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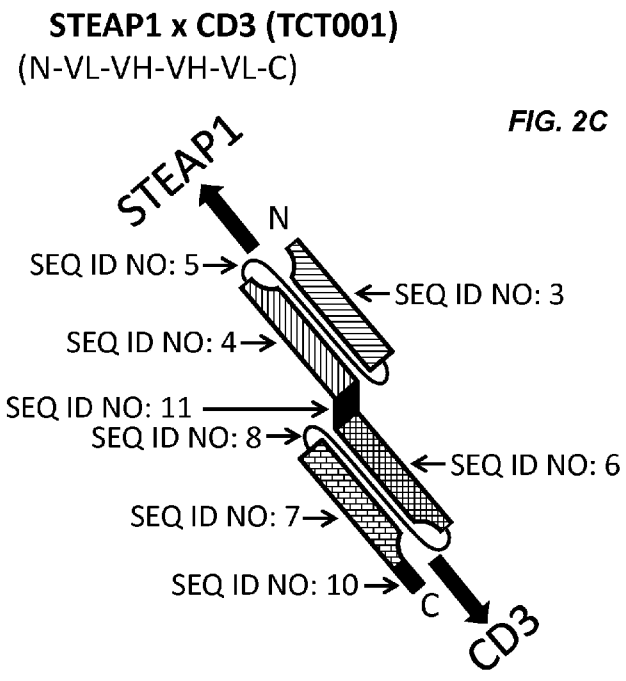



*FIG. 2C*

(57) Abstract: Compositions, methods, and kits are provided for treating or preventing prostate cancer. In certain aspects, the compositions, methods, and kits are designed to kill prostate cancer cells. The compositions, methods, and kits can be useful to prevent or treat metastatic prostate cancer. The present invention provides a bispecific binding reagent comprising at least a first domain and a second domain, wherein the first domain specifically binds to a prostate cancer cell surface antigen, and wherein the second domain specifically binds to a T cell surface antigen. The bispecific binding reagent can be used to stimulate immune cell activity against the prostate cancer cell. In certain aspects, the present invention provides a plurality of bispecific binding reagents for co-stimulation of immune cells. Methods for preventing and/or treating metastatic prostate cancer in a subject in need thereof using one or more bispecific binding reagents of the present invention are also provided. In other aspects, the present invention provides isolated nucleic acids, vectors, processes of production, pharmaceutical compositions, and kits related to the bispecific binding reagents described herein.

# COMPOSITIONS AND METHODS FOR PREVENTING AND TREATING PROSTATE CANCER

## CROSS-REFERENCES TO RELATED APPLICATIONS

**[0001]** This application claims priority to U.S. Provisional Application No. 61/809,205, filed April 5, 2013, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

## BACKGROUND OF THE INVENTION

**[0002]** With an estimated lifetime risk of 15.33%, approximately 1 in 7 men will be diagnosed with prostate cancer (PCa) (American Cancer Society, 2014). Fortunately, PCa progression is usually slow and the prognosis generally favorable when a suitable treatment is initiated early and the tumor is confined to prostate-tissue. On the other hand, for patients who are not cured after an effective primary intervention such as radical prostatectomy, androgen-deprivation therapy (ADT) may control the tumor burden for years, but frequently, castrate-resistant and eventually metastatic prostate cancer emerge. Prostate cancer claims tens of thousands of lives in the US each year.

**[0003]** For patients with castrate-resistant prostate cancer with detectable metastatic disease, standard of care treatment (chemotherapy with docetaxel and prednisone in combination) prolongs survival but is not curative (Adamo et al., *Front Endocrinol (Lausanne)*, 3:Article 73, 2012; Saad and Hotte, *Can Urol Assoc J*, 4(6):380-384, 2010). Underlying reasons may be related to the mechanism of action of docetaxel, a microtubule-stabilizer, which preferentially kills rapidly proliferating cells but likely spares slow cycling cancer cells. Among the latter cells may be cancer stem cells, which are proposed to give rise to the majority of the malignant cells within a tumor and appear to be particularly resistant to therapy (Kong et al., *Cancers (Basel)*, 3(1):716-729, 2011; Lang et al., *J Pathol*, 217(2):299-306, 2009). Thus, there is a need in the art for improved treatment and prevention strategies for prostate cancer. The present invention addresses this need and others.

## BRIEF SUMMARY OF THE INVENTION

**[0004]** In a first aspect, the present invention provides a bispecific binding reagent that simultaneously binds to an immune cell and a prostate cancer cell, wherein the bispecific

binding reagent comprises a polypeptide molecule having a first domain that binds to an antigen expressed by the immune cell and a second domain that binds to an antigen expressed by the prostate cancer cell. In some embodiments of the first aspect, the immune cell is selected from the group consisting of a T cell, a Natural Killer (NK) cell, and a Natural Killer T (NKT) cell. In some embodiments of the first aspect, the prostate cancer cell is selected from the group consisting of a metastatic prostate cancer cell and a castration-resistant prostate cancer cell. In some embodiments of the first aspect, the second domain binds to an antigen expressed on the surface of the prostate cancer cell.

**[0005]** In some cases, the first domain is derived from an antibody. For example, in some cases, the first domain comprises a single-chain variable fragment (scFv). In some cases, the second domain is derived from an antibody. For example, in some cases, the second domain comprises an scFv. In a further example of any of the foregoing aspects, embodiments, cases, or examples, the first domain comprises a first scFv and the second domain comprises a second scFv. In some cases, the bispecific binding reagent comprises a tandem single-chain variable fragment antibody molecule (taFv) comprising a first scFv and a second scFv.

**[0006]** In some embodiments of the first aspect, the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of ABCC4, ANO7, EPCAM, FOLH1, OR51E1, OR51E2, PSCA, SLC30A4, SLC45A3, SPON2, STEAP1, STEAP2, TARP, TMEFF2, TMPR8, TMPRSS2, and a gene encoding an NKG2D ligand. In some embodiments, the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of ABCC4, ANO7, EPCAM, FOLH1, OR51E1, OR51E2, PSCA, SLC45A3, SPON2, STEAP1, STEAP2, TARP, TMEFF2, TMPR8, and a gene encoding an NKG2D ligand. In some embodiments, the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of EPCAM, FOLH1, SPON2, STEAP1, and a gene encoding an NKG2D ligand. In some embodiments, the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of EPCAM, STEAP1, and a gene encoding an NKG2D ligand. In some embodiments, the antigen expressed by the prostate cancer cell is encoded by a gene encoding an NKG2D ligand. In some embodiments, the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of EPCAM and STEAP1. In some cases, the gene encoding the NKG2D ligand is selected from the group consisting of MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, and ULBP3.

**[0007]** In some embodiments of the first aspect, the antigen expressed by the immune cell is encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), CD28, CD2, CD27, IL7R, CD69, ICOS, IL2RB, PTPRC (CD45), CD48, SELL, CD137 (4-1BB, TNFRSF9), and CD134 (OX40, TNFRSF4). In some cases, the antigen expressed by the immune cell is encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), CD28, CD137 (4-1BB, TNFRSF9), and CD134 (OX40, TNFRSF4). In some cases, the antigen expressed by the immune cell is encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), and CD28.

**[0008]** In some embodiments of the first aspect, the first domain of the bispecific binding reagent specifically binds to the antigen CD3. In some cases, the first domain of the bispecific binding reagent comprises an anti-CD3 scFv. For example, in some cases, the anti-CD3 scFv comprises:

an anti-CD3 $V_H$ amino acid sequence of SEQ ID NO:6 and an anti-CD3 $V_L$ amino acid sequence of SEQ ID NO:7; or

an anti-CD3 $V_H$ amino acid sequence of SEQ ID NO:18 and an anti-CD3 $V_L$ amino acid sequence of SEQ ID NO:19.

**[0009]** In some embodiments of the first aspect, the second domain of the bispecific binding reagent specifically binds to the antigen STEAP1. In some cases, the second domain of the bispecific binding reagent comprises an anti-STEAP1 scFv. For example, in some cases, the anti-STEAP1 scFv comprises:

an anti-STEAP1 $V_L$ amino acid sequence of SEQ ID NO:3 and an anti-STEAP1 $V_H$ amino acid sequence of SEQ ID NO:4; or

an anti-STEAP1 $V_L$ amino acid sequence of SEQ ID NO:32 and an anti-STEAP1 $V_H$ amino acid sequence of SEQ ID NO:33.

**[0010]** In some embodiments of the first aspect, the bispecific binding reagent binds to both CD3 and STEAP1. In some embodiments, the bispecific binding reagent is an anti-CD3/anti-STEAP1 taFv comprising an anti-CD3 first scFv and an anti-STEAP1 second scFv. In some cases, the anti-CD3/anti-STEAP1 taFv comprises an inter-scFv linker with an amino acid sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NOS:63 to 76. In some cases, the anti-CD3/anti-STEAP1 taFv comprises the amino acid sequence of SEQ

ID NO:1. In some cases, the anti-CD3/anti-STEAP1 taFv comprises an amino acid sequence encoded by SEQ ID NO:2.

**[0011]** In some embodiments of the first aspect, the first domain of the bispecific binding reagent specifically binds to the antigen CD28. In some cases, the first domain of the bispecific binding reagent comprises an anti-CD28 scFv. In some cases, the anti-CD28 scFv comprises:

an anti-CD28 $V_H$ amino acid sequence of SEQ ID NO: 15 and an anti-CD28 $V_L$ amino acid sequence of SEQ ID NO: 14; or

an anti-CD28 $V_H$ amino acid sequence of SEQ ID NO: 27 and an anti-CD28 $V_L$ amino acid sequence of SEQ ID NO: 26.

**[0012]** In some embodiments of the first aspect, the second domain of the bispecific binding reagent specifically binds to the antigen EpCAM. In some cases, the second domain of the bispecific binding reagent comprises an anti-EpCAM scFv. In some cases, the anti-EpCAM scFv comprises:

an anti-EpCAM $V_H$ amino acid sequence of SEQ ID NO:16 and an anti-EpCAM $V_L$ amino acid sequence of SEQ ID NO:17; or

an anti-EpCAM $V_H$ amino acid sequence of SEQ ID NO:39 and an anti-EpCAM $V_L$ amino acid sequence of SEQ ID NO:38.

**[0013]** In some embodiments of the first aspect, the bispecific binding reagent binds to both CD28 and EpCAM. In some embodiments, the bispecific binding reagent is an anti-CD28/anti-EpCAM taFv comprising an anti-CD28 first scFv and an anti-EpCAM second scFv. In some cases, the anti-CD28/anti-EpCAM taFv comprises an inter-scFv linker with an amino acid sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NOS:63 to 76. In some cases, the anti-CD28/anti-EpCAM taFv comprises the amino acid sequence of SEQ ID NO:12. In some cases, the anti-CD28/anti-EpCAM taFv comprises an amino acid sequence encoded by SEQ ID NO:13.

**[0014]** In some embodiments of the first aspect, the second domain is derived from a ligand for a receptor, wherein the ligand is expressed on the surface of the prostate cancer cell. In some embodiments, the second domain is derived from a receptor that interacts with a ligand that is expressed on the surface of the prostate cancer cell. In some cases, the ligand for the receptor that is expressed on the surface of the prostate cancer cell is an NKG2D ligand. For example, in some cases, the NKG2D ligand is selected from the group consisting of MICA,

MICB, ULBP1, ULBP2, ULBP3, RAET1E, RAET1G, and RAET1L. In some cases, the receptor that interacts with a ligand that is expressed on the surface of the prostate cancer cell is NKG2D. In some cases, the second domain comprises the extracellular domain of NKG2D or a portion thereof, wherein the portion of the extracellular domain of NKG2D specifically binds to an NKG2D ligand. In some cases, the NKG2D ligand is selected from the group consisting of MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, and ULBP3. In some cases, the extracellular domain of NKG2D or a portion thereof comprises the amino acid sequence of SEQ ID NO:60. In some cases, the extracellular domain of the NKG2D receptor is linked to an scFv with a linker selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:6. The bispecific binding reagent can comprise the amino acid sequence of SEQ ID NO:61, or SEQ ID NO:62.

**[0015]** In some embodiments of the first aspect, the first domain and/or the second domain comprises one or more disulfide bridges.

**[0016]** In a second aspect, the present invention provides, an isolated nucleic acid encoding any one of the foregoing bispecific binding reagents. In a third aspect, the present invention provides an isolated vector comprising the foregoing nucleic acid. In some embodiments of the third aspect, the nucleic acid is operably linked to a promoter. In a fourth aspect, the present invention provides a host cell comprising any one of the foregoing nucleic acids or vectors.

**[0017]** In a fifth aspect, the present invention provides a process for the production of a bispecific binding reagent comprising:

culturing the isolated host cell of claim 58 under conditions suitable for the expression of the bispecific binding reagent; and

recovering the produced bispecific binding reagent from the cell or from the culture.

**[0018]** In a sixth aspect, the present invention provides a composition comprising a plurality of structurally different bispecific binding reagents. In some embodiments of the sixth aspect, the composition comprises:

a first bispecific binding reagent that binds to a first antigen expressed by an immune cell and a first antigen expressed by a prostate cancer cell, wherein the first antigen expressed by the immune cell is CD3; and

a second bispecific binding reagent that binds to a second antigen expressed by the immune cell and an antigen expressed by the prostate cancer cell.

**[0019]** In some embodiments of the sixth aspect, the first bispecific binding reagent binds CD3 and the second bispecific binding reagent binds CD28. In some cases, the first bispecific binding reagent and the second bispecific binding reagent bind the same antigen expressed by the prostate cancer cell. In some cases, the first bispecific binding reagent and the second bispecific binding reagent bind different antigens expressed by the prostate cancer cell. In some cases, the first bispecific binding reagent binds CD3 and STEAP1 and the second bispecific binding reagent binds CD28 and EpCAM. In some cases, the first bispecific binding reagent is an anti-CD3/anti-STEAP1 taFv and the second bispecific binding reagent is an anti-CD28/anti-EpCAM taFv. In some cases, the first bispecific binding reagent comprises the amino acid sequence SEQ ID NO:1 and the second bispecific binding reagent comprises the amino acid sequence SEQ ID NO:12. In some cases, the first bispecific binding reagent is a taFv encoded by SEQ ID NO:2 and the second bispecific binding reagent is a taFv encoded by SEQ ID NO:13.

**[0020]** In an eigth aspect, the present invention provides, a composition comprising a nucleic acid encoding any one or more of the foregoing bispecific binding reagents and a pharmaceutically acceptable excipient. In a ninth aspect, the present invention provides a composition comprising a plurality of structurally different nucleic acids, wherein the plurality of structurally different nucleic acids each encode a structurally different bispecific binding reagent, and a pharmaceutically acceptable excipient.

**[0021]** In a tenth aspect, the present invention provides a composition comprising any one or more of the foregoing bispecific binding reagents and a pharmaceutically acceptable excipient. In an eleventh aspect, the present invention provides a composition comprising a plurality of structurally different bispecific binding reagents and a pharmaceutically acceptable excipient. In some embodiments of the tenth or eleventh aspect, the composition further comprises a chemical compound or a biological agent capable of providing an activation signal for T cells.

**[0022]** In a twelfth aspect, the present invention provides, a method for treating a subject in need thereof comprising administering a therapeutically effective amount of a first bispecific binding reagent to the subject. In some embodiments of the twelfth aspect, the subject has prostate cancer. In some cases, the subject has prostate cancer selected from the group consisting of metastatic prostate cancer and castration-resistant prostate cancer.

**[0023]** In some embodiments of the twelfth aspect, the method further comprises simultaneously or sequentially administering a second bispecific binding reagent, wherein the second bispecific binding reagent specifically binds at least one antigen that is not specifically recognized by the first bispecific binding reagent. In some cases, the first and second bispecific binding reagents comprise a first bispecific binding reagent that binds to CD3 and a second bispecific binding reagent that binds to CD28. In some cases, the first and second bispecific binding reagents comprise a first bispecific binding reagent that binds to STEAP1 and a second bispecific binding reagent that binds to EpCAM. In some cases, the first and second bispecific binding reagents comprise a first bispecific binding reagent that binds to CD3 and STEAP1 and a second bispecific binding reagent that binds to CD28 and EpCAM. In some cases, the first and/or the second bispecific binding reagent is:

a bispecific binding reagent that comprises a second domain that is derived from a ligand for a receptor that is expressed on the surface of the prostate cancer cell; or

a bispecific binding reagent that comprises a second domain that is derived from a receptor that interacts with a ligand that is expressed on the surface of the prostate cancer cell.

**[0024]** In a thirteenth aspect, the present invention provides a kit comprising one or more of the foregoing bispecific binding reagents and optionally comprising a pharmaceutical excipient.

**[0025]** Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0026]** **Figure 1.** Schematic outline of tandem single-chain variable fragment (tandem scFv, taFv) bispecific antibodies.

**[0027]** **Figure 2.** Sequence elements of taFv antibodies. **A.** General outline. **B.** Cysteine-modified taFv antibody. Introduction of one or more cysteine residues in the light chain at positions that enable formation of disulfide bridges with one or more cysteine residues introduced into the heavy chain can stabilize the taFv and prevent aggregate formation. For example, introduction of a cysteine at positions $V_L100$ (variable light chain) and $V_H44$ (variable heavy chain) may promote the formation of disulfide bridges between the $V_L$ and $V_H$ segments. **C.** Structural outline of TCT001, an anti-STEAP1/anti-CD3 taFv antibody. TCT001 is composed of two scFv antibodies connected by a short glycine-rich linker, and a

C-terminal hexa histidine tag for purification. Whereas the N-terminal scFv is derived from a monoclonal antibody (mAb) specific for the prostate tumor-associated antigen (TAA) STEAP1, the C-terminal scFv is derived from a mAb recognizing a CD3 epitope on T cells. The variable region ($V_H$ and $V_L$) segments of each scFv are connected by glycine-rich linkers. **D.** Structural outline of TCT002, an anti-CD28/anti-EpCAM taFv antibody with $V_L$100 and $V_H$44 cysteine modifications in the anti-CD28 scFv domain. TCT002 is composed of two scFv antibodies connected by a short glycine-rich linker, and a C-terminal hexa histidine tag for purification. Whereas the N-terminal scFv is derived from a mAb recognizing a CD28 epitope on T cells, the C-terminal scFv is derived from a mAb specific for EpCAM, a TAA expressed in a variety of cancer types with particularly high expression levels in cancer stem cells. The $V_H$ and $V_L$ segments of each scFv are connected by glycine-rich linkers.

**[0028]** **Figure 3**. *STEAP1* mRNA expression in normal and tumor tissues. **A**. *STEAP1* mRNA expression in normal and malignant prostate cancer tissues versus normal (**A**) or nonprostatic malignant (**B**) tissues. For both **A** and **B**, mRNA expression data shown was extracted from the GENT database (U133Plus2 data only) (*available* on the web at medical-genome.kribb.re.kr/GENT/) (Shin et al., *Cancer Inform*, 10:149-157, 2011) and processed using Microsoft Excel. Processing included averaging of true technical replicate samples and removal of suspected accidental *in silico* "replicates." *Mean of all samples* refers to the average expression levels across all samples (gsm_id) from a particular tissue, and *Mean/Median of mean values* to the mean (average)/median expression levels across all studies (gse_id) conducted with a particular tissue.

**[0029]** **Figure 4.** Dual Stimulation of T cell (DuST) Technology. **A.** Agonistic CD28- and CD3-stimulating bispecific antibodies. **B.** Cotreatment with agonistic CD28- and CD3-stimulating bispecific antibodies may induce stronger cytotoxic effects than treatment with either antibody alone.

**[0030]** **Figure 5.** TCT001 purification by metal affinity chromatography. Shown is the absorbance at 280 nm for various elution fractions. TCT001 was predominantly eluted as a monomer.

**[0031]** **Figure 6**. Production of TCT001. SDS-PAGE (**A**, **C**) and Western Blot probed with an anti-His tag antibody (**B**, **D**). Data shown is from two different preparations (**A** and **B** from preparation 1, **C** and **D** from preparation 2). For both preparations, TCT001 displays

the expected size of approximately 60 kDa. **D**: denaturing condition, **ND**: non-denaturing condition, **P**: positive control (Multiple-tag (GenScript, Cat.No. M0101).

**[0032] Figure 7.** Cytotoxicity of TCT001. **A.** T cell growth curve obtained with human T cells (CD3+). **B.** E/T optimization. TCT001 of approximately 85% purity was coincubated with human prostate cancer LNCaP or human lung cancer NCI-H23 target cells with different numbers of preactivated human T cells. Cytotoxicity was assessed via an LDH release cell viability assay. **C.** At an effector:target (E/T) cell ratio of 20, preactivated human T cells and LNCaP or NCI-H23 target cells were coincubated with a dose-titration series of TCT001 of approximately 85% purity. Cytotoxicity was measured as in **B**. Whereas LNCaP cells were lysed with an $EC_{50}$ value of 1.2 ng/ml (approximately 20 pM), $EC_{50}$ lysis for NCI-H23 cells required 48.6 ng/ml (approximately 800 pM) of TCT001. $EC_{50}$ values were determined conservatively (not taking the approximately 15% impurities as non-TCT001 proteins into account) and are thus likely somewhat lower than indicated. Data is shown as mean values (n=3) with error bars showing the standard errors.

**[0033] Figure 8.** Production of TCT002. SDS-PAGE (**A**) and Western Blot probed with an anti-His tag antibody (**B**). TCT002 displays a size of around 60 kDa, and was produced with approximately 85% purity. No TCT002-TCT002 dimers are apparent. **D**: denaturing condition, **ND**: non-denaturing condition, **P**: positive control.

**[0034] Figure 9.** *Cytotoxicity by Single and Dual Stimulation of Effector Cells.* TCT001 and/or TCT002 were co-incubated for 6 hours with human prostate cancer LNCaP cells (T) and human PBMCs (E) pre-stimulated with IL-2 at the indicated E/T ratios. Cytotoxicity was thereafter assessed via an LDH release cell viability assay. Effector + target cells in the absence of TCT001 (2.5 µg/ml) and/or TCT002 (2.5 µg/ml) produced a high background signal which further increased with longer incubation times (up to 96 hours tested, data not shown). Under the assumption that this background signal at least in part comprises a signal derived from nonspecifically lysed LNCaP target cells, the percentage target cell lysis values shown are likely underestimated.

**[0035] Figure 10.** *Dose-Response Experiment for Bispecific Binding Reagent-Induced Target Cell Lysis.* At an effector:target (E/T) cell ratio of 10, human PBMCs not pre-stimulated overnight with IL-2 were co-incubated with a dose-titration series of TCT001 and/or TCT002 for 16 hours. For the combination condition, TCT002 was kept constant at 2.5 µg/ml, whereas TCT001 was added to concentrations up to 5 µg/ml. At the highest

concentration of TCT001, addition of TCT002 significantly enhanced the target cell killing effect (1-tailed *t*-test). Cytotoxicity was measured as in the experiment described in Figure 9. Data are shown as mean values (n=3) with error bars showing the standard deviations. Effector + target cells in the absence of TCT001 and/or TCT002 produced a high background signal which further increased with longer incubation times (up to 96 hours tested, data not shown). Under the assumption that this background signal at least in part comprises a signal derived from nonspecifically lysed LNCaP target cells, the percentage target cell lysis values shown are likely underestimated.

**[0036]** **Figure 11**. Exemplary variable and scFv sequences. **A-U**. Sequences of scFv modules suitable for the generation of taFv antibodies (**A-S**) or bispecific antibodies comprising an extracellular domain of a receptor such as NKG2D and an scFv domain (**T** and **U**). Variable sequence analysis including CDR identification or Kabat numbering was conducted via abYsis (*available* on the web at bioinf.org.uk/abysis/index.html) and/or Abnum (*available* on the web at bioinf.org.uk/abs/abnum/). Depicted are CDRs as defined by Chothia, and, if applicable, extended CDR sequences to additionally include the CDR elements as defined by Kabat.

## DEFINITIONS

**[0037]** Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. See, *e.g.*, Lackie, The Dictionary of Cell and Molecular Biology, 4th ed., Elsevier, 2007; Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY, 1989). Any methods, devices and materials similar or equivalent to those described herein can be used in the practice of this invention. The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.

**[0038]** The terms "binds to", "recognizes", and "interacts with" are used interchangeably herein to include the binding/interaction of a polypeptide motif which shows the capacity of specific interaction with a specific antigen or a specific group of antigens. The terms "specifically binds to", "specifically recognizes", and "specifically interacts with" in the context of the present invention refers to the ability of a binding reagent such as an antibody or a fragment or domain thereof to interact with and/or bind to a target antigen and without cross-reacting with molecules of similar sequences or structures. In some cases, a binding

reagent specifically binds to a target antigen when it binds to the target antigen with a substantially lower dissociation constant (*i.e.*, tighter binding) than a molecule of similar sequence or structure. For example, in some cases, a specific binding occurs when the binding reagent binds to the target antigen with an about 2, 3, 4, 5, 6, 8, 10, 15, 20, 25, 30, 40, 50, 100, or 1000-fold or greater affinity than a related molecule.

**[0039]** Cross-reactivity may be tested, for example, by assessing binding of the binding reagent under conventional conditions (*see, e.g.,* Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988; Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999) to the target antigen as well as to a number of more or less (*e.g.,* structurally and/or functionally) closely related molecules. These methods may include, without limitation, binding studies, blocking and competition studies with closely related molecules, FACS analysis, surface plasmon resonance (*e.g.,* with BIAcore), analytical ultracentrifugation, isothermal titration calorimetry, fluorescence anisotropy, fluorescence spectroscopy, radiolabeled ligand binding assays, and combinations thereof.

**[0040]** "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, and complements thereof. The term "polynucleotide" refers to a linear sequence of nucleotides. The term “nucleotide” typically refers to a single unit of a polynucleotide, *i.e.*, a monomer. Nucleotides can be ribonucleotides, deoxyribonucleotides, or modified versions thereof. Examples of polynucleotides contemplated herein include single- and double-stranded DNA, single- and double-stranded RNA (including siRNA), and hybrid molecules having mixtures of single- and double-stranded DNA and RNA.

**[0041]** The words "protein", "peptide", and "polypeptide" are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers, those containing modified residues, and non-naturally occurring amino acid polymer.

**[0042]** The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function similarly to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic

code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *e.g.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs may have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions similarly to a naturally occurring amino acid.

**[0043]** Amino acids may be referred to herein by their commonly used full name (*e.g.*, cysteine), their commonly known three letter symbols (*e.g.*, Cys), or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission (*e.g.*, C). Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

**[0044]** "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical or associated, *e.g.*, naturally contiguous, sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode most proteins. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to another of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes silent variations of the nucleic acid. One of skill will recognize that in certain contexts each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and UGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, often silent variations of a nucleic acid which encodes a polypeptide is implicit in a described sequence with respect to the expression product, but not with respect to actual probe sequences.

**[0045]** As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which

alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, paralogs, orthologs, and alleles of the invention. The following amino acids are typically conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see, e.g.,* Creighton, Proteins: Structures and Molecular Properties, W. H. Freeman and Company, 1984).

**[0046]** The terms "identical" or "percent identity," in the context of two or more nucleic acids, or two or more polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides, or amino acids, that are the same (*i.e.*, about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters, or by manual alignment and visual inspection. *See, e.g.*, the NCBI web site at ncbi.nlm.nih.gov/BLAST. Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the complement of a nucleotide test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the algorithms can account for gaps and the like. Typically, identity exists over a region comprising an antibody epitope, or a sequence that is at least about 25 amino acids or nucleotides in length, or over a region that is 50-100 amino acids or nucleotides in length, or over the entire length of the reference sequence.

**[0047]** The term "antigen" refers to a molecule bound by an "antibody" or a "bispecific binding reagent." Antigens may be proteins recognized by immunoglobulins, in which case the sites on the proteins bound by the immunoglobulins are referred to as "epitopes." Antigens may also, or alternatively, be recognized by non-immunoglobulin ligand binding domain of a bispecific binding reagent. In such cases, "antigen" refers to binding partners of the non-immunoglobulin ligand binding domain of the bispecific bidning reagent. In some

cases, the antigen can be a ligand of a cell surface receptor, *e.g.*, a ligand of NKG2D. In some cases, the bispecific binding reagent contains a cell surface receptor ligand, or recptor binding portion thereof, and the antigen is the ligand binding portion of the cell surface receptor.

**[0048]** The term "bispecific binding reagent" refers to a molecule that can specifically bind two different target antigens simultaneously. Bispecific binding reagents generally have two structurally distinct binding regions (domains), each of which specifically binds to a single target antigen. Bispecific binding reagents can be used to bind a target antigen on a cell and a different target antigen on a different cell. Bispecific binding reagents can include polypeptide sequences (domains) of one or more antibodies or antibody fragments (*e.g.*, one or more scFvs). An exemplary bispecific binding reagent is a tandem single-chain variable fragment antibody molecule (taFv) having a first scFv and a second scFv. Another exemplary bispecific binding reagent has a first domain derived from a cell surface receptor that specifically binds a ligand and a second domain that specifically binds a different target antigen. Yet another exemplary bispecific binding reagent has a first domain that is derived from a ligand for a cell surface receptor that specifically binds the cell surface receptor and a second domain that specifically binds to a different target antigen.

**[0049]** Bispecific binding reagents can include a polypeptide linker sequence that links the first domain and the second domain. Additionally, the bispecific binding reagent can contain linker sequences between subdomains of the first and/or second domain. For example, if the first and/or second domain are scFvs, then the bispecific binding reagent will have a linker between the $V_H$ and $V_L$ domains of the scFvs. Suitable linker sequences are known in the art, and the bispecific binding reagents are not limited to any one specific linker. In general, suitable linker sequences consist of relatively flexible and hydrophilic amino acid residues. Additionally, interdomain linker sequences (*e.g.*, scFv to scFv) are generally shorter than inter-subdomain linker sequences (*e.g.*, intra scFv linkers, such as $V_H$ to $V_L$ or $V_L$ to $V_H$ linkers). Suitable linker sequences however are not restricted to any one length or range of lengths. In some cases, a suitable linker sequence can contain a plurality of glycine and serine residues. Exemplary inter-subdomain linker sequences are provided by SEQ ID NOS: 5 and 8. Exemplary interdomain linker sequences are provided by SEQ ID NOS: 11, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, and 76.

**[0050]** Bispecific binding reagents of several different topologies are described herein. For example, as shown in Figure 1, The $V_H$ and $V_L$ domains of a first scFv can be oriented from the amino terminus (N) of the scFv coding sequence to the carboxy terminus (C). Alternatively, the $V_L$ and $V_H$ domains can be oriented N to C. Thus, for example, each first and second domain of a bispecific binding reagent comprising two scFvs can have four different topologies (**Figure 1** (inset)).

**[0051]** A bispecific binding reagent is "substantially pure" when it exists in solution as a substantially monodisperse population of molecules. For example, a bispecific binding reagent is substantially pure when it has a polydispersity index (PDI), a measure of the degree of mono- or polydispersity of a polymer in solution, as measured by photon correlation spectroscopy of less than about 0.2 (*e.g.*, less than about 0.15, 0.1, or 0.08). As another example, a bispecific binding reagent is substantially pure when at least about a majority proportion of the bispecific binding reagent is not aggregated (*e.g.*, at least about 50%, 60%, 75%, 80%, 90%, 95%, 99% or more). In some cases, aggregation or polydispersity of a bispecific binding reagent is minimized through the introduction of disulfide bridges between domains or subdomains of the bispecific binding reagents. In an exemplary embodiment, the disulfide bridges are within a taFv antibody and are between a $V_H$ subdomain of one scFv domain and a $V_L$ subdomain of another scFv domain.

**[0052]** An "antibody" can consist of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. An "antibody" functions as a binding protein and is structurally defined as comprising an amino acid sequence from or derived from the framework region of an immunoglobulin encoding gene of an animal producing antibodies.

**[0053]** A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" (about 50-70 kD) chain. The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ($V_L$) and variable heavy

chain ($V_H$) or light chain variable region and heavy chain variable region and the like refer to these light and heavy chains and their variable regions respectively.

**[0054]** The term "antibody" as used herein includes antibody fragments that retain binding activity. For example, there are a number of well characterized antibody fragments. Thus, for example, pepsin digests an antibody C-terminal to the disulfide linkages in the hinge region to produce $F(ab')_2$, a dimer of Fab which itself is a light chain joined to $V_H$-CH1 by a disulfide bond. The $F(ab')_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $F(ab')_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (see, *e.g.,* W. E. Paul, Fundamental Immunology, 3rd ed., Raven Press, N.Y., 1993 for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that fragments can be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized using recombinant DNA methodologies.

**[0055]** Antibodies include $V_H$-$V_L$ dimers, including those generated as single-chain antibodies (antibodies that exist as a single polypeptide chain) such as single-chain Fv antibodies (sFv or scFv) in which a variable heavy and a variable light region are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single-chain Fv antibody may be expressed from a nucleic acid in which the $V_H$- and $V_L$- encoding sequences are either joined directly or are joined by a peptide-encoding linker (*e.g.,* Huston et al., *Proc Natl Acad Sci USA*, 85(16):5879-5883, 1988). While the $V_H$ and $V_L$ are connected to each as a single polypeptide chain, the $V_H$ and $V_L$ subdomains associate non-covalently. In some cases, the non-covalent association of the $V_H$ and $V_L$ subdomains is further stabilized through one or more disulfide bridges between cysteine residues in the $V_H$ and $V_L$ subdomains. The $V_H$ and $V_L$ domains of an scFv can be oriented from the amino terminus (N) to the carboxy terminus (C) of the scFv coding sequence. Alternatively, the $V_L$ and $V_H$ domains can be oriented N to C. Thus, for example, an scFv can have two different possible topologies.

**[0056]** Alternatively, the antibody can be another fragment. Other fragments can also be generated, *e.g.,* using recombinant techniques, as soluble proteins or as fragments obtained from display methods. Antibodies can also include diantibodies and miniantibodies.

Antibodies of the invention also include heavy chain dimers, such as antibodies from camelids. In a preferred embodiment, the antibody is a tandem tandem single-chain variable fragment antibody molecule (taFv) having a first scFv and a second scFv.

**[0057]** As used herein, "V-region" refers to an antibody variable region domain comprising the segments of Framework 1, CDR1, Framework 2, CDR2, and Framework 3, including CDR3 and Framework 4, which segments are added to the V-segment as a consequence of rearrangement of the heavy chain and light chain V-region genes during B-cell differentiation.

**[0058]** As used herein, "complementarity-determining region (CDR)" refers to the three hypervariable regions in each chain of an antibody that interrupt the four "framework" regions established by the light and heavy chain variable regions. The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, a $V_H$ CDR3 is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a $V_L$ CDR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found.

**[0059]** The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three dimensional space.

**[0060]** The amino acid sequences of the CDRs and framework regions can be determined using various well known definitions in the art, *e.g.,* by Kabat, Chothia, or the IMGT®, the international ImMunoGeneTics information system® (*available at* imgt.org; founder and director: Marie-Paule Lefranc, Montpellier, France) (*see, e.g.,* Johnson and Wu, *Nucleic Acids Res*, 28(1):214-218, 2000; Johnson and Wu, *Nucleic Acids Res*, 29(1):205-206, 2001; Chothia and Lesk, *J Mol Biol*, 196(4):901-917, 1987; Chothia et al., *Nature*, 342(6252):877-883, 1989; Chothia et al., *J Mol Biol*, 227(3):799-817, 1992; Al-Lazikani et al., *J Mol Biol*, 273(4):927-948, 1997; Lefranc et al., *Dev Comp Immunol*, 27(1):55-77, 2003; and Lefranc, *Front Immunol*, 5:22, 2014). Definitions of antigen combining sites are also described in the following: Ruiz et al., *Nucleic Acids Res*, 28(1):219–221, 2000; Lefranc, *Nucleic Acids Res*, 29(1):207-209, 2001; MacCallum et al., *J Mol Biol*, 262(5):732-745, 1996; Martin et al., *Proc*

*Natl Acad Sci USA*, 86(23):9268–9272, 1989; Martin et al., *Methods Enzymol*, 203:121-153, 1991; Pedersen et al., *ImmunoMethods*, 1(2):126-136, 1992; and Rees et al., In Sternberg M.J.E. (ed.), Protein Structure Prediction, Oxford University Press, Oxford, 141–172, 1996).

**[0061]** An "isotype" is a class of antibodies defined by the heavy chain constant region. Immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the isotype classes, IgG, IgM, IgA, IgD and IgE, respectively.

**[0062]** A "monoclonal antibody" refers to a clonal preparation of antibodies. A "polyclonal antibody" refers to a preparation of antibodies that are raised against a single antigen, but with different complementarity determining regions.

**[0063]** As used herein, "chimeric antibody" refers to an immunoglobulin molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, drug, *etc.*; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region, or portion thereof, having a different or altered antigen specificity; or with corresponding sequences from another species or from another antibody class or subclass.

