “insert” sequence encoding

$\alpha$ CD74scFv::SLT-1A::KDEL was cloned into the pTxb1 vector (New England Biolabs, Ipswich, MA, U.S.) using standard procedures to produce a polynucleotide sequence encoding the cytotoxic protein  $\alpha$ CD74scFv::SLT-1A::KDEL ligated in frame to polynucleotide sequences encoding the amino-terminal intein of the vector. The plasmid insert polynucleotide sequence was verified by Sanger sequencing (Functional Biosciences, Madison, WI, U.S.) and transformed into T7 Shuffle® cells (New England Biolabs, Ipswich, MA, U.S.). The  $\alpha$ CD74scFv::SLT-1A::KDEL protein was produced and purified according to the IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system manual (New England Biolabs, Ipswich, MA, U.S.). Purification was accomplished using standard techniques known in the art, such as affinity chromatography.

#### Determining the Half-Maximal Inhibitory Concentration (IC<sub>50</sub>) of

$\alpha$ CD74scFv::SLT-1A::KDEL to Eukaryotic Ribosomes *in vitro*

[325] The ribosome inactivation capabilities of  $\alpha$ CD74scFv::SLT-1A::KDEL was determined in a cell-free, *in vitro* protein translation assay using the TNT® Quick Coupled Transcription/Translation Kit (L1170 Promega, Madison, WI, U.S.). The kit includes Luciferase T7 Control DNA and TNT® Quick Master Mix. The ribosome activity reaction was prepared according to the manufacturer's instructions to create "TNT" reaction mixtures.

[326] A series of 10-fold dilutions of  $\alpha$ CD74scFv::SLT-1A::KDEL to be tested was prepared in appropriate buffer and a series of identical TNT reaction mixture components was created for each dilution of  $\alpha$ CD74scFv::SLT-1A::KDEL. Each sample in the dilution series of  $\alpha$ CD74scFv::SLT-1A::KDEL protein was 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.) 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.), the half maximal inhibitory concentration (IC<sub>50</sub>) value was calculated for each sample.

[327] The inhibitory effect of  $\alpha$ CD74scFv::SLT-1A::KDEL on cell-free protein synthesis was strong. Dose dependence experiments determined that the IC<sub>50</sub> of  $\alpha$ CD74scFv::SLT-1A::KDEL on protein synthesis in this cell-free assay was about 3.1 pM (Table 9). This result was not substantially different from the IC<sub>50</sub> for the protein  $\alpha$ CD74scFv::SLT-1A, which lacks a KDEL motif, and was measured to be about 3.6 pM or equivalent of the SLT-1A-only positive control (Table 9).

10 **Table 9. KDEL Motif Had No Effect on Ribosome Inactivation:**  
Representative half-maximal inhibitory concentrations (IC<sub>50</sub>) for  $\alpha$ CD74scFv::SLT-1A::KDEL as compared to  $\alpha$ CD74scFv::SLT-1A

Cytotoxic Protein	IC <sub>50</sub> (pM)	IC <sub>50</sub> of SLT-1A only positive control (pM)
$\alpha$ CD74scFv::SLT-1A::KDEL	3.1	7.9
$\alpha$ CD74scFv::SLT-1A	3.6	7.9

15 Determining the Selective Cytotoxicity and Half-Maximal Cytotoxic Concentration (CD<sub>50</sub>) of  $\alpha$ CD74scFv::SLT-1A::KDEL Using a Cell-Kill Assay  
[328] The cytotoxicity characteristics of  $\alpha$ CD74scFv::SLT-1A::KDEL was determined by the following cell-kill assay. This assay determines the capacity of a cytotoxic protein to kill cells expressing the target biomolecule of its immunoglobulin-type binding region as compared to the SLT-1A protein that does not possess the binding region. Cells were plated (2 x 10<sup>3</sup> cells per well) in 20  $\mu$ L cell culture medium in 384-well plates. The  $\alpha$ CD74scFv::SLT-1A::KDEL protein was diluted 10-fold in buffer and 5  $\mu$ L of the dilutions were added to the cells. Control wells containing only cell culture medium were used for baseline correction. The cell samples were incubated with  $\alpha$ CD74scFv::SLT-1A::KDEL, or just buffer, for 3 days at 37°C and in an atmosphere of 5% CO<sub>2</sub>. The total cell survival or percent viability was determined using a luminescent readout using the CellTiter-Glo® Luminescent Cell Viability Assay (G7573 Promega Madison, WI, U.S.) according to the manufacturer's instructions. The Percent Viability of experimental wells was calculated using the following equation: (Test RLU - Average Media RLU) / (Average Cells RLU - Average Media RLU) \* 100. Log polypeptide

concentration versus Percent Viability was plotted in Prism (GraphPad Prism, San Diego, CA, U.S.) and log (inhibitor) vs. response (three parameter) analysis was used to determine the half-maximal cytotoxic concentration (CD<sub>50</sub>) value for  $\alpha$ CD74scFv::SLT-1A::KDEL.

5 [329] Dose dependence experiments determined that the CD<sub>50</sub> of the  $\alpha$ CD74scFv::SLT-1A::KDEL protein was about 29.9 nM for CD74 + Daudi cells (Table 10, Figure 6). The CD<sub>50</sub> for the SLT-1A-only negative control was 2026 nM and the same protein lacking the KDEL motif,  $\alpha$ CD74scFv::SLT-1A, was 95.3 nM (Table 10, Figure 6). These results exemplify the effect of adding  
 10 a KDEL signal motif on both cytotoxicity and selective cytotoxicity. The differences in cell-kill for these cytotoxic proteins were not predictable based on the *in vitro* results for protein synthesis inhibition and are not expected to be predictable based on target cell-binding characteristics.

15 **Table 10. KDEL motif Affected Cytotoxicity:** Representative half-maximal cytotoxic concentrations (CD<sub>50</sub>) of  $\alpha$ CD74scFv::SLT-1A::KDEL as compared to  $\alpha$ CD74scFv::SLT-1A

Cell Line	CD74 status	CD <sub>50</sub> (nM)		
		$\alpha$ CD74scFv::SLT-1A::KDEL	$\alpha$ CD74scFv::SLT-1A	SLT-1A only negative control
Daudi	positive	29.97	95.3	2026

20 Determining the *In Vivo* Effects of the Cytotoxic Protein  $\alpha$ CD74scFv::SLT-1A::KDEL Using Animal Models

[330] Animal models are used to determine the *in vivo* effects of the cytotoxic protein  $\alpha$ CD74scFv::SLT-1A::KDEL on neoplastic and/or immune cells. Various mice strains are used to test the effect of the cytotoxic protein after intravenous administration on xenograft tumors in mice resulting from the  
 25 injection into those mice of human neoplastic and/or immune cells which express CD74 on their cell surfaces.

**Example 5. A HER2-targeted, cytotoxic protein derived from Shiga-like toxin 1 A Subunit ( $\alpha$ HER2scFv::SLT-1A::RDEL)**

30 [331] In this example,  $\alpha$ HER2scFv::SLT-1A::RDEL was created and tested just as in Example 1, except for the carboxy-terminus ended with the peptide sequence RDEL instead of KDEL. The cell binding characteristics of the

cytotoxic protein  $\alpha$ HER2scFv::SLT-1A::RDEL was determined by a fluorescence-based flow cytometry assay as described in Example 1. The  $B_{\max}$  for the  $\alpha$ HER2scFv::SLT-1A::RDEL cytotoxic protein was measured to be about 130,000 MFI with a  $K_D$  of about 167 nM, whereas there was no meaningful binding to HER2- cells observed in this assay. The inhibitory effect of  $\alpha$ HER2scFv::SLT-1A::RDEL on cell-free protein synthesis was strong as shown by the measurement of an  $IC_{50}$  for  $\alpha$ HER2scFv::SLT-1A::RDEL on protein synthesis of about 36 pM.

[332] The cytotoxicity characteristics of  $\alpha$ HER2scFv::SLT-1A::RDEL are determined by the general cell-kill assay as described above in the previous examples using HER2+ cells. In addition, the selective cytotoxicity characteristics of  $\alpha$ HER2scFv::SLT-1A::RDEL are determined by the same general cell-kill assay using HER2- cells as a comparison to the HER2+ cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for HER2+ cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing HER2 on a cellular surface as compared to cells which do express HER2 on a cellular surface

**Example 6. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and the antibody  $\alpha$ Epstein-Barr-antigen**

[333] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). An immunoglobulin-type binding region  $\alpha$ Epstein-Barr-antigen is derived from a monoclonal antibody against an Epstein Barr antigen (Fang C et al., *J Immunol Methods* 287: 21-30 (2004)), which comprises an immunoglobulin-type binding region capable of binding a human cell infected by the Epstein-Barr virus or a transformed cell expressing an Epstein-Barr antigen. The Epstein-Barr antigen is expressed on multiple cell types, such as cells infected by an Epstein-Barr virus and cancer cells (e.g. lymphoma and nasopharyngeal cancer cells). In addition, Epstein-Barr infection is associated with other diseases, e.g., multiple sclerosis.

Construction, Production, and Purification of the Cytotoxic Protein  $\alpha$ EpsteinBarr::SLT-1A::KDEL

[334] The immunoglobulin-type binding region  $\alpha$ Epstein-Barr-antigen and Shiga toxin effector region are linked together, and a carboxy-terminal KDEL is added to form a protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the  $\alpha$ Epstein-Barr-antigen-binding protein  $\alpha$ EpsteinBarr::SLT-1A::KDEL. Expression of the  $\alpha$ EpsteinBarr::SLT-1A::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in the previous examples.

#### Determining the *In Vitro* Characteristics of the Cytotoxic Protein

##### 10 $\alpha$ EpsteinBarr::SLT-1A::KDEL

[335] The binding characteristics of the cytotoxic protein of this example for Epstein-Barr antigen positive cells and Epstein-Barr antigen negative cells is determined by a fluorescence-based, flow-cytometry assay as described above in the previous examples. The  $B_{max}$  for  $\alpha$ EpsteinBarr::SLT-1A::KDEL to Epstein-15 Barr antigen positive cells is measured to be approximately 50,000-200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no significant binding to Epstein-Barr antigen negative cells in this assay.

[336] The ribosome inactivation abilities of the  $\alpha$ EpsteinBarr::SLT-1A::KDEL cytotoxic protein is determined in a cell-free, *in vitro* protein translation as 20 described above in the previous examples. The inhibitory effect of the cytotoxic protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of  $\alpha$ EpsteinBarr::SLT-1A::KDEL on protein synthesis in this cell-free assay is approximately 0.1-100 pM.

##### 25 Determining the Cytotoxicity of the Cytotoxic Protein SLT-1A:: $\alpha$ EpsteinBarr::KDEL Using a Cell-Kill Assay

[337] The cytotoxicity characteristics of  $\alpha$ EpsteinBarr::SLT-1A::KDEL are determined by the general cell-kill assay as described above in the previous examples using Epstein-Barr antigen positive cells. In addition, the selective 30 cytotoxicity characteristics of  $\alpha$ EpsteinBarr::SLT-1A::KDEL are determined by the same general cell-kill assay using Epstein-Barr antigen negative cells as a comparison to the Epstein-Barr antigen positive cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for Epstein-Barr antigen positive cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is

approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing the Epstein-Barr antigen on a cellular surface as compared to cells which do express the Epstein-Barr antigen on a cellular surface.

5 Determining the *In Vivo* Effects of the Cytotoxic Protein  $\alpha$ EpsteinBarr::SLT-1A::KDEL Using Animal Models

[338] Animal models are used to determine the *in vivo* effects of the cytotoxic protein  $\alpha$ EpsteinBarr::SLT-1A::KDEL on neoplastic cells. Various mice strains are used to test the effect of the cytotoxic protein after intravenous  
10 administration on xenograft tumors in mice resulting from the injection into those mice of human neoplastic cells which express Epstein-Barr antigens on their cell surfaces.

15 **Example 7. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and the antibody  $\alpha$ Leishmania-antigen**

[339] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). An immunoglobulin-type binding region  $\alpha$ Leishmania-antigen is derived from an antibody generated, using techniques known in the art, to a cell-surface Leishmania antigen present on  
20 human cells harboring an intracellular trypanosomatid protozoa (*see* Berman J, Dwyer, *Clin Exp Immunol* 44: 342-348 (1981); Kenner J et al., *J Cutan Pathol* 26: 130-6 (1999); Silveira T et al., *Int J Parasitol* 31: 1451-8 (2001)).

25 Construction, Production, and Purification of the Cytotoxic Protein SLT-1A:: $\alpha$ Leishmania::KDEL

[340] The immunoglobulin-type binding region  $\alpha$ -Leishmania-antigen and Shiga toxin effector region are linked together, and a carboxy-terminal KDEL is added to form a protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the Leishmania-antigen-binding protein  
30 SLT-1A:: $\alpha$ Leishmania::KDEL. Expression of the SLT-1A:: $\alpha$ Leishmania::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in the previous examples.

