

PSMA TARGETING TRISPECIFIC PROTEINS AND METHODS OF USE**CROSS-REFERENCE**

[0001] This application claims the benefit of U.S. Provisional Application Nos. 62/426,069 filed November 23, 2016, and 62/426,077 filed November 23, 2016, which are incorporated by reference herein in their entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 22, 2017, is named 47517-708_601_SL.txt and is 150,911 bytes in size.

BACKGROUND OF THE INVENTION

[0003] The selective destruction of an individual cell or a specific cell type is often desirable in a variety of clinical settings. For example, it is a primary goal of cancer therapy to specifically destroy tumor cells, while leaving healthy cells and tissues intact and undamaged. One such method is by inducing an immune response against the tumor, to make immune effector cells such as natural killer (NK) cells or cytotoxic T lymphocytes (CTLs) attack and destroy tumor cells.

SUMMARY OF THE INVENTION

[0004] Provided herein are trispecific antigen-binding protein, pharmaceutical compositions thereof, as nucleic acids, recombinant expression vectors and host cells for making such trispecific antigen-binding proteins, and methods of use for the treatment of diseases, disorders, or conditions. In one aspect, described herein are prostate specific membrane antigen (PSMA) targeting trispecific proteins, wherein said proteins comprise (a) a first domain (A) which specifically binds to human CD3; (b) a second domain (B) which is a half-life extension domain; and (c) a third domain (C) which specifically binds to PSMA, wherein the domains are linked in the order H2N-(A)-(C)-(B)-COOH, H2N-(B)-(A)-(C)-COOH, H2N-(C)-(B)-(A)-COOH, or by linkers L1 and L2. In some embodiments, the first domain comprises a variable light chain and variable heavy chain each of which is capable of specifically binding to human CD3. In some embodiments, the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88. In some embodiments, the first domain is humanized or human. In some embodiments, the first domain has a KD binding of 150 nM or less to CD3 on CD3 expressing cells. In some embodiments, the second domain binds human serum albumin. In some embodiments, the second domain comprises a scFv, a variable heavy domain (VH), a

variable light domain (VL), a peptide, a ligand, or a small molecule. In some embodiments, the second domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 89-112. In some embodiments, the third domain comprises a scFv, a VH domain, a VL domain, a non-Ig domain, a ligand, a knottin, or a small molecule entity that specifically binds to PSMA. In some embodiments, the third domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 113-140.

[0005] In some embodiments, linkers L1 and L2 are each independently selected from (GS)_n (SEQ ID NO: 153), (GGS)_n (SEQ ID NO: 154), (GGGS)_n (SEQ ID NO: 155), (GGSG)_n (SEQ ID NO: 156), (GGSGG)_n (SEQ ID NO: 157), or (GGGGS)_n (SEQ ID NO: 158), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, linkers L1 and L2 are each independently (GGGGS)₄ (SEQ ID NO: 159) or (GGGGS)₃ (SEQ ID NO: 160). In some embodiments, the domains are linked in the order H₂N-(A)-(C)-(B)-COOH. In some embodiments, the domains are linked in the order H₂N-(B)-(C)-(A)-COOH.

[0006] In some embodiments, the protein is less than about 80 kDa. In some embodiments, the protein is about 50 to about 75 kDa. In some embodiments, the protein is less than about 60 kDa. In some embodiments, the protein has an elimination half-time of at least about 50 hours. In some embodiments, the protein has an elimination half-time of at least about 100 hours. In some embodiments, the protein has increased tissue penetration as compared to an IgG to the same PSMA.

[0007] In some embodiments, the protein comprises a sequence selected from the group consisting of SEQ ID NO: 140-152.

[0008] In another aspect, provided herein are pharmaceutical composition comprising (i) the PSMA targeting trispecific protein according to any one of the above embodiments and (ii) a pharmaceutically acceptable carrier.

[0009] Also provided herein are methods of treating an individual in need of treatment of cancer, the method comprising administration of an effective amount of the pharmaceutical composition or PSMA targeting trispecific proteins according to any of the above embodiments. In some embodiments, the cancer is prostate cancer or renal cancer.

[0010] One embodiment provides a PSMA targeting trispecific protein, wherein said protein comprises (a) a first domain (A) which specifically binds to human CD3; (b) a second domain (B) which is a half-life extension domain; and (c) a third domain (C) which specifically binds to PSMA, wherein the second domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 113-140. In some embodiments, domains are linked in the order H₂N-(A)-(C)-(B)-COOH, H₂N-(B)-(A)-(C)-COOH, H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2. In some embodiments, the first domain comprises one or more sequences selected from

the group consisting of SEQ ID NO: 1-88. In some embodiments, the second domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 89-112.

[0011] One embodiment provides a PSMA targeting trispecific protein, wherein said protein comprises a sequence selected from the group consisting of SEQ ID NO: 140-152. In some embodiments, said protein comprises a sequence selected from the group consisting of SEQ ID NO: 150-152.

[0012] One embodiment provides a prostate specific membrane antigen (PSMA) targeting trispecific protein, wherein said protein comprises (a) a first domain (A) which specifically binds to human CD3; (b) a second domain (B) which is a half-life extension domain; and (c) a third domain (C) which specifically binds to PSMA, wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2, and wherein the third domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 113-140.