**[0064]** As used herein, "humanized antibody" refers to an immunoglobulin molecule in which CDRs from a donor antibody are grafted onto human framework sequences. Humanized antibodies may also comprise residues of donor origin in the framework sequences. The humanized antibody can also comprise at least a portion of a human immunoglobulin constant region. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. Humanization can be performed using methods known in the art (*e.g.,* Jones et al., *Nature*, 321(6069):522-525, 1986; Riechmann et al., *Nature*, 332(6162):323-327, 1988; Verhoeyen et al., *Science*, 239(4847):1534-1536, 1988; Presta, *Curr Opin Struct Biol*, 2(4):593-596, 1992; U.S. Patent No. 4,816,567; U.S. Patent No. 5,225,539), including techniques such as "superhumanizing" antibodies (Tan et al., *J Immunol*, 169(2):1119-1125, 2002) and "resurfacing" (*e.g.,* Staelens et al., *Mol Immunol*, 43(8):1243-1257, 2006; and Roguska et al., *Proc Natl Acad Sci USA*, 91(3):969-973, 1994).

**[0065]** As used herein, the phrase "agonistic antibody" refers to an antibody or antibody fragment (*e.g.*, an scFv, taFv, *etc.*) that activates or stimulates an antigen upon binding. For example, an agonistic anti-CD28 antibody is capable of binding to CD28 and activating a co-stimulatory signal mediated by CD28. As another example, an agonistic CD3 antibody is capable of binding to CD3 and activating a co-stimulatory signal mediated by CD3. In some cases, the agonistic antibody provides a stronger activating signal than a natural ligand (*e.g.*, it is a superagonistic antibody). In other cases, the agonistic antibody is weaker than or substantially the same as a natural ligand.

**[0066]** An antibody can contain additional amino acids or molecules for purification or identification. For example, the antibody can contain an epitope or affinity tag. Exemplary epitope or affinity tags include, without limitation, peptide tags (*e.g.*, FLAG-tag, HA-tag, His-tag, Myc-tag, S-tag, SBP-tag, Strep-tag, eXact-tag), and protein tags (*e.g.*, GST-tag, MBP-tag, GFP-tag). In some case, the antibody is biotinylated. Other suitable epitope or affinity tags are known in the art.

**[0067]** The antibody binds to an "epitope" on the antigen. The epitope is the specific antibody binding interaction site on the antigen. If the antigen is a peptide, the epitope can include a few amino acids or portions of a few amino acids, *e.g.*, 5 or 6, or more, *e.g.*, 20 or more amino acids, or portions of those amino acids. In some cases, the epitope includes non-protein components, *e.g.*, from a carbohydrate, nucleic acid, or lipid. In some cases, the epitope is a three-dimensional moiety. Thus, for example, where the target is a protein, the epitope can be comprised of consecutive amino acids, or amino acids from different parts of the protein that are brought into proximity by protein folding (*e.g.*, a discontinuous epitope). The same is true for other types of target molecules that form three-dimensional structures.

**[0068]** Antibodies can be produced using any number of expression systems, including prokaryotic and eukaryotic expression systems. For example, the antibody can be produced in or by bacteria (*e.g.*, *E. coli*, *P. mirabilis*), fungi (*e.g.*, *S. cerevisiae*, *P. Pastoria*, *T. reesei*), plants or plant cells, insects or insect cells (*e.g.*, SF-9, SF21, Hi-5), or mammalian cells. In some embodiments, the expression system is a mammalian cell expression system, such as a hybridoma, or a CHO or 293 cell expression system. Many such systems are widely available from commercial suppliers.

**[0069]** In some cases, the antibody or antibody fragment can be conjugated to another molecule, *e.g.*, polyethylene glycol (PEGylation) or serum albumin, to provide an extended

half-life *in vivo* or to decrease non-specific binding. Examples of PEGylation of antibody fragments are provided in Knight et al., *Platelets*, 15(7):409-418, 2004 (for abciximab); Pedley et al., *Br J Cancer*, 70(6):1126-1130, 1994 (for an anti-CEA antibody); Chapman et al., *Nature Biotechnol*, 17(8):780-783, 1999; and Humphreys et al., *Protein Eng Des*, 20(5):227-234, 2007. The antibody or antibody fragment can also be labeled, or conjugated to a therapeutic agent as described herein.

**[0070]** As used herein, the phrase "tumor-associated antigen" or "TAA" refers to an antigen that is commonly associated with a cancer cell as compared to a noncancerous cell. In some cases, TAAs can be identified as those that have a high expression level in the cancer cell relative to the highest mean expression level among normal tissues of the same origin. For example, a prostate TAA can be identified as an antigen that has a high expression level in prostate cancer cells relative to the highest mean expression level among normal prostate tissue cells. In some cases, a prostate TAA may be highly expressed in both malignant and normal (benign) prostate cells but not in normal nonprostatic cells. Such TAAs are especially relevant after the prostate gland harboring the primary tumor(s) has been removed by, *e.g.*, radical prostatectomy because thereafter, in principle, only cancerous prostate cells – if any – remain in the body. The level of prostate cancer expression relative to highest mean expression level in normal nonprostatic male tissues is the PCa/MaxN value. In some cases, an antigen is identified as a prostate TAA if it has a PCa/MaxN value of at least about 2 (*e.g.*, at least about 2, 2.5, 3, 4, 5, 8, 10 or more). In some cases, prostate TAAs can be identified as those that have a high expression level in the cancer cell relative to the second highest mean expression level among normal nonprostatic male tissues of the same origin. This ratio is referred to as the PCa/2ndMaxN value. In some cases, an antigen is identified as a TAA if it has a PCa/2ndMaxN value of at least about 2 or 3 (*e.g.*, at least about 2, 2.5, 3, 4, 5, 8, 10 or more). In still other cases, an antigen can be identified as a TAA from a database, a scientific journal article, or a histological experiment. Exemplary methods for identifying prostate cancer TAAs are described herein.

**[0071]** As used herein, a first bispecific binding reagent, or an antigen-binding portion thereof, "competes" for binding to a target with a second bispecific binding reagent, or an antigen-binding portion thereof, when binding of the first bispecific binding reagent with the target is detectably decreased in the presence of the second bispecific binding reagent compared to the binding of the first bispecific binding reagent in the absence of the second

bispecific binding reagent. The alternative, where the binding of the second bispecific binding reagent to the target is also detectably decreased in the presence of the first bispecific binding reagent, can, but need not be the case. However, where each bispecific binding reagent detectably inhibits the binding of the other bispecific binding reagent to its cognate epitope or ligand, whether to the same, greater, or lesser extent, the bispecific binding reagents are said to "cross-compete" with each other for binding of their respective epitope(s). Both competing and cross-competing bispecific binding reagents are encompassed by the present invention. The term "competitor" bispecific binding reagent can be applied to the first or second bispecific binding reagent as can be determined by one of skill in the art. In some cases, the presence of the competitor bispecific binding reagent (*e.g.*, the second bispecific binding reagent) reduces binding of, *e.g.*, the first bispecific binding reagent to the target by at least 10%, *e.g.*, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or more, *e.g.*, so that binding of the first bispecific binding reagent to target is undetectable in the presence of the second (competitor) bispecific binding reagent. In some cases, a competitor bispecific binding reagent can be used to replace a first interaction between an immune cell and a target prostate cancer cell with a second interaction between the immune cell and the target prostate cancer cell as further described herein.

**[0072]** A "control" sample or value refers to a sample that serves as a reference, usually a known reference, for comparison to a test sample. For example, a test sample can be taken from a test condition, *e.g.*, in the presence of a test compound, and compared to samples from known conditions, *e.g.*, in the absence of the test compound (negative control), or in the presence of a known compound (positive control). A control can also represent an average value gathered from a number of tests or results. One of skill in the art will recognize that controls can be designed for assessment of any number of parameters. For example, a control can be devised to compare therapeutic benefit based on pharmacological data (*e.g.*, half-life) or therapeutic measures (*e.g.*, comparison of benefit and/or side effects, such as mean time of survival of a prostate cancer patient). Controls can be designed for *in vitro* applications. One of skill in the art will understand which controls are valuable in a given situation and be able to analyze data based on comparisons to control values. Controls are also valuable for determining the significance of data. For example, if values for a given parameter are widely variant in controls, variation in test samples may not be considered as significant.

**[0073]** A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include $^{32}P$, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins or other entities which can be made detectable, *e.g.*, by incorporating a radiolabel into a peptide or antibody specifically reactive with a target peptide. Any method known in the art for conjugating an antibody to the label may be employed, *e.g.*, using methods described in Hermanson, Bioconjugate Techniques, Academic Press/Elsevier, 1996.

**[0074]** A "labeled" molecule (*e.g.*, nucleic acid, protein, or antibody) is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the molecule may be detected by detecting the presence of the label bound to the molecule.

**[0075]** The terms "therapy," "treatment," and "amelioration" refer to any reduction in the severity of symptoms. In the case of treating prostate cancer, treatment can refer to, *e.g.*, reducing tumor size, number of prostate cancer cells, growth rate, metastatic activity, reducing cell death of non-cancer cells, reduced score or stabilized score as assessed by an accepted diagnostic or prognostic set of criteria (*e.g.*, reduced or stabilized Gleason Score, Gleason Pattern, TNM stage, Whitmore-Jewett stage, histological grade, PSA level or status, D'Amico classification, Partin outcome, CAPRA score, *etc.*), reduced nausea and other chemotherapy or radiotherapy side effects, *etc.* As used herein, the terms "treat" and "prevent" are not intended to be absolute terms. Treatment and prevention can refer to any delay in onset, amelioration of symptoms, improvement in patient survival, increase in survival time or rate, *etc.* Treatment and prevention can be complete (undetectable levels of neoplastic cells) or partial, such that fewer neoplastic cells are found in a patient than would have occurred without the present invention. The effect of treatment can be compared to an individual or pool of individuals not receiving the treatment, or to the same patient prior to treatment or at a different time during treatment. In some aspects, the severity of disease is reduced by at least 10%, as compared, *e.g.*, to the individual before administration or to a control individual not undergoing treatment. In some aspects, the severity of disease is reduced by at least 25%, 50%, 75%, 80%, or 90%, or in some cases, no longer detectable using standard diagnostic techniques. In some aspects of the current invention, treatment means mixing one or more types of bispecific binding reagent with target and effector cells *in*

*vitro*. For such treatments, the therapeutic effect may, *e.g.*, refer to the fraction of target cells lysed due to the presence of the bispecific binding reagent(s).

**[0076]** The terms "effective amount," "effective dose," "therapeutically effective amount," *etc.*, refer to that amount of the therapeutic agent sufficient to ameliorate a disorder, as described above. For example, for the given parameter (*e.g.*, mean time of survival), a therapeutically effective amount will show an increase or decrease of the therapeutic effect of at least 5%, 10%, 15%, 20%, 25%, 40%, 50%, 60%, 75%, 80%, 90%, or at least 100%. Therapeutic efficacy can also be expressed as "-fold" increase or decrease. For example, a therapeutically effective amount can have at least a 1.1-fold, 1.2-fold, 1.5-fold, 2-fold, 5-fold, or more effect over a control.

**[0077]** "Inhibits" or "inhibition" as used herein, *e.g.*, inhibition of characteristics such as cellular growth, proliferation, metabolic activity, viability, survival or division refers to a decrease in the characteristic relative to a control. In some cases, a bispecific binding reagent may inhibit one of the foregoing characteristics. For example, a prostate cancer cell treated with one or more bispecific binding reagents described herein may exhibit a decrease in one of the foregoing characteristics of approximately 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 12%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, 99.5%, 99.9% or more as compared to an untreated cell. In some cases, a prostate cancer cell treated with a one or more bispecific binding reagents described herein may exhibit a growth, proliferation, metabolic activity, viability, survival or division that is less than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 12%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, 99.5%, 99.9% of a control (*e.g.*, untreated) cell. In some cases, the inhibited prostate cancer cell is treated with one or more bispecific binding reagents described herein in the presence of immune cells. In some cases, the inhibited prostate cancer cell is also treated with one or more additional modulators of immune cell function (*e.g.*, one or more cytokines). In some cases, the inhibited prostate cancer cell is also treated with one or more additional chemotherapeutic agents.

**[0078]** The terms "dose" and "dosage" are used interchangeably herein. A dose refers to the amount of active ingredient given to an individual at each administration. For the present invention, the dose can refer to the concentration of the bispecific binding reagent or associated components, *e.g.*, the amount of therapeutic agent or dosage of radiolabel. The

dose will vary depending on a number of factors, including frequency of administration; size and tolerance of the individual; severity of the condition; risk of side effects; the route of administration; and the imaging modality of the detectable moiety (if present). One of skill in the art will recognize that the dose can be modified depending on the above factors or based on therapeutic progress. The term "dosage form" refers to the particular format of the pharmaceutical, and depends on the route of administration. For example, a dosage form can be in a liquid, *e.g.*, a saline solution for injection.

**[0079]** "Subject," "patient," "individual" and like terms are used interchangeably and refer to, except where indicated, mammals such as humans and non-human primates, as well as rabbits, rats, mice, goats, pigs, and other mammalian species. The term does not necessarily indicate that the subject has been diagnosed with a particular disease, but typically refers to an individual under medical supervision. A patient can be an individual that is seeking treatment, monitoring, adjustment or modification of an existing therapeutic regimen, *etc.* A "prostate cancer patient" can refer to an individual that has been diagnosed with prostate cancer, is currently following a therapeutic regimen, or is at risk of recurrence, *e.g.*, after surgery to remove a prostate tumor. In some embodiments, the prostate cancer patient has been diagnosed with prostate cancer and is a candidate for therapy. Prostate cancer patients can include individuals that have not received treatment, are currently receiving treatment, have had surgery, and those that have discontinued treatment.

**[0080]** In the context of treating prostate cancer, a subject in need of treatment can refer to an individual that has prostate cancer or a pre-cancerous condition, has had prostate cancer and is at risk of recurrence, is suspected of having prostate cancer, or is undergoing standard treatment for prostate cancer, such as radiotherapy or chemotherapy, *etc.* In a preferred embodiment, the subject in need of treatment has metastatic prostate cancer or CRPC (castrate-resistant prostate cancer, also referred to as castration-resistant prostate cancer).

**[0081]** The term "combination effect" in the context of this invention refers to the effect resulting from a treatment with at least two structurally different therapeutic agents, such as two structurally different bispecific binding reagents. Combination effects can be additive, synergistic, or antagonistic. Combination effects can, for instance, refer to the therapeutic efficacy. An *additive* effect means that the sum of the effects resulting from the individual therapeutic agents equals the effect resulting from the therapeutic agents applied in combination. A *synergistic* effect means that the combination effect is greater than the sum

of the effects resulting from the individual therapeutic agents. An a*ntagonistic* effect means that the effect of the combination treatment is smaller than the sum of the effects resulting from the individual therapeutic agents. In some cases, additive, synergistic, or antagonistic effects can be readily detected by manual inspection of individual and combination treatment effects. In some cases, data on individual and combination treatment outcomes can suggest the presence of synergism.

**[0082]** Additionally, or in the alternative, a widely accepted method to identify the type of effect includes determining the "combination index" (CI) as described by Chou and Talalay (Chou, *Pharmacol Rev*, 58(3):621-681, 2006; Chou, *Cancer Res*, 70(2):440-446, 2010; and US 2009/0324744), incorporated herein by reference. Drugs (*e.g.*, bispecific binding reagents) with a CI < 1 are thought to act synergistically, with a CI = 1 additively, and with a CI > 1 antagonistically. CI values for two mutually exclusive (similar mechanisms/modes of action) drugs are described by the classic isobologram formula $CI = (D)1/(Dx)1 + (D)2/(Dx)2$ (Formula 1), wherein (Dx)1 means the concentration of Drug 1 without Drug 2 that results in a particular effect, for instance, 50% cell killing, and (Dx)2 refers to the concentration of Drug 2 without Drug 1 that leads to the same effect. (D)1 and (D)2 are the individual concentrations of Drug 1 and Drug 2 in Drug 1-Drug 2 combinations.

**[0083]** Classic isobolograms are graphical representations of the concentrations of two drugs which in combination induce a particular effect, *e.g.*, 50% cell killing, under the assumption the effects of the two drugs are additive (CI = 1). If Drug 1 concentrations are shown on the X-axis, and Drug 2 concentrations on the Y-axis, such isobolograms may be generated by drawing a straight line from the position on the X-axis representing the concentration of Drug 1 by which the particular effect is achieved without Drug 2 to the position on the Y-axis representing the concentration of Drug 2 by which the same effect is achieved without Drug 1. Actual data points (with combinations of drugs at concentrations resulting in the particular effect) outside the triangle given by the X-axis, Y-axis, and the aforementioned "straight line" refer to drugs acting (at the tested concentrations) antagonistically (CI > 1), on the "straight line" additively (CI = 1), and inside the triangle synergistically (CI < 1).

**[0084]** To assess whether experimental data supports synergistic, additive, or antagonistic relationships between two therapeutic agents, CI values may be calculated via software such as CompuSyn (by ComboSyn, Inc., *available at* combosyn.com).

## DETAILED DESCRIPTION OF THE INVENTION

### I. Introduction

**[0085]** The present invention relates to bispecific binding reagents designed to activate immune cells against target prostate cancer cells. Immune cells are typically activated against target cells by interactions between immune cell receptors and target cell antigens. For example, T cells are typically activated by productive interactions between a T cell receptor (TCR) complex and a major histocompatibility (MHC) antigen complex on the surface of a target cell.

**[0086]** In some cases, immune cell activation against a target cell requires co-stimulation. For example, T cell activation can require stimulation through both the interaction between the TCR and MHC antigen complex (Signal 1) and interactions between one or more co-receptors and one or more target cell antigens (Signal 2). Failure to provide Signal 2 may result in T cell anergy/tolerance or apoptosis (Macian et al., *Curr Opin Immunol*, 16(2):209-216, 2004; Pardigon et al., *J Immunol*, 164(9):4493-4499, 2000; Ward, *Biochem J*, 318 ( Pt 2):361-377, 1996; Zhong et al., *Mol Ther*, 18(2):413-420, 2010).

**[0087]** In one embodiment of the present invention, immune cells are activated against target prostate cancer cells through a mechanism that is independent of MHC using a bispecific binding reagent. For example, bispecific binding reagents can be designed to cross-link a target prostate cancer cell to an immune cell by binding to both a component of the TCR complex and a prostate cancer cell antigen. Thus, the immune cell can be activated against the target prostate cancer cell by the signal (*e.g.*, Signal 1) provided by the bispecific binding reagent.

**[0088]** Clinical effects against B cell malignancies have been reported following treatment with a bispecific antibody. For example, the CD19 x CD3 bispecific T cell engager (BiTE) antibody blinatumomab has shown a clinical effect. However, an initial and temporary decline of the T cell counts was also observed (Nagorsen et al., *Pharmacol Ther*, 136(3):334-342, 2012). This drop may have been the result of T cell apoptosis induced by the lack of Signal 2 (Lacher & Provenzano, *Recent Pat Anticancer Drug Discov*, 8(3):239-254, 2013). Consequently, some embodiments of the present invention relate to combinations of bispecific antibodies that provide both Signal 1 and 2 and thus improve T cell activation and prevent T cell apoptosis. Thus, in such embodiments, a stronger target cell-directed cell killing effect is induced by application of a stimulatory and co-stimulatory signal in

comparison to approaches that only provide the stimulatory Signal 1. In one aspect, Signal 2 is provided by an agonistic bispecific binding reagent that binds to CD28. An exemplary agonistic bispecific binding reagent is a taFv having an scFv domain that binds to and activates CD28.

**[0089]** While numerous CD3-binding bispecific antibodies such as blinatumomab (CD19 x CD3) or solitomab (EpCAM x CD3) are well known in the art as mediators of anti-cancer cytotoxic effects, much fewer bispecific antibodies targeting T cell epitopes other than CD3 have been described. Among the latter constructs are NG2 or CD20-binding CD28 x TAA taFvs that activate T cells in the absence of a primary signal through the TCR/CD3 complex (Lacher and Provenzano, *Recent Pat Anticancer Drug Discov*, 8(3):239-254, 2013; Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003; Otz et al., *Leukemia*, 23(1):71-77, 2009 and U.S. Patent Application 7,538,196). However, target cell killing by such a supra-agonistic (superagonistic) antibody, CD28 x NG2 (rM28, also referred to as r28M), was not directly mediated by the activated T cells but rather by non-T cells stimulated by cytokines released from the activated T cells (Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003).

**[0090]** For reasons not entirely clear at present, dimeric/multimeric forms of supra-agonistic CD28 x TAA constructs exerted a more potent cytotoxic effect than their monomeric counterparts (Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003); Otz et al., *Leukemia*, 23(1):71-77, 2009; Otz, *Ph.D. Thesis, Neue Ansaetze fuer eine Antikoerper-basierte Therapie CD20+ lymphoider Neoplasien*, Eberhard Karls Universitaet Tuebingen, Tuebingen, Germany, 2008). A likely explanation is that dimeric/multimeric forms provide higher antibody concentrations at the tumor target sites and thereby stimulate higher numbers of CD28 molecules per T cell than monomeric forms. The same argument may also apply to the proposed lack of systemic T cell activation when using tumor-targeting CD28 x TAA taFv antibodies (Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003). On the other hand, in contrast to the monomeric form of the CD28 x NG2 antibody, high concentrations (>300 ng/ml) of the dimeric form activated T cells even in the absence of melanoma target cells (Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003). Anti-CD28 antibodies which activate T cells even in the absence of TCR stimulation may pose life-threatening risks in clinical applications. Such an effect, manifested by a cytokine storm and multiorgan

failure, was apparent while testing the superagonistic antibody (TGN1412) on healthy volunteers (Suntharalingam et al., *N Engl J Med*, 355(10):1018-1028, 2006).

**[0091]** Compositions, methods, and kits of the present invention can be designed to avoid a widespread/systemic T cell activation by providing Signals 1 and 2 only to T cells present in vicinity of defined target cells. In some embodiments, Signal 2 is provided without widespread/systemic T cell activation by means of stimulating a T cell co-receptor such as CD28 with an agonistic bispecific binding reagent not expected to activate T cells superagonistically. In some cases, the agonistic bispecific binding reagent contains an anti-CD28 scFv domain that does not superagonistically activate CD28. In some cases, the agonistic bispecific binding reagent that contains an anti-CD28 scFv domain that does not superagonistically activate CD28 can be provided in, and is stable in solution as, a predominantly monomeric form. In some cases, superagonistic activation, or lack thereof, can be detected *in vitro* by measuring T cell activation in the presence of the binding reagent. For example, detection of rapid proliferation of T cells and enhanced target cell lysis in the presence of an anti-CD28 reagent and in the absence of a T cell receptor-stimulating reagent indicates superagonistic activation.

## II. Compositions

### A. Bispecific Binding Reagents

**[0092]** Bispecific binding reagents are described herein. Bispecific binding reagents can be useful for activating an immune cell against a target prostate cancer cell. For example, the bispecific binding reagent can cross-link an immune cell to a target prostate cancer cell and induce cell-mediated cytotoxicity in the target prostate cancer cell.

**[0093]** In one embodiment, the bispecific binding reagent is a polypeptide having a first domain that binds to an antigen expressed by an immune cell and a second domain that binds to an antigen expressed by a prostate cancer cell. Such a bispecific binding reagent can therefore simultaneously bind to the immune cell and the prostate cancer cell. Typically, bispecific binding reagents described herein specifically bind two different, and structurally distinct, antigens.

**[0094]** Bispecific binding reagents that bind prostate cancer cells can be designed to target any cancer cell of the prostate. For example, bispecific binding reagents can bind to, and are useful for treatment of, without limitation, metastatic prostate cancer cells; castration resistant

prostate cancer cells; prostate cancer cells associated with stage T1, T2, T3, or T4 on the TNM scale; prostate cancer cells associated with Gleason patterns 1, 2, 3, 4, or 5 or associated with a Gleason Score of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; or prostate cancer cells associated with a Whitmore-Jewett stage of A1, A2, BIN, B1, B2, C1, C2, or D. In a preferred embodiment, the bispecific binding reagents bind to, and are useful for treatment of, metastatic prostate cancer cells; castration-resistant prostate cancer cells, prostate cancer cells associated with stage T3 or T4 on the TNM scale; prostate cancer cells associated with Gleason patterns 3, 4, or 5 or Gleason Score of 6, 7, 8, 9, or 10; or prostate cancer cells associated with a Whitmore-Jewett stage of C1, C2, or D.

**[0095]** The bispecific binding reagent can have a first domain and a second domain, wherein the first domain is derived from an antibody. For example, the first domain can be an antibody fragment, *e.g.*, scFv, Fab, Fab', or $F(ab')_2$, or a full-length antibody (*e.g.*, an IgA, IgG, IgE, IgM, or IgD). In a preferred embodiment, the first domain is an scFv. Similarly, the second domain can be derived from an antibody as described above. In a preferred embodiment, the second domain is an scFv. In some cases, the bispecific binding reagent has a first domain and a second domain, wherein both the first and second domain are derived from an antibody. For example, the first and second domain can be scFvs. In some cases, the bispecific binding reagent having a first and second scFv is a tandem single-chain variable fragment antibody molecule (taFv) as described herein. An exemplary generic structure of a taFv is depicted in **Figure 1**.

**[0096]** The bispecific binding reagent can bind to a prostate cancer cell and an immune cell. Exemplary target immune cells include, without limitation, T cells, Natural Killer (NK) cells, Natural Killer T (NKT) cells, monocytes, macrophages, neutrophils, B cells, dendritic cells, or mast cells. In a preferred embodiment, the immune cell is a T cell. In some cases, the T cell is a NKT cell. In another embodiment, the immune cell is an NK cell.

**[0097]** In some embodiments, the bispecific binding reagent binds to the target immune cell by specifically binding to an antigen expressed on the surface of the immune cell. For example, the bispecific binding reagent can specifically bind to a protein encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), CD28, CD2, CD27, IL7R, CD69, ICOS, IL2RB, PTPRC (CD45), CD48, SELL, CD137 (4-1BB, TNFRSF9), CD134 (OX40, TNFRSF4), and NKG2D. In some cases, the bispecific binding reagent specifically binds to a component of the TCR complex. In other

cases, the bispecific binding reagent specifically binds to a T cell co-receptor. In a preferred embodiment, the bispecific binding reagent specifically binds to CD3 or CD28. Consequently, in this embodiment, the bispecific binding reagent can have an anti-CD3 or an anti-CD28 scFv domain. In some cases, the anti-CD3 or anti-CD28 scFv domain is disulfide-stabilized with one or more disulfide bridges between $V_H$ and $V_L$ subdomains.

**[0098]** For example, the anti-CD3 scFv can have a $V_H$ sequence of SEQ ID NO:6 and a $V_L$ sequence of SEQ ID NO:7. As another example, the anti-CD3 scFv can have $V_H$ and $V_L$ subdomains with a high sequence identity to the $V_H$ and $V_L$ subdomains of SEQ ID NOs:6 and 7, but contain cysteine modifications to introduce stabilizing disulfide bridges between the $V_H$ and $V_L$ subdomains. For example, the anti-CD3 scFv can have a $V_H$ sequence of SEQ ID NO:18 and a $V_L$ sequence of SEQ ID NO:19. In some cases the anti-CD3 scFv has an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO:24, and SEQ ID NO:25.

**[0099]** An exemplary anti-CD28 scFv has a $V_H$ sequence of SEQ ID NO:15 and a $V_L$ sequence of SEQ ID NO:14. As another example, the anti-CD28 scFv can have $V_H$ and $V_L$ subdomains with a high sequence identity to the $V_H$ and $V_L$ subdomains of SEQ ID NOs:15 and 14, but contain cysteine modifications to introduce stabilizing disulfide bridges between the $V_H$ and $V_L$ subdomains. For example, the anti-CD28 scFv can have a $V_H$ sequence of SEQ ID NO:27 and a $V_L$ sequence of SEQ ID NO:26. In some cases the anti-CD28 scFv has an amino acid sequence selected from the group consisting of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, and SEQ ID NO: 31.

**[0100]** In some embodiments, the bispecific binding reagent specifically binds an antigen expressed on the surface of a prostate cancer cell, for instance, a transmembrane protein, a glycosylphosphatidylinositol (GPI)-anchored protein, or a secreted extracellular protein. In some cases, the antigen is a secreted extracellular protein that binds to, or associates with, a transmembrane or GPI-anchored protein. In some cases, the antigen is a tumor-associated antigen. Exemplary antigens expressed on the surface of prostate cancer cells include, without limitation, an antigen encoded by a gene selected from the group consisting of ABCC4, ANO7, EPCAM, FOLH1, OR51E1, OR51E2, PSCA, SLC30A4, SLC45A3, SPON2, STEAP1, STEAP2, TARP, TMEFF2, TMPR8, TMPRSS2, and a gene encoding an NKG2D ligand. In some cases, the antigen is specific to, or commonly associated with, prostate cancer cells (*e.g.*, STEAP1 or FOLH1). In other cases, the antigen is a cancer

antigen that is not generally restricted to prostate cancer cells (*e.g.*, EPCAM or an NKG2D ligand). In a preferred embodiment, the bispecific binding reagent has an anti-STEAP1 or an anti-EPCAM scFv domain. In some cases, the anti-STEAP1 or anti-EPCAM scFv domain is disulfide-stabilized with one or more disulfide bridges between $V_H$ and $V_L$ subdomains.

**[0101]** An exemplary anti-STEAP1 scFv has a $V_L$ sequence of SEQ ID NO:3 and a $V_H$ sequence of SEQ ID NO:4. As another example, the anti-STEAP1 scFv can have $V_L$ and $V_H$ subdomains with a high sequence identity to the $V_L$ and $V_H$ subdomains of SEQ ID NOs:3 and 4, but contain cysteine modifications to introduce stabilizing disulfide bridges between the $V_H$ and $V_L$ subdomains. For example, the anti-STEAP1 scFv can have a $V_L$ sequence of SEQ ID NO:32 and a $V_H$ sequence of SEQ ID NO:33. In some cases, the anti-STEAP1 scFv has an amino acid sequence selected from the group consisting of SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, and SEQ ID NO:37.

**[0102]** An exemplary anti-EPCAM scFv has a $V_H$ sequence of SEQ ID NO:16 and a $V_L$ sequence of SEQ ID NO:17. As another example, the anti-EPCAM scFv can have $V_L$ and $V_H$ subdomains with a high sequence identity to the $V_H$ and $V_L$ subdomains of SEQ ID NOs:16 and 17, but contain cysteine modifications to introduce stabilizing disulfide bridges between the $V_H$ and $V_L$ subdomains. For example, the anti-EPCAM scFv can have a $V_H$ sequence of SEQ ID NO:39 and a $V_L$ sequence of SEQ ID NO:38. In some cases, the anti-EPCAM scFv has an amino acid sequence selected from the group consisting of SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, and SEQ ID NO:43.

**[0103]** An exemplary anti-FOLH1 (PSMA) scFv has a $V_L$ sequence of SEQ ID NO:44 and a $V_H$ sequence of SEQ ID NO:45. As another example, the anti-FOLH1 scFv can have $V_L$ and VH subdomains with a high sequence identity to the $V_L$ and $V_H$ subdomains of SEQ ID NOs:44 and 45, but contain cysteine modifications to introduce stabilizing disulfide bridges between the $V_L$ and $V_H$ subdomains. For example, the anti-FOLH1 scFv can have a $V_L$ sequence of SEQ ID NO:46 and a $V_H$ sequence of SEQ ID NO:47. In some cases, the anti-PSMA scFv has an amino acid sequence selected from the group consisting of SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, and SEQ ID NO:51.

**[0104]** An exemplary anti-SPON2 scFv has a $V_L$ sequence of SEQ ID NO:52 and a $V_H$ sequence of SEQ ID NO:53. As another example, the anti-SPON2 scFv can have $V_L$ and $V_H$ subdomains with a high sequence identity to the $V_L$ and $V_H$ subdomains of SEQ ID NOs:52 and 53, but contain cysteine modifications to introduce stabilizing disulfide bridges between

the $V_L$ and $V_H$ subdomains. For example, the anti-SPON2 scFv can have a $V_L$ sequence of SEQ ID NO:54 and a $V_H$ sequence of SEQ ID NO:55. In some cases, the anti-SPON2 scFv has an amino acid sequence selected from the group consisting of SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, and SEQ ID NO:59.

**[0105]** In one embodiment, the bispecific binding reagent binds CD3 and STEAP1. In some cases, the anti-CD3/anti-STEAP1 bispecific binding reagent is a taFv having an anti-CD3 first scFv and an anti-STEAP1 second scFv. The anti-CD3/anti-STEAP1 taFv can have an inter-scFv linker with an amino acid sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NOS:63 to 76. An exemplary anti-CD3/anti-STEAP1 taFv has the amino acid sequence SEQ ID NO:2 or is encoded by SEQ ID NO:1.

**[0106]** In another embodiment, the bispecific binding reagent binds CD28 and EPCAM. In some cases, the anti-CD28/anti-EPCAM bispecific binding reagent is a taFv having an anti-CD3 first scFv and an anti-EPCAM second scFv. The anti-CD28/anti-EPCAM taFv can have an inter-scFv linker with an amino acid sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NOS:63 to 76. An exemplary anti-CD28/anti-EPCAM taFv has the amino acid sequence SEQ ID NO:12 or is encoded by SEQ ID NO:13.

**[0107]** In some preferred embodiments, the present invention comprises tandem single-chain variable fragment (taFv) bispecific antibodies (*e.g.*, **Figures 1**, **2A**, **2B**). In some particularly preferred embodiments, these taFv antibodies target CD3 and STEAP1 (*e.g.*, TCT001, **Figure 2C**) and/or CD28 and EpCAM (*e.g.*, TCT002, **Figure 2D**). **Figure 3** illustrates an mRNA expression profile demonstrating prostate and prostate cancer-specific expression of STEAP1.

**[0108]** In some embodiments, the bispecific binding reagent binds a ligand expressed on the surface of a prostate cancer cell, wherein the ligand binds a cell surface receptor of a different cell (*e.g.*, the ligand binds a cell surface receptor of an immune cell). As an example, the bispecific binding reagent can have a domain that is derived from the receptor for the ligand that is expressed on the surface of a prostate cancer cell. Alternatively, or in addition, the bispecific binding reagent can bind the receptor. For example, the bispecific binding reagent can have a domain that is derived from the ligand for the receptor. An exemplary cell surface receptor is NKG2D. An exemplary ligand expressed on the surface of a prostate cancer cell is an NKG2D ligand.

**[0109]** NKG2D is an activating receptor found on NK cells and some cytotoxic T cells. NKG2D binds to stress-induced ligands, which are often found overexpressed on the surface of cancer cells, such as prostate cancer cells. In some cases, the bispecific binding reagent binds NKG2D. In some cases, the NKG2D binding of the bispecific binding reagent is mediated by a domain derived from an antibody. In other cases, the NKG2D binding is mediated by a domain derived from an NKG2D ligand. Exemplary NKG2D ligands include, without limitation, MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, or ULBP3.

**[0110]** Similarly, the binding of a bispecific binding reagent to an NKG2D ligand can be mediated by a domain derived from an antibody. Alternatively, the binding to an NKG2D ligand can be mediated by a domain derived from NKG2D (*e.g.*, derived from the extracellular domain of NKG2D). In some cases, the domain derived from an antibody can provide specific binding to a specific NKG2D ligand (*e.g.*, MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, or ULBP3). In other cases, the domain derived from NKG2D can enable specific binding to NKG2D ligands in general (*e.g.*, MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, and ULBP3). In one embodiment, the bispecific binding reagent has a domain derived from the extracellular domain of NKG2D having the amino acid sequence of SEQ ID NO:60. In some cases, the extracellular domain of the NKG2D receptor is linked to a domain derived from an antibody (*e.g.*, scFv) that binds to an antigen expressed on the surface of an immune cell. In some cases, the extracellular domain of the NKG2D receptor is linked to a domain derived from an antibody with a linker selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:6.

**[0111]** Bispecific binding reagents can have a second domain derived from the extracellular domain of NKG2D and a first domain that specifically binds to an antigen expressed on an immune cell. For example, the bispecific binding reagent can have a first domain that binds to a protein encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), CD28, CD2, CD27, IL7R, CD69, ICOS, IL2RB, PTPRC (CD45), CD48, SELL, CD137 (4-1BB, TNFRSF9), and CD134 (OX40, TNFRSF4). In one embodiment, the bispecific binding reagent has an anti-CD3 or anti-CD28 first domain and a second domain derived from the extracellular domain of NKG2D. As one example, the bispecific binding reagent can have an anti-CD3 scFv linked to a domain derived from the extracellular domain of NKG2D. In a preferred

embodiment, the anti-CD3 scFv/anti-NKG2D bispecific binding reagent has the amino acid sequence SEQ ID NO:61. In another embodiment, the anti-CD28/anti-NKG2D bispecific binding reagent reagent has the amino acid sequence of SEQ ID NO:62.