Determining the *In Vitro* Characteristics of the Cytotoxic Protein SLT-1A:: $\alpha$ Leishmania::KDEL

[341] The binding characteristics of the cytotoxic protein of this example for Leishmania antigen positive cells and Leishmania antigen negative cells is  
5 determined by a fluorescence-based, flow-cytometry assay as described above in the previous examples. The  $B_{max}$  for SLT-1A:: $\alpha$ Leishmania::KDEL to Leishmania antigen positive cells is measured to be approximately 50,000-200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no significant binding to Leishmania antigen negative cells in this assay.

10 [342] The ribosome inactivation abilities of the SLT-1A:: $\alpha$ Leishmania::KDEL cytotoxic protein is determined in a cell-free, *in vitro* protein translation as described above in the previous examples. The inhibitory effect of the cytotoxic protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of SLT-1A:: $\alpha$ Leishmania::KDEL on protein synthesis in this cell-free assay is  
15 approximately 0.1-100 pM.

Determining the Cytotoxicity of the Cytotoxic Protein SLT-1A:: $\alpha$ Leishmania::KDEL Using a Cell-Kill Assay

[343] The cytotoxicity characteristics of SLT-1A:: $\alpha$ Leishmania::KDEL are  
20 determined by the general cell-kill assay as described above in the previous examples using Leishmania antigen positive cells. In addition, the selective cytotoxicity characteristics of SLT-1A:: $\alpha$ Leishmania::KDEL are determined by the same general cell-kill assay using Leishmania antigen negative cells as a comparison to the Leishmania antigen positive cells. The  $CD_{50}$  of the cytotoxic  
25 protein of this example is approximately 0.01-100 nM for Leishmania antigen positive cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing the Leishmania antigen on a cellular surface as compared to cells which do express the Leishmania antigen on a cellular surface.

30

**Example 8. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and immunoglobulin-type binding region  $\alpha$ Neurotensin-Receptor**

[344] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). An immunoglobulin-type binding

region  $\alpha$ Neurotensin-Receptor is derived from the DARPin™ (GenBank  
Accession: 2P2C\_R) or a monoclonal antibody (Ovigne J et al., *Neuropeptides*  
32: 247-56 (1998)) which binds the human neurotensin receptor. The  
neurotensin receptor is expressed by various cancer cells, such as breast cancer,  
5 colon cancer, lung cancer, melanoma, and pancreatic cancer cells.

#### Construction, Production, and Purification of the Cytotoxic Protein SLT- 1A:: $\alpha$ NeurotensinR::KDEL

[345] The immunoglobulin-type binding region  $\alpha$ NeurotensinR and Shiga toxin  
10 effector region are linked together, and a carboxy-terminal KDEL is added to  
form a protein. For example, a fusion protein is produced by expressing a  
polynucleotide encoding the neurotensin-receptor-binding protein SLT-  
1A:: $\alpha$ NeurotensinR::KDEL. Expression of the SLT-1A:: $\alpha$ NeurotensinR::KDEL  
cytotoxic protein is accomplished using either bacterial and/or cell-free, protein  
15 translation systems as described in the previous examples.

#### Determining the *In Vitro* Characteristics of the Cytotoxic Protein SLT- 1A:: $\alpha$ NeurotensinR::KDEL

[346] The binding characteristics of the cytotoxic protein of this example for  
20 neurotensin receptor positive cells and neurotensin receptor negative cells is  
determined by a fluorescence-based, flow-cytometry assay as described above in  
the previous examples. The  $B_{max}$  for SLT-1A:: $\alpha$ NeurotensinR::KDEL to  
neurotensin receptor positive cells is measured to be approximately 50,000-  
200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no  
25 significant binding to neurotensin receptor negative cells in this assay.

[347] The ribosome inactivation abilities of the SLT-  
1A:: $\alpha$ NeurotensinR::KDEL cytotoxic protein is determined in a cell-free, *in vitro*  
protein translation as described above in the previous examples. The inhibitory  
effect of the cytotoxic protein of this example on cell-free protein synthesis is  
30 significant. The  $IC_{50}$  of SLT-1A:: $\alpha$ NeurotensinR::KDEL on protein synthesis in  
this cell-free assay is approximately 0.1-100 pM.

#### Determining the Cytotoxicity of the Cytotoxic Protein SLT- 1A:: $\alpha$ NeurotensinR::KDEL Using a Cell-Kill Assay

[348] The cytotoxicity characteristics of SLT-1A:: $\alpha$ NeurotensinR::KDEL are determined by the general cell-kill assay as described above in the previous examples using neurotensin receptor positive cells. In addition, the selective cytotoxicity characteristics of SLT-1A:: $\alpha$ NeurotensinR::KDEL are determined  
5 by the same general cell-kill assay using neurotensin receptor negative cells as a comparison to the neurotensin receptor positive cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for neurotensin receptor positive cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing  
10 neurotensin receptor on a cellular surface as compared to cells which do express neurotensin receptor on a cellular surface.

Determining the *In Vivo* Effects of the Cytotoxic Protein SLT-1A:: $\alpha$ NeurotensinR::KDEL Using Animal Models

[349] Animal models are used to determine the *in vivo* effects of the cytotoxic protein SLT-1A:: $\alpha$ NeurotensinR::KDEL on neoplastic cells. Various mice strains are used to test the effect of the cytotoxic protein after intravenous administration on xenograft tumors in mice resulting from the injection into those mice of human neoplastic cells which express neurotensin receptors on  
20 their cell surfaces.

**Example 9. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and an immunoglobulin-type binding region  $\alpha$ EGFR**

[350] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). The binding region  $\alpha$ EGFR is derived  
25 from the AdNectin™ (GenBank Accession: 3QWQ\_B), the Affibody™ (GenBank Accession: 2KZI\_A; U.S. patent 8,598,113), or an antibody, all of which bind one or more human epidermal growth factor receptors. The expression of epidermal growth factor receptors are associated with human  
30 cancer cells, such as, *e.g.*, lung cancer cells, breast cancer cells, and colon cancer cells.

Construction, Production, and Purification of the Cytotoxic Protein SLT-1A:: $\alpha$ EGFR::KDEL

[351] The immunoglobulin-type binding region  $\alpha$ EGFR and Shiga toxin effector region are linked together, and a carboxy-terminal KDEL is added to form a protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the EGFR binding protein SLT-1A:: $\alpha$ EGFR::KDEL.  
5 Expression of the SLT-1A:: $\alpha$ EGFR::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in the previous examples.

Determining the *In Vitro* Characteristics of the Cytotoxic Protein SLT-1A:: $\alpha$ EGFR::KDEL  
10

[352] The binding characteristics of the cytotoxic protein of this example for EGFR+ cells and EGFR- cells is determined by a fluorescence-based, flow-cytometry assay as described above in the previous examples. The  $B_{max}$  for SLT-1A:: $\alpha$ EGFR::KDEL to EGFR+ cells is measured to be approximately  
15 50,000-200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no significant binding to EGFR- cells in this assay.

[353] The ribosome inactivation abilities of the SLT-1A:: $\alpha$ EGFR::KDEL cytotoxic protein is determined in a cell-free, *in vitro* protein translation as described above in the previous examples. The inhibitory effect of the cytotoxic  
20 protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of SLT-1A:: $\alpha$ EGFR::KDEL on protein synthesis in this cell-free assay is approximately 0.1-100 pM.

Determining the Cytotoxicity of the Cytotoxic Protein SLT-1A:: $\alpha$ EGFR::KDEL  
25 Using a Cell-Kill Assay

[354] The cytotoxicity characteristics of SLT-1A:: $\alpha$ EGFR::KDEL are determined by the general cell-kill assay as described above in the previous examples using EGFR+ cells. In addition, the selective cytotoxicity characteristics of SLT-1A:: $\alpha$ EGFR::KDEL are determined by the same general  
30 cell-kill assay using EGFR- cells as a comparison to the Leishmania antigen positive cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for EGFR+ cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is approximately 10-10,000 fold greater (less

cytotoxic) for cells not expressing EGFR on a cellular surface as compared to cells which do express EGFR on a cellular surface.

Determining the *In Vivo* Effects of the Cytotoxic Protein SLT-

5 1A:: $\alpha$ EGFR::KDEL Using Animal Models

[355] Animal models are used to determine the *in vivo* effects of the cytotoxic protein SLT-1A:: $\alpha$ EGFR::KDEL on neoplastic cells. Various mice strains are used to test the effect of the cytotoxic protein after intravenous administration on xenograft tumors in mice resulting from the injection into those mice of human  
10 neoplastic cells which express EGFR(s) on their cell surfaces.

**Example 10. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and the antibody  $\alpha$ CCR5**

[356] In this example, the Shiga toxin effector region is derived from the A  
15 subunit of Shiga-like Toxin 1 (SLT-1A). An immunoglobulin-type binding region  $\alpha$ CCR5 is derived from a monoclonal antibody against human CCR5 (CD195) (Bernstone L et al., *Hybridoma* 31: 7-19 (2012)). CCR5 is predominantly expressed on T-cells, macrophages, dendritic cells, and microglia. In addition, CCR5 plays a role in the pathogenesis and spread of the Human  
20 Immunodeficiency Virus (HIV).

Construction, Production, and Purification of the Cytotoxic Protein SLT-  
1A:: $\alpha$ CCR5::KDEL

[357] The immunoglobulin-type binding region  $\alpha$ CCR5 and Shiga toxin  
25 effector region are linked together, and a carboxy-terminal KDEL is added to form a protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the  $\alpha$ CCR5-binding protein SLT-1A:: $\alpha$ CCR5::KDEL. Expression of the SLT-1A:: $\alpha$ CCR5::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in  
30 the previous examples.

Determining the *In Vitro* Characteristics of the Cytotoxic Protein SLT-  
1A:: $\alpha$ CCR5

[358] The binding characteristics of the cytotoxic protein of this example for CCR5+ cells and CCR5- cells is determined by a fluorescence-based, flow-cytometry assay as described above in the previous examples. The  $B_{max}$  for SLT-1A:: $\alpha$ CCR5::KDEL to CCR5+ positive cells is measured to be  
5 approximately 50,000-200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no significant binding to CCR5- cells in this assay.

[359] The ribosome inactivation abilities of the SLT-1A:: $\alpha$ CCR5::KDEL cytotoxic protein is determined in a cell-free, *in vitro* protein translation as described above in the previous examples. The inhibitory effect of the cytotoxic  
10 protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of SLT-1A:: $\alpha$ CCR5::KDEL on protein synthesis in this cell-free assay is approximately 0.1-100 pM.

Determining the Cytotoxicity of the Cytotoxic Protein SLT-1A:: $\alpha$ CCR5::KDEL  
15 Using a Cell-Kill Assay

[360] The cytotoxicity characteristics of SLT-1A:: $\alpha$ CCR5::KDEL are determined by the general cell-kill assay as described above in the previous examples using CCR5+ cells. In addition, the selective cytotoxicity characteristics of SLT-1A:: $\alpha$ CCR5::KDEL are determined by the same general  
20 cell-kill assay using CCR5- cells as a comparison to the CCR5+ cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for CCR5+ cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing CCR5 on a cellular surface as compared to cells which do express CCR5 on a  
25 cellular surface.

Determining the *In Vivo* Effects of the Cytotoxic Protein SLT-1A:: $\alpha$ CCR5::KDEL Using Animal Models

[361] Animal models are used to determine the *in vivo* effects of the cytotoxic  
30 protein SLT-1A:: $\alpha$ CCR5::KDEL on depleting T-cells from donor materials (*see* Tsirigotis P et al., *Immunotherapy* 4: 407-24 (2012)). Non-human primates are used to determine *in vivo* effects of SLT-1A:: $\alpha$ CCR5. Graft-versus-host disease is analyzed in rhesus macaques after kidney transplantation when the donated organs are pretreated with SLT-1A:: $\alpha$ CCR5::KDEL (*see* Weaver T et al., *Nat*

*Med* 15: 746-9 (2009)). *In vivo* depletion of peripheral blood T lymphocytes in cynomolgus primates is observed after parenteral administration of different doses of SLT-1A:: $\alpha$ CCR5::KDEL. The use of SLT-1A:: $\alpha$ CCR5::KDEL to block HIV infection is tested by giving an acute dose of SLT-

5 1A:: $\alpha$ CCR5::KDEL to non-human primates in order to severely deplete circulating T-cells upon exposure to a simian immunodeficiency virus (SIV) (*see* Sellier P et al., *PLoS One* 5: e10570 (2010)).

**Example 11. A cytotoxic protein derived from the A Subunit of Shiga toxin  
10 and an anti-Env immunoglobulin domain**

[362] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga toxin (Stx-A). An immunoglobulin-type binding region  $\alpha$ Env is derived from existing antibodies that bind HIV envelope glycoprotein (Env), such as GP41, GP120, GP140, or GP160 (*see e.g.* Chen W et al., *J Mol Bio* 382:  
15 779-89 (2008); Chen W et al., *Expert Opin Biol Ther* 13: 657-71 (2013); van den Kerkhof T et al., *Retrovirology* 10: 102 (2013) or from antibodies generated using standard techniques (*see* Prabakaran et al., *Front Microbiol* 3: 277 (2012)). Envs are HIV surface proteins that are also displayed on the cell surfaces of  
20 infected cells predominantly in endosomal compartments, sufficient amounts of Envs could be present on a cell surface to be targeted by a highly potent cytotoxic protein of the invention. In addition, Env-targeting cytotoxic proteins might bind HIV virions and enter newly infected cells during the fusion of virions with a host cell.