[0013] One embodiment provides a PSMA targeting trispecific protein, wherein said protein comprises (a) a first domain (A) which specifically binds to human CD3; (b) a second domain (B) which is a half-life extension domain; and (c) a third domain (C) which specifically binds to PSMA, wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2, and wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.

[0014] One embodiment provides a method of treating prostate cancer, the method comprising administration of an effective amount of a PSMA targeting trispecific protein, wherein said protein comprises (a) a first domain (A) which specifically binds to human CD3; (b) a second domain (B) which is a half-life extension domain; and (c) a third domain (C) which specifically binds to PSMA, wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2, and wherein the third domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 113-140.

[0015] One embodiment provides a method of treating prostate cancer, the method comprising administration of an effective amount of a PSMA targeting trispecific protein, wherein said protein comprises (a) a first domain (A) which specifically binds to human CD3; (b) a second domain (B) which is a half-life extension domain; and (c) a third domain (C) which specifically binds to PSMA, wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2, and wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.

INCORPORATION BY REFERENCE

[0016] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0018] **Figure 1** is schematic representation of an exemplary PMSA targeting trispecific antigen-binding protein where the protein has an constant core element comprising an anti-CD3ε single chain variable fragment (scFv) and an anti-HSA variable heavy chain region; and a PMSA binding domain that can be a VH, scFv, a non-Ig binder, or ligand.

[0019] **Figures 2A-B** compare the ability of exemplary PSMA targeting trispecific proteins (PSMA targeting TriTAC molecules) with different affinities for CD3 to induce T cells to kill human prostate cancer cells. **Figure 2A** shows killing by different PMSA targeting TriTAC molecules in prostate cancer model LNCaP. **Figure 2B** shows killing by different PMSA targeting TriTAC molecules in prostate cancer model 22Rv1. **Figure 2C** shows EC50 values for PMSA targeting TriTAC in LNCaP and 22Rv1 prostate cancer models.

[0020] **Figure 3** shows the serum concentration of PSMA targeting TriTAC C236 in Cynomolgus monkeys after i.v. administration (100 µg/kg) over three weeks.

[0021] **Figure 4** shows the serum concentration of PSMA targeting TriTAC molecules with different CD3 affinities in Cynomolgus monkeys after i.v. administration (100 µg/kg) over three weeks.

[0022] **Figures 5A-C** show the ability of PSMA targeting TriTAC molecules with different affinities for PSMA to induce T cells to kill the human prostate cancer cell line LNCaP. **Figure 5A** shows the experiment performed in the absence of human serum albumin with a PSMA targeting BiTE as positive control. **Figure 5B** shows the experiment performed in the presence of human serum albumin with a PSMA targeting BiTE as positive control. **Figure 5C** shows EC50 values for PMSA targeting TriTAC in the presence or absence of HSA with a PSMA targeting BiTE as a positive control in LNCaP prostate cancer models.

[0023] **Figure 6** demonstrates the ability of PSMA targeting TriTAC molecules to inhibit tumor growth of human prostate cancer cells in a mouse xenograft experiment.

[0024] **Figures 7A-D** illustrates the specificity of TriTAC molecules in cell killing assays with target cell lines that do or do not express the target protein. **Figure 7A** shows EGFR and PSMA expression in LNCaP, KMS12BM, and OVCAR8 cell lines. **Figure 7B** shows killing of LNCaP tumor cells by PSMA, EGFR, and negative control TriTACs. **Figure 7C** shows killing of KMS12BM tumor cells by PSMA, EGFR, and negative control TriTACs. **Figure 7D** shows killing of OVCAR8 cells by PSMA, EGFR, and negative control TriTACs.

[0025] **Figures 8A-D** depict the impact of pre-incubation at 37°C and freeze/thaw cycles on TriTAC activity. **Figure 8A** shows PSMA TriTAC C235 activity after pre-incubation at 37°C or freeze/thaw cycles. **Figure 8B** shows PSMA TriTAC C359 activity after pre-incubation at 37°C or freeze/thaw cycles. **Figure 8C** shows PSMA TriTAC C360 activity after pre-incubation at 37°C or freeze/thaw cycles. **Figure 8D** shows PSMA TriTAC C361 activity after pre-incubation at 37°C or freeze/thaw cycles.

[0026] **Figures 9A-B** depict the activity of a PSMA targeting TriTAC molecule of this disclosure in redirected T cell killing in T cell dependent cellular cytotoxicity assays (TDCC). **Figure 9A** shows the impact of the PSMA targeting TriTAC molecule in redirecting cynomolgus peripheral blood mononuclear cells (PBMCs), from cynomolgus monkey donor G322, in killing LNCaP cells. **Figure 9B** shows the impact of the PSMA targeting TriTAC molecule in redirecting cynomolgus PBMCs, from cynomolgus monkey donor D173, to kill MDAPCa2b cells.

[0027] **Figure 10** depicts the impact of a PSMA targeting TriTAC molecule of this disclosure on expression of T cell activation markers CD25 and CD69.

[0028] **Figure 11** depicts the ability of a PSMA targeting TriTAC molecule of this disclosure to stimulate T cell proliferation in the presence of PSMA expressing target cells.