**[0112]** The present invention also provides a nucleic acid (*e.g.*, an isolated nucleic acid) encoding any of the $V_H$ or $V_L$ subdomains, scFvs, domains derived from NKG2D, taFvs, or bispecific binding reagents described herein. In some cases, a plurality of structurally different nucleic acids encoding any two or more of the $V_H$ or $V_L$ subdomains, scFvs, domains derived from NKG2D, taFvs, or bispecific binding reagents described herein is provided. In some cases, the nucleic acid(s) are in a pharmaceutical excipient.

**[0113]** The nucleic acids can be incorporated into a vector, such as an expression vector. For example, the expression vector can contain a regulatory sequence that is operably linked to and promotes the transcription of the gene encoded by the nucleic acid. In particular embodiments, the nucleic acid is cloned into a vector such as, for example, a mammalian expression vector. In other embodiments, the present invention provides an isolated host such as an isolated cell transformed or transfected or transduced with the nucleic acid or a vector comprising the nucleic acid. The vectors and/or host cells can be provided in a pharmaceutical excipient.

**B. Combination Compositions**

**[0114]** Compositions described herein can contain a plurality of (*e.g.*, at least about 2, 3, 4, 5, 6 or more) structurally different bispecific binding reagents. For example, the composition can contain two or more different bispecific binding reagents. In some case, the two different bispecific binding reagents each bind to two different antigens (*e.g.*, an anti-CD3/anti-STEAP1 taFv and an anti-CD28/anti-EPCAM taFv). In some cases, the two different bispecific binding reagents bind to at least one common antigen. For example, the two different bispecific binding reagents can bind the same antigen expressed by a target prostate cancer cell.

**[0115]** In one embodiment, the composition contains a first bispecific binding reagent that binds to a first antigen expressed by an immune cell and a first antigen expressed by a prostate cancer cell, wherein the first antigen expressed by the immune cell is CD3. The composition can further contain a second bispecific binding reagent that binds to a second antigen expressed by the immune cell and an antigen expressed by the prostate cancer cell. In some cases, the prostate cancer antigen bound by the first and second bispecific binding

reagent is the same. In some cases, the prostate cancer antigen bound by the first and second bispecific binding reagent in the composition is different. In some cases, the first bispecific binding reagent binds to the CD3 antigen by the immune cell and the second bispecific antigen binds to the CD28 antigen.

**[0116]** In certain embodiments, the composition further comprises a compound capable of providing an activation signal for T cells. Non-limiting examples of such compounds include compounds that bind CD3 and activate T cells, compounds that bind the T cell receptor (TCR) and activate T cells, superantigens, vaccines such as a VZV vaccine or components thereof such as immunogenic VZV peptides, as well as combinations thereof. In some cases, the pharmaceutical composition contains one or more additional modulators of immune cell function (*e.g.*, one or more cytokines). In some cases, the pharmaceutical composition contains one or more additional chemotherapeutic agents.

**[0117]** In an exemplary embodiment, the composition can contain a first bispecific binding reagent that binds CD3 and STEAP1 and a second bispecific binding reagent that binds CD28 and EpCAM. For example, the first bispecific binding reagent can be an anti-CD3/anti-STEAP1 taFv (*e.g.*, having an amino acid sequence of SEQ ID NO:1 or encoded by SEQ ID NO:2) and the second bispecific binding reagent can be an anti-CD28/anti-EpCAM taFv (*e.g.*, having an amino acid sequence of SEQ ID NO:12 or encoded by SEQ ID NO:13).

**[0118]** In some aspects, combinations of bispecific binding reagents are applied to a subject having prostate cancer. In preferred embodiments, collectively referred to as DuST (Dual Stimulation of T cell) technology, a combination of bispecific binding reagents are provided (*e.g.*, produced, contacted to a cell, or administered to a patient) that provides both stimulation and co-stimulation of immune cells against a target cell (*e.g.*, against a prostate cancer cell). In some cases, DuST comprises providing (*e.g.*, producing, contacting to a cell, or administering to a patient) a CD3-binding bispecific binding reagent and a bispecific binding reagent that binds to an activating immune cell co-receptor (*e.g.*, a CD28-binding bispecific binding reagent). **Table 1** outlines suitable nonlimiting examples of such combinations. Exemplary DuST combinations include, without limitation, anti-STEAP1/anti-CD3 and anti-CD28/anti-EpCAM, anti-CD3/anti-NKG2D and anti-CD28/anti-EpCAM, or anti-STEAP1/anti-CD3 and anti-CD28/anti-NKG2D bispecific binding reagents. In some cases, DuST comprises providing an anti-STEAP1/anti-CD3 bispecific binding reagent and an anti-CD28/anti-EpCAM bispecific binding reagent.

**Table 1**

(DuST combinations)

| CD28 x TAA#2: \ TAA#1 x CD3: | ABCC4 | ANO7 | EPCAM | FOLH1 | OR51E1 | OR51E2 | PSCA | SLC30A4 | SLC45A3 | SPON2 | STEAP1 | STEAP2 | TARP | TMEFF2 | TMPRSS2 | NKG2D-L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ABCC4 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| ANO7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| EPCAM | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| FOLH1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| OR51E1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| OR51E2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| PSCA | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| SLC30A4 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| SLC45A3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| SPON2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| STEAP1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| STEAP2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TARP | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TMEFF2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TMPRSS2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| NKG2D-L | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

**Table 1. DuST combinations.** Depicted are examples of combinations of bispecific binding reagents. Each "+" (plus) refers to a combination in which the CD3-binding reagent further binds the prostate tumor-associated antigen (TAA) represented by its gene symbol above the "+" (TAA#1) and the CD28-binding reagent further binds the prostate TAA represented by its gene symbol to the left of the "+" (TAA#2). NKG2D-L means NKG2D ligand and includes antigens represented by the gene symbols MICA, MICB, ULBP1, ULBP2, ULBP3, RAET1E, RAET1G, and RAET1L.

**[0119]** In some instances, it is desirable to apply more than two different bispecific binding reagents to a patient having prostate cancer. In some embodiments of the current invention, collectively referred to as MuST (Multiple Stimulations per T cell) technology, the current invention provides a treatment regimen for the patient comprising one or more structurally distinct CD3-binding bispecific binding reagents and one or more structurally distinct CD28-binding bispecific binding reagents. A particularly preferred combination consists of an anti-

STEAP1/anti-CD3 bispecific binding reagent, an anti-CD28/anti-EpCAM bispecific binding reagent, and an anti-CD3/anti-NKG2D bispecific binding reagent.

**[0120]** Bispecific binding reagents, or combinations thereof, can be produced and stored, and/or prepared for administration to a cell or a patient using methods known in the art. Nonlimiting examples of suitable formulations and storage conditions are described, *e.g.*, in US 2010/0303827, which is hereby incorporated by reference.

**[0087]** In other aspects, the present invention provides a kit comprising one or more of the bispecific binding reagents described herein. In some cases, the kit contains a pharmaceutical excipient.

**C. Pharmaceutically Acceptable Excipients and Pharmaceutical Compositions**

**[0121]** The foregoing binding reagents, nucleic acids, vectors, or host cells, or any combination thereof can be provided or produced in a pharmaceutically acceptable excipient or carrier. The term "carrier" refers to a typically inert substance used as a diluent or vehicle for a diagnostic or therapeutic agent. The term also encompasses a typically inert substance that imparts cohesive qualities to the composition. Physiologically acceptable carriers can be liquid, *e.g.*, physiological saline, phosphate buffer, normal buffered saline (135-150 mM NaCl), water, buffered water, 0.4% saline, 0.3% glycine, glycoproteins to provide enhanced stability (*e.g.*, albumin, lipoprotein, globulin, *etc.*), and the like. Since physiologically acceptable carriers are determined in part by the particular composition being administered as well as by the particular method used to administer the composition, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*See, e.g.*, Remington's Pharmaceutical Sciences, 18$^{th}$ ed., 1990).

**[0122]** The compositions of the present invention may be sterilized by conventional, well-known sterilization techniques or may be produced under sterile conditions. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, and the like, *e.g.*, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, and triethanolamine oleate. Sugars can also be included for stabilizing the compositions, such as a stabilizer for lyophilized antibody compositions.

**[0123]** Dosage forms can be prepared for mucosal (*e.g.*, nasal, sublingual, buccal, or rectal), parenteral (*e.g.*, subcutaneous, intravenous, intramuscular, or intraarterial injection, either bolus or infusion), oral, or transdermal administration to a patient. Examples of dosage forms include, but are not limited to: dispersions; suppositories; ointments; cataplasms (poultices); pastes; powders; dressings; creams; plasters; solutions; patches; aerosols (*e.g.*, nasal sprays or inhalers); gels; liquid dosage forms suitable for oral or mucosal administration to a patient, including suspensions (*e.g.*, aqueous or non-aqueous liquid suspensions, oil-in-water emulsions, or a water-in-oil liquid emulsions), solutions, and elixirs; liquid dosage forms suitable for parenteral administration to a patient; and sterile solids (*e.g.*, crystalline or amorphous solids) that can be reconstituted to provide liquid dosage forms suitable for parenteral administration to a patient.

**[0124]** Injectable (*e.g.*, intravenous) compositions can comprise a solution of the bispecific binding reagent or bispecific binding reagent-targeted composition suspended in an acceptable carrier, such as an aqueous carrier. Any of a variety of aqueous carriers can be used, *e.g.*, water, buffered water, 0.4% saline, 0.9% isotonic saline, 0.3% glycine, 5% dextrose, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Often, normal buffered saline (135-150 mM NaCl) will be used. The compositions can contain pharmaceutically acceptable auxiliary substances to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, *e.g.*, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* In some embodiments, the bispecific binding reagent-targeted composition can be formulated in a kit for intravenous administration.

**[0125]** Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intratumoral, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Injection solutions and suspensions can also be prepared from sterile powders, granules, and tablets. In the practice of the present invention, compositions can be administered, for example, by intravenous infusion, topically,

intraperitoneally, intravesically, or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of compositions can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials.

**[0126]** The composition of choice, alone or in combination with other suitable components, can be made into aerosol formulations ("nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, and nitrogen.

**[0127]** The pharmaceutical preparation can be packaged or prepared in unit dosage form. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the active component, *e.g.*, according to the dose of the therapeutic agent or concentration of bispecific binding reagent. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation. The composition can, if desired, also contain other compatible therapeutic agents.

## III. Methods

### A. Methods of Designing Bispecific Binding Reagents

**[0128]** In some embodiments, the present invention provides a method of designing and/or constructing a bispecific binding reagent. Design of bispecific binding reagents can include identification of one or more suitable target antigens (*e.g.*, target antigens expressed by an immune cell or expressed by a prostate cancer cell). Since prostate cancer patients often have their prostate gland harboring the primary tumor(s) removed to minimize the risk of metastatic disease, the potentially remaining cancer cells may be eliminated using bispecific antibodies that either target prostate cancer-specific antigens or antigens expressed on both normal and malignant prostate cells. Thus, for bispecific antibody-based approaches aimed at preventing metastatic outgrowth, prostate tissue-specific antigens may also be considered *bona fide* prostate tumor-associated antigens and serve as therapeutic targets.

**[0129]** Prostate tumor-associated antigens suitable for use as targets of a bispecific binding reagent can be identified from mRNA expression studies, immunohistochemistry, or any other method for identifying antigens that are likely to be expressed (*e.g.*, specifically expressed or highly expressed) on the surface of a prostate cell or a prostate cancer cell. In some embodiments, the present invention comprises bispecific antibodies targeting prostate tumor-associated antigens identified *in silico* using publically available mRNA expression

data. A nonlimiting strategy to identify such tumor-associated antigens involves a meta-analysis approach using data accessible via the Gene Expression across Normal and Tumor tissue (GENT) database (*available at* medical-genome.kribb.re.kr/GENT/) (Shin et al., *Cancer Inform*, 10:149-157, 2011) containing expression levels of thousands of genes in thousands of microarray samples deposited in databases such as Gene Expression Omnibus (GEO, NCBI). Gene symbols (NCBI) of transcripts identified by several means as encoding putative prostate tumor-associated antigens may serve as GENT input. Since the GENT database provides mRNA expression data, protein expression patterns may differ from those of the transcripts. *In silico* validation at the protein level may be attempted via the human protein atlas (*available at* proteinatlas.org/). In some cases, the human protein atlas can be used to identify suitable target antigens directly.

**[0130]** In certain aspects, bispecific binding reagents of the current invention may be designed by identifying suitable tumor-associated antigens in a database such as the GENT database with gene symbols of transcripts pre-selected from the Tissue-specific Gene Expression and Regulation (TiGER) (Liu et al., *BMC Bioinformatics*, 9:271, 2008; *available at* bioinfo.wilmer.jhu.edu/tiger/) and/or the Oncogenomics (Son et al., *Genome Res*, 15(3):443-450, 2005; *available at* pob.abcc.ncifcrf.gov/cgi-bin/JK) Normal Tissue Database. Both the TiGER and the Oncogenomics databases allow, for the skilled artisan, straightforward data analyses. Due to the smaller sample sizes compared to GENT, prostate expression levels relative to a variety of other normal tissues can be determined following data download and processing using spreadsheet-based approaches, and/or, for the Oncogenomics databases, via online software (*available at* pob.abcc.ncifcrf.gov/cgi-bin/JK). Alternatively, TiGER or the Oncogenomics database can be used to identify suitable target antigens directly.

**[0131]** GENT comprises a feature for basic visualization of the gene expression profiles for user-defined input gene queries. For additional data display, GENT expression data may be copied into a spreadsheet and processed accordingly. For instance, it may be desirable to visualize the data in several ways, such as by *means of all* [mean expression levels of all samples (gsm_id) from all studies (gse_id) conducted for a given tissue] and *means/medians of means* [mean/median values of the mean expression levels of the individual studies (gse_id) conducted for a given tissue]. While processing the GENT data, the skilled artisan may realize that for some samples, GENT comprises "accidental *in silico* replicates" (>1

identical expression value per gsm_id). The problem may be solved by averaging the expression levels for replicate samples and considering corresponding replicate samples as only one sample with an averaged expression level.

**[0132]** mRNA expression data, such as publicly available mRNA expression data (*e.g.*, the expression data provided by GENT) may provide a means to judge whether an antigen should be considered a prostate tumor-associated antigen. One way to quantitatively assess whether an antigen may qualify as a prostate TAA is by calculating the mean prostate cancer expression level relative to the highest mean expression level among all normal nonprostatic male tissues (PCa/MaxN value) in the GENT database. A high PCa/MaxN value suggests prostate (cancer)-specific gene expression (**Table 2**). For some antigens, the PCa/MaxN value may not be suitable. In these instances, it may be appropriate to consider less stringent criteria for defining prostate TAAs. For instance, antigens with PCa/MaxN < 2 may still qualify as prostate TAAs if they are associated with high PCa/2ndMaxN values (mean prostate cancer expression level relative to the *second highest* mean expression level among all normal nonprostatic male tissues in the GENT dataset) (**Table 2**). For some further antigens, even the PCa/2ndMaxN criterion may be too stringent **(Tables 3 and 4)**. For example, prostate stem cell antigen (PSCA) (both PCa/MaxN and PCa/2ndMax substantially lower than 2) was detected in most primary prostate carcinomas. However, expression levels increased with advanced disease/disease progression (high Gleason score, advanced stage, bone metastasis) (Feldmann et al., *J Immunol*, 189(6):3249-3259, 2012; Gu et al., *Oncogene*, 19(10):1288-1296, 2000). PSCA was targeted with bispecific antibodies comprising a CD3-binding domain (Feldmann et al., *J Immunol*, 189(6):3249-3259, 2012). TMPRSS2 is another example of a prostate TAA with both PCa/MaxN and PCa/2ndMax < 2, yet documented prostate cancer association (Leshem et al., *PLoS One*, 6(7):e21650, 2011; Wilson et al., Biochem J, 388(Pt 3):967-972, 2005).

**Table 2**
(Prostate/prostate cancer-selective transcripts)

| **Gene Symbols** | PCa/MaxN* | PCa/2ndMaxN* | *See Note below<br>Descriptions/Prostate cancer association | References |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| **ABCC4** | 3.5 | 4.4 | Transmembrane protein expressed in normal and malignant prostate tissue. | Ho et al., *Prostate*, 68(13):1421-1429, 2008 |
| **ANO7** | 3.0 | 3.2 | Large splice form encodes membrane protein expressed in normal prostate and prostate cancer. | Cereda et al., *Cancer Immunol Immunother*, 59(1):63-71, 2010 |
| **FOLH1** | 6.1 | 6.6 | Transmembrane protein expressed in normal prostate and prostate cancer. | Liu et al., *Cancer Res*, 57(17):3629-3634, 1997 |
| **OR51E1** | 7.7 | 10.2 | G-protein coupled receptor. Protein up-regulated in prostate cancer. | Wang et al., *Prostate*, 66(8):847-857, 2006 |
| **OR51E2** | 41.0 | 45.4 | G-protein coupled receptor. Protein up-regulated in prostate cancer. | Wang et al., *Prostate*, 66(8):847-857, 2006 |
| **SLC45A3** | 10.3 | 11.4 | Transmembrane protein expressed in normal prostate and prostate cancer. | Xu et al., *Cancer Res*, 61(4):1563-1568, 2001 |
| **SPON2** | 2.5 | 3.1 | Secreted extracellular protein expressed in normal prostate and prostate cancer. | Parry et al., *Cancer Res*, 65(18):8397-8405, 2005 |
| **STEAP1** | 4.2 | 6.4 | Transmembrane protein expressed in normal prostate and prostate cancer. | Moreaux et al., *Biochem Biophys Res Commun*, 429(3-4):148-155, 2012 |
| **STEAP2** | 6.9 | 7.5 | Transmembrane protein expressed in normal prostate and prostate cancer. | Porkka et al., *Lab Invest*, 82(11):1573-1582, 2002; proteinatlas.org |
| **TARP** | 3.8 | 5.4 | Transmembrane protein expressed in normal prostate and prostate cancer. | Hillerdal et al., *Proc Natl Acad Sci U S A*, 109(39):15877-15881, 2012 |
| **TMEFF2** | 2.8 | 2.9 | Transmembrane protein expressed in normal prostate and prostate cancer. | Afar et al., *Mol Cancer Ther*, 3(8):921-932, 2004 |
| **TRPM8** | 4.5 | 33.6 | Transmembrane protein. Up-regulated expression in prostate cancer. | Valero et al., *PLoS One*, 7(12):e51825, 2012 |
| **SLC30A4** | 1.9 | 4.5 | Expressed in PCa, but decreasing expression in progression from benign to invasive localized to metastatic PCa. | Henshall et al., *Oncogene*, 22(38):6005-6012, 2003 |
| *Due to the complexity of the data in the GENT datasets, which include apparent "***in silico*** duplication" errors, indicated PCa/MaxN and PCa/2ndMaxN values are considered estimated values. | | | | |

**Table 3**
(Known prostate cancer antigens with low PCa/MaxN and PCa/2ndMaxN scores)

| Gene Symbols | PCa/MaxN* | PCa/2ndMaxN* | * See Note below<br>Descriptions/Prostate cancer association | References |
|---|---|---|---|---|
| **PSCA** | 0.05 | 0.11 | GPI-anchored cell surface antigen. Expression in >80% of primary prostate cancers. Up-regulated at advanced stages. | Feldmann et al., *J Immunol*, 89(6):3249-3259, 2012; Gu et al., *Oncogene*, 19(10):1288-1296, 2000 |
| **EPCAM** | 0.27 | 0.59 | Transmembrane protein. Overexpressed in primary prostate cancer tissues and lymph node metastases. | Ni et al., *Int J Biochem Cell Biol*, 45(12):2736-2748, 2013 |
| **TMPRSS2** | 1.3 | 1.8 | Transmembrane protein expressed in normal prostate and prostate cancer. | Wilson et al., *Biochem J*, 388(Pt 3):967-972, 2005 |
| *Due to the complexity of the data in the GENT datasets, which include apparent "***in silico*** duplication" errors, indicated PCa/MaxN and PCa/2ndMaxN values are considered estimated values. | | | | |

**Table 4**
(NKG2D Ligands)

| Gene Symbols | PCa/MaxN* | PCa/2ndMaxN* | * See Note below<br>Descriptions/Prostate cancer association | References |
|---|---|---|---|---|
| **MICA, MICB** | Not found | Not found | MIC (MICA, MICB) up-regulated in early PCa and PCa cell lines. | Wu et al., *J Clin Invest*, 114(4):560-568, 2004 |
| **ULBP1 (RAET1I)** | 0.18 | 0.19 | Protein expressed in PCa tissue (low). | proteinatlas.org |
| **ULBP2 (RAET1H)** | 0.10 | 0.13 | Protein expressed in PCa tissue (low). | proteinatlas.org |
| **ULBP3 (RAET1N)** | 0.45 | 1.16 | RNA expressed in human prostate cancer PC-3 cells. | proteinatlas.org |
| **RAET1E (ULBP4)** | 0.05 | 0.05 | Protein expressed in PCa tissue (medium to high levels). | proteinatlas.org |
| **RAET1G (ULBP5)** | Not found | Not found | Protein expressed in PCa tissue (medium to high levels). | proteinatlas.org |

| | | | | |
|---|---|---|---|---|
| **RAET1L (ULBP6)** | Not found | Not found | RNA expressed in PC-3 cells (low abundance). | proteinatlas.org |
| *Due to the complexity of the data in the GENT datasets, which include apparent "***in silico*** duplication" errors, indicated PCa/MaxN and PCa/2ndMaxN values are considered estimated values. | | | | |

**[0133]** In certain aspects, the present invention may require *a priori* knowledge on whether an antigen may qualify as a prostate TAA. Such *a priori* knowledge may obsolete the need to pre-screen databases with small sample sizes such as TiGER or the Oncogenomics Normal Tissue Database. In such instances, the GENT database may serve as a tool to validate prostate/prostate cancer-specific gene expression. For example, STEAP1 is a well-known prostate TAA (Moreaux et al., *Biochem Biophys Res Commun*, 429(3-4):148-155, 2012). **Figure 3A** illustrates that GENT data confirms such a prostate/prostate cancer-specific expression profile for *STEAP1*.

**[0134]** Suitable immune cell antigens are known in the art. For example, any immune cell antigen having a cluster of differentiation classification (CD) may be suitable. As a specific example, any antigen expressed on the surface of a T cell, an NK cell, or an NKT cell can be selected as an immune cell target antigen for a bispecific binding reagent of the present invention. In a preferred embodiment, the target immune cell antigen is selected from the group consisting of CD3 delta (CD3D), CD3 epsilon (CD3E), CD3 zeta (CD247, CD3Z), CD28, CD137 (4-1BB, TNFRSF9), and CD134 (OX40, TNFRSF4). In another embodiment, the target immune cell antigen is NKG2D.

**[0135]** In some embodiments, nucleic acid sequences encoding known polypeptides that are specific for an immune cell antigen or a prostate cancer antigen can be utilized to generate a bispecific binding reagent. For example, in some cases, antibodies are known that specifically bind a target antigen (*e.g.*, specifically bind a target antigen on a prostate cell, a prostate cancer cell or an immune cell). The polypeptide sequence of the antigen binding region (*e.g.*, $V_H$ and $V_L$ sequences) can then be incorporated into a nucleic acid encoding a bispecific binding reagent. The bispecific binding reagent can include a nucleic acid encoding a linker between a nucleic acid encoding a first domain specific for an antigen expressed by an immune cell and a second nucleic acid encoding a second domain specific for an antigen expressed by a prostate cancer cell.

**[0136]** In some cases, a known interaction can be exploited in the design of a bispecific binding reagent. For example, stress antigens, such as MICA, MICB, RAET1E, RAET1G,

RAET1L, ULBP1, ULBP2, or ULBP3 are often expressed on prostate cancer cells. Such stress antigens are known to be ligands of the receptor NKG2D. Thus, a nucleic acid encoding the ligand binding domain of NKG2D can be incorporated into a bispecific binding reagent.

**[0137]** Alternatively or in addition, novel binding reagents can be generated, and their sequences can be incorporated into a bispecific binding reagent. For example, a suitable host can be immunized with prostate cancer cells, or an extract thereof (*e.g.*, cell extract or a purified antigen). Serum from the host can be assayed for production of suitable antibodies. The antibodies can be purified and/or cloned and the polypeptide or nucleic acid sequence utilized to design a domain of a bispecific binding reagent. As another example, a library of candidate binding reagents (*e.g.*, an scFv or Fab phage display library, a ribosome display library, a library of affimers or aptamers, a SELEX library, *etc.*) can be screened against a candidate antigen, and suitable binding sequences identified and incorporated into a bispecific binding reagent.

**[0138]** Nucleic acid sequences encoding antigen-specific polypeptides can be incorporated into a bispecific binding reagent using standard molecular biology tools. For example, a series of overlapping oligonucleotides encoding the antigen-specific polypeptides and one or more linkers can be assembled using standard nucleic acid amplification techniques.

### B. Methods of Production of Bispecific Binding Reagents

**[0139]** Bispecific binding reagents described herein can be produced using methods known in the art. In some embodiments, a bispecific binding reagent can be produced by culturing an isolated host cell that has been transformed with a nucleic acid encoding a bispecific binding reagent under conditions suitable for the expression of the bispecific binding reagent; and recovering the produced bispecific binding reagent from the cell or from the culture.

**[0140]** Suitable host cells include prokaryotic and eukaryotic expression systems. For example, the antibody can be produced in or by bacteria (*e.g.*, *E. coli*, *P. mirabilis*), fungi (*e.g.*, *S. cerevisiae*, *P. Pastoria*, *T. reesei*), plants or plant cells, insects or insect cells (*e.g.*, SF-9, SF21, Hi-5), or mammalian cells. In a preferred embodiment, eukaryotic expression systems are utilized to enable efficient disulfide bond formation and/or glycosylation. In some embodiments, the expression system is a mammalian cell expression system, such as a hybridoma, or a CHO or 293 cell expression system. Many such systems are widely available from commercial suppliers.

### C. Methods of Administration

**[0141]** In some embodiments, compositions can be administered to cells *in vitro*. For example, identification of cells from a sample provided by a patient that are effectively inhibited by compositions of the invention can suggest administering the inhibitory composition to a patient. In some cases, *in vitro* administration of one or more bispecific binding reagents to target cells of a prostate cancer cell line (*e.g.*, DU145, LNCaP, PC3, C4/C5, C4-2, C4-2B, *etc.*) can be utilized to identify bispecific binding reagents that effectively kill or inhibit such cells. *In vitro* administration can include administering one or more compositions of the invention to prostate cancer cells in the presence of effector cells (*e.g.*, immune cells such as T cells, NKT cells, or NK cells). Inhibition of target cells can be assayed by detecting killing of the target cells, decrease in growth or division of the target cells, or a reduction of metabolic activity of the target cells using methods known in the art.

**[0142]** Compositions of the invention can also be administered to an individual. For example, compositions of the invention can be administered to treat prostate cancer. In some embodiments, the method of treatment comprises administering to an individual a therapeutically effective amount of one or more bispecific binding reagents. For example, a therapeutically effective amount of one or more bispecific binding reagents in a pharmaceutically acceptable carrier can be administered. In some embodiments, the individual has been diagnosed with prostate cancer. In some embodiments, the individual is receiving or has received cancer therapy, *e.g.*, surgery, radiotherapy, or chemotherapy. In some embodiments, the individual has been diagnosed with metastatic prostate cancer, castration-resistant prostate cancer, androgen-independent prostate cancer, or prostate cancer that is resistant to one or more conventional treatments.

**[0143]** In some embodiments, the method further comprises monitoring the individual for progression of the prostate cancer. In some embodiments, the dose of the one or more bispecific binding reagents for each administration is determined based on the therapeutic progress of the individual, *e.g.*, where a higher dose of the composition is administered if the individual is not responding sufficiently to therapy.

**[0144]** Embodiments of the present invention relating to the stimulation of T cells via multiple (co)receptors are referred to as "Multiple Stimulations per T cell" (MuST) technology or, if carried out with only two types of bispecific binding reagents of which one

targets CD3 and the other a T cell co-receptor, as "Dual Stimulation of T cell" (DuST) technology (**Figure 4**).

**[0145]** In some embodiments, the invention can include a composition containing one or more structurally distinct bispecific binding reagents and a physiologically (*i.e.*, pharmaceutically) acceptable carrier. The term "carrier" refers to a typically inert substance used as a diluent or vehicle for a diagnostic or therapeutic agent. The term also encompasses a typically inert substance that imparts cohesive qualities to the composition. Physiologically acceptable carriers can be liquid, *e.g.*, physiological saline, phosphate buffer, normal buffered saline (135-150 mM NaCl), water, buffered water, 0.4% saline, 0.3% glycine, glycoproteins to provide enhanced stability (*e.g.*, albumin, lipoprotein, globulin, *etc.*), and the like. Since physiologically acceptable carriers are determined in part by the particular composition being administered as well as by the particular method used to administer the composition, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*See, e.g.*, Remington's Pharmaceutical Sciences, 18$^{th}$ ed., 1990).

**[0146]** The one or more bispecific binding reagents can be administered by injection or infusion through any suitable route including but not limited to intravenous, subcutaneous, intramuscular, intratumoral, or intraperitoneal routes.

**[0147]** An example of administration of a pharmaceutical composition includes storing the bispecific binding reagent at 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, 5, 7, or 10 mg/ml in a sterile aqueous solution at 4 °C, and diluting it to a concentration between about 25 microgram/ml and about 1 mg/ml in an injection buffer prior to administration to the patient. A suitable injection buffer comprises citrate (between about 25 and about 30 mM), trehalose dehydrate (between about 6 and about 15% (w/v)), lysine HCl (between about 50 and about 200 mM), and polysorbate 80 (between about 0.02 and about 0.1% (w/v)), and has a pH of about 7.0. While monitoring dose-limiting toxicity, the bispecific binding reagent(s) can be administered, for instance, once, twice, or three times per week by intravenous infusion over the course of 2-4 hours at a dose per infusion of, for instance, around 13 to around 130 microgram per m$^2$ (approximately 0.35 to approximately 3.5 microgram/kg using a $K_m$ factor of 37 as described by Reagan-Shaw et al., *FASEB J*, 22(3):659-661, 2008). In other embodiments, while monitoring dose-limiting toxicity, the bispecific binding reagent can be administered by continuous intravenous infusion via a portable minipump at a dose between around 0.5 microgram/m$^2$ per day and around 1 mg/m$^2$ per day (approximately 13.5

ng/kg/day to approximately 27 microgram/kg/day using a $K_m$ factor of 37). Continuous infusion can be performed in, for instance, 4 cycles, each with 4 weeks "on" (infusion) and 2 weeks "off" (no infusion). In further embodiments, the bispecific binding reagent is stored as a lyophilized formulation at 4 °C and reconsitutied in a suitable diluent (*e.g.*, a pH-buffered solution such as phosphate-buffered saline) prior to infusion. In still other embodiments, the administration procedure is via subcutaneous bolus injection.

**[0148]** In some cases, the bispecific binding reagent, or a combination of bispecific binding reagents, are administered to a patient as described in Nagorsen et al. (*Pharmacology & Therapeutics* 136 (2012) 334–342), incorporated herein by reference in its entirety for all purposes. In some cases, a bispecific binding reagent, or combination thereof, *e.g.*, TCT001 and TCT002 are dosed at about 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 2.5, 4, 5, 7, 10-fold any of the doses described in Nagorsen et al.

**[0149]** The dose of the one or more bispecific binding reagents can be chosen in order to provide effective therapy for the patient and is in the range of less than 5 microgram/$m^2$/day to about 1 g/$m^2$/day or in the range of 50 microgram/$m^2$/day - 100 mg/$m^2$/day per patient. In some cases, the dose is in the range of 5 microgram/$m^2$/day - 1 mg/$m^2$/day, or of approximately 50 microgram/$m^2$/day - 10 mg/$m^2$/day per patient. The dose may be repeated at an appropriate frequency which may entail continuous infusion by means of an external or implanted minipump or may be in the range of once or twice per day to once every three months, depending on the pharmacokinetics of the one or more bispecific binding reagents (*e.g.*, half-life of the bispecific binding reagents in the circulation) and the pharmacodynamic response (*e.g.*, the duration of the therapeutic effect of the one or more bispecific binding reagents). In some embodiments, the *in vivo* half-life of the bispecific binding reagent(s) is between about 7 and about 25 days and bispecific binding reagent(s) dosing is repeated between once per week and once every 3 months. In other embodiments, the *in vivo* half-life of the bispecific binding reagent(s) is between approximately 1 and approximately 2 hours and the bispecific binding reagent(s) are continuously infused for up to 4 weeks per treatment cycle of 6 weeks (4 weeks infusion followed by 2 weeks without) for a total of up to 5 treatment cycles.

**[0150]** The choice of target epitope may influence the effective dose, and may be used to modulate the system. In particular, should several bispecific binding reagents bind the same prostate cell target antigen (but differ in their T cell-stimulating domains), competition at the

target sites may require higher bispecific binding reagent concentrations to achieve a useful therapeutic effect. On the other hand, using the same target cell-binding domain may allow fine-modulation of the T cell stimulation by changing the relative amounts of the different T cell-stimulating domains at the target sites. For instance, it may be beneficial to first permit a substantial target site-restricted stimulation of CD28, and, upon T cell activation, to maintain the activated state, by competing out the anti-CD28 construct with a CD137 (4-1BB)-stimulating bispecific binding reagent with the same target cell-binding domain.

**[0151]** Administration can be periodic. Depending on the route of administration, the dose can be administered, *e.g.*, once every 1, 3, 5, 7, 10, 14, 21, or 28 days or longer (*e.g.*, once every 2, 3, 4, or 6 months). In some cases, administration is more frequent, *e.g.*, 2 or 3 times per day. In other cases, administration is continuous by means of an external or implantable minipump for, *e.g.*, 4 weeks per treatment cycle for a total of 4 treatment cycles. The patient can be monitored to adjust the dosage and frequency of administration depending on therapeutic progress and any adverse side effects, as will be recognized by one of skill in the art.

**[0152]** Thus, in some embodiments, additional administration is dependent on patient progress, *e.g.*, the patient is monitored between administrations. For example, after the first administration or round of administrations, the patient can be monitored for rate of tumor growth, recurrence (*e.g.*, in the case of a post-surgical patient), or general disease-related symptoms such as weakness, pain, nausea, *etc.*

**[0153]** In therapeutic use for the treatment of prostate cancer, a composition containing one or more bispecific binding reagents can be administered at the initial dosage of about 0.05 microgram/$m^2$ per day to about 1 g/$m^2$ per day and adjusted over time. A daily dose range of about 0.5 microgram/$m^2$ to about 100 mg/$m^2$, or about 0.5 microgram/$m^2$ to about 10 mg/$m^2$, or about 0.5 microgram/$m^2$ to about 1 mg/$m^2$, or about 0.5 microgram/$m^2$ to about 100 microgram/$m^2$, or about 0.5 microgram/$m^2$ to about 50 microgram/$m^2$ can be used. The dosage can be varied depending upon the requirements of the patient, the severity of the condition being treated, and the targeted composition being employed. For example, dosages can be empirically determined considering the type and stage of prostate cancer diagnosed in a particular patient.

**[0154]** The dose administered to a patient can be selected to be sufficient to affect a beneficial therapeutic response in the patient over time. The size of the dose can also be

determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular targeted composition in a particular patient, as will be recognized by the skilled practitioner. The dosage can further depend on the affinity of the one or more bispecific binding reagents for their target antigens. For example, compositions comprising bispecific binding reagents which bind with high affinity to their target cells are expected to be therapeutically effective at lower doses than those comprising low-affinity bispecific binding reagents.

**[0155]** Other factors expected to affect the therapeutically effective dose of bispecific binding reagents include the accessibility of both the reagents and the immune (effector) cells to the target cells. Even though it is reasible to assume that also solid tumors such as prostate cancers can be successfully treated with bispecific binding reagents by clearing cancer cells first in the regions closest to the tumor vasculature then in those further away, cancers not associated with tightly packed tumor masses (*e.g.*, B cell chronic lymphocytic leukemia) likely require lower doses of the bispecific binding reagents to achieve a therapeutic effect, even if the affinities of the bispecific binding reagents for the target antigens are identical. Similarly, therapeutic strategies aimed at preventing tumor recurrence or metastasis following removal of the primary tumor(s) (*e.g.*, by radical prostatectomy in the case of prostate cancer) likely also require lower therapeutic doses than treatments for large, established, tumors, since fewer cancer cells need to be cleared.

**[0156]** All patents, patent applications, and other publications, including GenBank Accession Numbers, cited in this application are incorporated by reference in their entirety for all purposes.

## EXAMPLES

### Example 1: TCT001 (anti-STEAP1scFv/anti-CD3scFv)

*A. Introduction*

**[0157]** TCT001 (anti-STEAP1scFv/anti-CD3scFv) is a taFv designed to inhibit prostate cancer cells by means of crosslinking the prostate tumor-associated antigen (TAA) STEAP1 to CD3 expressed on cytotoxic T cells (**Figure 2C**).