25 [363] Because HIV displays a high rate of mutation, it is preferable to use an immunoglobulin domain that binds a functional constrained part of an Env, such as shown by broadly neutralizing antibodies that bind Envs from multiple strains of HIV (van den Kerkhof T et al., *Retrovirology* 10: 102 (2013)). Because the Envs present on an infected cell's surface are believed to present sterically  
30 restricted epitopes (Chen W et al., *J Virol* 88: 1125-39 (2014), it is preferable to use binding regions smaller than 100 kD and ideally smaller than 25 kD, such as fragments of sdAbs like V<sub>H</sub>H domains.

Construction, Production, and Purification of the Cytotoxic Protein  $\alpha$ Env::SLT-1A::KDEL

[364] The immunoglobulin-type binding region  $\alpha$ Env and Shiga toxin effector region are linked together, and a carboxy-terminal KDEL is added to form a  
5 cytotoxic protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the  $\alpha$ Env-binding protein SLT-1A:: $\alpha$ Env::KDEL. Expression of the SLT-1A:: $\alpha$ Env::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in the previous examples.

10

Determining the *In Vitro* Characteristics of the Cytotoxic Protein SLT-1A:: $\alpha$ Env::KDEL

[365] The binding characteristics of the cytotoxic protein of this example for Env+ cells and Env- cells is determined by a fluorescence-based, flow-cytometry  
15 assay as described above in the previous examples. The  $B_{\max}$  for SLT-1A:: $\alpha$ Env::KDEL to Env+ positive cells is measured to be approximately 50,000-200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no significant binding to Env- cells in this assay.

[366] The ribosome inactivation abilities of the SLT-1A:: $\alpha$ Env::KDEL  
20 cytotoxic protein is determined in a cell-free, *in vitro* protein translation as described above in the previous examples. The inhibitory effect of the cytotoxic protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of SLT-1A:: $\alpha$ Env::KDEL on protein synthesis in this cell-free assay is approximately 0.1-100 pM.

25

Determining the Cytotoxicity of the Cytotoxic Protein SLT-1A:: $\alpha$ Env::KDEL Using a Cell-Kill Assay

[367] The cytotoxicity characteristics of SLT-1A:: $\alpha$ Env::KDEL are determined  
30 by the general cell-kill assay as described above in the previous examples using Env+ cells. In addition, the selective cytotoxicity characteristics of SLT-1A:: $\alpha$ Env::KDEL are determined by the same general cell-kill assay using Env- cells as a comparison to the Env+ cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for Env+ cells depending on the cell line and/or the HIV strain used to infect the cells to make them Env+. The  $CD_{50}$

of the cytotoxic protein is approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing Env on a cellular surface as compared to cells which do express Env on a cellular surface.

5 Determining the *In Vivo* Effects of the Cytotoxic Protein SLT-1A:: $\alpha$ Env::KDEL Using Animal Models

[368] The use of SLT-1A:: $\alpha$ Env::KDEL to inhibit HIV infection is tested by administering SLT-1A:: $\alpha$ Env::KDEL to simian immunodeficiency virus (SIV) infected non-human primates (*see* Sellier P et al., *PLoS One* 5: e10570 (2010)).

10

**Example 12. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and the antibody  $\alpha$ UL18**

[369] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin 1 (SLT-1A). An immunoglobulin-type binding region  $\alpha$ UL18 is derived from an antibody generated, using techniques known in  
15 the art, to the cell-surface cytomegalovirus protein UL18, which is present on human cells infected with cytomegalovirus (Yang Z, Bjorkman P, *Proc Natl Acad Sci USA* 105: 10095-100 (2008)). The human cytomegalovirus infection is associated with various cancers and inflammatory disorders.

20

Construction, Production, and Purification of the Cytotoxic Protein SLT-1A:: $\alpha$ UL18::KDEL

[370] The immunoglobulin-type binding region  $\alpha$ UL18 and Shiga toxin effector region are linked together, and a carboxy-terminal KDEL is added to  
25 form a protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the  $\alpha$ UL18-binding protein SLT-1A:: $\alpha$ UL18::KDEL. Expression of the SLT-1A:: $\alpha$ UL18::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in the previous examples.

30

Determining the *In Vitro* Characteristics of the Cytotoxic Protein SLT-1A:: $\alpha$ UL18::KDEL

[371] The binding characteristics of the cytotoxic protein of this example for cytomegalovirus protein UL18 positive cells and cytomegalovirus protein UL18

negative cells is determined by a fluorescence-based, flow-cytometry assay as described above in the previous examples. The  $B_{max}$  for SLT-1A:: $\alpha$ UL18::KDEL to cytomegalovirus protein UL18 positive cells is measured to be approximately 50,000-200,000 MFI with a  $K_D$  within the range of 0.01-100 nM, whereas there is no significant binding to cytomegalovirus protein UL18 negative cells in this assay.

[372] The ribosome inactivation abilities of the SLT-1A:: $\alpha$ UL18::KDEL cytotoxic protein is determined in a cell-free, *in vitro* protein translation as described above in the previous examples. The inhibitory effect of the cytotoxic protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of SLT-1A:: $\alpha$ UL18::KDEL on protein synthesis in this cell-free assay is approximately 0.1-100 pM.

#### Determining the Cytotoxicity of the Cytotoxic Protein SLT-1A:: $\alpha$ UL18::KDEL

##### Using a Cell-Kill Assay

[373] The cytotoxicity characteristics of SLT-1A:: $\alpha$ UL18::KDEL are determined by the general cell-kill assay as described above in the previous examples using cytomegalovirus protein UL18 positive cells. In addition, the selective cytotoxicity characteristics of SLT-1A:: $\alpha$ UL18::KDEL are determined by the same general cell-kill assay using cytomegalovirus protein UL18 negative cells as a comparison to the cytomegalovirus protein UL18 positive cells. The  $CD_{50}$  of the cytotoxic protein of this example is approximately 0.01-100 nM for cytomegalovirus protein UL18 positive cells depending on the cell line. The  $CD_{50}$  of the cytotoxic protein is approximately 10-10,000 fold greater (less cytotoxic) for cells not expressing the cytomegalovirus protein UL18 on a cellular surface as compared to cells which do express the cytomegalovirus protein UL18 on a cellular surface.

#### **Example 13. A cytotoxic protein derived from the A Subunit of Shiga-like toxin-1 and the antibody $\alpha$ helminth-intestinal-antigen**

[374] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin I (SLT-1A). An immunoglobulin-type binding region  $\alpha$ helminth-intestinal-antigen is derived from an antibody generated, using techniques known in the art, to the helminth ortholog of a human transferrin

receptor (see e.g. the nematode gene gcp-2.1 UniProt G8JYE4\_CAEEL; Rosa B et al., *Mol Cell Proteomics* M114.046227 (2015)).

#### Construction, Production, and Purification of the Cytotoxic Protein $\alpha$ Helminth-

##### 5 Intestinal-Antigen::SLT-1A::KDEL

[375] The immunoglobulin-type binding region  $\alpha$ helminth-intestinal-antigen and Shiga toxin effector region are linked, and a carboxy-terminal KDEL is added to form a protein. For example, a fusion protein is produced by expressing a polynucleotide encoding the  $\alpha$ helminth-intestinal-antigen-binding  
10 protein  $\alpha$ helminth-intestinal-antigen::SLT-1A::KDEL. Expression of the  $\alpha$ helminth-intestinal-antigen::SLT-1A::KDEL cytotoxic protein is accomplished using either bacterial and/or cell-free, protein translation systems as described in the previous examples.

##### 15 Determining the *In Vitro* Characteristics of the Cytotoxic Protein $\alpha$ Helminth-Intestinal-Antigen::SLT-1A::KDEL

[376] The binding characteristics of the cytotoxic protein of this example is determined by a molecular binding assay known in the art using a purified recombinant target protein. The  $K_D$  for  $\alpha$ helminth-intestinal-antigen::SLT-  
20 1A::KDEL to target protein is measured to be approximately 100 nM, whereas there is no significant binding to a negative control protein (e.g. purified, recombinant, human transferrin receptor) in this assay.

[377] The ribosome inactivation abilities of the  $\alpha$ helminth-intestinal-antigen::SLT-1A::KDEL cytotoxic protein is determined in a cell-free, *in vitro*  
25 protein translation as described above in the previous examples. The inhibitory effect of the cytotoxic protein of this example on cell-free protein synthesis is significant. The  $IC_{50}$  of  $\alpha$ helminth-intestinal-antigen::SLT-1A::KDEL on protein synthesis in this cell-free assay is approximately 0.1-100 pM.

##### 30 Determining the Toxicity of the Cytotoxic Protein $\alpha$ Helminth-Intestinal-Antigen::SLT-1A::KDEL Using Helminths

[378] The toxicity of  $\alpha$ helminth-intestinal-antigen::SLT-1A::KDEL to helminths is determined using model helminths (see e.g. Iatsenko I et al., *Toxins* 2050-63 (2014)). The helminth can be administered purified  $\alpha$ helminth-

intestinal-antigen::SLT-1A::KDEL by soaking or alternatively by feeding the helminth with bacteria expressing the SLT-1A:: $\alpha$ helminth-intestinal-antigen fusion protein.

[379] In addition, laboratory animals harboring helminths and/or displaying  
 5 helminth related diseases are administered  $\alpha$ helminth-intestinal-antigen::SLT-1A::KDEL and monitored for reduction or elimination of helminths and/or associated symptoms of parasitic helminth(s).

**Example 14. Cytotoxic proteins targeting various cell types**

10 [380] In this example, the Shiga toxin effector region is derived from the A subunit of Shiga-like Toxin 1 (SLT-1A), Shiga toxin (StxA), and/or Shiga-like Toxin 2 (SLT-2A). A binding region is derived from the molecules chosen from column 1 of Table 11 and which binds the extracellular target biomolecule indicated in column 2 of Table 11. The exemplary proteins of this example are  
 15 created with a carboxy-terminal KDEL-type signal motif using techniques known in the art and optionally linked with a detection promoting agent(s). The exemplary proteins of this example are tested as described in the previous examples using cells expressing the appropriate extracellular target biomolecules. The exemplary proteins of this example may be used, *e.g.*, to  
 20 labeling subcellular compartments of target cells and to diagnose and treat diseases, conditions, and/or disorders indicated in column 3 of Table 11.

**Table 11. Various Binding Regions for Cell Targeting of Cytotoxic Proteins**

Source of binding region	Extracellular target	Application(s)
alemtuzumab	CD52	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
basiliximab	CD25	T-cell disorders, such as prevention of organ transplant rejections, and some B-cell lineage cancers
brentuximab	CD30	hematological cancers, B-cell related immune disorders, and T-cell related immune disorders
catumaxomab	EpCAM	various cancers, such as ovarian cancer, malignant ascites, gastric cancer
cetuximab	EGFR	various cancers, such as colorectal cancer and

		head and neck cancer
daclizumab	CD25	B-cell lineage cancers and T-cell disorders, such as rejection of organ transplants
daratumumab	CD38	hematological cancers, B-cell related immune disorders, and T-cell related immune disorders
dinutuximab	ganglioside GD2	Various cancers, such as breast cancer, myeloid cancers, and neuroblastoma
efalizumab	LFA-1 (CD11a)	autoimmune disorders, such as psoriasis
ertumaxomab	HER2/neu	various cancers and tumors, such as breast cancer and colorectal cancer
gemtuzumab	CD33	myeloid cancer or immune disorder
ibritumomab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
ipilimumab	CD152	T-cell related disorders and various cancers, such as leukemia, melanoma
muromonab	CD3	prevention of organ transplant rejections
natalizumab	integrin $\alpha$ 4	autoimmune disorders, such as multiple sclerosis and Crohn's disease
obinutuzumab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
ocaratuzumab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
ocrelizumab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
ofatumumab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
palivizumab	F protein of respiratory syncytial virus	treat respiratory syncytial virus
panitumumab	EGFR	various cancers, such as colorectal cancer and head and neck cancer
pertuzumab	HER2/neu	various cancers and tumors, such as breast cancer and colorectal cancer
pro 140	CCR5	HIV infection and T-cell disorders
ramucirumab	VEGFR2	various cancers and cancer related disorders, such as solid tumors
rituximab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
tocilizumab or atlizumab	IL-6 receptor	autoimmune disorders, such as rheumatoid arthritis
tositumomab	CD20	B-cell cancers, such as lymphoma and