[0029] **Figures 12A-B** depict redirected T cell killing of LNCaP cells by PSMA targeting TriTAC molecules. **Figure 12A** shows redirected T cell killing of LNCaP cells by PSMA PH1T TriTAC (SEQ ID No: 150) and PSMA PH1 TriTAC (SEQ ID NO: 151) molecules. **Figure 12B** shows redirected T cell killing of LNCaP cells by PSMA Z2 TriTAC (SEQ ID NO: 152).

DETAILED DESCRIPTION OF THE INVENTION

[0030] Described herein are trispecific proteins that target prostate specific membrane antigen (PSMA), pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such proteins thereof. Also provided are methods of using the disclosed PSMA targeting trispecific proteins in the prevention, and/or treatment of diseases, conditions and disorders. The PSMA targeting trispecific proteins are capable of specifically binding to PSMA as well as CD3 and have a half-life extension domain, such as a domain

binding to human serum albumin (HSA). **Figure 1** depicts one non-limiting example of a trispecific antigen-binding protein.

[0031] In one aspect, the PSMA targeting trispecific proteins comprise a domain (A) which specifically binds to CD3, a domain (B) which specifically binds to human serum albumin (HSA), and a domain (C) which specifically binds to PSMA. The three domains in PSMA targeting trispecific proteins are arranged in any order. Thus, it is contemplated that the domain order of the PSMA targeting trispecific proteins are:

H₂N-(A)-(B)-(C)-COOH,
 H₂N-(A)-(C)-(B)-COOH,
 H₂N-(B)-(A)-(C)-COOH,
 H₂N-(B)-(C)-(A)-COOH,
 H₂N-(C)-(B)-(A)-COOH, or
 H₂N-(C)-(A)-(B)-COOH.

[0032] In some embodiments, the PSMA targeting trispecific proteins have a domain order of H₂N-(A)-(B)-(C)-COOH. In some embodiments, the PSMA targeting trispecific proteins have a domain order of H₂N-(A)-(C)-(B)-COOH. In some embodiments, the PSMA targeting trispecific proteins have a domain order of H₂N-(B)-(A)-(C)-COOH. In some embodiments, the PSMA targeting trispecific proteins have a domain order of H₂N-(B)-(C)-(A)-COOH. In some embodiments, the PSMA targeting trispecific proteins have a domain order of H₂N-(C)-(B)-(A)-COOH. In some embodiments, the PSMA targeting trispecific proteins have a domain order of H₂N-(C)-(A)-(B)-COOH.

[0033] In some embodiments, the PSMA targeting trispecific proteins have the HSA binding domain as the middle domain, such that the domain order is H₂N-(A)-(B)-(C)-COOH or H₂N-(C)-(B)-(A)-COOH. It is contemplated that in such embodiments where the HSA binding domain as the middle domain, the CD3 and PSMA binding domains are afforded additional flexibility to bind to their respective targets.

[0034] In some embodiments, the PSMA targeting trispecific proteins described herein comprise a polypeptide having a sequence described in Table 10 (SEQ ID NO: 140-152) and subsequences thereof. In some embodiments, the trispecific antigen binding protein comprises a polypeptide having at least 70%-95% or more homology to a sequence described in Table 10 (SEQ ID NO: 140-152). In some embodiments, the trispecific antigen binding protein comprises a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, or more homology to a sequence described in Table 10 (SEQ ID NO: 140-152). In some embodiments, the trispecific antigen binding protein has a sequence comprising at least a portion of a sequence described in Table 10 (SEQ ID NO: 140-152). In some embodiments, the PSMA trispecific antigen-binding

protein comprises a polypeptide comprising one or more of the sequences described in Table 10 (SEQ ID NO: 140-152). In further embodiments, the PSMA trispecific antigen-binding protein comprises one or more CDRs as described in the sequences in Table 10 (SEQ ID NO: 140-152).

[0035] The PSMA targeting trispecific proteins described herein are designed to allow specific targeting of cells expressing PSMA by recruiting cytotoxic T cells. This improves efficacy compared to ADCC (antibody dependent cell-mediated cytotoxicity), which is using full length antibodies directed to a sole antigen and is not capable of directly recruiting cytotoxic T cells. In contrast, by engaging CD3 molecules expressed specifically on these cells, the PSMA targeting trispecific proteins can crosslink cytotoxic T cells with cells expressing PSMA in a highly specific fashion, thereby directing the cytotoxic potential of the T cell towards the target cell. The PSMA targeting trispecific proteins described herein engage cytotoxic T cells via binding to the surface-expressed CD3 proteins, which form part of the TCR. Simultaneous binding of several PSMA trispecific antigen-binding protein to CD3 and to PSMA expressed on the surface of particular cells causes T cell activation and mediates the subsequent lysis of the particular PSMA expressing cell. Thus, PSMA targeting trispecific proteins are contemplated to display strong, specific and efficient target cell killing. In some embodiments, the PSMA targeting trispecific proteins described herein stimulate target cell killing by cytotoxic T cells to eliminate pathogenic cells (e.g., tumor cells expressing PSMA). In some of such embodiments, cells are eliminated selectively, thereby reducing the potential for toxic side effects.