**[0158]** TCT001 comprises an anti-CD3 epsilon single-chain variable fragment (scFv) domain with variable regions having the sequences set forth in SEQ ID NOs: 6 and 7. TCT001 further comprises anti-STEAP1 $V_L$ and $V_H$ domains having the sequences set forth in SEQ ID NOs: 3 and 4.

**[0159]** Following *in vitro* gene synthesis and cloning into a suitable expression vector, the TCT001 protein may be generated in, for instance CHO (Chinese Hamster Ovary) cells, and purified from the cell culture supernatant by immobilized-metal affinity chromatography (*See* (Block et al., *Methods Enzymol*, 463:439-473, 2009) taking advantage of the C-terminal hexa histidine tag. A corresponding optical density (OD280) plot for TCT001 elution fractions of example preparations is shown in **Figure 5**. Similarly, SDS-PAGE and Western blot data for two example preparations are shown in **Figure 6**.

**[0160]** The cytotoxic potential of one or more bispecific binding reagents such as TCT001 is assessed in T cell-mediated cytotoxicity assays using LNCaP and NCI-H23 cells as target cells. Here, a non-limiting example comprising a series of distinct experiments aimed at obtaining half-maximal effective concentration ($EC_{50}$) values for T cell-binding bispecific antibodies such as TCT001 is presented.

**[0161]** Bispecific binding reagents can permit the selective ablation of human prostate cancer cells *in vitro*. STEAP1 is highly expressed on the cell surface of the human prostate cancer cell line LNCaP. Compared to LNCaP cells, human lung cancer NCI-H23 (H23) cells seem to express substantially lower STEAP1 mRNA and protein levels. Fold-change differencs of the transcript levels may be calculated from the GENT U133A data (*available* at medical-genome.kribb.re.kr/GENT/) (Shin et al., *Cancer Inform*, 10:149-157, 2011): Based on four H23 samples (gsm_id: san_103, GSM171868, GSM372768, and GSM136306) and two (gsm_id: GSM310084 and san_630) LNCaP/LNCaP Clone FGC samples, STEAP1 mRNA levels are 25-fold higher in LNCaP than in H23 cells. By comparing the STEAP1 protein levels of H23 cells shown in Figure 3A of Hayashi et al., *J Transl Med*, 9:191, 2011 with those of LNCaP cells depiced in Figure 6A of WO 01/40276 (PC3 as "common denominator" reference sample), STEAP1 protein levels also appear to be substantially higher in LNCaP than in H23 cells. Given such differences, one would assume that TCT001 mediates a higher degree of cytotoxicity towards LNCaP than towards NCI-H23 cells. This hypothesis is confirmed by incubating serially diluted TCT001 with pre-activated human T cells and LNCaP or NCI-H23 target cells at a pre-established effector to target (E/T) cell ratio of 20:1. After four hours of treatment, cell viability was measured via a nonradioactive enzymatic assay.

**[0162]** The concentration of an anti-cancer agent that lyses 50% of the tumor cells relative to the maximal number of cancer cells that can be killed by the drug (at higher

concentrations) is referred to as $EC_{50}$ value. With an $EC_{50}$ value towards LNCaP cells of around 20 picomolar (1.2 ng/ml), and towards NCI-H23 of approximately 800 picomolar (48.6 ng/ml), TCT001 induced a strong STEAP1-selective cytotoxic effect.

**[0163]** $EC_{50}$ values can be determined using pre-established optimal effector:target (E/T) cell ratios. To identify such optimal E/T cell ratios, a constant number of target cells may be treated with a constant concentration of TCT001 and variable numbers of effector T cells. With the E/T ratio yielding the highest degree of specific cytotoxicity, dose-response studies may thereafter be conducted. Half-maximal effective concentration ($EC_{50}$) values may be determined by fitting the data to a Sigmoidal dose-response curve via non-linear regression using commonly available software such as Graphpad Prism.

*B. Materials and Methods*

*1. Reagents*

**[0164]** Consumables useful to assess the cytotoxic potential of bispecific binding reagents such as TCT001 may comprise Fetal Bovine Serum (FBS), RPMI-1640, Phosphate-Buffered Saline (PBS), Penicillin-Streptomycin, Phenol red-free MEM medium, 10 cm dishes, 96-well plates, recombinant human IL-2 (*e.g.*, Jiangsu Kingsley Pharmaceutical Co., Ltd., Production Lot#1205103), LNCaP cells (ATCC, cat# CRL-1740), NCI-H23 cells (ATCC, cat# CRL-5800), effector cells (CD3+) (neg. selected, e.g., 5-10 Mio/vial, Astarte Biologics, LLC, cat-lot#1017-1754OC12), T Cell Activation/Expansion Kit (Miltenyi Biotec Inc., cat# 130-091-441), Human AB Serum (*e.g.*, Innovative Research, cat# IPLA-SERAB), and a kit to measure lactate dehydrogenase (LDH) release (*e.g.*, Roche, cat# 11644793001).

*2. TCT001 Sequences*

SEQ ID NO: 1 (TCT001 PROTEIN SEQUENCE)
SEQ ID NO: 2 (TCT001 DNA SEQUENCE)

**FROM N- TO C-TERMINUS IN THIS ORDER:**

SEQ ID NO: 9 (SIGNAL PEPTIDE)
SEQ ID NO: 3 (ANTI-STEAP1 $V_L$)
SEQ ID NO: 5 (INTRA-scFv LINKER)
SEQ ID NO: 4 (ANTI-STEAP1 $V_H$)
SEQ ID NO: 11 (INTER-scFv LINKER)
SEQ ID NO: 6 (ANTI-CD3 $V_H$)
SEQ ID NO: 8 (INTRA-scFv LINKER)
SEQ ID NO: 7 (ANTI-CD3 $V_L$)
SEQ ID NO: 10 (HEXA HISTIDINE TAG)

*3. Culturing Effector T cells*

**[0165]** CD3+T cells are obtained from a commercial vendor, or magnetically isolated from whole blood/peripheral blood mononuclear cells (PBMCs) using magnetic beads conjugated to anti-CD3 antibodies. CD3+ cells are activated/expanded in RPMI-1640 medium supplemented with 10% human AB serum, and magnetic beats coated with anti-CD2, anti-CD3, and anti-CD28 antibodies (T Cell Activation/Expansion Kit, Miltenyi Biotec Inc.). For the expansion phase, the medium contains IL-2, and optionally penicillin-streptomycin. The protocol and recommendations from the T Cell Activation/Expansion Kit (Miltenyi Biotec Inc.) are followed. A T cell growth curve of CD3+ cells obtained from a commercial vendor and stimulated via the T Cell Activation/Expansion Kit is depicted in **Figure 7A**.

*4. Optimization of the E/T Ratio*

**[0166]** After T cell culture initiation, T cell counts are determined every 1 to 2 or 3 days. Target cells are seeded in a 96-well plate in triplicates at least one day before the cytotoxicity experiment is initiated. LNCaP or NCI-H23 target cells are seeded at 10000 cells per 100 µl per well.

**[0167]** The principle steps of the cytotoxicity experiment are as follows: **1.** Count target cells from several wells and calculate the total number of T cells needed. **2.** Remove a suitable aliquot of the T cell culture, centrifuge, and then suspend the T cell pellet in RPMI-1640 medium supplemented with 10% heat-inactivated FBS without antibiotics. Alternatively, the centrifugation step is substituted by magnetically removing the beads. Optionally, the cells are washed in plain RPMI-1640 medium or PBS prior to suspending them in RPMI-1640 medium supplemented with 10% heat-inactivated FBS without antibiotics. **3.** Suitable T cell dilutions are prepared in RPMI-1640 medium supplemented with 10% heat-inactivated FBS without antibiotics for several E/T ratios (*e.g.*, 20, 10, and 5), then carefully and slowly an appropriate volume of each T cell dilution is added to the target cells. **4.** TCT001 or PBS (control) is added for a final volume of no more than 200 µl per well. A suitable final IL-2 concentration is 20 U/mL. **5.** The cells are incubated at 37 °C / 5% $CO_2$. **6.** For instance, after 4 or more hours of treatment, a cytotoxicity assay is performed, *e.g.*, after microscopic confirmation of TCT001-associated target cell lysis. Cytotoxicity can be detected by measuring the release of lactate dehydrogenase (LDH) from damaged cells. For example, LDH activity in the medium can be detected by measuring enzymatic conversion of INT (iodotetrazolium chloride) (*e.g.*, Roche, cat# 11644793001).

The LDH activity is quantified by measuring the absorbance at 490 or 492 nm and at 650 nm (or another reference wavelength > 600 nm) on a microplate reader. The %(target cell lysis) values are calculated as described below.

*5. $EC_{50}$ Cytotoxicity Assay*

**[0168]** A dose-response study is conducted with a pre-determined optimal E/T ratio and a dose titration series of TCT001. The principle components of this type of experiment are described above.

**[0169]** The percentages of TCT001-mediated cell lysis is calculated according to Formula 2 wherein OD means the optical density (absorbance) at 492 nm minus the optical density at 650 nm, and OD(E+T) the OD of the control sample(s) comprising effector and target cells but lacking TCT001. OD(Maximum release) refers to the maximal LDH release from target cells following lysis of the cells with Triton X100, and OD(Minimum release) to the spontaneous basal LDH release of the target cells without the presence of effector cells and TCT001. The data is presented and analyzed via commonly available data analysis software (*e.g.*, GraphPad Prism). Formula 2: %(target cell lysis) = 100*[OD(Sample)-OD(E+T)]/[OD(Maximum release)-OD(Minimum release)].

*C. Results*

**[0170]** LNCaP and NCI-H23 target cells were incubated with T cells at E/T ratios of 20, 10 and 5 together with a constant amount of TCT001 (5 µg/mL) for 6 hours at 37 °C / 5% $CO_2$. Thereafter, LDH release was measured. As demonstrated in **Figure 7B**, TCT001 was most effective in eliciting T cell cytotoxicity towards LNCaP and NCI-H23 cells at an E/T ratio of 20.

**[0171]** The most effective E/T ratio (E/T = 20:1) was applied to dose-response studies with pre-activated T cells. LNCaP and NCI-H23 target cells were treated in triplicates with TCT001 at descending concentrations starting from 5 µg/mL for 4 and 24 hours at 37 °C / 5% $CO_2$. Thereafter, LDH release was measured. As demonstrated in **Figure 7C** and **Table 5**, after 4 hours of treatment, the $EC_{50}$ value for LNCaP cell lysis was 1.155 ng/ml (approx. 20 pM) and for NCI-H23 cell lysis 48.56 ng/ml (approx. 800 pM). The difference in the cytotoxic effect is likely explained by different STEAP1 target expression levels on LNCaP versus NCI-H23 cells. The 24-hour time point was associated with a high background lysis apparent in the absence of TCT001 but presence of effector cells and yielded no results.

**Table 5**: Dose-Response Curve Fitting Results (GraphPad Prism)

| | | NCI-H23 | LNCaP |
|---|---|---|---|
| Sigmoidal Dose-Response (variable slope) Best-fit values | | | |
| | Bottom % | 0 | 0 |
| | Top % | 39.8 | 65.44 |
| | $LOG_{EC50}$ | 1.686 | 0.06248 |
| | HILLSLOPE | 0.4613 | 0.5171 |
| | $EC_{50}$ (ng/mL) | 48.56 | 1.155 |
| Std. Error | | | |
| | TOP | 7.608 | 1.966 |
| | $LOG_{EC50}$ | 0.6281 | 0.1074 |
| | HILLSLOPE | 0.1635 | 0.0565 |
| 95% Confidence Intervals | | | |
| | TOP | 28.31-55.78 | 61.31-69.57 |
| | $LOG_{EC50}$ | 0.3667-3.006 | -0.1632-0.2882 |
| | HILLSLOPE | 0.1178-0.8048 | 0.3984-0.6358 |
| | $EC_{50}$ (ng/mL) | 2.327-1014 | 0.6867-1.942 |
| Goodness of Fit | | | |
| | Degrees of Freedom | 18 | 18 |
| | $R^2$ | 0.9099 | 0.9786 |
| | Absolute Sum of Squares | 384.7 | 282 |
| | Sy.x | 4.623 | 3.958 |
| | Constraints | Bottom 0 | Bottom 0 |
| Data | | | |
| | Number of X values | 7 | 7 |
| | Number of Y replicates | 3 | 3 |
| | Total Number of Values | 21 | 21 |
| | Number of Missing Values | 0 | 0 |

*D. Discussion*

**[0172]** With an $EC_{50}$ value of 1.2 ng/mL (around 20 pM) for LNCaP prostate cancer cell lysis, TCT001 exerted a similar or higher degree of cytotoxicity as BiTE constructs targeting solid tumors.

**[0173]** To compare $EC_{50}$ values among bispecific and other types of antibodies, it may be useful to convert "mass/volume" values (for instance, nanogram per mL) to "molar" concentrations (for example, picomolar). For taFv bispecific antibodies containing a first and a second scFv domain, this conversion may be carried out via estimated molecular weights of 60 kDa (60000 grams/mole).

**[0174]** $EC_{50}$ values for MEDI-565 (MT111), a CEA-targeting BiTE construct that entered clinical phase 1 testing for subjects with gastrointestinal adenocarcinomas, ranged from ≈0.2 (estimated from Figure 3 by Osada et al., *Br J Cancer*, 102(1):124-133, 2010) to ≈75 ng/ml (Peng et al., *PLoS One*, 7(5):e36412, 2012) (≈3 to ≈1250 pM). Solitomab (MT110), an EpCAM-targeting BiTE antibody associated with phase 1 evaluation in a clinical trial for various solid tumors, lysed MBA-MB-453 cells with an $EC_{50}$ value of around 0.54 ng/ml (≈9 pM) (Petsch et al., *MAbs*, 3(1):31-37, 2011). BiTE antibodies targeting the EphA2 or EGFR cell surface proteins, which are overexpressed/activated in multiple cancer types, were associated with $EC_{50}$ values in the approximate ranges of 1-9 ng/ml (17-150 pM) (EphA2) (Hammond et al., *Cancer Res*, 67(8):3927-3935, 2007) and 3.8-55 pg/ml (63-920 fM) (EGFR) (Lutterbuese et al., *Proc Natl Acad Sci U S A*, 107(28):12605-12610, 2010).

**[0175]** Also others have generated BiTEs targeting cell surface proteins associated primarily with prostate cancer, such as prostate-specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA). For instance, $EC_{50}$ values between 0.1 and 4 ng/ml (1.8 to 72 pM) were reported for AMG 212/BAY2010112 (Friedrich et al., *Mol Cancer Ther*, 11(12):2664-2673, 2012), which targets PSMA. Similarly, a PSCA-specific BiTE demonstrated cytotoxicity in the femtomolar range with $EC_{50}$ values likely in the low picomolar range (estimation from Figure 1 by Feldmann et al. (*J Immunol*, 189(6):3249-3259, 2012)).

**[0176]** Antibody-drug conjugates (ADCs) with variable sequences related to those of TCT001 have been described in U.S. Patent No. 8,436,147. In short, in cytotoxicity assays, such ADCs appear to lyse LNCaP target cells with $EC_{50}$ values of at best approximately 0.6 μg/mL (estimation from Figures 14C and 19C of U.S. Patent No. 8,436,147), which translates

to a concentration of no less than approximately 3.75 nM (assuming that an ADC molecule has a molecular weight of no more than 160 kDa). With an $EC_{50}$ value of approximately 20 pM, TCT001 exerted a substantially more potent cytotoxic effect towards LNCaP cells than these ADCs and thus demonstrates a major improvement in the art. Furthermore, in a clinical study, the anti-STEAP1 antibody-drug conjugate (ADC) DSTP3086S had an acceptable safety profile and demonstrated evidence of anti-tumor activity in patients with metastatic castration-resistant prostate cancer (Danila et al., *J Clin Oncol 31*, 2013 (suppl; abstr 5020), 2013 ASCO Annual Meeting). Therefore, approaches targeting STEAP1 with bispecific binding reagents to treat or prevent metastatic prostate cancer appear feasible.

**Example 2: TCT002 (anti-CD28scFv/anti-EpCAMscFv)**

*A. Introduction*

**[0177]** Whereas TCT001 was designed to target the prostate tumor-associated antigen (TAA) STEAP1 and the T cell antigen CD3, TCT002 is based on antibodies binding to EpCAM, a cell surface antigen expressed by a variety of cancers, and to the T cell antigen CD28. Given that the EpCAM expression levels may be high in cancer stem cells (Munz et al., *Cancer Res*, 69(14):5627-5629, 2009), TCT002 is an exceptionally effective anti-cancer agent.

**[0178]** TCT001 has demonstrated a substantial cytotoxic potential towards prostate cancer cells (**Figure 7**). Moreover, co-stimulation of cytotoxic T cells with TCT002 enhances this effect. Reasons include:

**[0179]** 1. Natural, MHC-based, T cell activation requires stimulation of CD3 and of co-receptors such as CD28. Without such co-stimulation, T cells may undergo apoptosis or become anergic/tolerant (Macian et al., *Curr Opin Immunol*, 16(2):209-216, 2004); Pardigon et al., *J Immunol*, 164(9):4493-4499, 2000; Ward, *Biochem J*, 318 ( Pt 2):361-377, 1996; Zhong et al., *Mol Ther*, 18(2):413-420, 2010). Thus, since TCT001 and TCT002 in combination activate both CD3 and CD28, treatment with both bispecific binding reagents in combination can result in a more robust anti-tumor effect than treatments carried out with only one of the bispecific binding reagents (**Figures 4, 9, and 10**).

**[0180]** 2. Since TCT001 and TCT002 target two different prostate TAAs and two different T cell antigens no competition for target binding occurs. This can translate into additive or synergistic prostate cancer cell killing effects.

*B. Materials and Methods*

*1. Reagents*

**[0181]** Consumables useful to assess the cytotoxic potential of bispecific binding reagents such as TCT002 may comprise Fetal Bovine Serum (FBS), RPMI-1640, Phosphate-Buffered Saline (PBS), Penicillin-Streptomycin, Phenol red-free MEM medium, 10 cm dishes, 96-well plates, recombinant human IL-2 (*e.g.*, Jiangsu Kingsley Pharmaceutical Co., Ltd., Production Lot#1205103), LNCaP cells (ATCC, cat# CRL-1740), NCI-H23 cells (ATCC, cat# CRL-5800), effector cells (CD3+) (neg. selected, *e.g.*, 5-10 Mio/vial, Astarte Biologics, LLC, cat-lot#1017-1754OC12), peripheral blood mononuclear cells (PBMCs), and a kit to measure cytotoxicity by, *e.g.*, lactate dehydrogenase (LDH) release (e.g., Roche, cat# 11644793001).

*2. TCT002 Sequences*

SEQ ID NO: 12 (TCT002 PROTEIN SEQUENCE)
SEQ ID NO: 13 (TCT002 DNA SEQUENCE)

**FROM N- TO C-TERMINUS IN THIS ORDER:**

SEQ ID NO: 9 (SIGNAL PEPTIDE)
SEQ ID NO: 14 (ANTI-CD28 $V_L$)
SEQ ID NO: 5 (INTER-scFv LINKER)
SEQ ID NO: 15 (ANTI-CD28 $V_H$)
SEQ ID NO: 11 (INTER-scFv LINKER)
SEQ ID NO: 16 (ANTI-EpCAM $V_H$)
SEQ ID NO: 5 (INTRA-scFv LINKER)
SEQ ID NO: 17 (ANTI-EpCAM $V_L$)
SEQ ID NO: 10 (HEXA HISTIDINE TAG)

*3. Culturing Effector T cells*

**[0182]** CD3+T cells are obtained from a commercial vendor, or magnetically isolated from whole blood/peripheral blood mononuclear cells (PBMCs) using magnetic beads conjugated to anti-CD3 antibodies. Since TCT002 was designed to stimulate T cells via CD28, T cells activated via CD28-stimulating beads (*e.g.*, as provided in the T Cell Activation/Expansion Kit from Miltenyi Biotec Inc.) may not constitute optimal effector cells to assess the cytotoxic potential of TCT002. Therefore, in some cases, it is more suitable to utilize unstimulated or interleukin 2 (IL-2)-only pre-stimulated peripheral blood mononuclear cells (PBMCs) as effector cells. For this purpose, PBMCs freshly isolated from human blood may be treated overnight with IL-2 and then used as effector cells to assess the potential for redirected lysis of bispecific binding reagent(s). A nonlimiting example illustrating the use of PBMCs as effector cells to assess the extent of prostate cancer cell lysis mediated by the

PSMA x CD3 BiTE AMG 212/BAY2010112 is included herein by reference (Friedrich et al., *Mol Cancer Ther*, 11(12):2664-2673, 2012). In some cases, however, it may be desirable to assess the activity of an anti-CD28 bispecific binding reagent (*e.g.*, TCT002), or a combination of an anti-CD28 bispecific binding reagent and one or more structurally distinct bispecific binding reagents (*e.g.*, TCT001 and TCT002), in the context of immune cells activated with CD28 stimulating beads. In some cases, the CD28 stimulating bead-activated immune cells can exhibit higher DuST-induced *in vitro* target cell lysis as compared to IL-2 activated or unstimulated PBMCs.

*4. $EC_{50}$ Cytotoxicity Assay*

**[0183]** Similarly to TCT001, the cytotoxic potential of TCT002 may be assessed *in vitro* using suitable assays. However, since TCT002 was designed to stimulate T cells primarily nonsuperagonistically via the CD28 co-receptor and not via, *e.g.*, CD3, TCT002 alone is not expected to exert the same high degree of cytotoxicity towards prostate cancer cells as TCT001. However, due to their complementary roles, TCT001 and TCT002 in combination comprise a composition with an exceptionally high prostate cancer-selective cytotoxic potential.

**[0184]** The assessment of the cytotoxic potential of TCT001, TCT002, and TCT001 and TCT002 in combination is performed using PBMCs as effector cells, and LNCaP human prostate cancer cells as target cells. As a nonlimiting example, this assessment is carried out by addressing three main parameters. **1**. Optimal effector:target (E/T) cell ratio. **2**. $EC_{50}$ values of TCT001 and TCT002 (individually). **3**. Combination indexes for TCT001 and TCT002, which indicate whether the combination of the two bispecific binding reagents may show synergistic, additive or antagonistic cytotoxic effects.

**[0185]** To identify an optimal E/T cell ratio, a constant number of target cells (*e.g.*, 10000 per well, 96-well plate) are treated with a constant concentration (*e.g.*, 2.5 microgram/mL) of TCT001, TCT002, or a combination of TCT001 and TCT002 and variable numbers of IL-2 pre-stimulated or nonstimulated PBMC effector cells. Using the Cytotoxicity Detection Kit (LDH) (Roche), %(target cell lysis) are measured at several time points following addition of the bispecific antibodies (for instance, at 6h, 24h, 48h, 96h). %(target cell lysis) may be calculated for each time point according to %(target cell lysis) = 100*[OD(Sample)-OD(E+T)]/[OD(Maximum release)-OD(Minimum release)] (Formula 2), wherein OD means the optical density (absorbance) at 490 or 492 nm minus the optical density at 650 nm (or

another wavelength > 600 nm), and OD(E+T) the OD of the control sample(s) comprising effector and target cells but lacking bispecific binding reagent(s). OD(Maximum release) refers to the maximal LDH release from target cells following lysis of the cells with Triton X100, and OD(Minimum release) to the spontaneous basal LDH release of the target cells without the presence of effector cells and TCT001 and/or TCT002. In general, dose-response studies are conducted with the E/T ratio yielding the highest degree of specific cytotoxicity, whereby half-maximal effective concentration ($EC_{50}$) values are determined based on the %(target cell lysis) values for different bispecific binding reagent concentrations and time points. Finally, with constant concentrations of TCT002 in combination with varying concentrations of TCT001 and vice versa, synergistic, antagonistic, or additive target cell killing effects can be identified.

*Results*

**[0186]** *Production of TCT002.* TCT002 was produced similarly to TCT001, *i.e.*, the TCT002 gene was synthesized *in vitro*, cloned into a mammalian expression vector, and expressed into CHO cells. TCT002 protein was purified from the culture supernatant by metal affinity chromatography. As demonstrated in **Figure 8A**, and predicted based on the design facilitating disulfide bridge-stabilization of the anti-CD28 scFv, TCT002 was obtained in a predominantly monomeric form. Therefore, in contrast to the reported superagonistic activation for dimeric forms of another anti-CD28 scFv-containing taFv antibody (Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003; Grosse-Hovest et al., *Int J Cancer*, 117(6):1060-1064, 2005), TCT002 is not expected to induce superagonistic target cell killing.

**[0187]** LNCaP cells together with freshly isolated peripheral blood mononuclear cells (PBMCs) that had been overnight stimulated with IL-2 were treated for 6 hours with 2.5 microgram/ml of TCT001, TCT002, or TCT001 and TCT002 in combination (each at 2.5 µg/ml) at E/T ratios of 50, 25, and 10 (**Figure 9**). Total LDH release of the E+T control condition (effector + target cells in absence of a bispecific binding reagent) was greater than the maximal signal obtained following target cell lysis with Triton X-100 thereby suggesting that substantial numbers of PBMC effector cells died in co-culture with LNCaP target cells. Under the assumption that this background signal at least in part comprises a signal derived from nonspecifically lysed LNCaP target cells, the percentage target cell lysis values shown are likely underestimated. A dose-response experiment was conducted with an E/T ratio of 10, using PBMCs not pre-stimulated with IL-2 as effector cells. As demonstrated in **Figure**

**10**, at lower concentrations of TCT001, target cell lysis was positively correlated with the TCT001 dose, and was not statistically significantly affected by the presense of TCT002 at 2.5 μg/ml ($p > 0.05$ by both 1- and 2-tailed *t*-test). On the other hand, at higher concentrations of TCT001, the percentage target cell lysis decreased. However, co-treatment with 2.5 μg/ml of TCT002 and 5 μg/ml of TCT001 resulted in a significantly higher proportion of lysed target cells than treatment with 5 μg/ml of TCT001 alone ($p < 0.05$ in 1-tailed *t*-test). These results are in agreement with a model stating that high doses of TCT001 induce T cell anergy and thus reduce effector function by providing high levels of Signal 1; and that co-stimulation with TCT002 provides Signal 2, thereby preventing anergy and the associated loss of effector function.

**[0188]** TCT002 was designed to contain cysteines at the variable region positions VH44 and VL100 of the anti-CD28 scFv domain to reduce aggregation (*e.g.*, dimerization) and thereby superagonistic T cell activation (Grosse-Hovest et al., *Eur J Immunol*, 33(5):1334-1340, 2003). As demonstrated in **Figures 9 and 10**, such superagonistic activation indeed did not occur; and it cannot simply be explained by a lack of cytotoxic potential of TCT002, since TCT002 can enhance the cytotoxic effect induced by TCT001 (**Figures 9 and 10**) and thereby likely by other bispecific binding reagents, including those that agonistically stimulate T cells by binding to CD3.

**Example 3: Generation of Bispecific Binding Reagents**

**[0189]** Single-chain variable fragment domains for (taFv) bispecific antibodies and other bispecific binding reagents that target a tumor-associated antigen (TAA) and a T cell surface antigen such as CD3 or CD28 (**Figures 1 and 2**), can be generated using methods for the production of monoclonal antibodies (mAbs). Alternatively, for some TAAs, mAbs suitable for the generation of bispecific binding reagents that already exist are utilized. **Figure 11** provides the sequences of a number of exemplary scFvs and variable sequences generated using $V_L$ and $V_H$ sequences from mAbs.

**[0190]** $V_L$ and $V_H$ sequences derived from mAbs that bind target antigens may be made by a number of different methods (for instance, summarized in U.S. Patent No. 8,436,147) such as those employed to generate hybridomas (Kohler and Milstein, *Nature*, 256(5517):495-497, 1975; Harlow and Lane, Antibodies: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1988; Hämmerling et al., Monoclonal Antibodies and T-cell Hybridomas: Perspectives and Technical Advances, 563-681, Elsevier/North-Holland Biomedical Press,

1981), by recombinant DNA techniques (for instance, see U.S. Patent No. 4,816,567), via phage display methods (Clackson et al., *Nature*, 352(6336):624-628, 1991; Fellouse et al., *Proc Natl Acad Sci U S A*, 101(34):12467-12472, 2004; Lee et al., *J Mol Biol*, 340(5):1073-1093, 2004); Lee et al., *J Immunol Methods*, 284(1-2):119-132, 2004; Marks et al., *J Mol Biol*, 222(3):581-597, 1991); Sidhu et al., *J Mol Biol*, 338(2):299-310, 2004; Marks et al., *Biotechnology (N Y)*, 10(7):779-783, 1992), and by methods based on transgenic animals expressing human antibodies (for example, see WO 98/24893, WO 96/34096, WO 96/33735, WO 91/10741, U.S. Patent Nos.: 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and Jakobovits et al., *Proc Natl Acad Sci U S A*, 90(6):2551-2555, 1993; Jakobovits et al., *Nature*, 362(6417):255-258, 1993; Bruggemann et al., *Year Immunol*, 7:33-40, 1993; Lonberg et al., *Nature*, 368(6474):856-859, 1994; Morrison, *Nature*, 368(6474):812-813, 1994; Fishwild et al., *Nat Biotechnol*, 14(7):845-851, 1996; Neuberger, *Nat Biotechnol*, 14(7):826, 1996; Lonberg and Huszar, *Int Rev Immunol*, 13(1):65-93, 1995).

**[0191]** The following nonlimiting example illustrates a common procedure to generate suitable taFvs encompassing the following steps: **1.** Immunization of mice with an extracellular portion of the TAA or with a TAA-expressing cell line. **2.** Harvest of spleen cells and fusion with myeloma cells to generate hybridomas. **3.** Screening of hybridomas for production of antibodies with high affinity for the TAA. **4.** Cloning and sequencing of the $V_L$ and $V_H$ regions from selected hybridomas. **5.** Generation of taFv constructs using conventional cloning techniques or *in silico* design followed by *in vitro* gene synthesis and cloning of the resulting taFv genes into suitable eukaryotic expression vectors. **6.** Production of recombinant taFv proteins in CHO (Chinese Hamster Ovary) or HEK293 (Human Embryonic Kidney 293) cells and purification from culture supernatant.

**[0192]** The following nonlimiting example, derived from U.S. Patent No. 6,136,311, illustrates how monoclonal antibodies may be raised against unknown cell surface tumor-associated antigens (TAAs). Female BALB/c mice are immunized intraperitoneally with a suitable cancer cell line every 2 weeks for a total of 6 weeks followed by a booster immunization with fresh prostate epithelial cells grown *in vitro*. Three days afterwards, spleen cells are fused with SP-2 mouse myeloma cells following standard techniques (Kohler and Milstein, *Nature*, 256(5517):495-497, 1975; Ueda et al., *Proc Natl Acad Sci U S A*, 78(8):5122-5126, 1981; and U.S. Patent No. 6,136,311). Cell culture supernatants of the resulting hybridoma clones are screened, for instance, via complement cytotoxicity assays

against the cancer cell line used for immunization. Further screening may include immunohistochemical assays to assess target tissue selectivity.

**[0193]** Bispecific binding reagents can contain human or humanized $V_L$ and $V_H$ domains. Compared to their murine counterparts, human or humanized domains are expected to result in no or less severe adverse immune reactions in humans. A preferred method to obtain $V_L$ and $V_H$ sequences comprises their isolation from nonimmunized human phage display libraries using methods herein incorporated by nonlimiting references (Clackson et al., *Nature*, 352(6336):624-628, 1991; Fellouse et al., *Proc Natl Acad Sci U S A*, 101(34):12467-12472, 2004; Lee et al., *J Mol Biol*, 340(5):1073-1093, 2004; Lee et al., *J Immunol Methods*, 284(1-2):119-132, 2004; Marks et al., *J Mol Biol*, 222(3):581-597, 1991; Sidhu et al., *J Mol Biol*, 338(2):299-310, 2004; Marks et al., *Biotechnology (N Y)*, 10(7):779-783, 1992).

**[0194]** taFv antibodies can contain glycine-rich linkers between the $V_L$ and $V_H$ domains within an scFv, and between the two scFvs (**Figure 1**). It is well known in the art that such linkers, *e.g.*, SEQ ID NOs: 5 and 8 (intra-scFv linker), and 11 (inter-scFv linker), are both flexible and, due to the presence of serine residue(s), soluble in an aqueous environment.

**Example 4: Cloning or Synthesis of taFvs**

*A. Synthesis*

**[0195]** taFv antibodies and other bispecific binding reagents are generated by expression of *in vitro* synthesized taFv genes in CHO or HEK293 cells. Methods related to *in vitro* gene synthesis have been described elsewhere and are herein incorporated by the following nonlimiting references: U.S. Pat. Nos.: 8,053,191; 6,110,668; 6,008,031; and 5,922,539; and Agarwal et al., *Nature*, 227(5253):27-34, 1970; Ma et al., *Curr Opin Chem Biol*, 16(3-4):260-267, 2012; Matzas et al., *Nat Biotechnol*, 28(12):1291-1294, 2010; Hoover and Lubkowski, *Nucleic Acids Res*, 30(10):e43, 2002; Rouillard et al., *Nucleic Acids Res*, 32(Web Server issue):W176-180, 2004; Bode et al., *Nucleic Acids Res*, 37(Web Server issue):W214-221, 2009; Marchand and Peccoud, *Methods Mol Biol*, 852:3-10, 2012; Sandhu et al., *Biotechniques*, 12(1):14-16, 1992; Horton et al., *Methods Enzymol*, 217:270-279, 1993; Stemmer et al., *Gene*, 164(1):49-53, 1995; Wooddell and Burgess, *Genome Res*, 6(9):886-892, 1996; Gao et al., *Nucleic Acids Res*, 31(22):e143, 2003; Young and Dong, *Nucleic Acids Res*, 32(7):e59, 2004; Tian et al., *Nature*, 432(7020):1050-1054, 2004; Wu et al., *J Biotechnol*, 124(3):496-503, 2006; Kong et al., *Nucleic Acids Res*, 35(8):e61, 2007; Ben Yehezkel et al., *Nucleic Acids Res*, 36(17):e107, 2008; Huang et al., *Methods Mol Biol*,

852:23-34, 2012; Eren and Swenson, *J Biol Chem*, 264(25):14874-14879, 1989; Mehta et al., *Protein Expr Purif*, 11(1):86-94, 1997; Au et al., *Biochem Biophys Res Commun*, 248(1):200-203, 1998; Li and Elledge, *Methods Mol Biol*, 852:51-59, 2012; Gibson et al., *Science*, 319(5867):1215-1220, 2008; Chalmers and Curnow, *Biotechniques*, 30(2):249-252, 2001; Gibson, *Methods Mol Biol*, 852:11-21, 2012; Hughes et al., *Methods Enzymol*, 498:277-309, 2011; Ma et al., *Trends Biotechnol*, 30(3):147-154, 2012; Kelly et al., *J Biol Eng*, 3:4, 2009; Ho-Shing et al., *Methods Mol Biol*, 852:61-76, 2012. Following *in vitro* gene synthesis, taFv genes are cloned into expression vectors by standard methods (for instance, see Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989; Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1988; Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2012).

*B. Generation of taFv Genes via Conventional Cloning Strategies*

**[0196]** taFv antibodies and other bispecific binding reagents are generated by procedures comprising cloning of the $V_L$ and $V_H$ regions into an expression vector after amplification by polymerase chain reaction (PCR) using nucleic acid from hybridoma cells as template. Corresponding methods are well known in the art (for instance, see Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989; Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1988; Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 2012).

*C. Generation of Bispecific Antibodies with Human or Humanized Variable Regions*

**[0197]** Bispecific binding reagents containing one or more antibody domains utilize human $V_L$ and $V_H$ sequences or are humanized to reduce antigenicity. While fully human domains are directly isolated by, for instance, phage (Marks et al., *J Mol Biol*, 222(3):581-597, 1991) or ribosome (Hanes et al., *Nat Biotechnol*, 18(12):1287-1292, 2000) display technologies using human antibody (*e.g.*, scFv format) libraries, humanization is performed by replacing the framework regions of antibodies raised in animals, generally in mice, with suitable human sequences. This essentially results in human variable domains comprising rodent hypervariable regions (complementarity determining regions, CDRs). Furthermore, it is often desirable to improve the affinity of nonhuman, human, or humanized antibodies. This can be accomplished by mutagenesis and selection of those antibodies with improved

affinity. Several methods are known in the art. Among the most suitable ones are those based on phage, yeast or ribosome display technologies (Bradbury et al., *Nat Biotechnol*, 29(3):245-254, 2011).