		leukemia, and B-cell related immune disorders, such as autoimmune disorders
trastuzumab	HER2/neu	various cancers and tumors, such as breast cancer and colorectal cancer
ublrituximab	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders
vedolizumab	integrin $\alpha4\beta7$	autoimmune disorders, such as Crohn's disease and ulcerative colitis
CD20 binding antibodies and scFv(s)	CD20	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders ( <i>see e.g. Geng S et al., Cell Mol Immunol 3: 439-43 (2006); Olafesn T et al., Protein Eng Des Sel 23: 243-9 (2010)</i> )
CD22 binding scFv(s)	CD22	B-cell cancers or B-cell related immune disorders ( <i>see e.g. Kawas S et al., MAbs 3: 479-86 (2011)</i> )
CD25 binding scFv(s)	CD25	various cancers of the B-cell lineage and immune disorders related to T-cells ( <i>see e.g. Muramatsu H et al., Cancer Lett 225: 225-36 (2005)</i> )
CD30 binding monoclonal antibody(s)	CD30	B-cell cancers or B-cell/T-cell related immune disorders ( <i>see e.g. Klimka A et al., Br J Cancer 83: 252-60 (2000)</i> )
CD33 binding monoclonal antibody(s)	CD33	myeloid cancer or immune disorder ( <i>see e.g. Benedict C et al., J Immunol Methods 201: 223-31 (1997)</i> )
CD38 binding immunoglobulin domains	CD38	hematological cancers, B-cell related immune disorders, and T-cell related immune disorders ( <i>see e.g. U.S. patent 8,153,765</i> )
CD40 binding scFv(s)	CD40	various cancers and immune disorders ( <i>see e.g. Ellmark P et al., Immunology 106: 456-63 (2002)</i> )
CD52 binding monoclonal antibody(s)	CD52	B-cell cancers, such as lymphoma and leukemia, and B-cell related immune disorders, such as autoimmune disorders ( <i>see e.g. U.S. Patent 7,910,104 B2</i> )
CD56 binding monoclonal antibody(s)	CD56	immune disorders and various cancers, such as lung cancer, Merkel cell carcinoma, myeloma ( <i>see e.g. Shin J et al., Hybridoma 18: 521-7 (1999)</i> )
CD79 binding monoclonal antibody(s)	CD79	B-cell cancers or B-cell related immune disorders ( <i>see e.g. Zhang L et al., Ther Immunol 2: 191-202 (1995)</i> )
CD133	CD133	various cancers, hematologic malignancies,

binding monoclonal antibodies and scFv(s)		and immune disorders ( <i>see e.g.</i> Bidlingmaier S et al., <i>J Mol Med</i> 86: 1025-32 (2008); Pavlon L et al., <i>J Microsc</i> 231: 374-83 (2008); Rappa G et al., <i>Stem Cells</i> 26: 3008-17 (2008); Swaminathan S et al., <i>J Immunol Methods</i> 361: 110-5 (2010); Wang J et al., <i>Hybridoma</i> 29: 241-9 (2010); Zhu X et al., <i>Mol Cancer Ther</i> 9: 2131-41 (2010); Xia J et al., <i>Sci Rep</i> 3: 3320 (2013))
CD248 binding scFv(s)	CD248	various cancers, such as inhibiting angiogenesis ( <i>see e.g.</i> Zhao A et al., <i>J Immunol Methods</i> 363: 221-32 (2011))
EpCAM binding monoclonal antibody(s)	EpCAM	various cancers, such as ovarian cancer, malignant ascites, gastric cancer ( <i>see e.g.</i> Schanzer J et al., <i>J Immunother</i> 29: 477-88 (2006))
PSMA binding monoclonal antibody(s)	PSMA	prostate cancer ( <i>see e.g.</i> Frigerio B et al., <i>Eur J Cancer</i> 49: 2223-32 (2013))
Eph-B2 binding monoclonal antibody(s)	Eph-B2	various cancers such as colorectal cancer and prostate cancer ( <i>see e.g.</i> Abéngozar M et al., <i>Blood</i> 119: 4565-76 (2012))
Endoglin binding monoclonal antibody(s)	Endoglin	various cancers, such as breast cancer and colorectal cancers ( <i>see e.g.</i> Völkel T et al., <i>Biochim Biophys Res Acta</i> 1663: 158-66 (2004))
FAP binding monoclonal antibody(s)	FAP	various cancers, such as sarcomas and bone cancers ( <i>see e.g.</i> Zhang J et al., <i>FASEB J</i> 27: 581-9 (2013))
CEA binding antibody(s) and scFv(s)	CEA	various cancers, such as gastrointestinal cancer, pancreatic cancer, lung cancer, and breast cancer ( <i>see e.g.</i> Neumaier M et al., <i>Cancer Res</i> 50: 2128-34 (1990); Pavoni E et al., <i>BMC Cancer</i> 6: 4 (2006); Yazaki P et al., <i>Nucl Med Biol</i> 35: 151-8 (2008); Zhao J et al., <i>Oncol Res</i> 17: 217-22 (2008))
CD24 binding monoclonal antibody(s)	CD24	various cancers, such as bladder cancer ( <i>see e.g.</i> Kristiansen G et al., <i>Lab Invest</i> 90: 1102-16 (2010))
LewisY antigen binding scFv(s)	LewisY antigens	various cancers, such as cervical cancer and uterine cancer ( <i>see e.g.</i> Power B et al., <i>Protein Sci</i> 12: 734-47 (2003); monoclonal antibody BR96 Feridani A et al., <i>Cytometry</i> 71: 361-70 (2007))
adalimumab	TNF- $\alpha$	various cancers and immune disorders, such as Rheumatoid arthritis, Crohn's Disease, Plaque Psoriasis, Psoriatic Arthritis,

		Ankylosing Spondylitis, Juvenile Idiopathic Arthritis, Hemolytic disease of the newborn
afelimomab	TNF- $\alpha$	various cancers and immune disorders
ald518	IL-6	various cancers and immune disorders, such as rheumatoid arthritis
anrukinzumab or ima-638	IL-13	various cancers and immune disorders
briakinumab	IL-12, IL-23	various cancers and immune disorders, such as psoriasis, rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis
brodalumab	IL-17	various cancers and immune disorders, such as inflammatory diseases
canakinumab	IL-1	various cancers and immune disorders, such as rheumatoid arthritis
certolizumab	TNF- $\alpha$	various cancers and immune disorders, such as Crohn's disease
fezakinumab	IL-22	various cancers and immune disorders, such as rheumatoid arthritis, psoriasis
ganitumab	IGF-I	various cancers
golimumab	TNF- $\alpha$	various cancers and immune disorders, such as rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis
infliximab	TNF- $\alpha$	various cancers and immune disorders, such as rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis, Crohn's disease, ulcerative colitis
ixekizumab	IL-17A	various cancers and immune disorders, such as autoimmune diseases
mepolizumab	IL-5	various immune disorders and cancers, such as B-cell cancers
nerelimomab	TNF- $\alpha$	various cancers and immune disorders
olokizumab	IL6	various cancers and immune disorders
ozoralizumab	TNF- $\alpha$	inflammation
perakizumab	IL17A	various cancers and immune disorders, such as arthritis
placulumab	human TNF	various immune disorders and cancers
sarilumab	IL6	various cancers and immune disorders, such as rheumatoid arthritis, ankylosing spondylitis
siltuximab	IL-6	various cancers and immune disorders
sirukumab	IL-6	various cancers and immune disorders, such as rheumatoid arthritis
tabalumab	BAFF	B-cell cancers
ticilimumab or tremelimumab	CTLA-4	various cancers
tildrakizumab	IL23	immunologically mediated inflammatory disorders
tnx-650	IL-13	various cancers and immune disorders, such

		as B-cell cancers
tocilizumab or atlizumab	IL-6 receptor	various cancers and immune disorders, such as rheumatoid arthritis
ustekinumab	IL-12, IL-23	various cancers and immune disorders, such as multiple sclerosis, psoriasis, psoriatic arthritis
Various growth factors: VEGF, EGF1, EGF2, FGF	VEGFR, EGFR, FGFR	various cancer, such as breast cancer and colon cancer, and to inhibit vascularization
Various cytokines: IL-2, IL-6, IL-23, CCL2, BAFFs, TNFs, RANKL	IL-2R, IL-6R, IL-23R, CD80/CD86, TNFRSF13/ TNFRSF17, TNFR	various immune disorders and cancers
Broadly neutralizing antibodies identified from patient samples	Influenza surface antigens (e.g. hemagglutinins and matrix protein 2)	viral infections ( <i>see e.g.</i> Prabakaran et al., <i>Front Microbiol</i> 3: 277 (2012))
Broadly neutralizing antibodies identified from patient samples	Coronavirus surface antigens	viral infections ( <i>see e.g.</i> Prabakaran et al., <i>Front Microbiol</i> 3: 277 (2012))
Various antibodies	Filovirus surface antigens (e.g. VP35, VP40, and glycoprotein )	viral infections ( <i>see e.g.</i> Olinger G et al., <i>Proc Natl Acad Sci USA</i> 109: 18030-5 (2012); Pettitt J et al., <i>Sci Transl Med</i> 5: 199ra113 (2013); Stahelin R, <i>Expert Opin Ther Targets</i> 18: 115-20 (2014); Becquart P et al., <i>PLoS One</i> 9: e96360 (2014); Stahelin R, <i>Front Microbiol</i> 5: 300 (2014); Tran E et al., <i>J Virol</i> 88: 10958-62 (2014); Murin C et al., <i>Proc Natl Acad Sci USA</i> 111: 17182-7 (2014))
Broadly neutralizing antibodies identified from patient samples	Henipavirus surface antigens	viral infections ( <i>see e.g.</i> Prabakaran et al., <i>Front Microbiol</i> 3: 277 (2012))
Various	HIV surface	viral infections ( <i>see e.g.</i> Kitidee K et al., <i>BMC</i>

antibodies including broadly neutralizing antibodies and scFvs	antigens (e.g. matrix protein Map17)	<i>Biotechnol</i> 10: 80 (2010); Yu L, Guan Y, <i>Front Immunol</i> 5: 250 (2014))
Broadly neutralizing antibodies identified from patient samples	Influenza surface antigens (e.g. hemagglutinins and matrix protein 2)	viral infections (see e.g. Prabakaran et al., <i>Front Microbiol</i> 3: 277 (2012))

[381] 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, or exceeding the, scope of the invention or claims.

[382] The international patent application publications WO 2014164680 A1 and WO 2014164693 A2, the disclosures of U.S. provisional patent applications 61/951,110, 61/951,121, and 62/010,918, the disclosures of international PCT patent application serial numbers WO 2014/164680, WO 2014/164693, WO 2015/113005, WO 2015/113007, and WO 2015/120058 and 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 referred to herein.

Sequence Listing		
ID Number	Text Description	Biological Sequence
SEQ ID NO:1	Shiga-like toxin 1 Subunit A (SLT-1A)	KEFTLDFSTAKTYVDSLNVIRS AIGTPLQTISSGGTSLLMIDSGS GDNLFAVDVRGIDPEEGRFNN LRLIVERNNLYVTGFVNRTNN VFYRFADFSHVTFPGTTAVTLS GDSSYTTLQRVAGISRTGMQIN RHSLTTSYLDLMSHSGTSLTQS VARAMLRFVTVTAEALRFRQI QRGFRTTLDDL SGRSYVMTAE DVDLTLNWGR LSSVLPDYHGQ DSVRVGRISFGSINAILGSVALI LNCHHHASRVARMASDEFPSM CPADGRVVRGITHNKILWDSSTL GAILMRRTISS
SEQ ID NO:2	Shiga toxin Subunit A	KEFTLDFSTAKTYVDSLNVIRS AIGTPLQTISSGGTSLLMIDSGT GDNLFAVDVRGIDPEEGRFNN LRLIVERNNLYVTGFVNRTNN VFYRFADFSHVTFPGTTAVTLS GDSSYTTLQRVAGISRTGMQIN RHSLTTSYLDLMSHSGTSLTQS VARAMLRFVTVTAEALRFRQI QRGFRTTLDDL SGRSYVMTAE DVDLTLNWGR LSSVLPDYHGQ DSVRVGRISFGSINAILGSVALI LNCHHHASRVARMASDEFPSM CPADGRVVRGITHNKILWDSSTL GAILMRRTISS
SEQ ID NO:3	Shiga-like toxin 2 Subunit A (SLT-2A)	DEFTVDFSSQKSYVDSLNSIRS AISTPLGNISQGGVSVSVINHVL GGNYISLNVRGLDPYSERFNHL RLIMERNNLYVAGFINTETNIF YRFSDFSHISVPDVITVSMTTDS SYSSLQRIADLERTGMQIGRHS LVGSYLDLMEFRGRSMTRASS RAMLRFVTVIAEALRFRQIQRG FRPALSEASPLYTMTAQDVDLT LNWGRISNVLPEYRGEEGVRIG RISFNLSAILGSVAVILNCHST GSYSVRSVSQKQKTECQIVGD RAAIKVNNVLWEANTIAALLN RKPQDLTEPNQ
SEQ ID NO:4	$\alpha$ HER2scFv::SLT- 1A::KDEL variant 1	MDIQMTQSPSSLSASVGDRVTI TCRASQDVNTAVAWYQQKPG KAPKLLIYASFLYSGVPSRFSG SRSGTDFTLTISSLQPEDFATYY CQQHYTTPPTFGQGKVEIKRT GSTSGSGKPGSGEGSEVQLVES