[0036] The PSMA targeting trispecific proteins described herein confer further therapeutic advantages over traditional monoclonal antibodies and other smaller bispecific molecules. Generally, the effectiveness of recombinant protein pharmaceuticals depends heavily on the intrinsic pharmacokinetics of the protein itself. One such benefit here is that the PSMA targeting trispecific proteins described herein have extended pharmacokinetic elimination half-time due to having a half-life extension domain such as a domain specific to HSA. In this respect, the PSMA targeting trispecific proteins described herein have an extended serum elimination half-time of about two, three, about five, about seven, about 10, about 12, or about 14 days in some embodiments. This contrasts to other binding proteins such as BiTE or DART molecules which have relatively much shorter elimination half-times. For example, the BiTE CD19×CD3 bispecific scFv-scFv fusion molecule requires continuous intravenous infusion (i.v.) drug delivery due to its short elimination half-time. The longer intrinsic half-times of the PSMA targeting trispecific proteins solve this issue thereby allowing for increased therapeutic potential such as low-dose pharmaceutical formulations, decreased periodic administration and/or novel pharmaceutical compositions.

[0037] The PSMA targeting trispecific proteins described herein also have an optimal size for enhanced tissue penetration and tissue distribution. Larger sizes limit or prevent penetration or distribution of the protein in the target tissues. The PSMA targeting trispecific proteins described herein avoid this by having a small size that allows enhanced tissue penetration and distribution. Accordingly, the PSMA targeting trispecific proteins described herein, in some embodiments have a size of about 50 kD to about 80 kD, about 50 kD to about 75 kD, about 50 kD to about 70 kD, or about 50 kD to about 65 kD. Thus, the size of the PSMA targeting trispecific proteins is advantageous over IgG antibodies which are about 150 kD and the BiTE and DART diabody molecules which are about 55 kD but are not half-life extended and therefore cleared quickly through the kidney.

[0038] In further embodiments, the PSMA targeting trispecific proteins described herein have an optimal size for enhanced tissue penetration and distribution. In these embodiments, the PSMA targeting trispecific proteins are constructed to be as small as possible, while retaining specificity toward its targets. Accordingly, in these embodiments, the PSMA targeting trispecific proteins described herein have a size of about 20 kD to about 40 kD or about 25 kD to about 35 kD to about 40 kD, to about 45 kD, to about 50 kD, to about 55 kD, to about 60 kD, to about 65 kD. In some embodiments, the PSMA targeting trispecific proteins described herein have a size of about 50kD, 49, kD, 48 kD, 47 kD, 46 kD, 45 kD, 44 kD, 43 kD, 42 kD, 41 kD, 40 kD, about 39 kD, about 38 kD, about 37 kD, about 36 kD, about 35 kD, about 34 kD, about 33 kD, about 32 kD, about 31 kD, about 30 kD, about 29 kD, about 28 kD, about 27 kD, about 26 kD, about 25 kD, about 24 kD, about 23 kD, about 22 kD, about 21 kD, or about 20 kD. An exemplary approach to the small size is through the use of single domain antibody (sdAb) fragments for each of the domains. For example, a particular PSMA trispecific antigen-binding protein has an anti-CD3 sdAb, anti-HSA sdAb and an sdAb for PSMA. This reduces the size of the exemplary PSMA trispecific antigen-binding protein to under 40 kD. Thus in some embodiments, the domains of the PSMA targeting trispecific proteins are all single domain antibody (sdAb) fragments. In other embodiments, the PSMA targeting trispecific proteins described herein comprise small molecule entity (SME) binders for HSA and/or the PSMA. SME binders are small molecules averaging about 500 to 2000 Da in size and are attached to the PSMA targeting trispecific proteins by known methods, such as sortase ligation or conjugation. In these instances, one of the domains of PSMA trispecific antigen-binding protein is a sortase recognition sequence, e.g., LPETG (SEQ ID NO: 57). To attach a SME binder to PSMA trispecific antigen-binding protein with a sortase recognition sequence, the protein is incubated with a sortase and a SME binder whereby the sortase attaches the SME binder to the recognition sequence. Known SME binders include MIP-1072 and MIP-1095 which bind to prostate-

specific membrane antigen (PSMA). In yet other embodiments, the domain which binds to PSMA of PSMA targeting trispecific proteins described herein comprise a knottin peptide for binding PSMA. Knottins are disulfide-stabilized peptides with a cysteine knot scaffold and have average sizes about 3.5 kD. Knottins have been contemplated for binding to certain tumor molecules such as PSMA. In further embodiments, domain which binds to PSMA of PSMA targeting trispecific proteins described herein comprise a natural PSMA ligand.

[0039] Another feature of the PSMA targeting trispecific proteins described herein is that they are of a single-polypeptide design with flexible linkage of their domains. This allows for facile production and manufacturing of the PSMA targeting trispecific proteins as they can be encoded by single cDNA molecule to be easily incorporated into a vector. Further, because the PSMA targeting trispecific proteins described herein are a monomeric single polypeptide chain, there are no chain pairing issues or a requirement for dimerization. It is contemplated that the PSMA targeting trispecific proteins described herein have a reduced tendency to aggregate unlike other reported molecules such as bispecific proteins with Fc-gamma immunoglobulin domains.