**[0198]** Nonlimiting examples of methods for the generation of human antibodies are outlined in U.S. Patent No. 8,436,147 and are herein incorporated by reference, and include methods based on transgenic animals expressing human antibodies (for example, see WO 98/24893, WO 96/34096, WO 96/33735,WO 91/10741, U.S. Patent Nos.: 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and Jakobovits et al., *Proc Natl Acad Sci U S A*, 90(6):2551-2555, 1993; Jakobovits et al., *Nature*, 362(6417):255-258, 1993; Bruggemann et al., *Year Immunol*, 7:33-40, 1993; Lonberg et al., *Nature*, 368(6474):856-859, 1994; Morrison, *Nature*, 368(6474):812-813, 1994; Fishwild et al., *Nat Biotechnol*, 14(7):845-851, 1996; Neuberger, *Nat Biotechnol*, 14(7):826, 1996; Lonberg and Huszar, *Int Rev Immunol*, 13(1):65-93, 1995), and procedures comprising isolation and identification of human $V_L$ and $V_H$ domains from antibody libraries (for instance, see Marks et al., *J Mol Biol*, 222(3):581-597, 1991; Marks et al., *Biotechnology (N Y)*, 10(7):779-783, 1992).

**[0199]** As outlined in U.S. Patent No. 8,436,147, humanization is performed as described by Winter and colleagues (Jones et al., *Nature*, 321(6069):522-525, 1986; Riechmann et al., *Nature*, 332(6162):323-327, 1988; Verhoeyen et al., *Science*, 239(4847):1534-1536, 1988). As discussed elsewhere (for example, in U.S. Patent No. 8,436,147), humanization is conducted, for instance, via the "best-fit" or the "consensus framework" method. In the former case, the sequence of the rodent variable domain to be humanized is compared to a library of human variable domains. The human framework most similar to the rodent framework is then chosen (Sims et al., *J Immunol*, 151(4):2296-2308, 1993; Chothia and Lesk, *J Mol Biol*, 196(4):901-917, 1987). In contrast, for the "consensus framework" method, a consensus framework sequence derived from multiple human sequences of a particular heavy or light chain subgroup is selected (Carter et al., *Proc Natl Acad Sci U S A*, 89(10):4285-4289, 1992; Presta et al., *J Immunol*, 151(5):2623-2632, 1993; and U.S. Patent No. 8,436,147).

**[0200]** Further considerations for the selection of the optimal framework sequence relate to the framework's potential effect on the affinity of the humanized antibody for the target antigen (for instance, see U.S. Patent No. 8,436,147). Therefore, in some cases, three-dimensional *in silico* modeling of both the parental (*e.g.*, rodent) antibody and hypothetical

humanized candidate antibodies. With commonly available computer programs, individual amino acid residues are tested for their potential effect on the affinity of the humanized antibody for the target antigen. With this method, individual residues are identified which, when substituted by those of another human framework sequence or by residues found in the parental antibody, improve the affinity of the humanized antibody. Usually, the CDR elements contribute the most to the affinity of an antibody for its antigen. However, also residues closely underlying the CDRs in the beta-sheet framework that do not directly contribute to the binding of the antigen may affect the affinity. Around 30 of such "Vernier positions" that affect $V_L$:$V_H$ domain packing and CDR conformation have been identified (Foote and Winter, *J Mol Biol*, 224(2):487-499, 1992 and U.S. Patent No. 8,436,147).

**[0201]** A nonlimiting example illustrating the concept of substituting residues at particular Vernier positions relates to the humanization of the murine anti-STEAP1 antibody 120.545 (see U.S. Patent No. 8,436,147). Briefly, by comparing the 120.545 $V_L$ sequence with the consensus human kappa I $V_L$ domain, and the 120.545 $V_H$ sequence with the consensus human subgroup III consensus $V_H$ domain, differences at 6 key Vernier positions were identified (positions 24, 37, 48, 67, 73, and 78). Replacing the alanine residue at Vernier position 24 by a valine residue (A24V) has a particularly strong effect on STEAP1 binding, as demonstrated by a FACS experiment in which this variant (120.v24) displayed on a phage as a Fab antibody is used to stain STEAP1-expressing cells.

**[0202]** Another method for the humanization of antibodies is referred to as "superhumanization". In contrast to the above illustrated standard approaches in which human framework sequences are sought with minimal deviations from the parental non-human antibody, "superhumanization" comprises the identification of human genomic antibody sequences with *CDRs* similar to those of the parental antibody and the selection of the corresponding frameworks for humanization. This concept is exemplified by the superhumanization of the anti-CD28 mAb 9.3 and is herein incorporated by reference (Tan et al., *J Immunol*, 169(2):1119-1125, 2002).

**Example 5: Bispecific Binding Reagents Targeting Stress-induced Antigens**

**[0203]** NKG2D is an activating C-type lectin-like receptor expressed on the surface of immune cells such as natural killer (NK) cells and CD8+ T cells. NKG2D ligands (*e.g.*, the MICs MICA and MICB, and the ULBPs ULBP1, ULBP2, ULBP3, RAET1E, RAET1G, and RAET1L) are thought to be specifically expressed on the surface of a wide variety of cells in

response to cellular stress resulting from, for instance, malignant transformation/tumorigenesis, DNA damage, pathogen infection, Toll-like receptor signaling, and proteasome inhibition (Fernandez-Messina et al., *J Biol Chem*, 285(12):8543-8551, 2010; Nausch and Cerwenka, *Oncogene*, 27(45):5944-5958, 2008; Ohashi et al., *J Biol Chem*, 285(22):16408-16415, 2010). While NK cells may directly eliminate their target cells following engagement with the latter cells' NKG2D ligands, it is unclear whether the same holds true for T cells (Cao et al., *J Biol Chem*, 282(26):18922-18928, 2007).

**[0204]** A key function of the immune system is the ablation of NKG2D ligand-expressing cancer cells by NKG2D receptor-expressing effector cells to prevent tumor development and to ablate cancer cells in existing tumors. In agreement, NKG2D ligand expression correlated with improved survival of cancer patients (Cao et al., *J Biol Chem*, 282(26):18922-18928, 2007). However, even though the NKG2D receptor-ligand system is instrumental for immune surveillance, it is at times not sufficient as malignancies establish despite the expression of NKG2D ligands. For instance, MIC expression was induced in 95% of prostate carcinomas (Wu et al., *J Clin Invest*, 114(4):560-568, 2004).

**[0205]** An escape mechanism from the NKG2D receptor-ligand system well known in the art comprises the shedding of NKG2D ligands from the surface of cancer cells. The reduction in the numbers of both the remaining, intact, ligands on the cancer cells and the free (not bound to competing soluble NKG2D ligand) NKG2D receptors on the effector cells may translate into a reduced anti-tumor response (Fernandez-Messina et al., *J Biol Chem*, 285(12):8543-8551, 2010; Cao et al., *J Biol Chem*, 282(26):18922-18928, 2007; Wu et al., *J Clin Invest*, 114(4):560-568, 2004; Liu et al., *J Clin Invest*, 123(10):4410-4422, 2013). Furthermore, soluble NKG2D ligands produced from RNA splice forms lacking features required for cell surface localization may be secreted by cancer cells (Cao et al., *J Biol Chem*, 282(26):18922-18928, 2007). A second prevailing concept by which the tumor-directed cytotoxic response may be down-modulated suggests that NKG2D receptors with chronic ligand exposure become down-regulated. Thus, high levels of soluble NKG2D ligands may exhaust NKG2D function by providing such a chronic stimulation (Fernandez-Messina et al., *J Biol Chem*, 285(12):8543-8551, 2010; Liu et al., *J Clin Invest*, 123(10):4410-4422, 2013).

**[0206]** In this example, a bispecific binding reagent containing an NKG2D ligand-binding domain of NKG2D and a domain interacting with a T cell surface antigen such as CD3 or CD28 are created. The NKG2D ligand-binding domain of NKG2D is derived from an

extracellular domain of an NKG2D receptor (*e.g.*, a human NKG2D receptor). The human NKG2D receptor sequence is known, *e.g.*, NCBI NP_031386.2. The T cell surface antigen-binding domain is an scFv domain interacting with a T cell antigen such as, *e.g.,* CD3 and CD28 (**Figure 11T and 11U**).

**[0207]** NKG2D ligands, and cells that express such ligands, are targeted by means of bispecific binding reagents having an extracellular domain of an NKG2D receptor and a T cell-binding domain. These bispecific binding reagents overcome the limitations imposed by soluble NKG2D ligands addressed above, as they may be utilized to "titrate out" the soluble ligands thereby avoiding the down-regulation of the NKG2D receptor. Additionally, since shedding of NKG2D ligands on cancer cells does not affect 100% of the ligands, CD3 scFv-NKG2D and CD28 scFv-NKG2D bispecific binding reagents crosslink cytotoxic T cells to NKG2D ligand-expressing prostate cancer cells thereby initiating the ablation of the target prostate cancer cells.

**[0208]** Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference.

# INFORMAL SEQUENCE LISTING

SEQ ID NO: 1 (TCT001 PROTEIN SEQUENCE):
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QLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWVRQAPGKGLEWVGYISNSGSTSYNPSLKSRFTISRDNSKN
TLYLQMNSLRAEDTAVYYCARERNYDYDDYYYAMDYWGQGTLVTVSSGGGGSDIKLQQSGAELARPGASVKMSCK
TSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCAR
YYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQ
QKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKHHHHH
H

SEQ ID NO: 2 (TCT001 DNA SEQUENCE):
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AGCCCTTCAAGCCTGAGCGCCTCTGTGGGCGATAGAGTGACAATCACCTGCAAGAGCAGCCAGAGCCTGCTGTAC
CGGAGCAACCAGAAGAACTACCTGGCCTGGTACCAGCAGAAGCCTGGAAAGGCCCCTAAGCTGCTGATCTACTGG
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ACCAGCGGCTACACCTTCACCCGGTACACCATGCACTGGGTCAAGCAGAGGCCAGGACAGGGACTGGAGTGGATC
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TACTACGACGACCACTACTGCCTGGACTACTGGGGACAGGGAACCACACTGACCGTGTCTAGCGTGGAGGGAGGA
TCTGGAGGATCTGGAGGAAGCGGAGGATCTGGAGGCGTGGATGACATCCAGCTGACCCAGAGCCCTGCCATCATG
TCTGCCTCTCCTGGCGAGAAAGTGACCATGACCTGCAGGGCTAGCAGCAGCGTGTCCTACATGAACTGGTACCAG
CAGAAGAGCGGCACCAGCCCTAAGAGATGGATCTACGACACCAGCAAGGTGGCCAGCGGAGTGCCTTACCGGTTC
AGCGGCAGCGGCAGCGGAACCAGCTACAGCCTGACCATCAGCAGCATGGAGGCCGAGGATGCTGCCACATACTAC
TGCCAGCAGTGGAGCTCTAACCCTCTGACATTCGGGGCTGGGACAAAACTGGAACTGAAACACCACCACCACCAC
CATTGATAA

SEQ ID NO: 3 (ANTI-STEAP1 VL):
DIQMTQSPSSLSASVGDRVTITCKSSQSLLYRSNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGT
DFTLTISSLQPEDFATYYCQQYYNYPRTFGQGTKVEIKR

SEQ ID NO: 4 (ANTI-STEAP1 VH):
EVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWVRQAPGKGLEWVGYISNSGSTSYNPSLKSRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCARERNYDYDDYYYAMDYWGQGTLVTVSS

SEQ ID NO: 5 (INTRA-scFv LINKER):
GGGGSGGGGSGGGGS

SEQ ID NO: 6 (ANTI-CD3 VH):
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSS

SEQ ID NO: 7 (ANTI-CD3 VL):
DIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTIS
SMEAEDAATYYCQQWSSNPLTFGAGTKLELK

SEQ ID NO: 8 (INTRA-scFv LINKER):
VEGGSGGSGGSGGSGGVD

SEQ ID NO: 9 (SIGNAL PEPTIDE):

MGWSCIILFLVATATGVHS

SEQ ID NO: 10 (HEXA HISTIDINE TAG):
HHHHHH

SEQ ID NO: 11 (INTER-scFv LINKER):
GGGGS

SEQ ID NO: 12 (TCT002 PROTEIN SEQUENCE):
MGWSCIILFLVATATGVHSDIELTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAAS
NVESGVPARFSGSGSGTNFSLNIHPVDEDDVAMYFCQQSRKVPYTFGCGTKLEIKRGGGGSGGGGSGGGGSQVKL
QQSGPGLVTPSQSLSITCTVSGFSLSDYGVHWVRQSPGQCLEWLGVIWAGGGTNYNSALMSRKSISKDNSKSQVF
LKMNSLQADDTAVYYCARDKGYSYYYSMDYWGQGTTVTVSSGGGGSQIQLVQSGPELKKPGETVKISCKASGYTF
TKYGMNWVKQAPGKGLKWMGWINTYTEEPTYGDDFKGRFAFSLETSASTANLQINNLKSEDTATYFCARFGSAVD
YWGQGTSVTVSSGGGGSGGGGSGGGGSDIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQS
PQLLIYQMSNLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCAQNLELPRTFGGGTKLEMKRTVHHHHHH

SEQ ID NO: 13 (TCT002 DNA SEQUENCE)
ATGGGGTGGTCCTGTATTATTCTGTTCCTGGTGGCTACCGCTACCGGCGTGCATTCCGACATCGAGCTGACTCAG
TCTCCCGCATCACTGGCCGTGAGTCTGGGCCAGAGGGCAACTATCTCATGCCGCGCCTCAGAGAGCGTGGAATAC
TATGTCACCTCCCTGATGCAGTGGTACCAGCAGAAACCCGGCCAGCCCCCTAAGCTGCTGATCTTCGCCGCTAGT
AACGTGGAGTCAGGGGTCCCTGCTCGGTTCTCCGGGTCTGGAAGTGGCACCAACTTTTCTCTGAATATTCACCCA
GTGGACGAAGACGATGTCGCAATGTACTTCTGCCAGCAGAGCCGAAAGGTGCCCTATACCTTTGGGTGTGGAACA
AAGCTGGAGATCAAAAGAGGAGGAGGAGGAAGCGGAGGAGGAGGCTCCGGAGGCGGGGGATCTCAGGTGAAGCTG
CAGCAGAGCGGACCTGGACTGGTCACCCCATCACAGAGCCTGTCCATTACTTGCACCGTGTCTGGATTCTCTCTG
AGTGACTACGGCGTGCATTGGGTCAGACAGAGCCCTGGGCAGTGTCTGGAATGGCTGGGAGTGATCTGGGCTGGC
GGGGGAACTAACTATAATAGCGCACTGATGTCCAGGAAGTCAATTAGCAAAGATAACTCCAAGTCTCAGGTGTTT
CTGAAAATGAATAGCCTGCAGGCCGACGATACAGCTGTCTACTATTGCGCTAGAGACAAGGGCTACTCTTACTAT
TACAGTATGGATTATTGGGGACAGGGCACCACAGTGACAGTCTCCTCAGGAGGAGGAGGATCCCAGATCCAGCTG
GTGCAGTCTGGCCCCGAGCTGAAGAAACCTGGGGAAACTGTCAAAATTTCCTGTAAGGCAAGCGGATACACATTC
ACTAAATATGGCATGAACTGGGTGAAGCAGGCCCCAGGGAAGGGACTGAAATGGATGGGCTGGATCAATACCTAC
ACAGAGGAACCCACCTATGGCGACGACTTCAAGGGGCGCTTCGCCTTTAGCCTGGAGACAAGTGCTTCAACTGCA
AATCTGCAGATTAACAATCTGAAGTCCGAAGACACTGCAACCTACTTCTGCGCCCGATTTGGCTCTGCTGTGGAT
TATTGGGGCCAGGGGACAAGTGTGACTGTCTCTAGTGGGGGAGGAGGAAGTGGAGGAGGAGGATCTGGAGGAGGA
GGCAGCGACATCGTGATGACCCAGGCAGCCTTTAGCAACCCCGTCACCCTGGGGACAAGCGGCTCCATCTCTTGT
AGGTCAAGCAAATCCCTGCTGCACTCTAATGGGATTACATATCTGTACTGGTATCTGCAGAAGCCAGGACAGTCC
CCCCAGCTGCTGATCTACCAGATGAGTAACCTGGCATCAGGCGTGCCTGACAGGTTCTCCTCTAGTGGAAGCGGC
ACTGACTTCACCCTGCGGATTTCCAGAGTGGAGGCCGAAGATGTGGGCGTCTATTACTGTGCTCAGAATCTGGAG
CTGCCACGCACCTTCGGCGGAGGAACTAAACTGGAAATGAAACGGACTGTGCATCACCACCACCACCAC

SEQ ID NO: 14 (ANTI-CD28 VL WITH VL100 CYSTEINE MODIFICATION):
DIELTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNF
SLNIHPVDEDDVAMYFCQQSRKVPYTFGCGTKLEIKR

SEQ ID NO: 15 (ANTI-CD28 VH WITH VH44 CYSTEINE MODIFICATION):
QVKLQQSGPGLVTPSQSLSITCTVSGFSLSDYGVHWVRQSPGQCLEWLGVIWAGGGTNYNSALMSRKSISKDNSK
SQVFLKMNSLQADDTAVYYCARDKGYSYYYSMDYWGQGTTVTVSS

SEQ ID NO: 16 (ANTI-EpCAM VH):
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKGLKWMGWINTYTEEPTYGDDFKGRFAFSLETS
ASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSS

SEQ ID NO: 17 (ANTI-EpCAM VL):
DIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLASGVPDRFSSSGSGTD
FTLRISRVEAEDVGVYYCAQNLELPRTFGGGTKLEMKRTV

SEQ ID NO: 18 (ANTI-CD3 VH WITH VH44 CYSTEINE MODIFICATION):
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQCLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSS

SEQ ID NO: 19 (ANTI-CD3 VL WITH VL100 CYSTEINE MODIFICATION):
DIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTIS
SMEAEDAATYYCQQWSSNPLTFGCGTKLELK

SEQ ID NO: 20 (ANTI-CD3 scFv, VH-VL, AS IN TCT001):
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSA
SPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQ
QWSSNPLTFGAGTKLELK

SEQ ID NO: 21 (ANTI-CD3 scFv, VH-VL, WITH VH44-VL100 CYSTEINE MODIFICATIONS):
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQCLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSA
SPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQ
QWSSNPLTFGCGTKLELK

SEQ ID NO: 22 (ALTERNATIVE ANTI-CD3 scFv, VH-VL):
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMSASPG
EKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWS
SNPLTFGAGTKLELK

SEQ ID NO: 23 (ALTERNATIVE ANTI-CD3 scFv, VH-VL, WITH VH44-VL100 CYSTEINE MODIFICATIONS):
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQCLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMSASPG
EKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWS
SNPLTFGCGTKLELK

SEQ ID NO: 24 (ANTI-CD3 scFv, VL-VH):
DIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTIS
SMEAEDAATYYCQQWSSNPLTFGAGTKLELKGGGGSGGGGSGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTF
TRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHY
CLDYWGQGTTLTVSS

SEQ ID NO: 25 (ANTI-CD3 scFv, VL-VH, WITH VL100-VH44 CYSTEINE MODIFICATIONS):
DIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTIS
SMEAEDAATYYCQQWSSNPLTFGCGTKLELKGGGGSGGGGSGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTF
TRYTMHWVKQRPGQCLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHY
CLDYWGQGTTLTVSS

SEQ ID NO: 26 (ANTI-CD28 VL):
DIELTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNF
SLNIHPVDEDDVAMYFCQQSRKVPYTFGGGTKLEIKR

SEQ ID NO: 27 (ANTI-CD28 VH):
QVKLQQSGPGLVTPSQSLSITCTVSGFSLSDYGVHWVRQSPGQGLEWLGVIWAGGGTNYNSALMSRKSISKDNSK
SQVFLKMNSLQADDTAVYYCARDKGYSYYYSMDYWGQGTTVTVSS

SEQ ID NO: 28 (ANTI-CD28 scFv, VL-VH):
DIELTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNF
SLNIHPVDEDDVAMYFCQQSRKVPYTFGGGTKLEIKRGGGGSGGGGSGGGGSQVKLQQSGPGLVTPSQSLSITCT
VSGFSLSDYGVHWVRQSPGQGLEWLGVIWAGGGTNYNSALMSRKSISKDNSKSQVFLKMNSLQADDTAVYYCARD
KGYSYYYSMDYWGQGTTVTVSS

SEQ ID NO: 29 (ANTI-CD28 scFv, VL-VH, WITH VL100-VH44 CYSTEINE MODIFICATIONS, AS IN TCT002):

DIELTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNF
SLNIHPVDEDDVAMYFCQQSRKVPYTFGCGTKLEIKRGGGGSGGGGSGGGGSQVKLQQSGPGLVTPSQSLSITCT
VSGFSLSDYGVHWVRQSPGQCLEWLGVIWAGGGTNYNSALMSRKSISKDNSKSQVFLKMNSLQADDTAVYYCARD
KGYSYYYSMDYWGQGTTVTVSS

SEQ ID NO: 30 (ANTI-CD28 scFv, VH-VL):
QVKLQQSGPGLVTPSQSLSITCTVSGFSLSDYGVHWVRQSPGQGLEWLGVIWAGGGTNYNSALMSRKSISKDNSK
SQVFLKMNSLQADDTAVYYCARDKGYSYYYSMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPASLAVSL
GQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNFSLNIHPVDEDDVAMY
FCQQSRKVPYTFGGGTKLEIKR

SEQ ID NO: 31 (ANTI-CD28 scFv, VH-VL, WITH VH44-VL100 CYSTEINE
MODIFICATIONS):
QVKLQQSGPGLVTPSQSLSITCTVSGFSLSDYGVHWVRQSPGQCLEWLGVIWAGGGTNYNSALMSRKSISKDNSK
SQVFLKMNSLQADDTAVYYCARDKGYSYYYSMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPASLAVSL
GQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNFSLNIHPVDEDDVAMY
FCQQSRKVPYTFGCGTKLEIKR

SEQ ID NO: 32 (ANTI-STEAP1 VL, WITH VL100 CYSTEINE MODIFICAITON):
DIQMTQSPSSLSASVGDRVTITCKSSQSLLYRSNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGT
DFTLTISSLQPEDFATYYCQQYYNYPRTFGCGTKVEIKR

SEQ ID NO: 33 (ANTI-STEAP1 VH, WITH VH44 CYSTEINE MODIFICATION):
EVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWVRQAPGKCLEWVGYISNSGSTSYNPSLKSRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCARERNYDYDDYYYAMDYWGQGTLVTVSS

SEQ ID NO: 34 (ANTI-STEAP1 scFv, VL-VH, AS IN TCTOO1):
DIQMTQSPSSLSASVGDRVTITCKSSQSLLYRSNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGT
DFTLTISSLQPEDFATYYCQQYYNYPRTFGQGTKVEIKRGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLS
CAVSGYSITSDYAWNWVRQAPGKGLEWVGYISNSGSTSYNPSLKSRFTISRDNSKNTLYLQMNSLRAEDTAVYYC
ARERNYDYDDYYYAMDYWGQGTLVTVSS

SEQ ID NO: 35 (ANTI-STEAP1 scFv, VL-VH, WITH VL100-VH44 CYSTEINE
MODIFICATIONS):
DIQMTQSPSSLSASVGDRVTITCKSSQSLLYRSNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGT
DFTLTISSLQPEDFATYYCQQYYNYPRTFGCGTKVEIKRGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLS
CAVSGYSITSDYAWNWVRQAPGKCLEWVGYISNSGSTSYNPSLKSRFTISRDNSKNTLYLQMNSLRAEDTAVYYC
ARERNYDYDDYYYAMDYWGQGTLVTVSS

SEQ ID NO: 36 (ANTI-STEAP1 scFv, VH-VL):
EVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWVRQAPGKGLEWVGYISNSGSTSYNPSLKSRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCARERNYDYDDYYYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSL
SASVGDRVTITCKSSQSLLYRSNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGTDFTLTISSLQP
EDFATYYCQQYYNYPRTFGQGTKVEIKR

SEQ ID NO: 37 (ANTI-STEAP1 scFv, VH-VL, WITH VH44-VL100 CYSTEINE
MODIFICATIONS):
EVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWVRQAPGKCLEWVGYISNSGSTSYNPSLKSRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCARERNYDYDDYYYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSL
SASVGDRVTITCKSSQSLLYRSNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGTDFTLTISSLQP
EDFATYYCQQYYNYPRTFGCGTKVEIKR

SEQ ID NO: 38 (ANTI-EpCAM VL, WITH VL100 CYSTEINE MODIFICATION):
DIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLASGVPDRFSSSGSGTD
FTLRISRVEAEDVGVYYCAQNLELPRTFGCGTKLEMKRTV

SEQ ID NO: 39 (ANTI-EpCAM VH, WITH VH44 CYSTEINE MODIFICATION):
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKCLKWMGWINTYTEEPTYGDDFKGRFAFSLETS
ASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSS

SEQ ID NO: 40 (ANTI EpCAM scFv, VH-VL, AS IN TCTOO2):
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKGLKWMGWINTYTEEPTYGDDFKGRFAFSLETS
ASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVMTQAAFSNPVTLGTSG
SISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCA
QNLELPRTFGGGTKLEMKRTV

SEQ ID NO: 41 (ANTI EpCAM scFv, VH-VL, WITH VH44-VL100 CYSTEINE
MODIFICATIONS):
QIQLVQSGPELKKPGETVKISCKASGYTFTKYGMNWVKQAPGKCLKWMGWINTYTEEPTYGDDFKGRFAFSLETS
ASTANLQINNLKSEDTATYFCARFGSAVDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVMTQAAFSNPVTLGTSG
SISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLASGVPDRFSSSGSGTDFTLRISRVEAEDVGVYYCA
QNLELPRTFGCGTKLEMKRTV

SEQ ID NO: 42 (ANTI EpCAM scFv, VL-VH):
DIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLASGVPDRFSSSGSGTD
FTLRISRVEAEDVGVYYCAQNLELPRTFGGGTKLEMKRTVGGGGSGGGGSGGGGSQIQLVQSGPELKKPGETVKI
SCKASGYTFTKYGMNWVKQAPGKGLKWMGWINTYTEEPTYGDDFKGRFAFSLETSASTANLQINNLKSEDTATYF
CARFGSAVDYWGQGTSVTVSS

SEQ ID NO: 43 (ANTI EpCAM scFv, VL-VH, WITH VL100-VH44 CYSTEINE
MODIFICATIONS):
DIVMTQAAFSNPVTLGTSGSISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLASGVPDRFSSSGSGTD
FTLRISRVEAEDVGVYYCAQNLELPRTFGCGTKLEMKRTVGGGGSGGGGSGGGGSQIQLVQSGPELKKPGETVKI
SCKASGYTFTKYGMNWVKQAPGKCLKWMGWINTYTEEPTYGDDFKGRFAFSLETSASTANLQINNLKSEDTATYF
CARFGSAVDYWGQGTSVTVSS

SEQ ID NO: 44 (ANTI-PSMA VL):
DIVMTQSHKFMSTSVGDRVSIICKASQDVGTAVDWYQQKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTI
TNVQSEDLADYFCQQYNSYPLTFGAGTKLEIK

SEQ ID NO: 45 (ANTI-PSMA VH):
EVQLQQSGPELVKPGTSVRISCKTSGYTFTEYTIHWVKQSHGKSLEWIGNINPNNGGTTYNQKFEDKATLTVDKS
SSTAYMELRSLTSEDSAVYYCAAGWNFDYWGQGTTLTVSS

SEQ ID NO: 46 (ANTI-PSMA VL, WITH VL100 CYSTEINE MODIFICATION):
DIVMTQSHKFMSTSVGDRVSIICKASQDVGTAVDWYQQKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTI
TNVQSEDLADYFCQQYNSYPLTFGCGTKLEIK

SEQ ID NO: 47 (ANTI-PSMA VH, WITH VH44 CYSTEINE MODIFICATION):
EVQLQQSGPELVKPGTSVRISCKTSGYTFTEYTIHWVKQSHGKCLEWIGNINPNNGGTTYNQKFEDKATLTVDKS
SSTAYMELRSLTSEDSAVYYCAAGWNFDYWGQGTTLTVSS

SEQ ID NO: 48 (ANTI-PSMA scFv, VL-VH):
DIVMTQSHKFMSTSVGDRVSIICKASQDVGTAVDWYQQKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTI
TNVQSEDLADYFCQQYNSYPLTFGAGTKLEIKGGGGSGGGGSGGGGSEVQLQQSGPELVKPGTSVRISCKTSGYT
FTEYTIHWVKQSHGKSLEWIGNINPNNGGTTYNQKFEDKATLTVDKSSSTAYMELRSLTSEDSAVYYCAAGWNFD
YWGQGTTLTVSS

SEQ ID NO: 49 (ANTI-PSMA scFv, VL-VH, WITH VL100-VH44 CYSTEINE
MODIFICATIONS):
DIVMTQSHKFMSTSVGDRVSIICKASQDVGTAVDWYQQKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTI
TNVQSEDLADYFCQQYNSYPLTFGCGTKLEIKGGGGSGGGGSGGGGSEVQLQQSGPELVKPGTSVRISCKTSGYT
FTEYTIHWVKQSHGKCLEWIGNINPNNGGTTYNQKFEDKATLTVDKSSSTAYMELRSLTSEDSAVYYCAAGWNFD
YWGQGTTLTVSS

SEQ ID NO: 50 (ANTI-PSMA scFv, VH-VL):
EVQLQQSGPELVKPGTSVRISCKTSGYTFTEYTIHWVKQSHGKSLEWIGNINPNNGGTTYNQKFEDKATLTVDKS
SSTAYMELRSLTSEDSAVYYCAAGWNFDYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSDIVMTQSHKFMSTSVGDRVS

IICKASQDVGTAVDWYQQKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTITNVQSEDLADYFCQQYNSYP
LTFGAGTMLDLK

SEQ ID NO: 51 (ANTI-PSMA scFv, VH-VL, WITH VH44-VL100 CYSTEINE
MODIFICATIONS):
EVQLQQSGPELVKPGTSVRISCKTSGYTFTEYTIHWVKQSHGKCLEWIGNINPNNGGTTYNQKFEDKATLTVDKS
SSTAYMELRSLTSEDSAVYYCAAGWNFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQSHKFMSTSVGDRVS
IICKASQDVGTAVDWYQQKPGQSPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTITNVQSEDLADYFCQQYNSYP
LTFGCGTKLEIK

SEQ ID NO: 52 (ANTI-SPON2 VL):
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLT
ISRLEPEDFAVYYCQQYSSSLTFGGGTKVEIK

SEQ ID NO: 53 (ANTI-SPON2 VH):
EVQLVQSGGGLVMPGGSLRLSCAGSGFTFSSYVMHWLRQAPGKGLEWVSVIGTGGVTHYADSVKGRFMISRDNAK
NSLYLQMNSLRAEDTAMYYCARWGYYGSGSYENDAFDIWGQGTMVTVSS

SEQ ID NO: 54 (ANTI-SPON2 VL, WITH VL100 CYSTEINE MODIFICATION):
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLT
ISRLEPEDFAVYYCQQYSSSLTFGCGTKVEIK

SEQ ID NO: 55 (ANTI-SPON2 VH, WITH VH44 CYSTEINE MODIFICAITON):
EVQLVQSGGGLVMPGGSLRLSCAGSGFTFSSYVMHWLRQAPGKCLEWVSVIGTGGVTHYADSVKGRFMISRDNAK
NSLYLQMNSLRAEDTAMYYCARWGYYGSGSYENDAFDIWGQGTMVTVSS

SEQ ID NO: 56 (ANTI-SPON2 scFv, VL-VH):
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLT
ISRLEPEDFAVYYCQQYSSSLTFGGGTKVEIKGGGGSGGGGSGGGGSGGGGSEVQLVQSGGGLVMPGGSLRLSCAGSGFT
FSSYVMHWLRQAPGKGLEWVSVIGTGGVTHYADSVKGRFMISRDNAKNSLYLQMNSLRAEDTAMYYCARWGYYGS
GSYENDAFDIWGQGTMVTVSS

SEQ ID NO: 57 (ANTI-SPON2 scFv, VL-VH, WITH VL100-VH44 CYSTEINE
MODIFICATIONS):
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLT
ISRLEPEDFAVYYCQQYSSSLTFGCGTKVEIKGGGGSGGGGSGGGGSGGGGSEVQLVQSGGGLVMPGGSLRLSCAGSGFT
FSSYVMHWLRQAPGKCLEWVSVIGTGGVTHYADSVKGRFMISRDNAKNSLYLQMNSLRAEDTAMYYCARWGYYGS
GSYENDAFDIWGQGTMVTVSS

SEQ ID NO: 58 (ANTI-SPON2 scFv, VH-VL):
EVQLVQSGGGLVMPGGSLRLSCAGSGFTFSSYVMHWLRQAPGKGLEWVSVIGTGGVTHYADSVKGRFMISRDNAK
NSLYLQMNSLRAEDTAMYYCARWGYYGSGSYENDAFDIWGQGTMVTVSSGGGGSGGGGSGGGGSEIVLTQSPGTL
SLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV
YYCQQYSSSLTFGGGTKVEIK

SEQ ID NO: 59 (ANTI-SPON2 scFv, VH-VL, WITH VH44-VL100 CYSTEINE
MODIFICATIONS):
EVQLVQSGGGLVMPGGSLRLSCAGSGFTFSSYVMHWLRQAPGKCLEWVSVIGTGGVTHYADSVKGRFMISRDNAK
NSLYLQMNSLRAEDTAMYYCARWGYYGSGSYENDAFDIWGQGTMVTVSSGGGGSGGGGSGGGGSEIVLTQSPGTL
SLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV
YYCQQYSSSLTFGCGTKVEIK

SEQ ID NO: 60 (76-216 OF HUMAN NKG2D AS IN NP_031386.2):
AVFLNSLFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYSKEDQDLLKLVK
SYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTV

SEQ ID NO: 61 (ANTI-CD3 scFv LINKED TO NKG2D):
MGWSCIILFLVATATGVHSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRG
YTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSG
GSGGVDDIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTS

YSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKGGGGSGGGGSGGGGSAVFLNSLFNQEVQIPLTESYCGP
CPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSI
LSPNLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTVHHHHHH

SEQ ID NO: 62 (ANTI-CD28 scFv LINKED TO NKG2D):
DIELTQSPASLAVSLGQRATISCRASESVEYYVTSLMQWYQQKPGQPPKLLIFAASNVESGVPARFSGSGSGTNF
SLNIHPVDEDDVAMYFCQQSRKVPYTFGCGTKLEIKRGGGGSGGGGSGGGGSQVKLQQSGPGLVTPSQSLSITCT
VSGFSLSDYGVHWVRQSPGQCLEWLGVIWAGGGTNYNSALMSRKSISKDNSKSQVFLKMNSLQADDTAVYYCARD
KGYSYYYSMDYWGQGTTVTVSSGGGGSGGGGSGGGGSAVFLNSLFNQEVQIPLTESYCGPCPKNWICYKNNCYQF
FDESKNWYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGD
CALYASSFKGYIENCSTPNTYICMQRTVHHHHHH

SEQ ID NO: 63 (ALTERNATIVE INTER-scFv LINKER)
GGGSG

SEQ ID NO: 64 (ALTERNATIVE INTER-scFv LINKER)
GGSGG

SEQ ID NO: 65 (ALTERNATIVE INTER-scFv LINKER)
GSGGG

SEQ ID NO: 66 (ALTERNATIVE INTER-scFv LINKER)
SGGGG

SEQ ID NO: 67 (ALTERNATIVE INTER-scFv LINKER)
GGGSS

SEQ ID NO: 68 (ALTERNATIVE INTER-scFv LINKER)
GGSGS

SEQ ID NO: 69 (ALTERNATIVE INTER-scFv LINKER)
GSGGS

SEQ ID NO: 70 (ALTERNATIVE INTER-scFv LINKER)
SGGGS

SEQ ID NO: 71 (ALTERNATIVE INTER-scFv LINKER)
GGSSG

SEQ ID NO: 72 (ALTERNATIVE INTER-scFv LINKER)
GSGSG

SEQ ID NO: 73 (ALTERNATIVE INTER-scFv LINKER)
SGGSG

SEQ ID NO: 74 (ALTERNATIVE INTER-scFv LINKER)
GSSGG

SEQ ID NO: 75 (ALTERNATIVE INTER-scFv LINKER)
SGSGG

SEQ ID NO: 76 (ALTERNATIVE INTER-scFv LINKER)
SSGGG

WHAT IS CLAIMED IS:

1. A bispecific binding reagent that simultaneously binds to an immune cell and a prostate cancer cell, wherein said bispecific binding reagent comprises a polypeptide molecule having a first domain that binds to an antigen expressed by the immune cell and a second domain that binds to an antigen expressed by the prostate cancer cell.

2.. The bispecific binding reagent of claim 1, wherein the prostate cancer cell is selected from the group consisting of a metastatic prostate cancer cell and a castration-resistant prostate cancer cell.

3. The bispecific binding reagent of claim 1 or 2, wherein the first domain is derived from an antibody.

4. The bispecific binding reagent of claim 3, wherein the first domain comprises a single-chain variable fragment (scFv).