		GGGLVQPGGSLRLSCAASGFNI KDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTA VYYCSRWGGDGFYAMDVWG QGTLVTVSSEFPKPSTPPGSSGG APKEFTLDFSTAKTYVDSLNI RSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFN NLRLIVERNNLYVTGFVNRTN NVFYRFADFSHVTFPGTTAVTL SGDSSYTTLQRVAGISRTGMQI NRHSLTTSYLDLMSHSGTSLTQ SVARAMLRFVTVTAELRFRQI QRGFRTTLDLSDGRSYVMTAE DVDLTLNWGRLLSVLPDYHGQ DSVRVGRISFGSINAILGVALI LNCHHHASRVARKDEL
SEQ ID NO:5	$\alpha$ HER2scFv::SLT-1A::KDEL variant 2	MDIQMTQSPSSLSASVGDRVTI TCRASQDVNTAVAWYQQKPG KAPKLLIYASFLYSGVPSRFSG SRSGTDFTLTISSLQPEDFATYY CQQHYTTPPTFGQGTKVEIKRT GSTSGSGKPGSGEGSEVQLVES GGGLVQPGGSLRLSCAASGFNI KDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTA VYYCSRWGGDGFYAMDVWG QGTLVTVSSEFPKPSTPPGSSGG APGILGFVFTLKEFTLDFSTAKT YVDSLNVIRSAIGTPLQTISSGG TSLLMIDSGSGDNLFAVDVRGI DPEEGRFNRLRLIVERNNLYVT GFVNRTNNVFYRFADFSHVTFP GTTAVTLSGDSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSH SGTSLTQSVARAMLRFVTVTA EALRFRQIQRGFRTTLDLSDGR SYVMTAEDVDLTLNWGRLLSV LPDYHGQDSVRVGRISFGSINAI LGSVALILNCHHHASRVARKD EL
SEQ ID NO:6	$\alpha$ HER2scFv::SLT-1A::KDEL variant 3	MDIQMTQSPSSLSASVGDRVTI TCRASQDVNTAVAWYQQKPG KAPKLLIYASFLYSGVPSRFSG SRSGTDFTLTISSLQPEDFATYY CQQHYTTPPTFGQGTKVEIKRT GSTSGSGKPGSGEGSEVQLVES GGGLVQPGGSLRLSCAASGFNI

		KDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTA VYYCSRWGGDGFYAMDVWG QGTLVTVSSEFPKPSTPPGSSGG APKEFTLDFSTAKTYVDSLNI RSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFN NRLLIVERNNLYVTGFVNRTN NVFYRFADFSHVTFPGTTAVTL SGDSSYTTLQRVAGISRTGMQI NRHSLTTSYLDLMSHSGTSLTQ SVARAMLRFVTVTAELRFRQI QRGFRTTLDLSSGRSYVMTAE DVDLTLNWGRLLSSVLPDYHGQ DSVRVGRISFGSINAILGVALI LNCHHHASRVARKDEL
SEQ ID NO:7	$\alpha$ HER2scFv::SLT-1A::KDEL variant 4	MDIQMTQSPSSLSASVGDRTI TCRASQDVNTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSG SRSGTDFTLTISSLQPEDFATYY CQQHYTTPPTFGGQTKVEIKRT GSTSGSGKPGSGEGSEVQLVES GGGLVQPGSLRLSCAASGFNI KDTYIHWVRQAPGKGLEWVA RIYPTNGYTRYADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTA VYYCSRWGGDGFYAMDVWG QGTLVTVSSEFPKPSTPPGSSGG APGILGFVFTLKEFTLDFSTAKT YVDSLNVIRSAIGTPLQTISSGG TSLLMIDSGSGDNLFAVDVIRGI DPEEGRFNRLLIVERNNLYVT GFVNRTNNVFYRFADFSHVTFP GTTAVTLSGDSSYTTLQRVAGI SRTGMQINRHSLTTSYLDLMSH SGTSLTQSVARAMLRFVTVTA EALRFRQIQRGFRTTLDLSSGR SYVMTAEDVDLTLNWGRLLSSV LPDYHGQDSVRVGRISFGSINAI LGSVALIILNCHHHASRVARKD EL
SEQ ID NO:8	$\alpha$ CD38scFv::SLT-1A::KDEL variant 1	MDIELTQSPSSFSVSLGDRVTIT CKASEDIYNRLAWYQQKPGNA PRLISGATSLETGVPSRFSGSG SGKDYTLSITSLQTEDVATYYC QQYWSTPTFGGGTKLEIKGSTS GSGKPGSGEGSKVQLQESGPSL VQPSQRLSITCTVSGFSLISYGV HWVRQSPGKGLEWLGVIRG

		GSTDYNAAFMSRLSITKDNSKS QVFFKMNSLQADDTAIYFCAK TLITTYAMDYWGQGTTVTVS SEFPKPSTPPGSSGGAPKEFTLD FSTAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNNLRLIVER NNLYVTGFVNRTNNVFYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAELRFRQIQRGFRTT LDDLGRSYVMTAEDVDLTLN WGRLSSVLPDYHGQDSVRVGR ISFGSINAILGSVALILNCHHHA SRVARKDEL
SEQ ID NO:9	αCD38scFv::SLT-1A::KDEL variant 2	MDIELTQSPSSFSVSLGDRVTIT CKASEDIYNRLAWYQQKPGNA PRLISGATSLETGVPSRFGSGG SGKDYTLSITSLQTEDVATYYC QQYWSTPTFGGGTKLEIKGSTS GSGKPGSGEGSKVQLQESGPSL VQPSQRLSITCTVSGFSLISYGV HWVRQSPGKGLEWLGVIWRG GSTDYNAAFMSRLSITKDNSKS QVFFKMNSLQADDTAIYFCAK TLITTYAMDYWGQGTTVTVS SEFPKPSTPPGSSGGAPGILGFV FTLKEFTLDFSTAKTYVDSLNV IRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFN NRLIVERNNLYVTGFVNRTN NVFYRFADFSHVTFPGTTAVTL SGDSSYTTLQRVAGISRTGMQI NRHSLTTSYLDLMSHSGTSLTQ SVARAMLRFVTVTAELRFRQI QRGFRTTLDDLGRSYVMTAE DVDLTLNWGRLSSVLPDYHGQ DSVRVGRISFGSINAILGSVALI LNCHHHASRVARKDEL
SEQ ID NO:10	αCD38scFv::SLT-1A::KDEL variant 3	MDIELTQSPSSFSVSLGDRVTIT CKASEDIYNRLAWYQQKPGNA PRLISGATSLETGVPSRFGSGG SGKDYTLSITSLQTEDVATYYC QQYWSTPTFGGGTKLEIKGSTS GSGKPGSGEGSKVQLQESGPSL VQPSQRLSITCTVSGFSLISYGV HWVRQSPGKGLEWLGVIWRG GSTDYNAAFMSRLSITKDNSKS QVFFKMNSLQADDTAIYFCAK

		<p>TLITTYGYAMDYWGQGTTVTVS SEFPKPSTPPGSSGGAPKEFTLD FSTAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNRLRIVER NNLYVTGFVNRTNNVFYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAEALRFRQIQRGFRTT LDDLSSVLPDYHGQDSVRVGR ISFGSINAILGVALILNCHHHA SRVARKDEL</p>
SEQ ID NO:11	$\alpha$ CD38scFv::SLT-1A::KDEL variant 4	<p>MDIELTQSPSSFSVSLGDRVTIT CKASEDIYNRLAWYQQKPGNA PRLISGATSLETGVPSRFSGSG SGKDYTLSTSLQTEDVATYYC QQYWSTPTFGGGTKLEIKGSTS GSGKPGSGEGSKVQLQESGPSL VQPSQRLSITCTVSGFSLISYGV HWVRQSPGKGLEWLGVIWRG GSTDYNAAFMSRLSITKDNSKS QVFFKMNSLQADDTAIYFCAK TLITTYGYAMDYWGQGTTVTVS SEFPKPSTPPGSSGGAPGILGFV FTLKEFTLDFSTAKTYVDSLNV IRSAIGTPLQTISSGGTSLLMIDS GSGDNLFAVDVRGIDPEEGRFN NRLRIVERNNLYVTGFVNRTN NVFYRFADFSHVTFPGTTAVTL SGDSSYTTLQRVAGISRTGMQI NRHSLTTSYLDLMSHSGTSLTQ SVARAMLRFVTVTAEALRFRQI QRGFRTTLDDLSSVLPDYHGQ DSVRVGRISFGSINAILGVALI LNCHHHASRVARKDEL</p>
SEQ ID NO:12	$\alpha$ CD19scFv::SLT-1A::KDEL variant 1	<p>MDIVMTQAAPSIPVTPGESVSIS CRSSKSLNSNGNTYLYWFLQ RPGQSPQLLIYRMSNLAGVDP RFGSGSGTAFTLRISRVEAED VGVYYCMQHLEYPFTFGAGTK LELKGSTSGSGKPGSGEGSEVQ LQQSGPELIKPGASVKMSCKAS GYTFTSYVMHWVKQKPGQGL EWIGYINPYNDGTYNEKFKG KATLTSKSSSTAYMELSSLTS EDSAVYYCARGTYYYGSRVFD YWGQGTTLTVSSAEFPKPSTPP</p>

		GSSGGAPKEFTLDFSTAKTYVD SLNVIRSAIGTPLQTISSGGTSLL MIDSGSGDNLFAVDVRGIDPEE GRFNRLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTT AVTLSGDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFQIQRGFRITLDDLSGRSY VMTAEDVDLTLNWGRLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNCHHHASRVARKDE L
SEQ ID NO:13	$\alpha$ CD19scFv::SLT-1A::KDEL variant 2	MDIVMTQAAPSIPVTPGESVSIS CRSSKSLNSNGNTYLYWFLQ RPGQSPQLLIYRMSNLASGVPD RFSGSGSGTAFTLRISRVEAED VGVYYCMQHLEYPFTFGAGTK LELKGSTSGSGKPGSGEGSEVQ LQQSGPELIKPGASVKMSCKAS GYTFTSYVMHWVKQKPGQGL EWIGYINPYNDGTKYNEKFKG KATLTSDKSSSTAYMELSSLTS EDSAVYYCARGTYYYGSRVFD YWGQGTTLTVSSAEFPKPSTPP GSSGGAPGILGFVFTLKEFTLDF STAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNRLRLIVER NNLYVTGFVNRTNNVFYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAEALRFQIQRGFRIT LDDLSGRSYVMTAEDVDLTLN WGRLSSVLPDYHGQDSVRVGR ISFGSINAILGSVALILNCHHHA SRVARKDEL
SEQ ID NO:14	$\alpha$ CD19scFv::SLT-1A::KDEL variant 3	MDIVMTQAAPSIPVTPGESVSIS CRSSKSLNSNGNTYLYWFLQ RPGQSPQLLIYRMSNLASGVPD RFSGSGSGTAFTLRISRVEAED VGVYYCMQHLEYPFTFGAGTK LELKGSTSGSGKPGSGEGSEVQ LQQSGPELIKPGASVKMSCKAS GYTFTSYVMHWVKQKPGQGL EWIGYINPYNDGTKYNEKFKG KATLTSDKSSSTAYMELSSLTS EDSAVYYCARGTYYYGSRVFD YWGQGTTLTVSSAEFPKPSTPP

		GSSGGAPKEFTLDFSTAKTYVD SLNVIRSAIGTPLQTISSGGTSLL MIDSGSGDNLFAVDVRGIDPEE GRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTT AVTLSGDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFQIQRGFRTTLDLSDGRSY VMTAEDVDLTLNWGRLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNCHHHASRVARKDE L
SEQ ID NO:15	$\alpha$ CD19scFv::SLT-1A::KDEL variant 4	MDIVMTQAAPSIPVTPGESVVIS CRSSKSLNSNGNTYLYWFLQ RPGQSPQLLIYRMSNLAGVDP RFSGSGSGTAFTLRISRVEAED VGVYYCMQHLEYPFTFGAGTK LELKGSTSGSGKPGSGEGSEVQ LQQSGPELIKPGASVKMCKAS GYTFTSYVMHWVKQKPGQGL EWIGYINPYNDGTYNEKFKG KATLTSKSSSTAYMELSSLTS EDSAVYYCARGTYYYGSRVFD YWGQGTTLTVSSAEFPKPSTPP GSSGGAPGILGFVFTLKEFTLDF STAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNNLRLIVER NNLYVTGFVNRTNNVFYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAEALRFQIQRGFRTT LDDLSGRSYVMTAEDVDLTLN WGRLSSVLPDYHGQDSVRVGR ISFGSINAILGSVALILNCHHHA SRVARKDEL
SEQ ID NO:16	$\alpha$ CD74scFv::SLT-1A::KDEL variant 1	MDIQLTQSPSLPVTLGQPASIS CRSSQSLVHRNGNTYLHWFQQ RPGQSPRLLIYTVSNRFSGVDP RFSGSGSGTDFTLKISRVEAED VGVYFCSQSSHVPPTFGAGTRL EIKGSTSGSGKPGSGEGSTKGQ VQLQQSGSELKKPGASVKVSC KASGYTFTNYGVNWIKQAPGQ GLQWMGWINPNTGEPTFDDDF KGRFAFSLDTSVSTAYLQISSL KADDTAVYFCRSRSGKNEAWF AYWGQGTTLTVSSEFPKPSTPP