[0040] In the PSMA targeting trispecific proteins described herein, the domains are linked by internal linkers L1 and L2, where L1 links the first and second domain of the PSMA targeting trispecific proteins and L2 links the second and third domains of the PSMA targeting trispecific proteins. Linkers L1 and L2 have an optimized length and/or amino acid composition. In some embodiments, linkers L1 and L2 are the same length and amino acid composition. In other embodiments, L1 and L2 are different. In certain embodiments, internal linkers L1 and/or L2 are "short", *i.e.*, consist of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 amino acid residues. Thus, in certain instances, the internal linkers consist of about 12 or less amino acid residues. In the case of 0 amino acid residues, the internal linker is a peptide bond. In certain embodiments, internal linkers L1 and/or L2 are "long", *i.e.*, consist of 15, 20 or 25 amino acid residues. In some embodiments, these internal linkers consist of about 3 to about 15, for example 8, 9 or 10 contiguous amino acid residues. Regarding the amino acid composition of the internal linkers L1 and L2, peptides are selected with properties that confer flexibility to the PSMA targeting trispecific proteins, do not interfere with the binding domains as well as resist cleavage from proteases. For example, glycine and serine residues generally provide protease resistance. Examples of internal linkers suitable for linking the domains in the PSMA targeting trispecific proteins include but are not limited to (GS)_n (SEQ ID NO: 153), (GGS)_n (SEQ ID NO: 154), (GGGS)_n (SEQ ID NO: 155), (GGSG)_n (SEQ ID NO: 156), (GGSGG)_n (SEQ ID NO: 157), or (GGGGS)_n (SEQ ID NO: 158), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In one embodiment, internal linker L1 and/or L2 is (GGGGS)₄ (SEQ ID NO: 159) or (GGGGS)₃ (SEQ ID NO: 160).

CD3 Binding Domain

[0041] The specificity of the response of T cells is mediated by the recognition of antigen (displayed in context of a major histocompatibility complex, MHC) by the TCR. As part of the TCR, CD3 is a protein complex that includes a CD3 γ (gamma) chain, a CD3 δ (delta) chain, and two CD3 ϵ (epsilon) chains which are present on the cell surface. CD3 associates with the α (alpha) and β (beta) chains of the TCR as well as CD3 ζ (zeta) altogether to comprise the complete TCR. Clustering of CD3 on T cells, such as by immobilized anti-CD3 antibodies leads to T cell activation similar to the engagement of the T cell receptor but independent of its clone-typical specificity.

[0042] In one aspect, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds to CD3. In one aspect, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds to human CD3. In some embodiments, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds to CD3 γ . In some embodiments, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds to CD3 δ . In some embodiments, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds to CD3 ϵ .

[0043] In further embodiments, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds to the TCR. In certain instances, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds the α chain of the TCR. In certain instances, the PSMA targeting trispecific proteins described herein comprise a domain which specifically binds the β chain of the TCR.

[0044] In certain embodiments, the CD3 binding domain of the PSMA targeting trispecific proteins described herein exhibit not only potent CD3 binding affinities with human CD3, but show also excellent crossreactivity with the respective cynomolgus monkey CD3 proteins. In some instances, the CD3 binding domain of the PSMA targeting trispecific proteins are cross-reactive with CD3 from cynomolgus monkey. In certain instances, human:cynomolgus K_D ratios for CD3 are between 5 and 0.2.

[0045] In some embodiments, the CD3 binding domain of the PSMA trispecific antigen-binding protein can be any domain that binds to CD3 including but not limited to domains from a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody. In some instances, it is beneficial for the CD3 binding domain to be derived from the same species in which the PSMA trispecific antigen-binding protein will ultimately be used in. For example, for use in humans, it may be beneficial for the CD3 binding

domain of the PSMA trispecific antigen-binding protein to comprise human or humanized residues from the antigen binding domain of an antibody or antibody fragment.

[0046] Thus, in one aspect, the antigen-binding domain comprises a humanized or human antibody or an antibody fragment, or a murine antibody or antibody fragment. In one embodiment, the humanized or human anti-CD3 binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a humanized or human anti-CD3 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a humanized or human anti-CD3 binding domain described herein, e.g., a humanized or human anti-CD3 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs.

[0047] In some embodiments, the humanized or human anti-CD3 binding domain comprises a humanized or human light chain variable region specific to CD3 where the light chain variable region specific to CD3 comprises human or non-human light chain CDRs in a human light chain framework region. In certain instances, the light chain framework region is a λ (lamda) light chain framework. In other instances, the light chain framework region is a κ (kappa) light chain framework.

[0048] In some embodiments, the humanized or human anti-CD3 binding domain comprises a humanized or human heavy chain variable region specific to CD3 where the heavy chain variable region specific to CD3 comprises human or non-human heavy chain CDRs in a human heavy chain framework region.

[0049] In certain instances, the complementary determining regions of the heavy chain and/or the light chain are derived from known anti-CD3 antibodies, such as, for example, muromonab-CD3 (OKT3), oteelixizumab (TRX4), teplizumab (MGA031), visilizumab (Nuvion), SP34, TR-66 or X35-3, VIT3, BMA030 (BW264/56), CLB-T3/3, CRIS7, YTH12.5, F111-409, CLB-T3.4.2, TR-66, WT32, SPv-T3b, 11D8, XIII-141, XIII-46, XIII-87, 12F6, T3/RW2-8C8, T3/RW2-4B6, OKT3D, M-T301, SMC2, F101.01, UCHT-1 and WT-31.