5. The bispecific binding reagent of any one of claims 1-4, wherein the second domain is derived from an antibody.

6. The bispecific binding reagent of claim 6, wherein the second domain comprises an scFv.

7. The bispecific binding reagent of any one of claims 1-6, wherein the first domain comprises a first scFv and the second domain comprises a second scFv.

8. The bispecific binding reagent of any one of claims 1-7, wherein the bispecific binding reagent is a tandem single-chain variable fragment antibody molecule (taFv) comprising a first scFv and a second scFv.

9. The bispecific binding reagent of any one of claims 1-8, wherein the immune cell is selected from the group consisting of a T cell, a Natural Killer (NK) cell, and a Natural Killer T (NKT) cell.

10. The bispecific binding reagent of any one of claims 1-9, wherein the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of ABCC4, ANO7, EPCAM, FOLH1, OR51E1, OR51E2, PSCA, SLC30A4,

SLC45A3, SPON2, STEAP1, STEAP2, TARP, TMEFF2, TMPR8, TMPRSS2, and a gene encoding an NKG2D ligand.

11. The bispecific binding reagent of any one of claims 1-9, wherein the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of ABCC4, ANO7, EPCAM, FOLH1, OR51E1, OR51E2, PSCA, SLC45A3, SPON2, STEAP1, STEAP2, TARP, TMEFF2, TMPR8, and a gene encoding an NKG2D ligand.

12. The bispecific binding reagent of any one of claims 1-9, wherein the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of EPCAM, FOLH1, SPON2, STEAP1, and a gene encoding an NKG2D ligand.

13. The bispecific binding reagent of any one of claims 1-9, wherein the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of EPCAM, STEAP1, and a gene encoding an NKG2D ligand.

14. The bispecific binding reagent of any one of claims 1-9, wherein the antigen expressed by the prostate cancer cell is encoded by a gene encoding an NKG2D ligand.

15. The bispecific binding reagent of any one of claims 10-14, wherein the gene encoding the NKG2D ligand is selected from the group consisting of MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, and ULBP3.

16. The bispecific binding reagent of any one of claims 1-9, wherein the antigen expressed by the prostate cancer cell is encoded by a gene selected from the group consisting of EPCAM and STEAP1.

17. The bispecific binding reagent of any one of claims 1-16, wherein the second domain binds to an antigen expressed on the surface of the prostate cancer cell.

18. The bispecific binding reagent of any one of claims 1-17, wherein the antigen expressed by the immune cell is encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), CD28, CD2, CD27, IL7R, CD69, ICOS, IL2RB, PTPRC (CD45), CD48, SELL, CD137 (4-1BB, TNFRSF9), and CD134 (OX40, TNFRSF4).

19. The bispecific binding reagent of any one of claims 1-17, wherein the antigen expressed by the immune cell is encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), CD28, CD137 (4-1BB, TNFRSF9), and CD134 (OX40, TNFRSF4).

20. The bispecific binding reagent of any one of claims 1-17, wherein the antigen expressed by the immune cell is encoded by a gene selected from the group consisting of CD3D (CD3 delta), CD3E (CD3 epsilon), CD247 (CD3 zeta, CD3Z), and CD28.

21. The bispecific binding reagent of any one of claims 1-20, wherein the first domain of the bispecific binding reagent specifically binds to the antigen CD3.

22. The bispecific binding reagent of claim 21, wherein the first domain of the bispecific binding reagent comprises an anti-CD3 scFv.

23. The bispecific binding reagent of claim 22, wherein the anti-CD3 scFv comprises:

an anti-CD3 $V_H$ amino acid sequence of SEQ ID NO:6 and an anti-CD3 $V_L$ amino acid sequence of SEQ ID NO:7; or

an anti-CD3 $V_H$ amino acid sequence of SEQ ID NO:18 and an anti-CD3 $V_L$ amino acid sequence of SEQ ID NO:19.

24. The bispecific binding reagent of any one of claims 1-23, wherein the second domain of the bispecific binding reagent specifically binds to the antigen STEAP1.

25. The bispecific binding reagent of claim 24, wherein the second domain of the bispecific binding reagent comprises an anti-STEAP1 scFv.

26. The bispecific binding reagent of claim 25, wherein the anti-STEAP1 scFv comprises:

an anti-STEAP1 $V_L$ amino acid sequence of SEQ ID NO:3 and an anti-STEAP1 $V_H$ amino acid sequence of SEQ ID NO:4; or

an anti-STEAP1 $V_L$ amino acid sequence of SEQ ID NO:32 and an anti-STEAP1 $V_H$ amino acid sequence of SEQ ID NO:33.

27. The bispecific binding reagent of any one of claims 1-13 and 17-26, wherein the bispecific binding reagent binds to both CD3 and STEAP1.

28. The bispecific binding reagent of any one of claims 8-13 and 17-27, wherein the bispecific binding reagent is an anti-CD3/anti-STEAP1 taFv comprising an anti-CD3 first scFv and an anti-STEAP1 second scFv.

29. The bispecific binding reagent of claim 28, wherein the anti-CD3/anti-STEAP1 taFv comprises an inter-scFv linker with an amino acid sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NOS:63 to 76.

30. The bispecific binding reagent of claim 28, wherein the anti-CD3/anti-STEAP1 taFv comprises the amino acid sequence of SEQ ID NO:1.

31. The bispecific binding reagent of claim 28, wherein the anti-CD3/anti-STEAP1 taFv comprises an amino acid sequence encoded by SEQ ID NO:2.

32. The bispecific binding reagent of any one of claims 1-20 and 24-26, wherein the first domain of the bispecific binding reagent specifically binds to the antigen CD28.

33. The bispecific binding reagent of claim 32, wherein the first domain of the bispecific binding reagent comprises an anti-CD28 scFv.

34. The bispecific binding reagent of claim 33, wherein the anti-CD28 scFv comprises:

an anti-CD28 $V_H$ amino acid sequence of SEQ ID NO:15 and an anti-CD28 $V_L$ amino acid sequence of SEQ ID NO:14; or

an anti-CD28 $V_H$ amino acid sequence of SEQ ID NO:27 and an anti-CD28 $V_L$ amino acid sequence of SEQ ID NO:26.

35. The bispecific binding reagent of any one of claims 1-10, 16-23, and 32-34, wherein the second domain of the bispecific binding reagent specifically binds to the antigen EpCAM.

36. The bispecific binding reagent of claim 35, wherein the second domain of the bispecific binding reagent comprises an anti-EpCAM scFv.

37. The bispecific binding reagent of claim 36, wherein the anti-EpCAM scFv comprises:

an anti-EpCAM $V_H$ amino acid sequence of SEQ ID NO:16 and an anti-EpCAM $V_L$ amino acid sequence of SEQ ID NO:17; or

an anti-EpCAM $V_H$ amino acid sequence of SEQ ID NO:39 and an anti-EpCAM $V_L$ amino acid sequence of SEQ ID NO:38.

38. The bispecific binding reagent of any one of claims 1-10, 16-20, and 32-37, wherein the bispecific binding reagent binds to both CD28 and EpCAM.

39. The bispecific binding reagent of claim 8-10, 16-20, and 32-38, wherein the bispecific binding reagent is an anti-CD28/anti-EpCAM taFv comprising an anti-CD28 first scFv and an anti-EpCAM second scFv.

40. The bispecific binding reagent of claim 39, wherein the anti-CD28/anti-EpCAM taFv comprises an inter-scFv linker with an amino acid sequence selected from the group consisting of SEQ ID NO:11 and SEQ ID NOS:63 to 76.

41. The bispecific binding reagent of claim 40, wherein the anti-CD28/anti-EpCAM taFv comprises the amino acid sequence of SEQ ID NO:12.

42. The bispecific binding reagent of claim 40, wherein the anti-CD28/anti-EpCAM taFv comprises an amino acid sequence encoded by SEQ ID NO:13.

43. The bispecific binding reagent of any one of claims 1-10, wherein the second domain is derived from a ligand for a receptor, wherein the ligand is expressed on the surface of the prostate cancer cell.

44. The bispecific binding reagent of any one of claims 1-10, wherein the second domain is derived from a receptor that interacts with a ligand that is expressed on the surface of the prostate cancer cell.

45. The bispecific binding reagent of claim 43, wherein the ligand for the receptor that is expressed on the surface of the prostate cancer cell is an NKG2D ligand.

46. The bispecific binding reagent of claim 45, wherein the NKG2D ligand is selected from the group consisting of MICA, MICB, ULBP1, ULBP2, ULBP3, RAET1E, RAET1G, and RAET1L.

47. The bispecific binding reagent of claim 44, wherein the receptor that interacts with a ligand that is expressed on the surface of the prostate cancer cell is NKG2D.

48. The bispecific binding reagent of claim 47, wherein the second domain comprises the extracellular domain of NKG2D or a portion thereof, wherein the portion of the extracellular domain of NKG2D specifically binds to an NKG2D ligand.

49. The bispecific binding reagent of claim 48, wherein the NKG2D ligand is selected from the group consisting of MICA, MICB, RAET1E, RAET1G, RAET1L, ULBP1, ULBP2, and ULBP3.

50. The bispecific binding reagent of claim 48 or 49, wherein the extracellular domain of NKG2D or a portion thereof comprises the amino acid sequence of SEQ ID NO:60.

51. The bispecific binding reagent of claim 50, wherein the extracellular domain of the NKG2D receptor is linked to an scFv with a linker selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:6.

52. The bispecific binding reagent of any one of claims 47-51, wherein the bispecific binding reagent comprises the amino acid sequence of SEQ ID NO:61.

53. The bispecific binding reagent of any one of claims 47-51, wherein the bispecific binding reagent comprises the amino acid sequence of SEQ ID NO:62.

54. The bispecific binding reagent of any one of claims 1-53, wherein the first domain and/or the second domain comprises one or more disulfide bridges.

55. An isolated nucleic acid encoding a bispecific binding reagent of any one of claims 1-54.

56. An isolated vector comprising the nucleic acid of claim 55.

57. The vector of claim 56, wherein the nucleic acid is operably linked to a promoter.

58. A host cell comprising the nucleic acid of claim 55 or the vector of claim 56 or 57.

59. A process for the production of a bispecific binding reagent comprising:

culturing the isolated host cell of claim 58 under conditions suitable for the expression of the bispecific binding reagent; and

recovering the produced bispecific binding reagent from the cell or from the culture.

60. A composition comprising a plurality of structurally different bispecific binding reagents of any one of claims 1-54.

61. The composition of claim 60, wherein the composition comprises:

a first bispecific binding reagent that binds to a first antigen expressed by an immune cell and a first antigen expressed by a prostate cancer cell, wherein the first antigen expressed by the immune cell is CD3; and

a second bispecific binding reagent that binds to a second antigen expressed by the immune cell and a second antigen expressed by the prostate cancer cell.

62. The composition of claim 61, wherein the first bispecific binding reagent binds CD3 and the second bispecific binding reagent binds CD28.

63. The composition of claim 61 or 62, wherein the first bispecific binding reagent and the second bispecific binding reagent bind the same antigen expressed by the prostate cancer cell.

64. The composition of claim 61 or 62, wherein the first bispecific binding reagent and the second bispecific binding reagent bind different antigens expressed by the prostate cancer cell.

65. The composition of claim 64, wherein the first bispecific binding reagent binds CD3 and STEAP1 and the second bispecific binding reagent binds CD28 and EpCAM.

66. The composition of claim 64, wherein the first bispecific binding reagent is an anti-CD3/anti-STEAP1 taFv and the second bispecific binding reagent is an anti-CD28/anti-EpCAM taFv.

67. The composition of claim 66, wherein the first bispecific binding reagent comprises the amino acid sequence SEQ ID NO:1 and the second bispecific binding reagent comprises the amino acid sequence SEQ ID NO:12.

68. The composition of claim 66, wherein the first bispecific binding reagent is a taFv encoded by SEQ ID NO:2 and the second bispecific binding reagent is a taFv encoded by SEQ ID NO:13.

69. A composition comprising a nucleic acid of claim 55 and a pharmaceutically acceptable excipient.

70. A composition comprising a plurality of structurally different nucleic acids of claim 55 and a pharmaceutically acceptable excipient.

71. A composition comprising a bispecific binding reagent of any one of claims 1-54 and a pharmaceutically acceptable excipient.

72. A composition comprising a plurality of structurally different bispecific binding reagents of any one of claims 1-54 and a pharmaceutically acceptable excipient.

73. The composition of claim 71 or 72, wherein the composition further comprises a chemical compound or a biological agent capable of providing an activation signal for T cells.

74. A method for treating a subject in need thereof comprising administering a therapeutically effective amount of a first bispecific binding reagent of any one of claims 1-54 to the subject.

75. The method of claim 74, wherein the subject has prostate cancer.

76. The method of claim 74, wherein the subject has prostate cancer selected from the group consisting of metastatic prostate cancer and castration-resistant prostate cancer.

77. The method of any one of claims 74-76, wherein the method further comprises simultaneously or sequentially administering a second bispecific binding reagent, wherein the second bispecific binding reagent specifically binds at least one antigen that is not specifically recognized by the first bispecific binding reagent.

78. The method of claim 77, wherein the first and second bispecific binding reagents comprise a first bispecific binding reagent that binds to CD3 and a second bispecific binding reagent that binds to CD28.

79. The method of claim 77 or 78, wherein the first and second bispecific binding reagents comprise a first bispecific binding reagent that binds to STEAP1 and a second bispecific binding reagent that binds to EpCAM.

80. The method of claim 77 or 78, wherein the first and second bispecific binding reagents comprise a first bispecific binding reagent that binds to CD3 and STEAP1 and a second bispecific binding reagent that binds to CD28 and EpCAM.

81. The method of claim 77 or 78, wherein the first and/or the second bispecific binding reagent is:

a bispecific binding reagent that comprises a second domain that is derived from a ligand for a receptor that is expressed on the surface of the prostate cancer cell; or

a bispecific binding reagent that comprises a second domain that is derived from a receptor that interacts with a ligand that is expressed on the surface of the prostate cancer cell.

82. A kit comprising one or more of the bispecific binding reagents of any one of claims 1-54 and optionally comprising a pharmaceutical excipient.

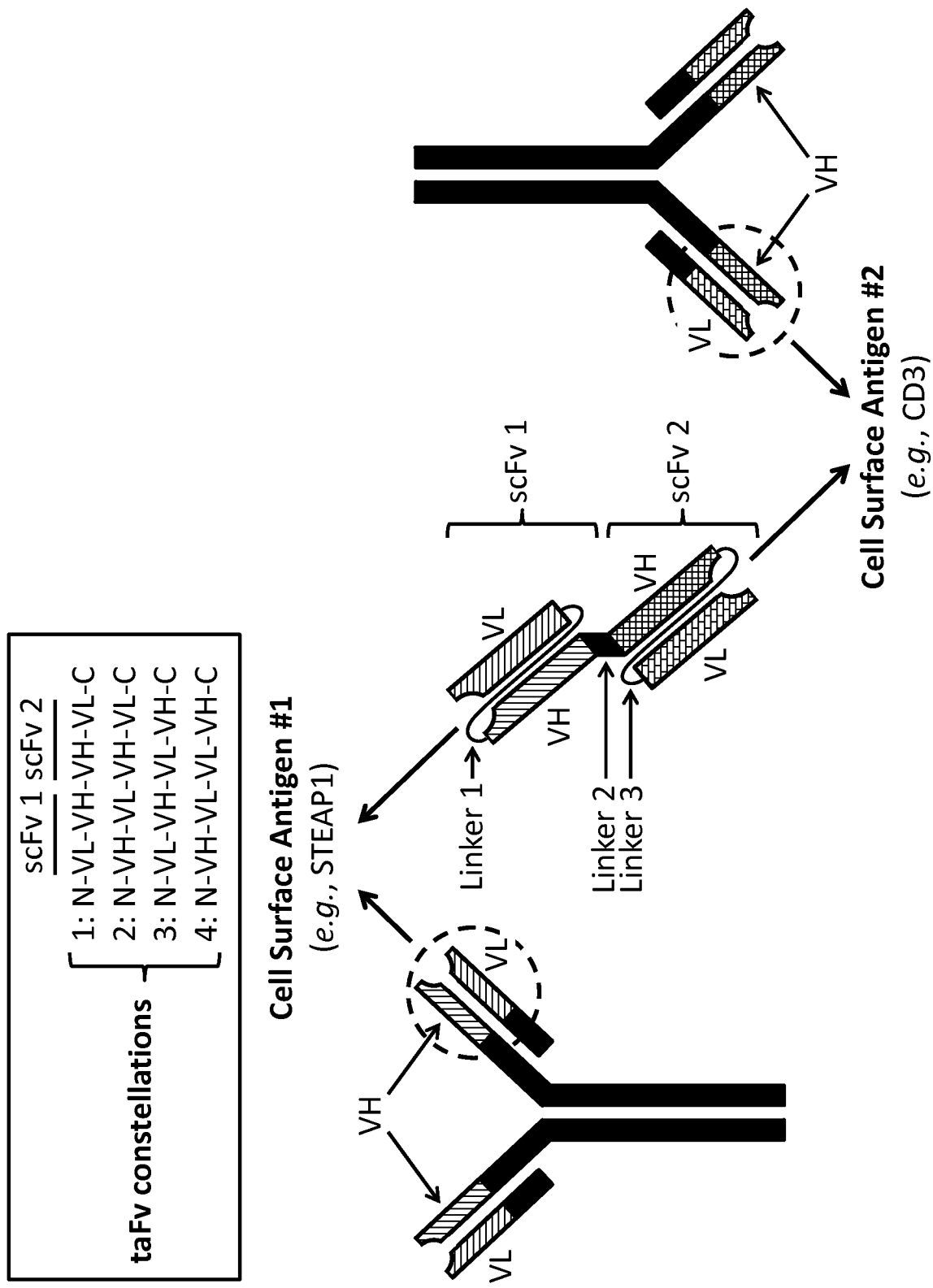

taFv constellations
scFv 1 scFv 2
1: N-VL-VH-VH-VL-C
2: N-VH-VL-VH-VL-C
3: N-VL-VH-VL-VH-C
4: N-VH-VL-VL-VH-C
Cell Surface Antigen #1
(e.g., STEAP1)
Cell Surface Antigen #2
(e.g., CD3)
scFv 1
scFv 2
Linker 1
Linker 2
Linker 3
VH
VL

FIG. 1

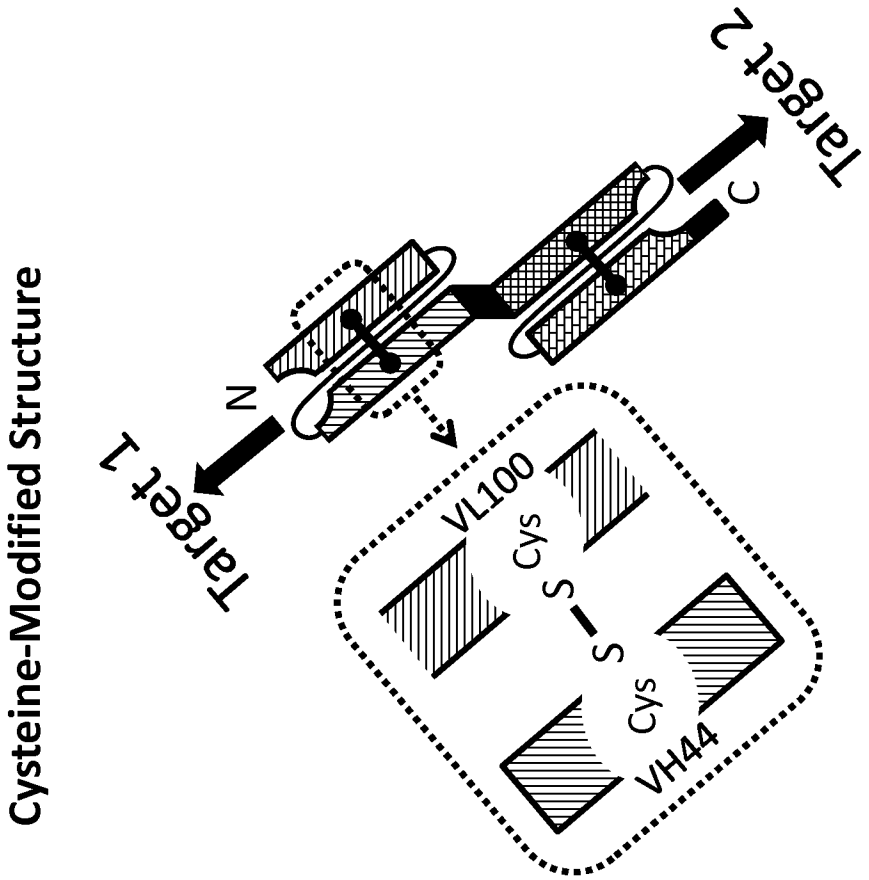

Cysteine-Modified Structure
Target 2
C
N
Target 1
VL100
Cys
S
S
Cys
VH44

FIG. 2B

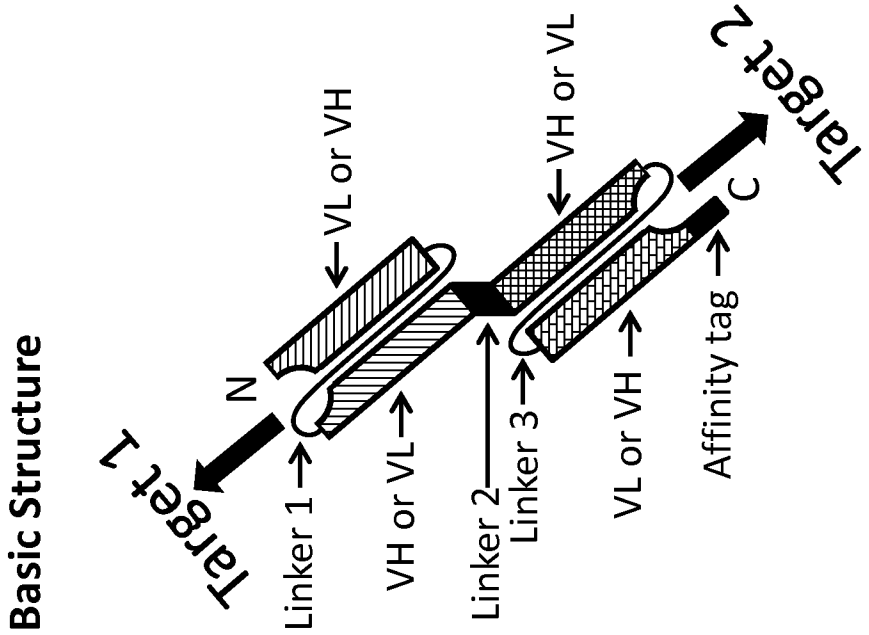

Basic Structure
Target 2
C
VL or VH
VH or VL
N
Target 1
Linker 1
VH or VL
Linker 2
Linker 3
VL or VH
Affinity tag

FIG. 2A

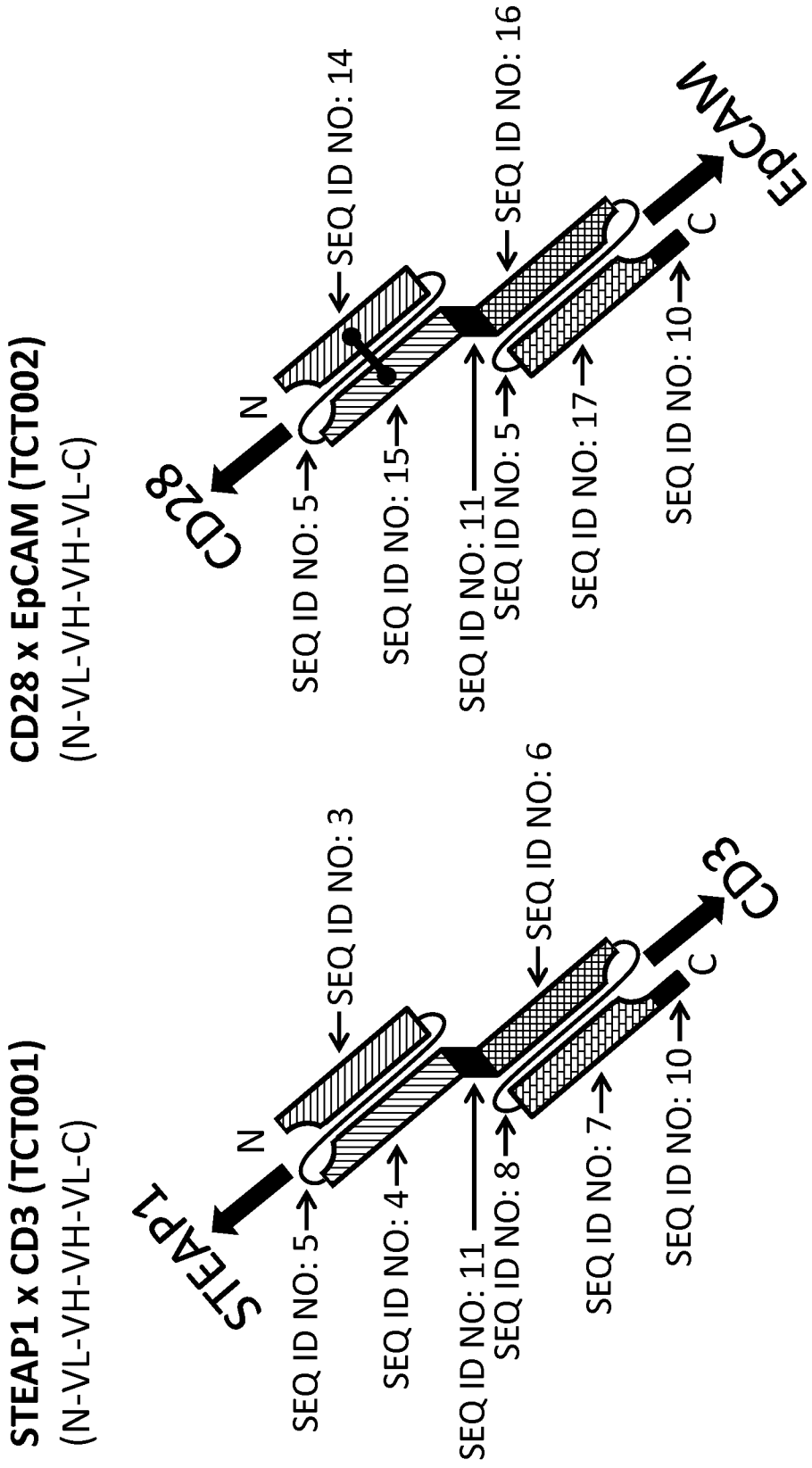

CD28 x EpCAM (TCT002)
(N-VL-VH-VH-VL-C)
N
CD28
SEQ ID NO: 5
SEQ ID NO: 14
SEQ ID NO: 15
SEQ ID NO: 11
SEQ ID NO: 5
SEQ ID NO: 16
SEQ ID NO: 17
SEQ ID NO: 10
C
EPCAM
STEAP1 x CD3 (TCT001)
(N-VL-VH-VH-VL-C)
N
STEAP1
SEQ ID NO: 5
SEQ ID NO: 3
SEQ ID NO: 4
SEQ ID NO: 11
SEQ ID NO: 8
SEQ ID NO: 6
SEQ ID NO: 7
SEQ ID NO: 10
C
CD3

*FIG. 2D*

*FIG. 2C*

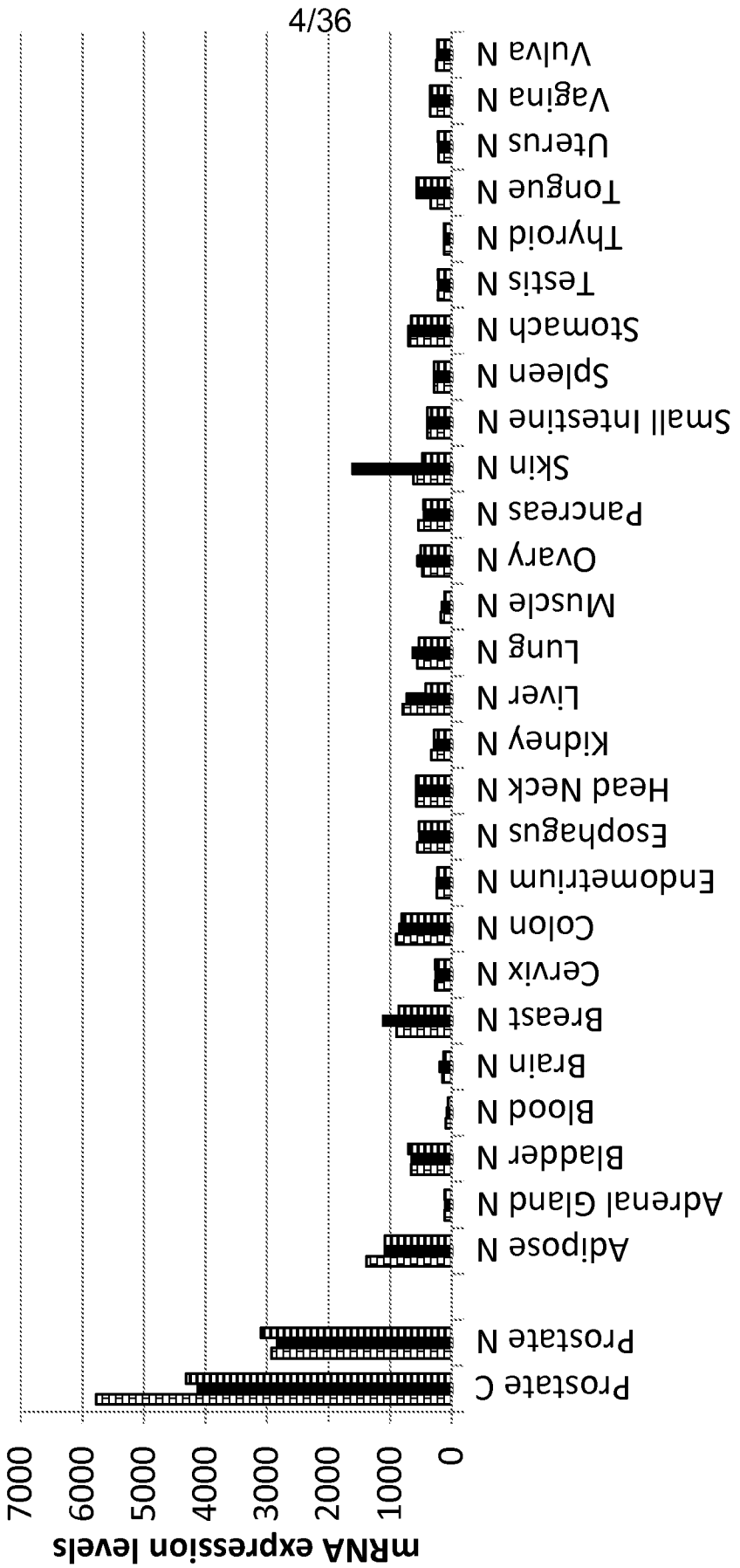

STEAP1
Mean of all samples
Mean of mean values
Median of mean values
mRNA expression levels
0
1000
2000
3000
4000
5000
6000
7000
Prostate C
Prostate N
Adipose N
Adrenal Gland N
Bladder N
Blood N
Brain N
Breast N
Cervix N
Colon N
Endometrium N
Esophagus N
Head Neck N
Kidney N
Liver N
Lung N
Muscle N
Ovary N
Pancreas N
Skin N
Small Intestine N
Spleen N
Stomach N
Testis N
Thyroid N
Tongue N
Uterus N
Vagina N
Vulva N

FIG. 3A

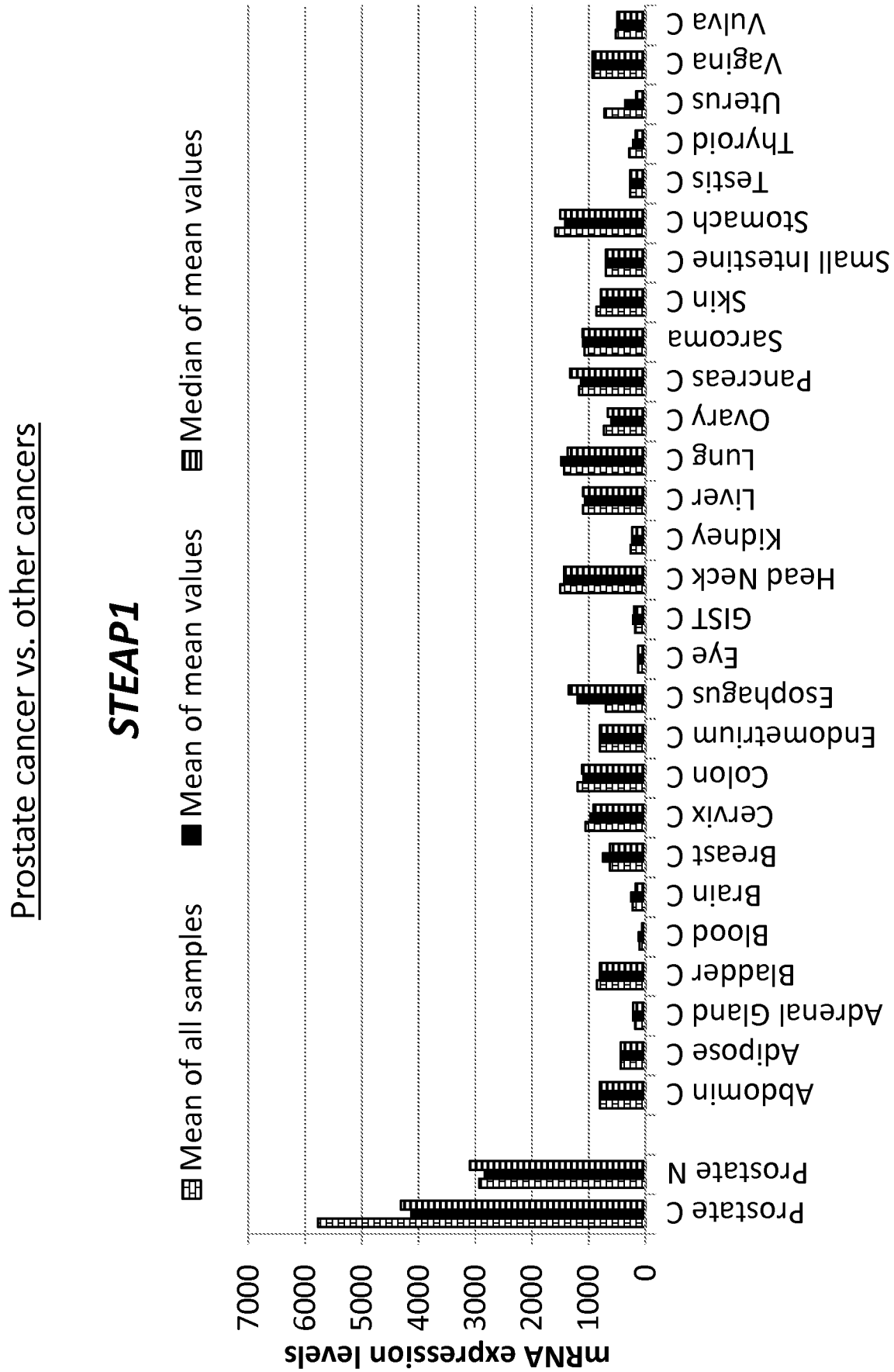

Prostate cancer vs. other cancers
STEAP1
Mean of all samples
Mean of mean values
Median of mean values
mRNA expression levels
7000
6000
5000
4000
3000
2000
1000
0
Prostate C
Prostate N
Abdomin C
Adipose C
Adrenal Gland C
Bladder C
Blood C
Brain C
Breast C
Cervix C
Colon C
Endometrium C
Esophagus C
Eye C
GIST C
Head Neck C
Kidney C
Liver C
Lung C
Ovary C
Pancreas C
Sarcoma
Skin C
Small Intestine C
Stomach C
Testis C
Thyroid C
Uterus C
Vagina C
Vulva C