		GSSGGAPKEFTLDFSTAKTYVD SLNVIRSAIGTPLQTISSGGTSL MIDSGSGDNLFAVDVRGIDPEE GRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTT AVTLSGDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFQIQRGFRITLDDLSGRSY VMTAEDVDLTLNWGRLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNCHHHASRVARKDE L
SEQ ID NO:17	$\alpha$ CD74scFv::SLT-1A::KDEL variant 2	MDIQLTQSPLSLPVTLGQPASIS CRSSQSLVHRNGNTYLHWFQQ RPGQSPRLLIYTVSNRFSGVPD RFSGSGSGTDFTLKISRVEAED VGVYFCSQSSHVPPTFGAGTRL EIKGSTSGSGKPGSGEGSTKGQ VQLQQSGSELKKPGASVKVSC KASGYTFTNYGVNWIKQAPGQ GLQWMGWINPNTGEPTFDDDF KGRFAFSLDTSVSTAYLQISSL KADDTAVYFCRSRSGKNEAWF AYWGQGLVTVSSEFPKPSTPP GSSGGAPGILGFVFTLKEFTLDF STAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNNLRLIVER NNLYVTGFVNRTNNVFYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAEALRFQIQRGFRIT LDDLSGRSYVMTAEDVDLTLN WGRLSSVLPDYHGQDSVRVGR ISFGSINAILGSVALILNCHHHA SRVARKDEL
SEQ ID NO:18	$\alpha$ CD74scFv::SLT-1A::KDEL variant 3	MDIQLTQSPLSLPVTLGQPASIS CRSSQSLVHRNGNTYLHWFQQ RPGQSPRLLIYTVSNRFSGVPD RFSGSGSGTDFTLKISRVEAED VGVYFCSQSSHVPPTFGAGTRL EIKGSTSGSGKPGSGEGSTKGQ VQLQQSGSELKKPGASVKVSC KASGYTFTNYGVNWIKQAPGQ GLQWMGWINPNTGEPTFDDDF KGRFAFSLDTSVSTAYLQISSL KADDTAVYFCRSRSGKNEAWF AYWGQGLVTVSSEFPKPSTPP

		GSSGGAPKEFTLDFSTAKTYVD SLNVIRSAIGTPLQTISSGGTSLL MIDSGSGDNLFAVDVRGIDPEE GRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTT AVTLSGDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFQIQRGFRITLDDLSGRSY VMTAEDVDLTLNWGRLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNCHHHASRVARKDE L
SEQ ID NO:19	$\alpha$ CD74scFv::SLT-1A::KDEL variant 4	MDIQLTQSPLSLPVTLGQPASIS CRSSQSLVHRNGNTYLHWFQQ RPGQSPRLLIYTVSNRFSGVPD RFSGSGSGTDFTLKISRVEAED VGVYFCSQSSHVPPTFGAGTRL EIKGSTSGSGKPGSGEGSTKGG VQLQQSGSELKKPGASVKVSC KASGYTFTNYGVNWIQAPGQ GLQWMGWINPNTGEPTFDDDF KGRFAFSLDTSVSTAYLQISSL KADDTAVYFCRSRSGKNEAWF AYWGQGLVTVSSEFPKPSTPP GSSGGAPGILGFVFTLKEFTLDF STAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNNLRLIVER NNLYVTGFVNRTNNVFYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAEALRFQIQRGFRIT LDDLSGRSYVMTAEDVDLTLN WGRLSSVLPDYHGQDSVRVGR ISFGSINAILGSVALILNCHHA SRVARKDEL
SEQ ID NO:20	$\alpha$ HER2-V <sub>H</sub> H::SLT-1A::KDEL variant 1	MEVQLVESGGGLVQAGGSLRL SCAASGITFSINTMGWYRQAPG KQRELVALISSIGDTYYADSVK GRFTISRDNKNTVYLQMNLSL KPEDTAVYYCKRFRTAAQGTD YWGQGTQVTVSSAHHSEDPSS KAPKAPKEFTLDFSTAKTYVDS LNVIRSAIGTPLQTISSGGTSLL MIDSGSGDNLFAVDVRGIDPEE GRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTT AVTLSGDSSYTTLQRVAGISRT

		GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFRIQIRGFRTTLDLSDGRSY VMTAEDVDLTLNWGRLLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNCHHHASRVARKDE L
SEQ ID NO:21	$\alpha$ HER2-V <sub>H</sub> H::SLT-1A::KDEL variant 2	MEVQLVESGGGLVQAGGSLRL SCAASGITFSINTMGWYRQAPG KQRELVALISSIGDTYYADSVK GRFTISRDNKNTVYVYLMNSL KPEDTAVYYCKRFRTAAQGTD YWGQGTQVTVSSEFPKSTPPG SSGAPKEFTLDFSTAKTYVDS LNVIRSAIGTPLQTISSGGTSL MIDSGSGDNLFAVDVRGIDPEE GRFNNLRLIVERNNLYVTGFV NRTNNVFYRFADFSHVTFPGTT AVTLSDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFRIQIRGFRTTLDLSDGRSY VMTAEDVDLTLNWGRLLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNCHHHASRVARKDE L
SEQ ID NO:22	$\alpha$ HER2-V <sub>H</sub> H::SLT-1A::KDEL variant 3	MEVQLVESGGGLVQAGGSLRL SCAASGITFSINTMGWYRQAPG KQRELVALISSIGDTYYADSVK GRFTISRDNKNTVYVYLMNSL KPEDTAVYYCKRFRTAAQGTD YWGQGTQVTVSSAHHSEDPSS KAPKAPGILGFVFTLKEFTLDFS TAKTYVDSLNVIRSAIGTPLQTI SSGGTSLLMIDSGSGDNLFAVD VRGIDPEEGRFNNLRLIVERNN LYVTGFVNRTNNVFYRFADFS HVTFPGTTAVTLSDSSYTTLQ RVAGISRTGMQINRHSLTTSYL DLMSHSGTSLTQSVARAMLRF VTVTAEALRFRIQIRGFRTTLD DLSGRSYVMTAEDVDLTLNW GRLLSSVLPDYHGQDSVRVGRIS FGSINAILGSVALILNCHHHASR VARKDEL
SEQ ID NO:23	$\alpha$ HER2-V <sub>H</sub> H::SLT-1A::KDEL variant 4	MEVQLVESGGGLVQAGGSLRL SCAASGITFSINTMGWYRQAPG KQRELVALISSIGDTYYADSVK GRFTISRDNKNTVYVYLMNSL KPEDTAVYYCKRFRTAAQGTD

		<p>YWGQGTQVTVSSEFPKPSTPPG  SSGGAPGILGFVFTLKEFTLDFS  TAKTYVDSLNVIRSAIGTPLQTI  SSGGTSLLMIDSGSGDNLFAVD  VRGIDPEEGRFNNLRLIVERNN  LYVTGFVNRNTNNVFYRFADFS  HVTFFPGTTAVTLSGDSSYTTLQ  RVAGISRTGMQINRHSLTTSYL  DLMSHSGTSLTQSVARAMLRF  VTVTAEALRFRQIQRGFRTTLD  DLSGRSYVMTAEDVDLTLNW  GRLSSVLPDYHGQDSVRVGRIS  FGSINAILGSVALILNCHHHASR  VARKDEL</p>
SEQ ID NO:24	<p><math>\alpha</math>HER2-  V<sub>H</sub>H::StxA::KDEL  variant 1</p>	<p>MEVQLVESGGGLVQAGGSLRL  SCAASGITFSINTMGWYRQAPG  KQRELVALISSIGDTYYADSVK  GRFTISRDNKNTVYLMNSL  KPEDTAVYYCKRFRTAAQGTD  YWGQGTQVTVSSAHHSEDPSS  KAPKAPKEFTLDFSTAKTYVDS  LNVIRSAIGTPLQTISSGGTSLL  MIDSGTGDNLFAVDVRGIDPEE  GRFNNLRLIVERNNLYVTGFV  NRTNNVFYRFADFSHVTFFPGTT  AVTLSGDSSYTTLQRVAGISRT  GMQINRHSLTTSYLDLMSHSG  TSLTQSVARAMLRFVTVTAEA  LFRQIQRGFRTTLDLSDGRSY  VMTAEDVDLTLNWGRLSSVLP  DYHGQDSVRVGRISFGSINAIL  GSVALILNSHHHASRVARKE  L</p>
SEQ ID NO:25	<p><math>\alpha</math>HER2-  V<sub>H</sub>H::StxA::KDEL  variant 2</p>	<p>MEVQLVESGGGLVQAGGSLRL  SCAASGITFSINTMGWYRQAPG  KQRELVALISSIGDTYYADSVK  GRFTISRDNKNTVYLMNSL  KPEDTAVYYCKRFRTAAQGTD  YWGQGTQVTVSSEFPKPSTPPG  SSGGAPKEFTLDFSTAKTYVDS  LNVIRSAIGTPLQTISSGGTSLL  MIDSGTGDNLFAVDVRGIDPEE  GRFNNLRLIVERNNLYVTGFV  NRTNNVFYRFADFSHVTFFPGTT  AVTLSGDSSYTTLQRVAGISRT  GMQINRHSLTTSYLDLMSHSG  TSLTQSVARAMLRFVTVTAEA  LFRQIQRGFRTTLDLSDGRSY  VMTAEDVDLTLNWGRLSSVLP  DYHGQDSVRVGRISFGSINAIL</p>



		AVTLSGDSSYTTLQRVAGISRT GMQINRHSLTTSYLDLMSHSG TSLTQSVARAMLRFVTVTAEA LRFQIQRGFRTTLLDLSGRSY VMTAEDVDLTLNWGRLSSVLP DYHGQDSVRVGRISFGSINAIL GSVALILNSHHASRVARKDE L
SEQ ID NO:29	StxA::αCD20- FN3::KDEL	MKEFTLDFSTAKTYVDSLNVIR SAIGTPLQTISSGGTSLLMIDSG TGDNLFAVDVRGIDPEEGRFN NRLRIVERNNLYVTGFVNRTN NVFYRFADFSHVTFPGTTAVTL SGDSSYTTLQRVAGISRTGMQI NRHSLTTSYLDLMSHSGTSLTQ SVARAMLRFVTVTAEALRFQI QRGFRTTLLDLSGRSYVMTAE DVDLTLNWGRLSSVLPDYHGQ DSVRVGRISFGSINAILGSVALI LNCHHHASRVAREFPKPSTPPG SSGGAPASVSDVPRDLEVVA TPTSLISWCRQCADSYRITY GETGGNSPVQFETVPGSWKTA TISGLKPGVDYITIVYVTHYY GWDYRSHPIINRYRTGSKDEL
SEQ ID NO:30	IL-2::SLT-1A::KDEL variant 1	MAPTSSSTKKTQLQLEHLLLDL QMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEEL KPLEEVLNLAQSKNFHLRPRDL ISNINVIVLELKGSETTFMCEYA DETATIVEFLNRWITFCQSIISTL TEFPKPSTPPGSSGGAPKEFTLD FSTAKTYVDSLNVIRSAIGTPLQ TISSGGTSLLMIDSGSGDNLFA VDVRGIDPEEGRFNRLRIVER NNLYVTGFVNRTNNVYRFAD FSHVTFPGTTAVTLSGDSSYTT LQRVAGISRTGMQINRHSLTTS YLDLMSHSGTSLTQSVARAML RFVTVTAEALRFQIQRGFRTT LDDLSGRSYVMTAEDVDLTLN WGRLSSVLPDYHGQDSVRVGR ISFGSINAILGSVALILNCHHHA SRVARKDEL
SEQ ID NO:31	IL-2::SLT-1A::KDEL variant 2	MAPTSSSTKKTQLQLEHLLLDLQ MILNGINNYKNPKLTRMLTFKFY MPKKATELKHLQCLEEELKPLEE VLNLAQSKNFHLRPRDLISNINVIV LELKGSETTFMCEYADETATIVEF LNRWITFCQSIISTLTFPKPSTPPG SSGGAPGILGFVFTLKEFTLDFSTA

		<p>KTYVDSLNVIRSAIGTPLQTISSGG  TSLLMIDSGSGDNLFAVDVRGIDP  EEGRFNNLRLIVERNNLYVTGFVN  RTNNVFYRFADFSHVTFPGTTAVT  LSGDSSYTTLQRVAGISRTGMQIN  RHSLTTSYLDLMSHSGTSLTQSVA  RAMLRFVTVTAELRFRQIQRGF  RTTLDDLSGRSYVMTAEDVDLTL  NWGRLLSSVLPDYHGQDSVRVGR  SFGSINAILGSVALILNCHHHASRV  ARKDEL</p>
SEQ ID NO:32	IL-2::StxA::KDEL variant 1	<p>MAPTSSSTKKTQLQLEHLLLDL  QMILNGINNYKNPKLTRMLTF  KFYMPKKATELKHLQCLEEEL  KPLEEVLNLAQSKNFHLRPRDL  ISNINVIVLELKGSETTFMCEYA  DETATIVEFLNRWITFCQSIISTL  TEFPKPSTPPGSSGGAPKEFTLD  FSTAKTYVDSLNVIRSAIGTPLQ  TISSGGTSLLMIDSGTGDNLFA  VDVRGIDPEEGRFNNLRLIVER  NNLYVTGFVNRTNNVFYRFAD  FSHVTFPGTTAVTLSGDSSYTT  LQRVAGISRTGMQINRHSLTTS  YLDLMSHSGTSLTQSVARAML  RFVTVTAELRFRQIQRGFRRT  LDDLSGRSYVMTAEDVDLTLN  WGRLLSSVLPDYHGQDSVRVGR  ISFGSINAILGSVALILNCHHHA  SRVARKDEL</p>
SEQ ID NO:33	IL-2::StxA::KDEL variant 2	<p>MAPTSSSTKKTQLQLEHLLLDL  QMILNGINNYKNPKLTRMLTF  KFYMPKKATELKHLQCLEEEL  KPLEEVLNLAQSKNFHLRPRDL  ISNINVIVLELKGSETTFMCEYA  DETATIVEFLNRWITFCQSIISTL  TEFPKPSTPPGSSGGAPGILGFV  FTLKEFTLDFSTAKTYVDSLNV  IRSAIGTPLQTISSGGTSLLMIDS  GTGDNLFAVDVRGIDPEEGRF  NNLRLIVERNNLYVTGFVNRT  NNVFYRFADFSHVTFPGTTAVT  LSGDSSYTTLQRVAGISRTGMQ  INRHSLTTSYLDLMSHSGTSLT  QSVARAMLRFVTVTAELRFR  QIQRGFRRTLDDLSGRSYVMT  AEDVDLTLNWGRLLSSVLPDYH  GQDSVRVGRISFGSINAILGSA  LILNSHHHASRVARKDEL</p>