[0050] In one embodiment, the anti-CD3 binding domain is a single chain variable fragment (scFv) comprising a light chain and a heavy chain of an amino acid sequence provided herein. As used herein, "single chain variable fragment" or "scFv" refers to an antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked via a short flexible polypeptide linker, and capable of being expressed as a

single polypeptide chain, and wherein the scFv retains the specificity of the intact antibody from which it is derived. In an embodiment, the anti-CD3 binding domain comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided herein, or a sequence with 95-99% identity with an amino acid sequence provided herein; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided herein, or a sequence with 95-99% identity to an amino acid sequence provided herein. In one embodiment, the humanized or human anti-CD3 binding domain is a scFv, and a light chain variable region comprising an amino acid sequence described herein, is attached to a heavy chain variable region comprising an amino acid sequence described herein, via a scFv linker. The light chain variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region- scFv linker-heavy chain variable region or heavy chain variable region- scFv linker-light chain variable region.

[0051] In some instances, scFvs which bind to CD3 are prepared according to known methods. For example, scFv molecules can be produced by linking VH and VL regions together using flexible polypeptide linkers. The scFv molecules comprise a scFv linker (e.g., a Ser-Gly linker) with an optimized length and/or amino acid composition. Accordingly, in some embodiments, the length of the scFv linker is such that the VH or VL domain can associate intermolecularly with the other variable domain to form the CD3 binding site. In certain embodiments, such scFv linkers are "short", i.e. consist of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 amino acid residues. Thus, in certain instances, the scFv linkers consist of about 12 or less amino acid residues. In the case of 0 amino acid residues, the scFv linker is a peptide bond. In some embodiments, these scFv linkers consist of about 3 to about 15, for example 8, 9 or 10 contiguous amino acid residues. Regarding the amino acid composition of the scFv linkers, peptides are selected that confer flexibility, do not interfere with the variable domains as well as allow inter-chain folding to bring the two variable domains together to form a functional CD3 binding site. For example, scFv linkers comprising glycine and serine residues generally provide protease resistance. In some embodiments, linkers in a scFv comprise glycine and serine residues. The amino acid sequence of the scFv linkers can be optimized, for example, by phage-display methods to improve the CD3 binding and production yield of the scFv. Examples of peptide scFv linkers suitable for linking a variable light chain domain and a variable heavy chain domain in a scFv include but are not limited to (GS)_n (SEQ ID NO: 153), (GGS)_n (SEQ ID NO: 154), (GGGS)_n

(SEQ ID NO: 155), (GGSG)_n (SEQ ID NO: 156), (GGSGG)_n (SEQ ID NO: 157), or (GGGGS)_n (SEQ ID NO: 158), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In one embodiment, the scFv linker can be (GGGGS)₄ (SEQ ID NO: 159) or (GGGGS)₃ (SEQ ID NO: 160). Variation in the linker length may retain or enhance activity, giving rise to superior efficacy in activity studies.

[0052] In some embodiments, CD3 binding domain of PSMA trispecific antigen-binding protein has an affinity to CD3 on CD3 expressing cells with a K_D of 1000 nM or less, 500 nM or less, 200 nM or less, 100 nM or less, 80 nM or less, 50 nM or less, 20 nM or less, 10 nM or less, 5 nM or less, 1 nM or less, or 0.5 nM or less. In some embodiments, the CD3 binding domain of PSMA trispecific antigen-binding protein has an affinity to CD3 ϵ , γ , or δ with a K_D of 1000 nM or less, 500 nM or less, 200 nM or less, 100 nM or less, 80 nM or less, 50 nM or less, 20 nM or less, 10 nM or less, 5 nM or less, 1 nM or less, or 0.5 nM or less. In further embodiments, CD3 binding domain of PSMA trispecific antigen-binding protein has low affinity to CD3, i.e., about 100 nM or greater.

[0053] The affinity to bind to CD3 can be determined, for example, by the ability of the PSMA trispecific antigen-binding protein itself or its CD3 binding domain to bind to CD3 coated on an assay plate; displayed on a microbial cell surface; in solution; etc. The binding activity of the PSMA trispecific antigen-binding protein itself or its CD3 binding domain of the present disclosure to CD3 can be assayed by immobilizing the ligand (e.g., CD3) or the PSMA trispecific antigen-binding protein itself or its CD3 binding domain, to a bead, substrate, cell, etc. Agents can be added in an appropriate buffer and the binding partners incubated for a period of time at a given temperature. After washes to remove unbound material, the bound protein can be released with, for example, SDS, buffers with a high pH, and the like and analyzed, for example, by Surface Plasmon Resonance (SPR).

[0054] In some embodiments, CD3 binding domains described herein comprise a polypeptide having a sequence described in Table 7 (SEQ ID NO: 1-88) and subsequences thereof. In some embodiments, the CD3 binding domain comprises a polypeptide having at least 70%-95% or more homology to a sequence described in Table 7 (SEQ ID NO: 1-88). In some embodiments, the CD3 binding domain comprises a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, or more homology to a sequence described in Table 7 (SEQ ID NO: 1-88). In some embodiments, the CD3 binding domain has a sequence comprising at least a portion of a sequence described in Table 7 (SEQ ID NO: 1-88). In some embodiments, the CD3 binding domain comprises a polypeptide comprising one or more of the sequences described in Table 7 (SEQ ID NO: 1-88).