FIG. 3B

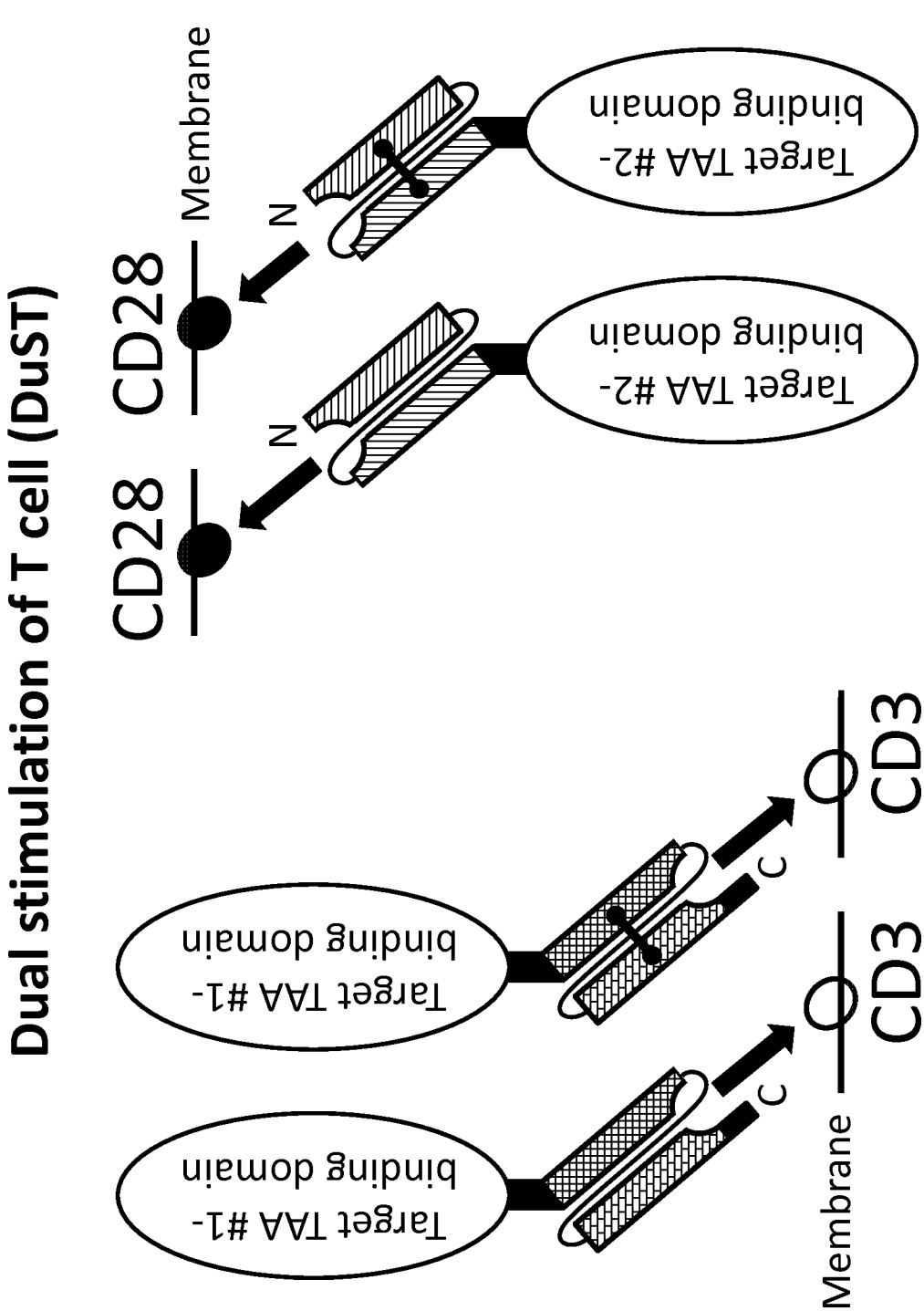

Dual stimulation of T cell (DuST)
Membrane
CD28
CD28
N
N
Target TAA #2-binding domain
Target TAA #2-binding domain
Representative:
TCT002 (CD28 x EpCAM)
Target TAA #1-binding domain
Target TAA #1-binding domain
Membrane
CD3
CD3
C
C
Representative:
TCT001 (STEAP1 x CD3)

*FIG. 4A*

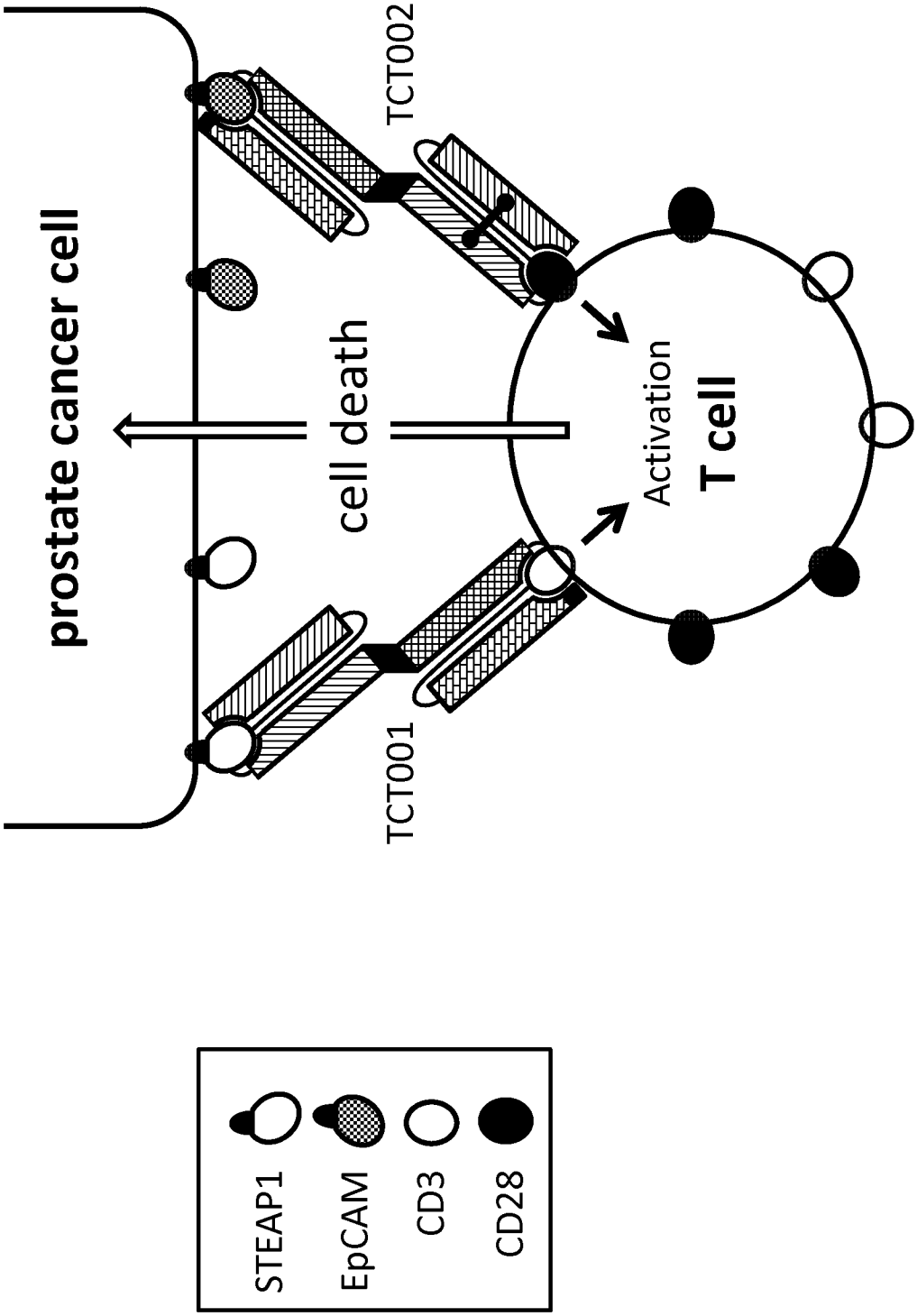

prostate cancer cell
cell death
TCT002
TCT001
Activation
T cell
STEAP1
EpCAM
CD3
CD28

FIG. 4B

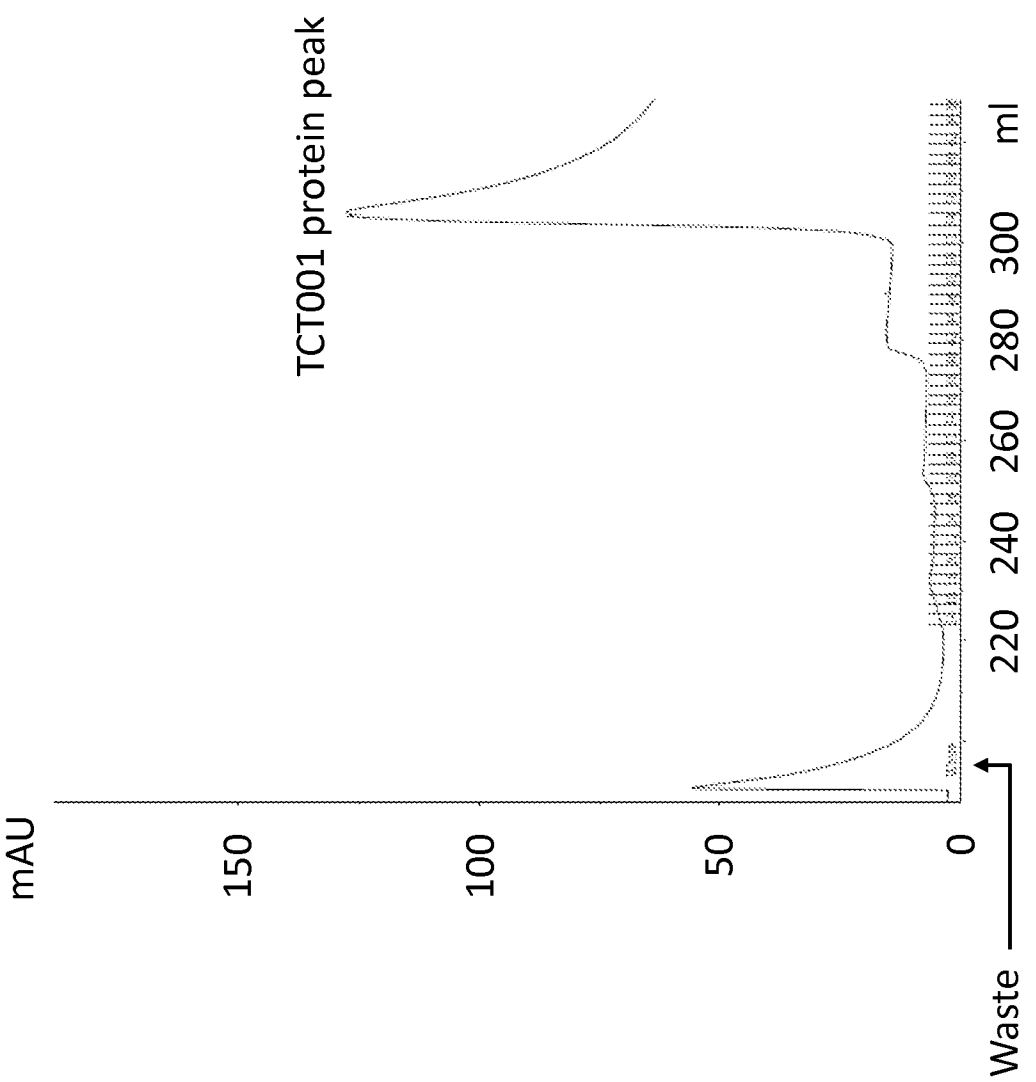

Chromatography of TCT001 (OD280)
mAU
150
100
50
0
TCT001 protein peak
Waste
220
240
260
280
300
ml

*FIG. 5*

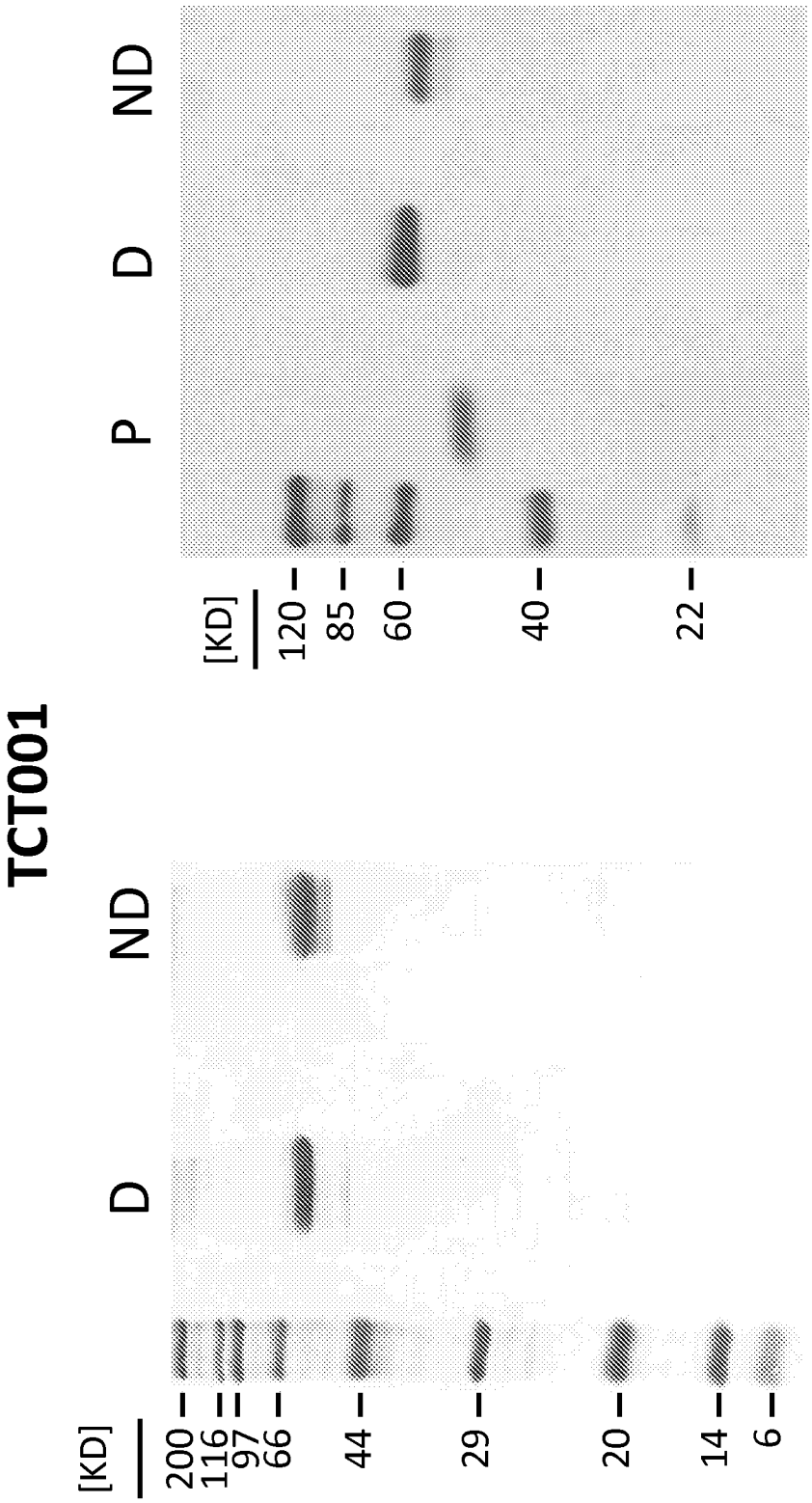

TCT001
D
ND
[KD]
200
116
97
66
44
29
20
14
6
SDS-PAGE
P
D
ND
[KD]
120
85
60
40
22
Western Blot (anti-His tag)

*FIG. 6A*

*FIG. 6B*

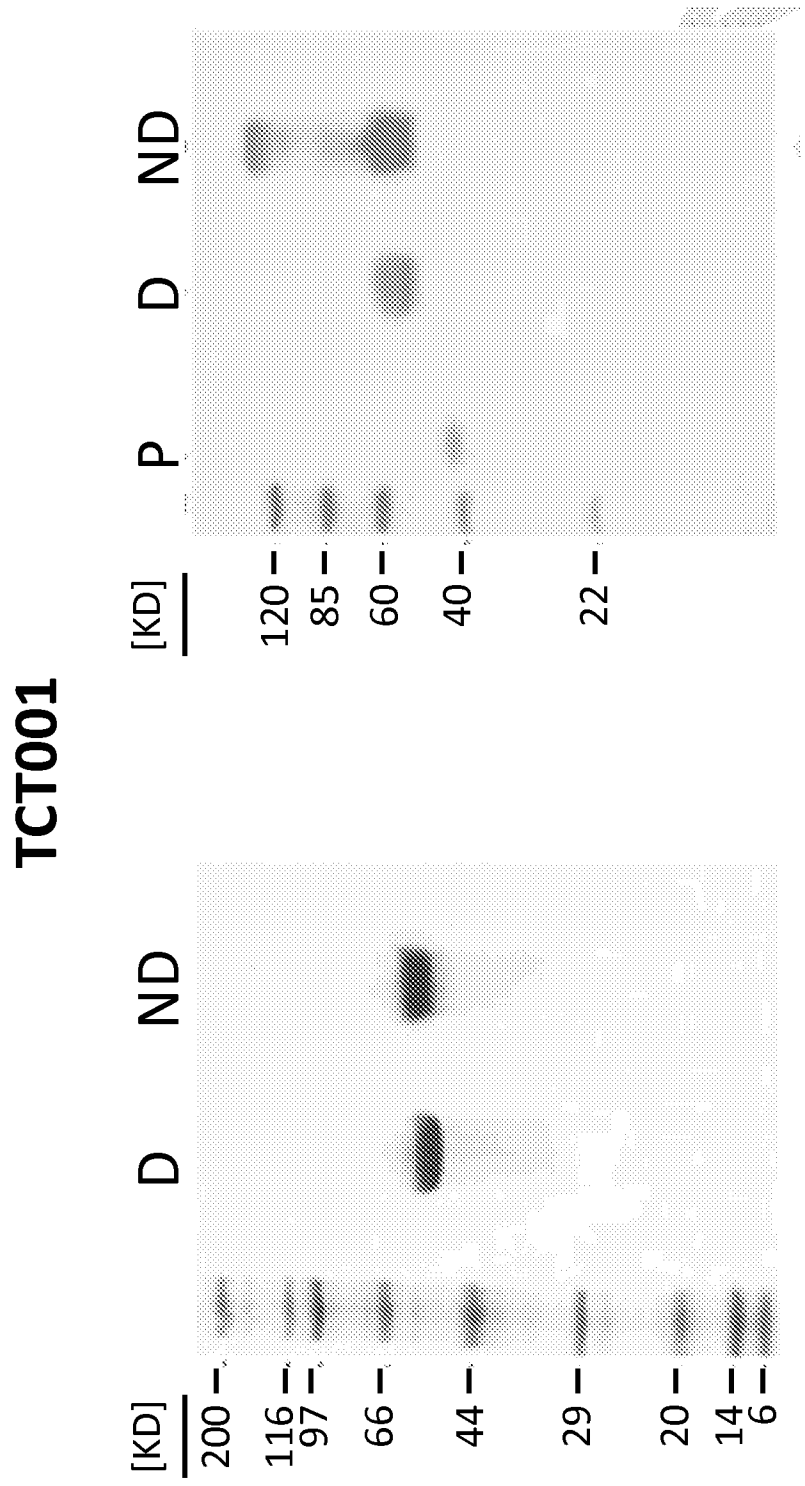

TCT001
[KD]
200
116
97
66
44
29
20
14
6
D
ND
SDS-PAGE
[KD]
120
85
60
40
22
P
D
ND
Western Blot (anti-His tag)

*FIG. 6C*

*FIG. 6D*

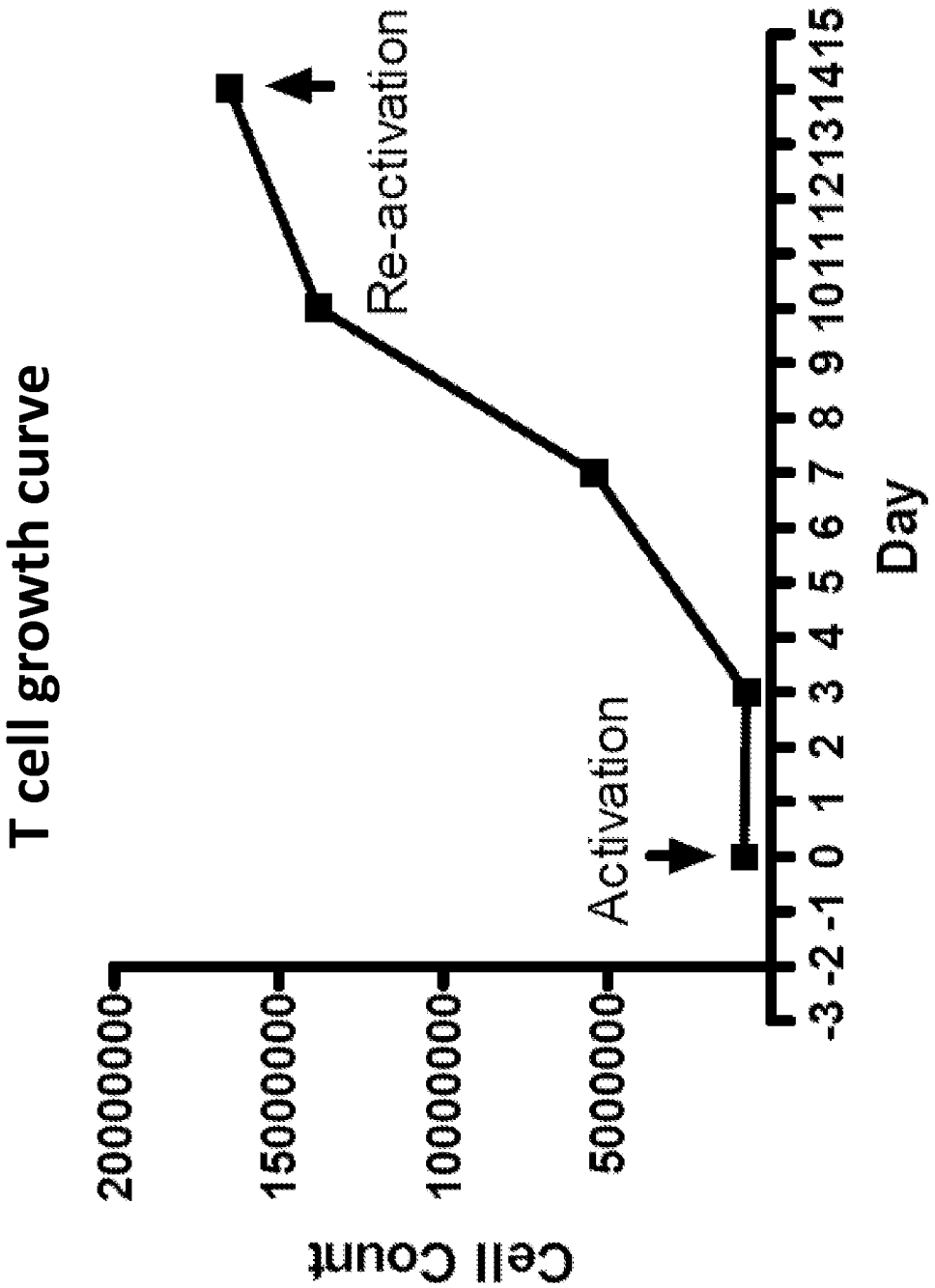

T cell growth curve
Cell Count
20000000
15000000
10000000
5000000
Activation
Re-activation
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day

FIG. 7A

# TCT001

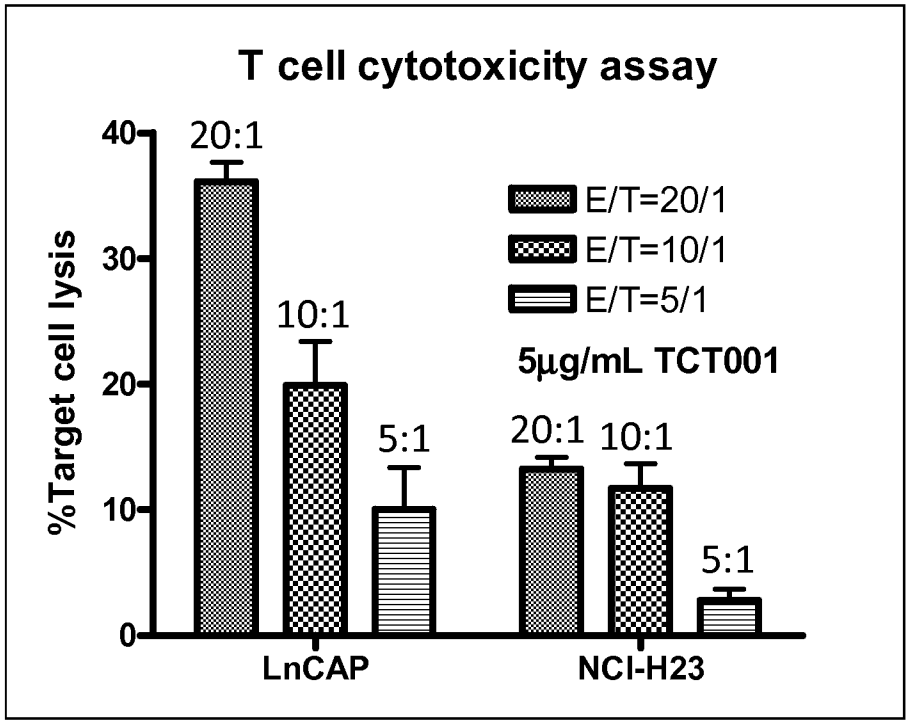
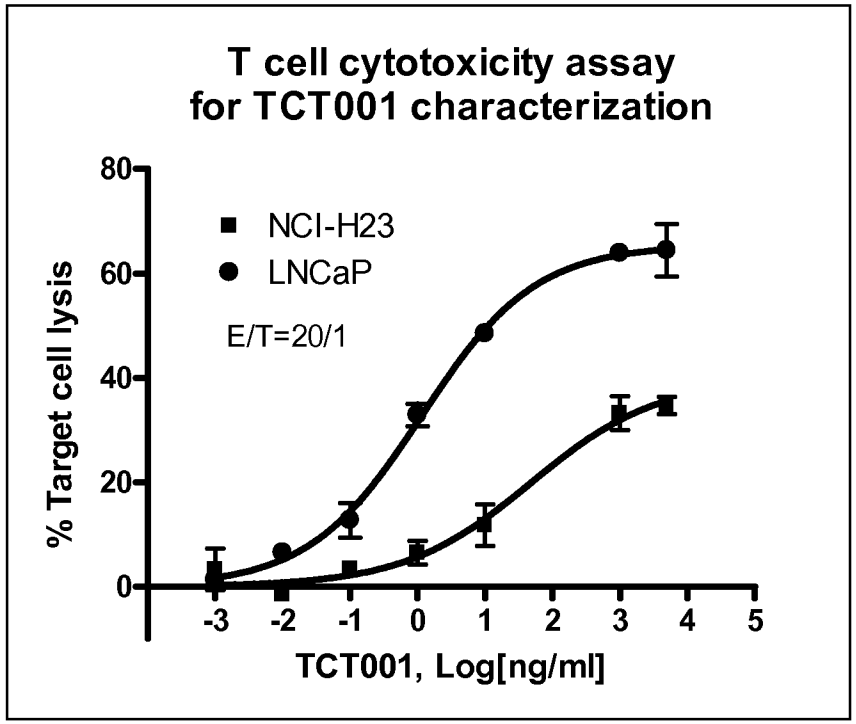



*FIG. 7B*




$EC_{50}$ (LNCaP) = 1.2 ng/ml (approx. 20 pM)
$EC_{50}$ (NCI-H23) = 48.6 ng/ml (approx. 800 pM)

*FIG. 7C*

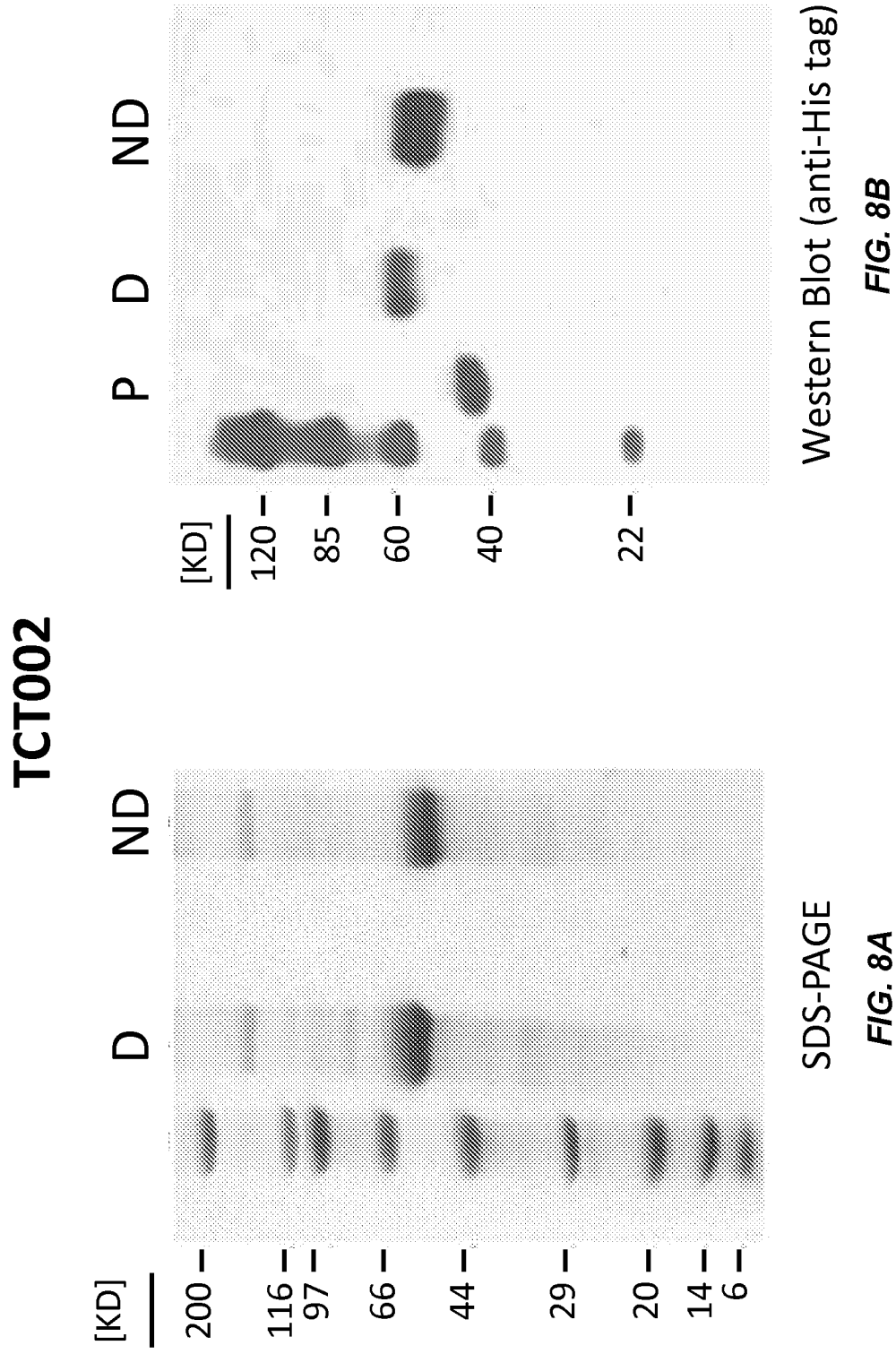

TCT002
[KD]
200
116
97
66
44
29
20
14
6
D
ND
SDS-PAGE
[KD]
120
85
60
40
22
P
D
ND
Western Blot (anti-His tag)

*FIG. 8A*

*FIG. 8B*

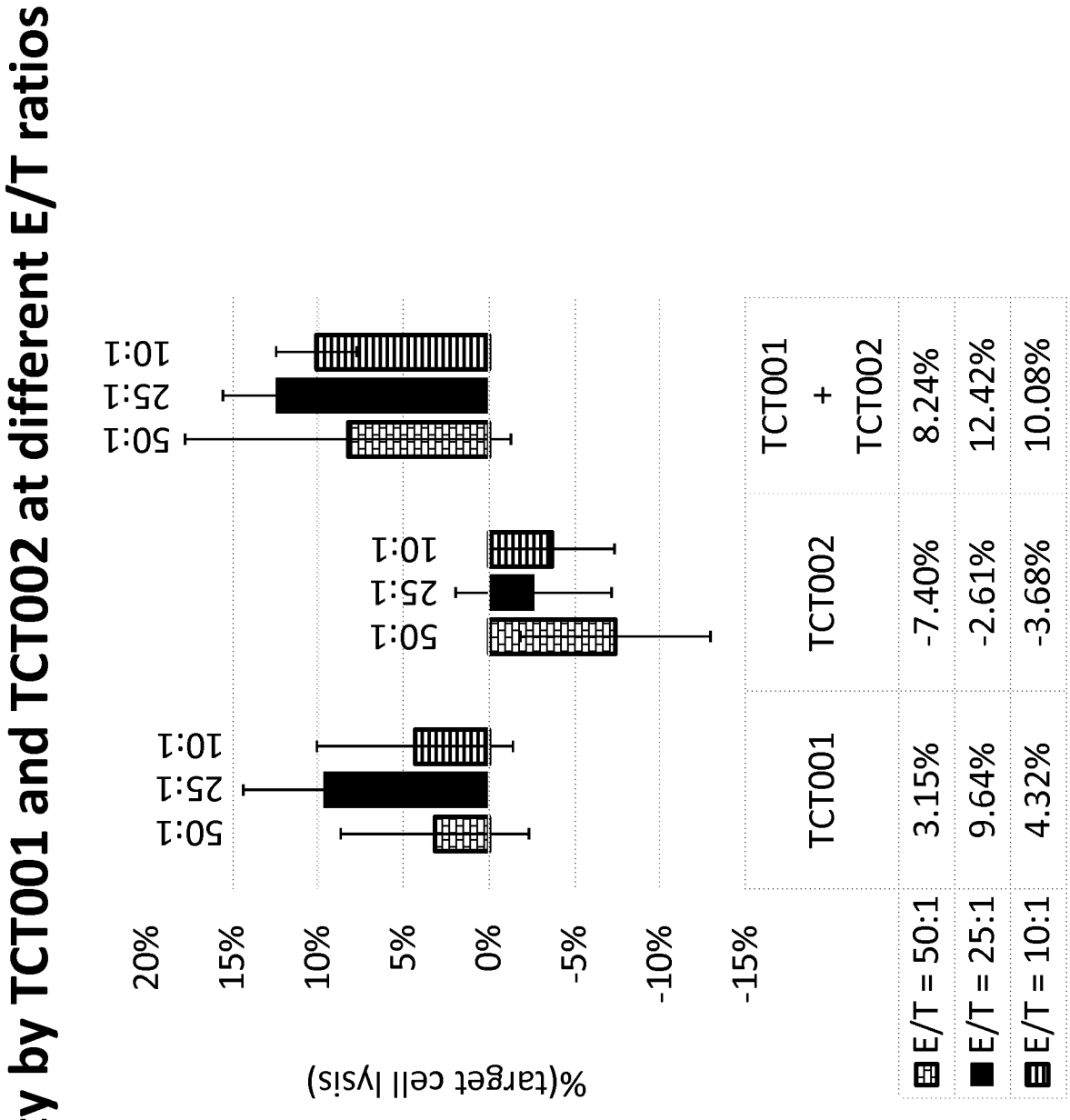
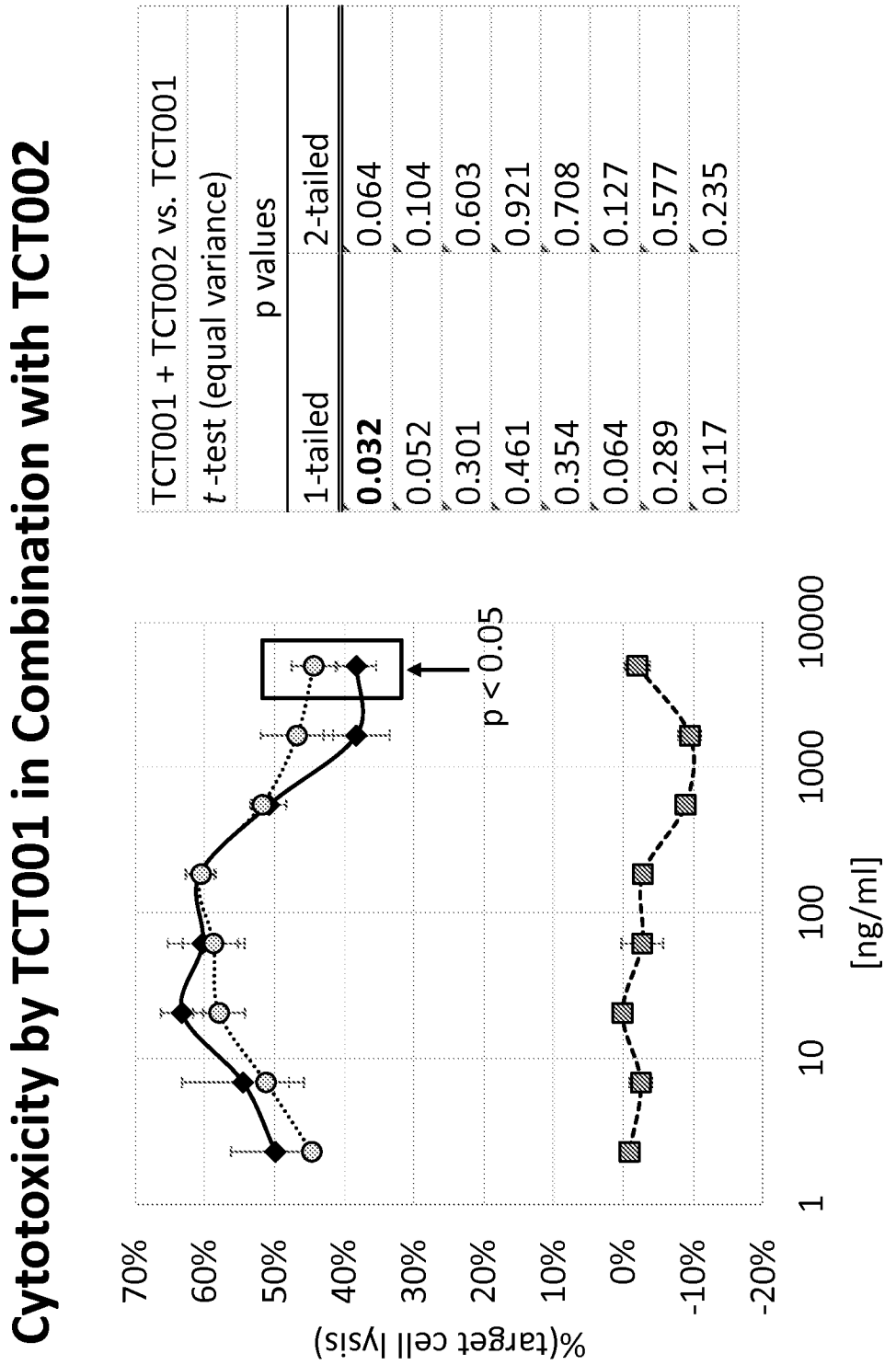

Cytotoxicity by TCT001 and TCT002 at different E/T ratios
%(target cell lysis)
20%
15%
10%
5%
0%
-5%
-10%
-15%
50:1
25:1
10:1
50:1
25:1
10:1
50:1
25:1
10:1
E/T = 50:1
E/T = 25:1
E/T = 10:1
TCT001
3.15%
9.64%
4.32%
TCT002
-7.40%
-2.61%
-3.68%
TCT001 + TCT002
8.24%
12.42%
10.08%

*FIG. 9*




| TCT001 + TCT002 vs. TCT001 | |
|---|---|
| *t*-test (equal variance) | |
| p values | |
| 1-tailed | 2-tailed |
| **0.032** | 0.064 |
| 0.052 | 0.104 |
| 0.301 | 0.603 |
| 0.461 | 0.921 |
| 0.354 | 0.708 |
| 0.064 | 0.127 |
| 0.289 | 0.577 |
| 0.117 | 0.235 |

*FIG. 10*

# Anti-CD3 variable sequences

Anti-CD3 VH as in TCT001 (SEQ ID NO: 6)

DIKLQQSGAELARPGASVKMSCKTS*GYTFTR*
*Ytmh*WVKQRPGQGLEWIG*yiNPSRGYtnynq*
*kfkd*KATLTTDKSSSTAYMQLSSLTSEDSAV
YYCAR*YYDDHYCLDY*WGQGTTLTVSS

Anti-CD3 VH with VL100 Cys (SEQ ID NO: 18)

DIKLQQSGAELARPGASVKMSCKTS*GYTFTR*
*Ytmh*WVKQRPGQ**C**LEWIG*yiNPSRGYtnynq*
*kfkd*KATLTTDKSSSTAYMQLSSLTSEDSAV
YYCAR*YYDDHYCLDY*WGQGTTLTVSS

Anti-CD3 VL as in TCT001 (SEQ ID NO: 7)

DIQLTQSPAIMSASPGEKVTMTC*RASSSVSY*
*MN*WYQQKSGTSPKRWIY*DTSKVAS*GVPYRFS
GSGSGTSYSLTISSMEAEDAATYYC*QQWSSN*
*PLT*FGAGTKLELK

Anti-CD3 VL with VH44 Cys (SEQ ID NO: 19)

DIQLTQSPAIMSASPGEKVTMTC*RASSSVSY*
*MN*WYQQKSGTSPKRWIY*DTSKVAS*GVPYRFS
GSGSGTSYSLTISSMEAEDAATYYC*QQWSSN*
*PLT*FG**C**GTKLELK

*Italic, underscored*: Chothia + Kabat CDRs.
*Italic, underscored, CAPITAL letters*: Chothia CDRs.
**Bold, underscored**: VL100 Cys and VH44 Cys.