The invention is claimed as follows:

1. A protein comprising:

5 a) a binding region comprising one or more polypeptides selected from: single-domain antibody fragment, single-chain variable fragment, antibody variable fragment, Fd fragment, and antigen-binding fragment (Fab), wherein the binding region specifically binds at least one extracellular target biomolecule,

10 b) a Shiga toxin effector region comprising a polypeptide exhibiting Shiga toxin A Subunit cytotoxicity, wherein the Shiga toxin effector region comprises an amino acid sequence that is at least 90% identical to amino acids 1 to 251 of SEQ ID NO:1 or SEQ ID NO:2;

15 wherein the amino acid residue corresponding to position 75 of SEQ ID NO: 1 or 2 is asparagine, the amino acid residue corresponding to position 77 of SEQ ID NO: 1 or 2 is tyrosine, the amino acid residue corresponding to position 114 of SEQ ID NO: 1 or 2 is tyrosine, the amino acid residue corresponding to position 167 of SEQ ID NO: 1 or 2 is glutamate, the amino acid residue corresponding to position 170 of SEQ ID NO: 1 or 2 is arginine, the amino acid residue corresponding to position 176 of SEQ ID NO: 1 or 2 is arginine, and the amino acid residue corresponding to position 203 of SEQ ID NO: 1 or 2 is tryptophan;

20 and

c) a carboxy-terminal endoplasmic reticulum retention/retrieval signal motif of a member of the KDEL family.

25 2. The protein of claim 1, wherein the binding region comprises a single-domain antibody fragment or a single-chain variable fragment.

30 3. The protein of claim 1 or claim 2, wherein the binding region comprises a heavy-chain antibody domain derived from a camelid, a camelid V<sub>H</sub>H, a heavy-chain antibody domain derived from a cartilaginous fish, an immunoglobulin new antigen receptor, or a V<sub>NAR</sub> fragment.

4. The protein of any one of claims 1-3, which exhibits a cytotoxicity that is at least 3-fold greater to a first population of cells whose members are physically coupled to extracellular target biomolecule bound by the binding region of the protein compared to a second population of cells whose members are not physically coupled to the extracellular target biomolecule bound by the binding region.

5. The protein of any one of claims 1 to 4, wherein the extracellular target biomolecule is selected from the group consisting of:

CD20, CD22, CD40, CD74, CD79, CD25, CD30, HER2/neu/ErbB2, EGFR, EpCAM, EphB2, prostate-specific membrane antigen, Cripto, CDCP1, endoglin/CD105, fibroblast activation protein, Lewis-Y, CD19, CD21, CS1/SLAMF7, CD33, CD52, CD133, gpA33, mucin, TAG-72, tyrosine-protein kinase transmembrane receptor, carbonic anhydrase IX, folate binding protein, ganglioside GD2, ganglioside GD3, ganglioside GM2, ganglioside Lewis-Y2, VEGFR, Alpha Vbeta3, Alpha5beta1, ErbB1/EGFR, Erb3, c-MET, IGF1R, EphA3, TRAIL-R1, TRAIL-R2, RANK, tenascin, CD64, mesothelin, BRCA1, MART-1/MelanA, gp100, tyrosinase, TRP-1, TRP-2, MAGE-1, MAGE-3, GAGE-1/2, BAGE, RAGE, NY-ESO-1, CDK-4, beta-catenin, MUM-1, caspase-8, KIAA0205, HPVE6, SART-1, PRAME, carcinoembryonic antigen, prostate specific antigen, prostate stem cell antigen, human aspartyl (asparaginy) beta-hydroxylase, EphA2, HER3/ErbB-3, MUC1, tyrosinase associated antigen, HPV-E7, Epstein-Barr virus antigen, Bcr-Abl, alpha-fetoprotein antigen, 17-A1, bladder tumor antigen, CD38, CD15, CD23, CD53, CD88, CD129, CD183, CD191, CD193, CD244, CD294, CD305, C3AR, FcεRIa, galectin-9, PD-L1, mrp-14, Siglec-8, Siglec-10, CD49d, CD13, CD44, CD54, CD63, CD69, CD123, CD193, TLR4, IgE, CD107a, CD203c, CD14, CD68, CD80, CD86, CD115, F4/80, ILT-3, galectin-3, CD11a-c, GITRL, MHC class I molecule, MHC class II molecule, CD284/TLR4, CD107/Mac3, CD195/CCR5, HLA-DR, CD16/32, CD282/TLR2, CD11c, and any immunogenic fragment of any one of the foregoing.

6. The protein of any one of claims 1 to 5, wherein the Shiga toxin effector region comprises or consists of an amino acid sequence that is at least 95% identical to amino acids 1 to 251 of SEQ ID NO:1 or SEQ ID NO:2.

5 7. The protein of claim 6, wherein the Shiga toxin effector region comprises or consists of amino acids 1 to 251 of SEQ ID NO:1 or SEQ ID NO:2.

8. The protein of claim 7, wherein the Shiga toxin effector region comprises or consists of amino acids 1 to 251 of SEQ ID NO:1.

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9. The protein of any one of claims 1 to 8, wherein the carboxy-terminal endoplasmic reticulum retention/retrieval signal motif is selected from the group consisting of:

15 KDEL (SEQ ID NO:34), HDEF (SEQ ID NO:35), HDEL (SEQ ID NO:36), RDEF (SEQ ID NO:37), RDEL (SEQ ID NO:38), WDEL (SEQ ID NO:39), YDEL (SEQ ID NO:40), HEEF (SEQ ID NO:41), HEEL (SEQ ID NO:42), KEEL (SEQ ID NO:43), REEL (SEQ ID NO:44), KAEL (SEQ ID NO:45), KCEL (SEQ ID NO:46), KFEL (SEQ ID NO:47), KGEL (SEQ ID NO:48), KHEL (SEQ ID NO:49), KLEL (SEQ ID NO:50), KNEL (SEQ ID NO:51), KQEL (SEQ ID NO:52), KREL (SEQ ID NO:53), KSEL (SEQ ID NO:54), KVEL (SEQ ID NO:55), KWEL (SEQ ID NO:56), KYEL (SEQ ID NO:57), KEDL (SEQ ID NO:58), KIEL (SEQ ID NO:59), DKEL (SEQ ID NO:60), FDEL (SEQ ID NO:61), KDEF (SEQ ID NO:62), KKEL (SEQ ID NO:63), HADL (SEQ ID NO:64), HAEL (SEQ ID NO:65), HIEL (SEQ ID NO:66), HNEL (SEQ ID NO:67), HTEL (SEQ ID NO:68), KTEL (SEQ ID NO:69), HVEL (SEQ ID NO:70), NDEL (SEQ ID NO:71), QDEL (SEQ ID NO:72), REDL (SEQ ID NO:73), RNEL (SEQ ID NO:74), RTDL (SEQ ID NO:75), RTEL (SEQ ID NO:76), SDEL (SEQ ID NO:77), TDEL (SEQ ID NO:78), SKEL (SEQ ID NO:79), STEL (SEQ ID NO:80), and EDEL (SEQ ID NO:81).

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10. The protein of any one of claims 1-9, wherein the binding region comprises or consists of amino acids 2-241 of any one of SEQ ID NOs:4-16.
- 5 11. The protein of any one of claims 1-10, wherein the protein comprises or consists of the polypeptide shown in any one of SEQ ID NOs: 4-27.
12. The protein of any one of claims 1-11, in the form of a homo-multimer or a hetero-multimer.
- 10 13. The protein of any one of claims 1-12, in the form of a pharmaceutically acceptable solvate or salt.
14. A pharmaceutical composition comprising the protein of any one of claims 1 to 13 and at least one pharmaceutically acceptable excipient or carrier.
- 15 15. The pharmaceutical composition of claim 14, wherein the pharmaceutically acceptable carrier comprises a physiologically acceptable solvent or dispersion medium.
16. The pharmaceutical composition of claim 14 or claim 15, wherein the
- 20 pharmaceutically acceptable carrier comprises water, alcohol, a polyol, vegetable oil, or an injectable organic ester.
17. The pharmaceutical composition of any one of claims 14 to 16, further comprising an adjuvant; a coating; an antimicrobial, antibacterial, or antifungal agent; an isotonic
- 25 agent; a polyalcohol; an absorption delaying agent; a stabilizer; a buffer; a surfactant; or a pharmaceutically acceptable antioxidant and combinations thereof.
18. The pharmaceutical composition of claim 17, wherein the adjuvant is a preservative, wetting agent, emulsifying agent, or dispersing agent.

30

19. The pharmaceutical composition of claim 17, wherein the coating is lecithin.
20. The pharmaceutical composition of claim 17, wherein the antibacterial or antifungal agent is a paraben, chlorobutanol, phenol, or sorbic acid.
- 5
21. The pharmaceutical composition of claim 17, wherein the isotonic agent is a sugar or sodium chloride.
22. The pharmaceutical composition of claim 17, wherein the polyalcohol is mannitol or sorbitol.
- 10
23. The pharmaceutical composition of claim 17, wherein the pharmaceutically acceptable antioxidant is a water-soluble antioxidant, an oil-soluble antioxidant, or a metal-chelating agent.
- 15
24. The pharmaceutical composition of claim 23, wherein the water-soluble antioxidant is ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, or sodium sulfite.
- 20
25. The pharmaceutical composition of claim 23, wherein the oil-soluble antioxidant is ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propylgallate, or alpha-tocopherol.
26. The pharmaceutical composition of claim 23, wherein the metal chelating agent is citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, or phosphoric acid.
- 25
27. A polynucleotide encoding the protein of any one of claims 1 to 13, or a complement thereof.
- 30
28. An expression vector comprising the polynucleotide of claim 27.

29. A host cell comprising the polynucleotide of claim 27 or the expression vector of claim 28.

5 30. Use of a therapeutically effective amount of the protein of any one of claims 1 to 13 or the pharmaceutical composition of any one of claims 14 to 26 for treating a disease, disorder, or condition in a patient in need thereof, wherein the disease, disorder, or condition involves cells that express the extracellular target biomolecule bound by the binding region of the protein.

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31. The use of claim 30, wherein the disease, disorder, or condition is selected from the group consisting of: cancer, tumor, immune disorder, and microbial infection.

32. The use of claim 31, wherein the cancer is selected from the group consisting of:

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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, or

wherein the immune disorder is associated with a disease, and the disease is selected

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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.