[0055] In certain embodiments, CD3 binding domain comprises an scFv with a heavy chain CDR1 comprising SEQ ID NO: 16, and 22-33. In certain embodiments, CD3 binding domain

comprises an scFv with a heavy chain CDR2 comprising SEQ ID NO: 17, and 34-43. In certain embodiments, CD3 binding domain comprises an scFv with a heavy chain CDR3 comprising SEQ ID NO: 18, and 44-53. In certain embodiments, CD3 binding domain comprises an scFv with a light chain CDR1 comprising SEQ ID NO: 19, and 54-66. In certain embodiments, CD3 binding domain comprises an scFv with a light chain CDR2 comprising SEQ ID NO: 20, and 67-79. In certain embodiments, CD3 binding domain comprises an scFv with a light chain CDR3 comprising SEQ ID NO: 21, and 80-86.

Half-Life Extension Domain

[0056] Contemplated herein are domains which extend the half-life of an antigen-binding domain. Such domains are contemplated to include but are not limited to HSA binding domains, Fc domains, small molecules, and other half-life extension domains known in the art.

[0057] Human serum albumin (HSA) (molecular mass ~67 kDa) is the most abundant protein in plasma, present at about 50 mg/ml (600 μ M), and has a half-life of around 20 days in humans. HSA serves to maintain plasma pH, contributes to colloidal blood pressure, functions as carrier of many metabolites and fatty acids, and serves as a major drug transport protein in plasma.

[0058] Noncovalent association with albumin extends the elimination half-time of short lived proteins. For example, a recombinant fusion of an albumin binding domain to a Fab fragment resulted in an *in vivo* clearance of 25- and 58-fold and a half-life extension of 26- and 37-fold when administered intravenously to mice and rabbits respectively as compared to the administration of the Fab fragment alone. In another example, when insulin is acylated with fatty acids to promote association with albumin, a protracted effect was observed when injected subcutaneously in rabbits or pigs. Together, these studies demonstrate a linkage between albumin binding and prolonged action.

[0059] In one aspect, the PSMA targeting trispecific proteins described herein comprise a half-life extension domain, for example a domain which specifically binds to HSA. In some embodiments, the HSA binding domain of PSMA trispecific antigen-binding protein can be any domain that binds to HSA including but not limited to domains from a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody. In some embodiments, the HSA binding domain is a single chain variable fragments (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (VHH) of camelid derived single domain antibody, peptide, ligand or small molecule entity specific for HSA. In certain embodiments, the HSA binding domain is a single-domain antibody. In other embodiments, the HSA binding domain is a peptide. In further embodiments, the HSA binding domain is a small molecule. It is contemplated that the HSA binding domain of PSMA trispecific antigen-binding protein is fairly small and no more

than 25 kD, no more than 20 kD, no more than 15 kD, or no more than 10 kD in some embodiments. In certain instances, the HSA binding is 5 kD or less if it is a peptide or small molecule entity.

[0060] The half-life extension domain of PSMA trispecific antigen-binding protein provides for altered pharmacodynamics and pharmacokinetics of the PSMA trispecific antigen-binding protein itself. As above, the half-life extension domain extends the elimination half-time. The half-life extension domain also alters pharmacodynamic properties including alteration of tissue distribution, penetration, and diffusion of the trispecific antigen-binding protein. In some embodiments, the half-life extension domain provides for improved tissue (including tumor) targeting, tissue distribution, tissue penetration, diffusion within the tissue, and enhanced efficacy as compared with a protein without an half-life extension domain. In one embodiment, therapeutic methods effectively and efficiently utilize a reduced amount of the trispecific antigen-binding protein, resulting in reduced side effects, such as reduced non-tumor cell cytotoxicity.

[0061] Further, the binding affinity of the half-life extension domain can be selected so as to target a specific elimination half-time in a particular trispecific antigen-binding protein. Thus, in some embodiments, the half-life extension domain has a high binding affinity. In other embodiments, the half-life extension domain has a medium binding affinity. In yet other embodiments, the half-life extension domain has a low or marginal binding affinity. Exemplary binding affinities include K_D concentrations at 10 nM or less (high), between 10 nM and 100 nM (medium), and greater than 100 nM (low). As above, binding affinities to HSA are determined by known methods such as Surface Plasmon Resonance (SPR).

[0062] In some embodiments, HSA binding domains described herein comprise a polypeptide having a sequence described in Table 8 (SEQ ID NO: 89-112) and subsequences thereof. In some embodiments, the HSA binding domain comprises a polypeptide having at least 70%-95% or more homology to a sequence described in Table 8 (SEQ ID NO: 89-112). In some embodiments, the HSA binding domain comprises a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, or more homology to a sequence described in Table 8 (SEQ ID NO: 89-112). In some embodiments, the HSA binding domain has a sequence comprising at least a portion of a sequence described in Table 8 (SEQ ID NO: 89-112). In some embodiments, the HSA binding domain comprises a polypeptide comprising one or more of the sequences described in Table 8 (SEQ ID NO: 89-112).