***FIG. 11A***

# Anti-CD3 scFv (VH-VL)

Anti-CD3 scFv (as in TCT001)

**Bold, underscored**: VH44 **G** and VL100 **A**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIKLQQSGAELARPGASVKMSCKTS*GYTFTR*
*Ytmh*WVKQRPGQ**G**LEWIG*yiNPSRGYtnynq*
*kfkd*KATLTTDKSSSTAYMQLSSLTSEDSAV
YYCAR*YYDDHYCLDY*WGQGTTLTVSSVEGGS
GGSGGSGGSGGVDDIQLTQSPAIMSASPGEK
VTMTC*RASSSVSYMN*WYQQKSGTSPKRWIY*D*
*TSKVAS*GVPYRFSGSGSGTSYSLTISSMEAE
DAATYYC*QQWSSNPLT*FG**A**GTKLELK

SEQ ID NO: 20

Cysteine-modified anti-CD3 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIKLQQSGAELARPGASVKMSCKTS*GYTFTR*
*Ytmh*WVKQRPGQ**C**LEWIG*yiNPSRGYtnynq*
*kfkd*KATLTTDKSSSTAYMQLSSLTSEDSAV
YYCAR*YYDDHYCLDY*WGQGTTLTVSSVEGGS
GGSGGSGGSGGVDDIQLTQSPAIMSASPGEK
VTMTC*RASSSVSYMN*WYQQKSGTSPKRWIY*D*
*TSKVAS*GVPYRFSGSGSGTSYSLTISSMEAE
DAATYYC*QQWSSNPLT*FG**C**GTKLELK

SEQ ID NO: 21

***FIG. 11B***

# Alternative anti-CD3 scFv (VH-VL)

Anti-CD3 scFv

**Bold, underscored**: VH44 **G** and VL100 **A**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIKLQQSGAELARPGASVKMSCKTS*GYTFTR*
*Ytmh*WVKQRPGQ**G**LEWIG*yiNPSRGYtnynq*
*kfkd*KATLTTDKSSSTAYMQLSSLTSEDSAV
YYCAR*YYDDHYCLDY*WGQGTTLTVSSGGGGS
GGGGSGGGGSDIQLTQSPAIMSASPGEKVTM
TC*RASSSVSYMN*WYQQKSGTSPKRWIY*DTSK*
*VAS*GVPYRFSGSGSGTSYSLTISSMEAEDAA
TYYC*QQWSSNPLT*FG**A**GTKLELK

SEQ ID NO: 22

Cysteine-modified anti-CD3 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIKLQQSGAELARPGASVKMSCKTS*GYTFTR*
*Ytmh*WVKQRPGQ**C**LEWIG*yiNPSRGYtnynq*
*kfkd*KATLTTDKSSSTAYMQLSSLTSEDSAV
YYCAR*YYDDHYCLDY*WGQGTTLTVSSGGGGS
GGGGSGGGGSDIQLTQSPAIMSASPGEKVTM
TC*RASSSVSYMN*WYQQKSGTSPKRWIY*DTSK*
*VAS*GVPYRFSGSGSGTSYSLTISSMEAEDAA
TYYC*QQWSSNPLT*FG**C**GTKLELK

SEQ ID NO: 23



*FIG. 11C*

# Anti-CD3 scFv (VL-VH)

Anti-CD3 scFv

**Bold, underscored**: VH44 **G** and VL100 **A**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIQLTQSPAIMSASPGEKVTMTC*RASSSVSY*
*MN*WYQQKSGTSPKRWIY*DTSKVAS*GVPYRFS
GSGSGTSYSLTISSMEAEDAATYYC*QQWSSN*
*PLT*FG**A**GTKLELKGGGGSGGGGSGGGGSDIK
LQQSGAELARPGASVKMSCKTS*GYTFTRYtm*
*h*WVKQRPGQ**G**LEWIG*yiNPSRGYtnynqkfk*
*d*KATLTTDKSSSTAYMQLSSLTSEDSAVYYC
AR*YYDDHYCLDY*WGQGTTLTVSS

SEQ ID NO: 24

Cysteine-modified anti-CD3 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIQLTQSPAIMSASPGEKVTMTC*RASSSVSY*
*MN*WYQQKSGTSPKRWIY*DTSKVAS*GVPYRFS
GSGSGTSYSLTISSMEAEDAATYYC*QQWSSN*
*PLT*FG**C**GTKLELKGGGGSGGGGSGGGGSDIK
LQQSGAELARPGASVKMSCKTS*GYTFTRYtm*
*h*WVKQRPGQ**C**LEWIG*yiNPSRGYtnynqkfk*
*d*KATLTTDKSSSTAYMQLSSLTSEDSAVYYC
AR*YYDDHYCLDY*WGQGTTLTVSS

SEQ ID NO: 25

*FIG. 11D*

# Anti-CD28 variable sequences

Sequences in TCT002

Anti-CD28 VL (SEQ ID NO: 26)

DIELTQSPASLAVSLGQRATISC*RASESVEY*
*YVTSLMQ*WYQQKPGQPPKLLIF*AASNVES*GV
PARFSGSGSGTNFSLNIHPVDEDDVAMYFC*Q*
*QSRKVPYT*FGGGTKLEIKR

Anti-CD28 VL with VL100 Cys (SEQ ID NO: 14)

DIELTQSPASLAVSLGQRATISC*RASESVEY*
*YVTSLMQ*WYQQKPGQPPKLLIF*AASNVES*GV
PARFSGSGSGTNFSLNIHPVDEDDVAMYFC*Q*
*QSRKVPYT*FG**C**GTKLEIKR

Anti-CD28 VH (SEQ ID NO: 27)

QVKLQQSGPGLVTPSQSLSITCTVS*GFSLSD*
*Ygvh*WVRQSPGQGLEWLG*viWAGGGtnynsa*
*lms*RKSISKDNSKSQVFLKMNSLQADDTAVY
YCAR*DKGYSYYYSMDY*WGQGTTVTVSS

Anti-CD28 VH with VH44 Cys (SEQ ID NO: 15)

QVKLQQSGPGLVTPSQSLSITCTVS*GFSLSD*
*Ygvh*WVRQSPGQ**C**LEWLG*viWAGGGtnynsa*
*lms*RKSISKDNSKSQVFLKMNSLQADDTAVY
YCAR*DKGYSYYYSMDY*WGQGTTVTVSS

*Italic, underscored*: Chothia + Kabat CDRs.
*Italic, underscored, CAPITAL letters*: Chothia CDRs.
**Bold, underscored**: VL100 Cys and VH44 Cys.

***FIG. 11E***

# anti-CD28 scFv (VL-VH)

Anti-CD28 scFv

**Bold, underscored**: VH44 **G** and VL100 **G**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIELTQSPASLAVSLGQRATISC*RASESVEY*
*YVTSLMQ*WYQQKPGQPPKLLIF*AASNVES*GV
PARFSGSGSGTNFSLNIHPVDEDDVAMYFC*Q*
*QSRKVPYT*FG**G**GTKLEIKRGGGGSGGGGSGG
GGSQVKLQQSGPGLVTPSQSLSITCTVS*GFS*
*LSDYgvh*WVRQSPGQ**G**LEWLG*viWAGGGtny*
*nsalms*RKSISKDNSKSQVFLKMNSLQADDT
AVYYCAR*DKGYSYYYSMDY*WGQGTTVTVSS

SEQ ID NO: 28

Cysteine-modified anti-CD28 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIELTQSPASLAVSLGQRATISC*RASESVEY*
*YVTSLMQ*WYQQKPGQPPKLLIF*AASNVES*GV
PARFSGSGSGTNFSLNIHPVDEDDVAMYFC*Q*
*QSRKVPYT*FG**C**GTKLEIKRGGGGSGGGGSGG
GGSQVKLQQSGPGLVTPSQSLSITCTVS*GFS*
*LSDYgvh*WVRQSPGQ**C**LEWLG*viWAGGGtny*
*nsalms*RKSISKDNSKSQVFLKMNSLQADDT
AVYYCAR*DKGYSYYYSMDY*WGQGTTVTVSS

SEQ ID NO: 29



*FIG. 11F*

# anti-CD28 scFv (VH-VL)

Anti-CD28 scFv

**Bold, underscored**: VH44 **G** and VL100 **G**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

QVKLQQSGPGLVTPSQSLSITCTVS*GFSLSD*
*Ygvh*WVRQSPGQ**G**LEWLG*viWAGGGtnynsa*
*lms*RKSISKDNSKSQVFLKMNSLQADDTAVY
YCAR*DKGYSYYYSMDY*WGQGTTVTVSSGGGG
SGGGGSGGGGSDIELTQSPASLAVSLGQRAT
ISC*RASESVEYYVTSLMQ*WYQQKPGQPPKLL
IF*AASNVES*GVPARFSGSGSGTNFSLNIHPV
DEDDVAMYFC*QQSRKVPYT*FG**G**GTKLEIKR

SEQ ID NO: 30

Cysteine-modified anti-CD28 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

QVKLQQSGPGLVTPSQSLSITCTVS*GFSLSD*
*Ygvh*WVRQSPGQ**C**LEWLG*viWAGGGtnynsa*
*lms*RKSISKDNSKSQVFLKMNSLQADDTAVY
YCAR*DKGYSYYYSMDY*WGQGTTVTVSSGGGG
SGGGGSGGGGSDIELTQSPASLAVSLGQRAT
ISC*RASESVEYYVTSLMQ*WYQQKPGQPPKLL
IF*AASNVES*GVPARFSGSGSGTNFSLNIHPV
DEDDVAMYFC*QQSRKVPYT*FG**C**GTKLEIKR

SEQ ID NO: 31

***FIG. 11G***

# Anti-STEAP1 variable sequences

Anti-STEAP1 VL as in TCT001 (SEQ ID NO: 3)

DIQMTQSPSSLSASVGDRVTITC*KSSQSLLY*
*RSNQKNYLA*WYQQKPGKAPKLLIY*WASTRES*
GVPSRFSGSGSGTDFTLTISSLQPEDFATYY
C*QQYYNYPRT*FGQGTKVEIKR

Anti-STEAP1 VL - VL100 Cys (SEQ ID NO: 32)

DIQMTQSPSSLSASVGDRVTITC*KSSQSLLY*
*RSNQKNYLA*WYQQKPGKAPKLLIY*WASTRES*
GVPSRFSGSGSGTDFTLTISSLQPEDFATYY
C*QQYYNYPRT*FG**C**GTKVEIKR

Anti-STEAP1 VH as in TCT001 (SEQ ID NO: 4)

EVQLVESGGGLVQPGGSLRLSCAVS*GYSITS*
*DYawn*WVRQAPGKGLEWVG*yiSNSGStsynp*
*slks*RFTISRDNSKNTLYLQMNSLRAEDTAV
YYCAR*ERNYDYDDYYYAMDY*WGQGTLVTVSS

Anti-STEAP1 VH - VH44 Cys (SEQ ID NO: 33)

EVQLVESGGGLVQPGGSLRLSCAVS*GYSITS*
*DYawn*WVRQAPGK**C**LEWVG*yiSNSGStsynp*
*slks*RFTISRDNSKNTLYLQMNSLRAEDTAV
YYCAR*ERNYDYDDYYYAMDY*WGQGTLVTVSS

*Italic, underscored*: Chothia + Kabat CDRs.
*Italic, underscored, CAPITAL letters*: Chothia CDRs.
**Bold, underscored**: VL100 Cys and VH44 Cys.

***FIG. 11H***

# anti-STEAP1 scFv (VL-VH)

Anti-STEAP1 scFv (as in TCT001)

**Bold, underscored**: VH44 **G** and VL100 **Q**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIQMTQSPSSLSASVGDRVTITC*KSSQSLLY*
*RSNQKNYLA*WYQQKPGKAPKLLIY*WASTRES*
GVPSRFSGSGSGTDFTLTISSLQPEDFATYY
C*QQYYNYPRT*FG**Q**GTKVEIKRGGGGSGGGGS
GGGGSEVQLVESGGGLVQPGGSLRLSCAVS*G*
*YSITSDYawn*WVRQAPGK**G**LEWVG*yiSNSGS*
*tsynpslks*RFTISRDNSKNTLYLQMNSLRA
EDTAVYYCAR*ERNYDYDDYYYAMDY*WGQGTL
VTVSS

SEQ ID NO: 34

Cysteine-modified anti-STEAP1 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIQMTQSPSSLSASVGDRVTITC*KSSQSLLY*
*RSNQKNYLA*WYQQKPGKAPKLLIY*WASTRES*
GVPSRFSGSGSGTDFTLTISSLQPEDFATYY
C*QQYYNYPRT*FG**C**GTKVEIKRGGGGSGGGGS
GGGGSEVQLVESGGGLVQPGGSLRLSCAVS*G*
*YSITSDYawn*WVRQAPGK**C**LEWVG*yiSNSGS*
*tsynpslks*RFTISRDNSKNTLYLQMNSLRA
EDTAVYYCAR*ERNYDYDDYYYAMDY*WGQGTL
VTVSS

SEQ ID NO: 35



***FIG. 11I***

# anti-STEAP1 scFv (VH-VL)

Anti-STEAP1 scFv

**Bold, underscored**: VH44 **G** and VL100 **Q**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EVQLVESGGGLVQPGGSLRLSCAVS*GYSITS*
*DYawn*WVRQAPGK**G**LEWVG*yiSNSGStsynp*
*slks*RFTISRDNSKNTLYLQMNSLRAEDTAV
YYCAR*ERNYDYDDYYYAMDY*WGQGTLVTVSS
GGGGSGGGGSGGGGSDIQMTQSPSSLSASVG
DRVTITC*KSSQSLLYRSNQKNYLA*WYQQKPG
KAPKLLIY*WASTRES*GVPSRFSGSGSGTDFT
LTISSLQPEDFATYYC*QQYYNYPRT*FG**Q**GTK
VEIKR

SEQ ID NO: 36

Cysteine-modified anti-STEAP1 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EVQLVESGGGLVQPGGSLRLSCAVS*GYSITS*
*DYawn*WVRQAPGK**C**LEWVG*yiSNSGStsynp*
*slks*RFTISRDNSKNTLYLQMNSLRAEDTAV
YYCAR*ERNYDYDDYYYAMDY*WGQGTLVTVSS
GGGGSGGGGSGGGGSDIQMTQSPSSLSASVG
DRVTITC*KSSQSLLYRSNQKNYLA*WYQQKPG
KAPKLLIY*WASTRES*GVPSRFSGSGSGTDFT
LTISSLQPEDFATYYC*QQYYNYPRT*FG**C**GTK
VEIKR

SEQ ID NO: 37



***FIG. 11J***

# Anti-EpCAM variable sequences

Anti-EpCAM VL as in TCT002 (SEQ ID NO: 17)

DIVMTQAAFSNPVTLGTSGSISC*RSSKSLLH*
*SNGITYLY*WYLQKPGQSPQLLIY*QMSNLAS*G
VPDRFSSSGSGTDFTLRISRVEAEDVGVYYC
*AQNLELPRT*FGGGTKLEMKRTV

Anti-EpCAM VL - VL100 Cys (SEQ ID NO: 38)

DIVMTQAAFSNPVTLGTSGSISC*RSSKSLLH*
*SNGITYLY*WYLQKPGQSPQLLIY*QMSNLAS*G
VPDRFSSSGSGTDFTLRISRVEAEDVGVYYC
*AQNLELPRT*FG**C**GTKLEMKRTV

Anti-EpCAM VH as in TCT001 (SEQ ID NO: 16)

QIQLVQSGPELKKPGETVKISCKAS*GYTFTK*
*Ygmn*WVKQAPGKGLKWMG*wiNTYTEEptygd*
*dfkg*RFAFSLETSASTANLQINNLKSEDTAT
YFCAR*FGSAVDY*WGQGTSVTVSS

Anti-EpCAM VH - VH44 Cys (SEQ ID NO: 39)

QIQLVQSGPELKKPGETVKISCKAS*GYTFTK*
*Ygmn*WVKQAPGK**C**LKWMG*wiNTYTEEptygd*
*dfkg*RFAFSLETSASTANLQINNLKSEDTAT
YFCAR*FGSAVDY*WGQGTSVTVSS

*Italic, underscored*: Chothia + Kabat CDRs.
*Italic, underscored, CAPITAL letters*: Chothia CDRs.
**Bold, underscored**: VL100 Cys and VH44 Cys.



***FIG. 11K***

# anti-EpCAM scFv (VH-VL)

Anti-EpCAM scFv (as in TCT002)

**Bold, underscored**: VH44 **G** and VL100 **G**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

QIQLVQSGPELKKPGETVKISCKAS*GYTFTK*
*Ygmn*WVKQAPGK**G**LKWMG*wiNTYTEEptygd*
*dfkg*RFAFSLETSASTANLQINNLKSEDTAT
YFCAR*FGSAVDY*WGQGTSVTVSSGGGGSGGG
GSGGGGSDIVMTQAAFSNPVTLGTSGSISC*R*
*SSKSLLHSNGITYLY*WYLQKPGQSPQLLIY*Q*
*MSNLAS*GVPDRFSSSGSGTDFTLRISRVEAE
DVGVYYC*AQNLELPRT*FG**G**GTKLEMKRTV

SEQ ID NO: 40

Cysteine-modified anti-EpCAM scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

QIQLVQSGPELKKPGETVKISCKAS*GYTFTK*
*Ygmn*WVKQAPGK**C**LKWMG*wiNTYTEEptygd*
*dfkg*RFAFSLETSASTANLQINNLKSEDTAT
YFCAR*FGSAVDY*WGQGTSVTVSSGGGGSGGG
GSGGGGSDIVMTQAAFSNPVTLGTSGSISC*R*
*SSKSLLHSNGITYLY*WYLQKPGQSPQLLIY*Q*
*MSNLAS*GVPDRFSSSGSGTDFTLRISRVEAE
DVGVYYC*AQNLELPRT*FG**C**GTKLEMKRTV

SEQ ID NO: 41



*FIG. 11L*

# anti-EpCAM scFv (VL-VH)

Anti-EpCAM scFv

**Bold, underscored**: VH44 **G** and VL100 **G**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIVMTQAAFSNPVTLGTSGSISC*RSSKSLLH*
*SNGITYLY*WYLQKPGQSPQLLIY*QMSNLAS*G
VPDRFSSSGSGTDFTLRISRVEAEDVGVYYC
*AQNLELPRT*FG**G**GTKLEMKRTVGGGGSGGGG
SGGGGSQIQLVQSGPELKKPGETVKISCKAS
*GYTFTKYgmn*WVKQAPGK**G**LKWMG*wiNTYTE*
*Eptygddfkg*RFAFSLETSASTANLQINNLK
SEDTATYFCAR*FGSAVDY*WGQGTSVTVSS

SEQ ID NO: 42

Cysteine-modified anti-EpCAM scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIVMTQAAFSNPVTLGTSGSISC*RSSKSLLH*
*SNGITYLY*WYLQKPGQSPQLLIY*QMSNLAS*G
VPDRFSSSGSGTDFTLRISRVEAEDVGVYYC
*AQNLELPRT*FG**C**GTKLEMKRTVGGGGSGGGG
SGGGGSQIQLVQSGPELKKPGETVKISCKAS
*GYTFTKYgmn*WVKQAPGK**C**LKWMG*wiNTYTE*
*Eptygddfkg*RFAFSLETSASTANLQINNLK
SEDTATYFCAR*FGSAVDY*WGQGTSVTVSS

SEQ ID NO: 43

***FIG. 11M***

# Anti-PSMA variable sequences

Anti-PSMA VL (SEQ ID NO: 44)

DIVMTQSHKFMSTSVGDRVSIIC*KASQDVGT*
*AVD*WYQQKPGQSPKLLIY*WASTRHT*GVPDRF
TGSGSGTDFTLTITNVQSEDLADYFC*QQYNS*
*YPLT*FGAGTKLEIK

Anti-PSMA VL with VL100 Cys (SEQ ID NO: 46)

DIVMTQSHKFMSTSVGDRVSIIC*KASQDVGT*
*AVD*WYQQKPGQSPKLLIY*WASTRHT*GVPDRF
TGSGSGTDFTLTITNVQSEDLADYFC*QQYNS*
*YPLT*FG**C**GTKLEIK

Anti-PSMA VH (SEQ ID NO: 45)

EVQLQQSGPELVKPGTSVRISCKTS*GYTFTE*
*Ytih*WVKQSHGKSLEWIG*niNPNNGGttynq*
*kfed*KATLTVDKSSSTAYMELRSLTSEDSAV
YYCAA*GWNFDY*WGQGTTLTVSS

Anti-PSMA VH with VH44 Cys (SEQ ID NO: 47)

EVQLQQSGPELVKPGTSVRISCKTS*GYTFTE*
*Ytih*WVKQSHGK**C**LEWIG*niNPNNGGttynq*
*kfed*KATLTVDKSSSTAYMELRSLTSEDSAV
YYCAA*GWNFDY*WGQGTTLTVSS

*Italic, underscored*: Chothia + Kabat CDRs.
*Italic, underscored, CAPITAL letters*: Chothia CDRs.
**Bold, underscored**: VL100 Cys and VH44 Cys.



***FIG. 11N***

# anti-PSMA scFv (VL-VH)

Anti-PSMA scFv

**Bold, underscored**: VH44 **S** and VL100 **A**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIVMTQSHKFMSTSVGDRVSIIC*KASQDVGT*
*AVD*WYQQKPGQSPKLLIY*WASTRHT*GVPDRF
TGSGSGTDFTLTITNVQSEDLADYFC*QQYNS*
*YPLT*FG**A**GTKLEIKGGGGSGGGGSGGGGSEV
QLQQSGPELVKPGTSVRISCKTS*GYTFTEYt*
*ih*WVKQSHGK**S**LEWIG*niNPNNGGttynqkf*
*ed*KATLTVDKSSSTAYMELRSLTSEDSAVYY
CAA*GWNFDY*WGQGTTLTVSS

SEQ ID NO: 48

Cysteine-modified anti-PSMA scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

DIVMTQSHKFMSTSVGDRVSIIC*KASQDVGT*
*AVD*WYQQKPGQSPKLLIY*WASTRHT*GVPDRF
TGSGSGTDFTLTITNVQSEDLADYFC*QQYNS*
*YPLT*FG**C**GTKLEIKGGGGSGGGGSGGGGSEV
QLQQSGPELVKPGTSVRISCKTS*GYTFTEYt*
*ih*WVKQSHGK**C**LEWIG*niNPNNGGttynqkf*
*ed*KATLTVDKSSSTAYMELRSLTSEDSAVYY
CAA*GWNFDY*WGQGTTLTVSS

SEQ ID NO: 49

***FIG. 11O***

# anti-PSMA scFv (VH-VL)

Anti-PSMA scFv

**Bold, underscored**: VH44 **S** and VL100 **A**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EVQLQQSGPELVKPGTSVRISCKTS*GYTFTE*
*Ytih*WVKQSHGK**S**LEWIG*niNPNNGGttynq*
*kfed*KATLTVDKSSSTAYMELRSLTSEDSAV
YYCAA*GWNFDY*WGQGTTLTVSSGGGGSGGGG
SGGGGSDIVMTQSHKFMSTSVGDRVSIIC*KA*
*SQDVGTAVD*WYQQKPGQSPKLLIY*WASTRHT*
GVPDRFTGSGSGTDFTLTITNVQSEDLADYF
C*QQYNSYPLT*FG**A**GTKLEIK

SEQ ID NO: 50

Cysteine-modified anti-PSMA scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EVQLQQSGPELVKPGTSVRISCKTS*GYTFTE*
*Ytih*WVKQSHGK**C**LEWIG*niNPNNGGttynq*
*kfed*KATLTVDKSSSTAYMELRSLTSEDSAV
YYCAA*GWNFDY*WGQGTTLTVSSGGGGSGGGG
SGGGGSDIVMTQSHKFMSTSVGDRVSIIC*KA*
*SQDVGTAVD*WYQQKPGQSPKLLIY*WASTRHT*
GVPDRFTGSGSGTDFTLTITNVQSEDLADYF
C*QQYNSYPLT*FG**C**GTKLEIK

SEQ ID NO: 51



***FIG. 11P***

# Anti-SPON2 variable sequences

Anti-SPON2 VL (SEQ ID NO: 52)

EIVLTQSPGTLSLSPGERATLSC*RASQSVSS*
*SYLA*WYQQKPGQAPRLLIY*GASSRAT*GIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYC*QQYS*
*SSLT*FGGGTKVEIK

Anti-SPON2 VL - VL100 Cys (SEQ ID NO: 54)

EIVLTQSPGTLSLSPGERATLSC*RASQSVSS*
*SYLA*WYQQKPGQAPRLLIY*GASSRAT*GIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYC*QQYS*
*SSLT*FG**C**GTKVEIK

Anti-SPON2 VH (SEQ ID NO: 53)

EVQLVQSGGGLVMPGGSLRLSCAGS*GFTFSS*
*Yvmh*WLRQAPGKGLEWVS*viGTGGVthyads*
*vkg*RFMISRDNAKNSLYLQMNSLRAEDTAMY
YCAR*WGYYGSGSYENDAFDI*WGQGTMVTVSS

Anti-SPON2 VH - VH44 Cys (SEQ ID NO: 55)

EVQLVQSGGGLVMPGGSLRLSCAGS*GFTFSS*
*Yvmh*WLRQAPGK**C**LEWVS*viGTGGVthyads*
*vkg*RFMISRDNAKNSLYLQMNSLRAEDTAMY
YCAR*WGYYGSGSYENDAFDI*WGQGTMVTVSS

*Italic, underscored*: Chothia + Kabat CDRs.
*Italic, underscored, CAPITAL letters*: Chothia CDRs.
**Bold, underscored**: VL100 Cys and VH44 Cys.

***FIG. 11Q***



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# anti-SPON2 scFv (VL-VH)

Anti-SPON2 scFv

**Bold, underscored**: VH44 **G** and VL100 **G**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EIVLTQSPGTLSLSPGERATLSC*RASQSVSS*
*SYLA*WYQQKPGQAPRLLIY*GASSRAT*GIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYC*QQYS*
*SSLT*FG**G**GTKVEIKGGGGSGGGGSGGGGSEV
QLVQSGGGLVMPGGSLRLSCAGS*GFTFSSYv*
*mh*WLRQAPGK**G**LEWVS*viGTGGVthyadsvk*
*g*RFMISRDNAKNSLYLQMNSLRAEDTAMYYC
AR*WGYYGSGSYENDAFDI*WGQGTMVTVSS

SEQ ID NO: 56

Cysteine-modified anti-SPON2 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EIVLTQSPGTLSLSPGERATLSC*RASQSVSS*
*SYLA*WYQQKPGQAPRLLIY*GASSRAT*GIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYC*QQYS*
*SSLT*FG**C**GTKVEIKGGGGSGGGGSGGGGSEV
QLVQSGGGLVMPGGSLRLSCAGS*GFTFSSYv*
*mh*WLRQAPGK**C**LEWVS*viGTGGVthyadsvk*
*g*RFMISRDNAKNSLYLQMNSLRAEDTAMYYC
AR*WGYYGSGSYENDAFDI*WGQGTMVTVSS

SEQ ID NO: 57



*FIG. 11R*

# anti-SPON2 scFv (VH-VL)

Anti-SPON2 scFv

**Bold, underscored**: VH44 **G** and VL100 **G**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EVQLVQSGGGLVMPGGSLRLSCAGS*GFTFSS*
*Yvmh*WLRQAPGK**G**LEWVS*viGTGGVthyads*
*vkg*RFMISRDNAKNSLYLQMNSLRAEDTAMY
YCAR*WGYYGSGSYENDAFDI*WGQGTMVTVSS
GGGGSGGGGSGGGGSEIVLTQSPGTLSLSPG
ERATLSC*RASQSVSSSYLA*WYQQKPGQAPRL
LIY*GASSRAT*GIPDRFSGSGSGTDFTLTISR
LEPEDFAVYYC*QQYSSSLT*FG**G**GTKVEIK

SEQ ID NO: 58

Cysteine –modified anti-SPON2 scFv

**Bold, underscored**: VH44 **C** and VL100 **C**.
*Italic, underscored*: CDRs (Chothia + Kabat).
*Italic, undersc., CAPITAL letters:* Chothia CDRs.

EVQLVQSGGGLVMPGGSLRLSCAGS*GFTFSS*
*Yvmh*WLRQAPGK**C**LEWVS*viGTGGVthyads*
*vkg*RFMISRDNAKNSLYLQMNSLRAEDTAMY
YCAR*WGYYGSGSYENDAFDI*WGQGTMVTVSS
GGGGSGGGGSGGGGSEIVLTQSPGTLSLSPG
ERATLSC*RASQSVSSSYLA*WYQQKPGQAPRL
LIY*GASSRAT*GIPDRFSGSGSGTDFTLTISR
LEPEDFAVYYC*QQYSSSLT*FG**C**GTKVEIK

SEQ ID NO: 59

***FIG. 11S***

# CD3 (VL-VH)-NKG2D

NKG2D

**Bold underscored**: transmembrane region.
*Italic underscored*: SEQ ID NO: 60.

MGWIRGRRSRHSWEMSEFHNYNLDLKKSDFS
TRWQKQRCPVVKSKCRENAS**PFFFCCFIAVA**
**MGIRFIIMVT**IWS*AVFLNSLFNQEVQIPLTE*
*SYCGPCPKNWICYKNNCYQFFDESKNWYESQ*
*ASCMSQNASLLKVYSKEDQDLLKLVKSYHWM*
*GLVHIPTNGSWQWEDGSILSPNLLTIIEMQK*
*GDCALYASSFKGYIENCSTPNTYICMQRTV*

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ANTI-CD3-NKG2D

MGWSCIILFLVATATGVHSDIKLQQSGAELA
RPGASVKMSCKTSGYTFTRYTMHWVKQRPGQ
GLEWIGYINPSRGYTNYNQKFKDKATLTTDK
SSSTAYMQLSSLTSEDSAVYYCARYYDDHYC
LDYWGQGTTLTVSSGGGGSGGGGSGGGGSDI
QLTQSPAIMSASPGEKVTMTCRASSSVSYMN
WYQQKSGTSPKRWIYDTSKVASGVPYRFSGS
GSGTSYSLTISSMEAEDAATYYCQQWSSNPL
TFGAGTKLELKGGGGSGGGGSGGGGSAVFLN
SLFNQEVQIPLTESYCGPCPKNWICYKNNCY
QFFDESKNWYESQASCMSQNASLLKVYSKED
QDLLKLVKSYHWMGLVHIPTNGSWQWEDGSI
LSPNLLTIIEMQKGDCALYASSFKGYIENCS
TPNTYICMQRTVHHHHHH

SEQ ID NO: 61



*FIG. 11T*

# CD28 (VL-VH)-NKG2D

NKG2D

**Bold underscored**: transmembrane region.
*Italic underscored*: SEQ ID NO: 60.

MGWIRGRRSRHSWEMSEFHNYNLDLKKSDFS
TRWQKQRCPVVKSKCRENAS**PFFFCCFIAVA**
**MGIRFIIMVT**IWS*AVFLNSLFNQEVQIPLTE*
*SYCGPCPKNWICYKNNCYQFFDESKNWYESQ*
*ASCMSQNASLLKVYSKEDQDLLKLVKSYHWM*
*GLVHIPTNGSWQWEDGSILSPNLLTIIEMQK*
*GDCALYASSFKGYIENCSTPNTYICMQRTV*

NCBI NP_031386

ANTI-CD28-NKG2D

MGWSCIILFLVATATGVHSDIELTQSPASLA
VSLGQRATISCRASESVEYYVTSLMQWYQQK
PGQPPKLLIFAASNVESGVPARFSGSGSGTN
FSLNIHPVDEDDVAMYFCQQSRKVPYTFGCG
TKLEIKRGGGGSGGGGSGGGGSQVKLQQSGP
GLVTPSQSLSITCTVSGFSLSDYGVHWVRQS
PGQCLEWLGVIWAGGGTNYNSALMSRKSISK
DNSKSQVFLKMNSLQADDTAVYYCARDKGYS
YYYSMDYWGQGTTVTVSSGGGGSGGGGSGGG
GSAVFLNSLFNQEVQIPLTESYCGPCPKNWI
CYKNNCYQFFDESKNWYESQASCMSQNASLL
KVYSKEDQDLLKLVKSYHWMGLVHIPTNGSW
QWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVHHHHHH

SEQ ID NO: 62



*FIG. 11U*