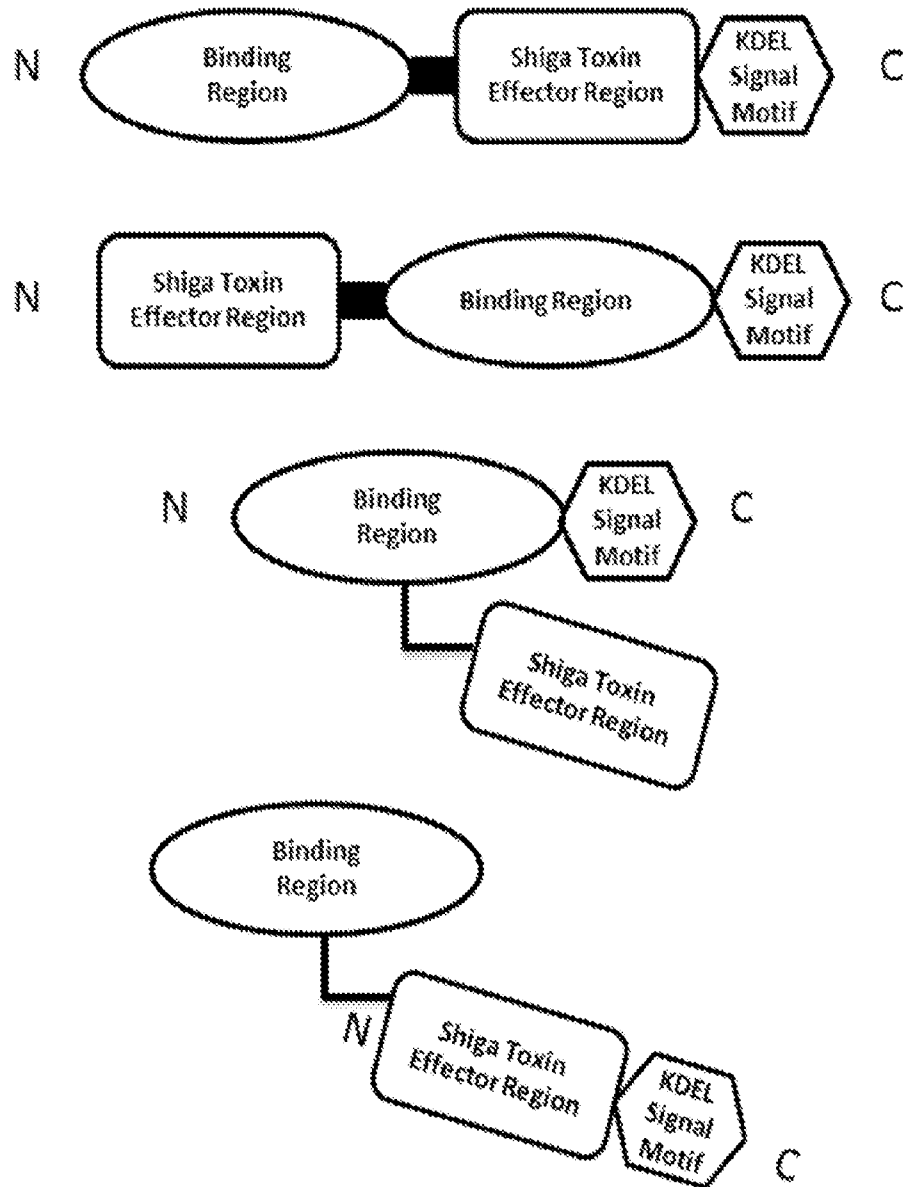
25

33. A composition of matter of any one of claims 1 to 29 for the treatment or prevention of cancer, a tumor, an immune disorder, or a microbial infection.

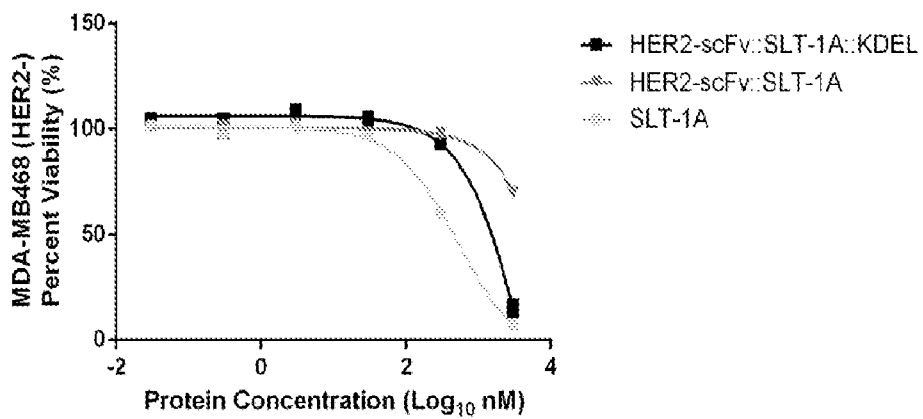
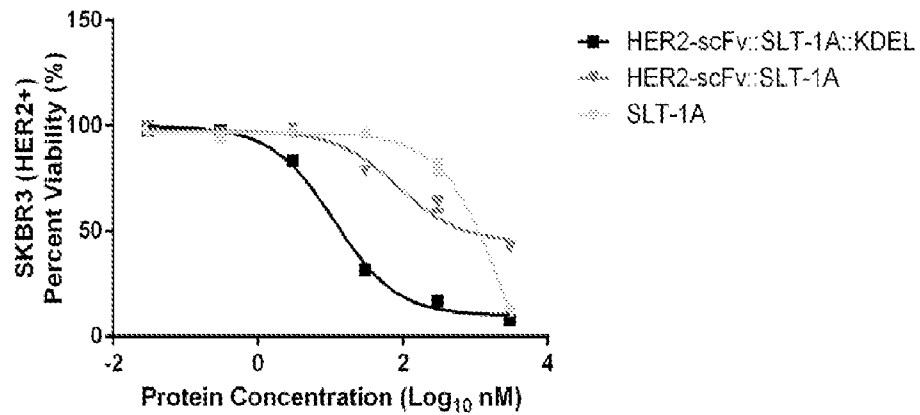
34. Use of a composition of matter of any one of claims 1 to 29 in the manufacture of a medicament for the treatment or prevention of a cancer, tumor, immune disorder, or microbial infection.

5

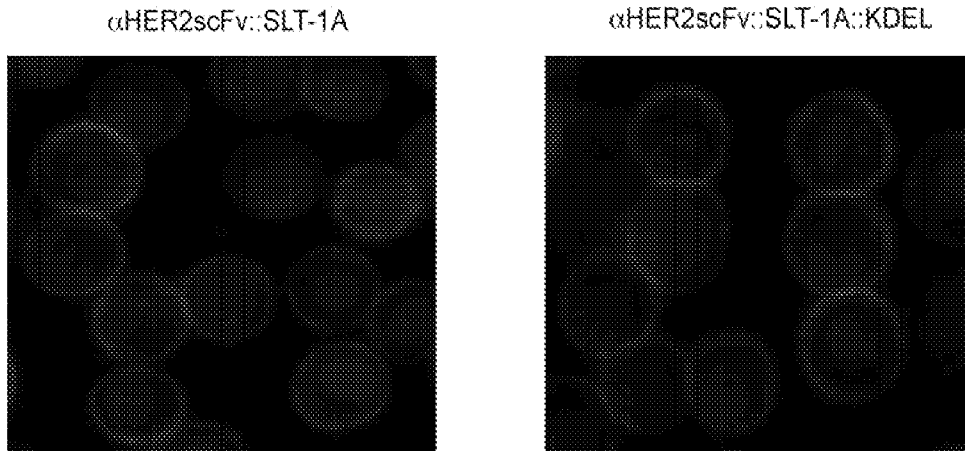
Figure 1. Schematic Drawing of the General Architecture of Exemplary Cytotoxic Proteins



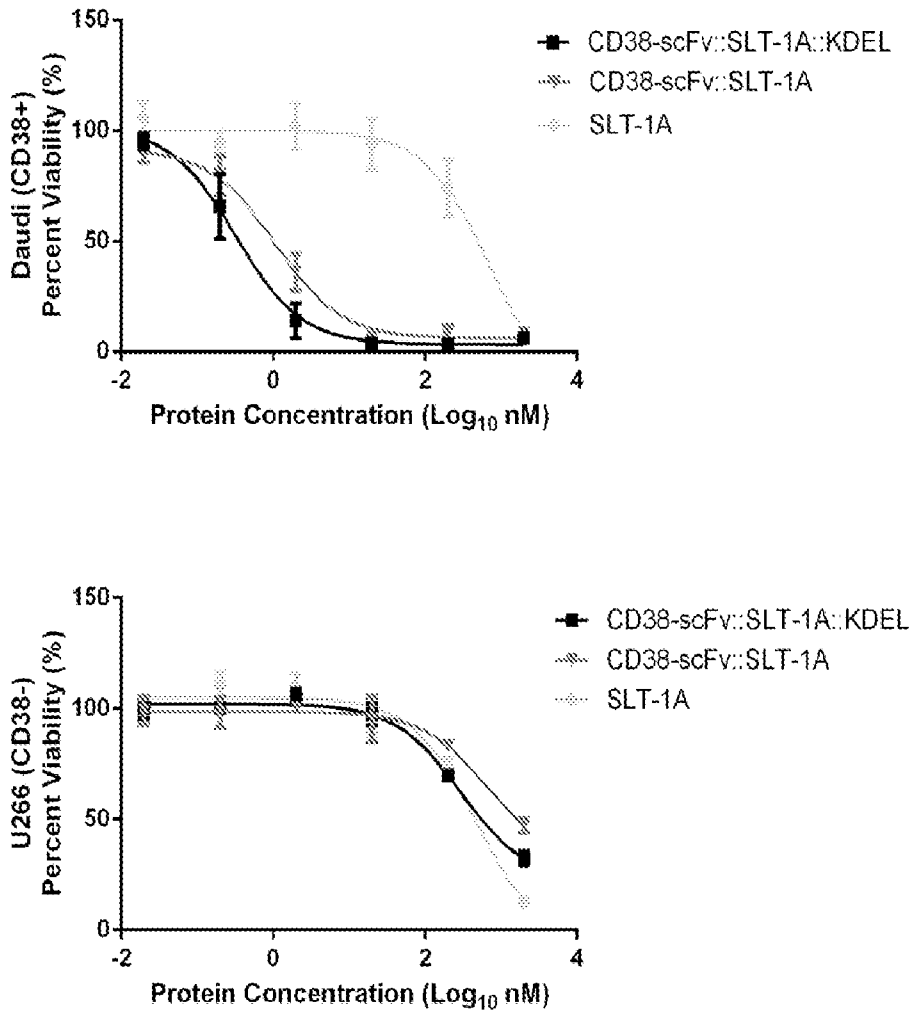
**Figure 2. Addition of KDEL Improved Targeted Cytotoxicity:**  
 **$\alpha$ HER2scFv::SLT-1A::KDEL showed improved cytotoxicity as compared to its ancestor which lacked a KDEL signal motif**



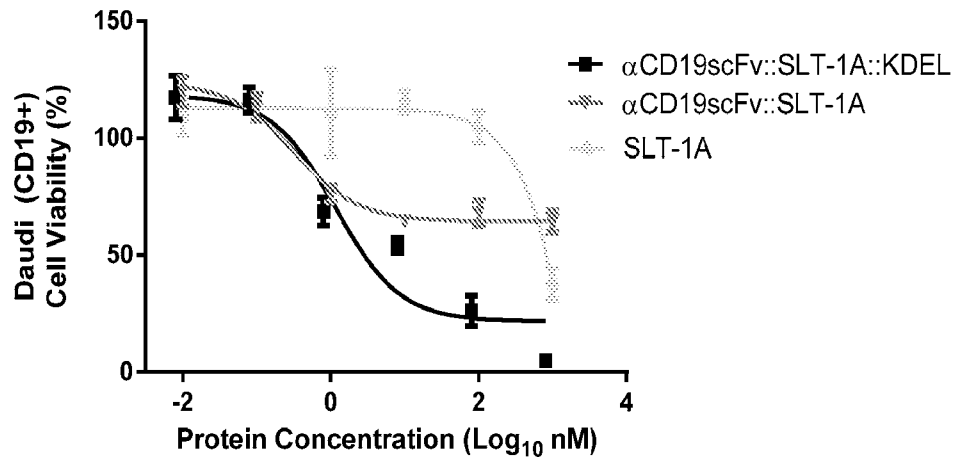
**Figure 3. Cell Entry: Similar cellular internalization of  $\alpha$ HER2scFv::SLT-1A and the carboxy-KDEL bearing variant  $\alpha$ HER2scFv::SLT-1A::KDEL**



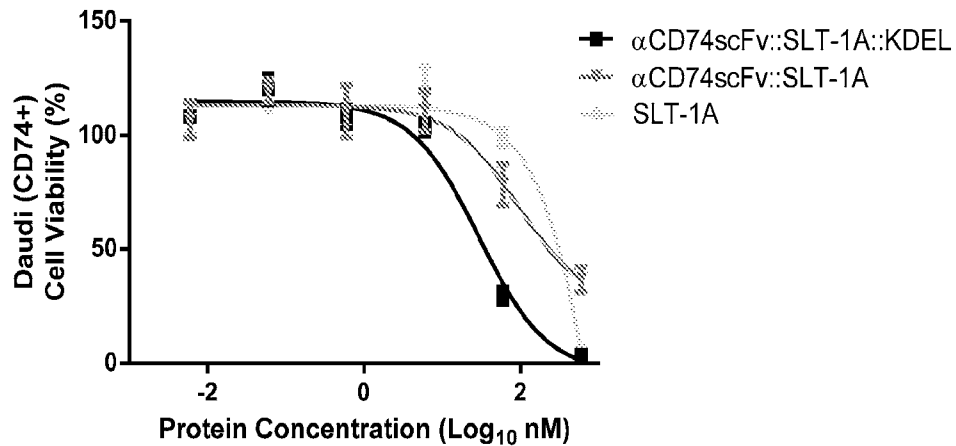
**Figure 4. Addition of KDEL Improved Targeted Cytotoxicity:**  
 **$\alpha$ CD38scFv::SLT-1A::KDEL showed improved cytotoxicity compared to its**  
**ancestor which lacked a KDEL signal motif**



**Figure 5. Addition of KDEL Improved Targeted Cytotoxicity:**  
 $\alpha$ CD19scFv::SLT-1A::KDEL showed improved cytotoxicity compared to its ancestor which lacked a KDEL signal motif



**Figure 6. Addition of KDEL Improved Targeted Cytotoxicity:**  
 $\alpha$ CD74scFv::SLT-1A::KDEL showed improved cytotoxicity compared to its ancestor which lacked a KDEL signal motif



**Schematic Drawing of the General Architecture of Exemplary Cytotoxic Proteins**