[0063] In some embodiments, HSA binding domains described herein comprise a single domain antibody with a CDR1 comprising SE ID NO: 96, and 99-101. In some embodiments, HSA binding domains described herein comprise a single domain antibody with a CDR1 comprising

SE ID NO: 97, and 102-107. In some embodiments, HSA binding domains described herein comprise a single domain antibody with a CDR1 comprising SE ID NO: 98, 108 and 109.

Prostate Specific Membrane Antigen (PSMA) Binding Domain

[0064] Prostate specific membrane antigen (PSMA) is a 100 kD Type II membrane glycoprotein expressed in prostate tissues having sequence identity with the transferrin receptor with NAALADase activity. PSMA is expressed in increased amounts in prostate cancer, and elevated levels of PSMA are also detectable in the sera of these patients. PSMA expression increases with disease progression, becoming highest in metastatic, hormone-refractory disease for which there is no present therapy.

[0065] In addition to the described CD3 and half-life extension domains, the PSMA targeting trispecific proteins described herein also comprise a domain that binds to PSMA. The design of the PSMA targeting trispecific proteins described herein allows the binding domain to PSMA to be flexible in that the binding domain to PSMA can be any type of binding domain, including but not limited to, domains from a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody. In some embodiments, the binding domain to PSMA is a single chain variable fragments (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (VHH) of camelid derived single domain antibody. In other embodiments, the binding domain to PSMA is a non-Ig binding domain, i.e., antibody mimetic, such as anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPin, fynomers, kunitz domain peptides, and monobodies. In further embodiments, the binding domain to PSMA is a ligand or peptide that binds to or associates with PSMA. In yet further embodiments, the binding domain to PSMA is a knottin. In yet further embodiments, the binding domain to PSMA is a small molecular entity.

[0066] In some embodiments, the PSMA binding domain comprises the following formula: f1-r1-f2-r2-f3-r3-f4, wherein r1, r2, and r3 are complementarity determining regions CDR1, CDR2, and CDR3, respectively, and f1, f2, f3, and f4 are framework residues, and wherein r1 comprises SEQ ID No. 114, SEQ ID No. 115, SEQ ID No. 116, or SEQ ID NO: 125, r2 comprises SEQ ID No. 117, SEQ ID NO. 118, SEQ ID No. 119, SEQ ID No. 120, SEQ ID No. 121, SEQ ID No. 122, SEQ ID No. 123, or SEQ ID NO: 126, and r3 comprises SEQ ID No. 124, or SEQ ID NO: 127.

[0067] In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX4X₅TDYAEX6VKG), and (c) the amino acid sequence of CDR3 is as set forth in

SEQ ID No. 164 (DX₇YGY). In some embodiments, the amino acid residues X₁, X₂, X₃, X₄, X₅, X₆, and X₇ are independently selected from glutamic acid, proline, serine, histidine, threonine, aspartic acid, glycine, lysine, threonine, glutamine, and tyrosine. In some embodiments, X₁ is proline. In some embodiments, X₂ is histidine. In some embodiments, X₃ is aspartic acid. In some embodiments, X₄ is lysine. In some embodiments, X₅ is glutamine. In some embodiments, X₆ is tyrosine. In some embodiments, X₇ is serine. The PSMA binding protein of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is histidine, X₃ is aspartic acid, X₄ is glycine, X₅ is threonine, X₆ is serine, and X₇ is serine.

[0068] In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and (c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₁ is proline. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and (c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₅ is glutamine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and (c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₆ is tyrosine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and (c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₄ is lysine, and X₇ is serine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and (c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₂ is histidine, X₃ is aspartic acid, X₄ is lysine, and X₇ is serine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163

(X₃INPAX₄X₅TDYAEX₆VKG), and(c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₁ is proline, X₂ is histidine, X₃ is aspartic acid, and X₇ is serine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and(c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₂ is histidine, X₃ is aspartic acid, X₅ is glutamine, and X₇ is serine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and(c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₂ is histidine, X₃ is aspartic acid, X₆ is tyrosine, and X₇ is serine. In some embodiments, the PSMA binding domain comprises a CDR1, CDR2, and CDR3, wherein (a) the amino acid sequence of CDR1 is as set forth in SEQ ID No. 162 (RFMISX₁YX₂MH), (b) the amino acid sequence of CDR2 is as set forth in SEQ ID No. 163 (X₃INPAX₄X₅TDYAEX₆VKG), and(c) the amino acid sequence of CDR3 is as set forth in SEQ ID No. 164 (DX₇YGY), wherein X₂ is histidine, X₃ is aspartic acid, and X₇ is serine.

[0069] The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is histidine, X₃ is threonine, X₄ is glycine, X₅ is threonine, X₆ is serine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is histidine, X₃ is threonine, X₄ is glycine, X₅ is threonine, X₆ is serine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is serine, X₃ is threonine, X₄ is lysine, X₅ is threonine, X₆ is serine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is proline, X₂ is serine, X₃ is threonine, X₄ is glycine, X₅ is threonine, X₆ is serine, and X₇ is glycine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is serine, X₃ is threonine, X₄ is glycine, X₅ is glutamine, X₆ is serine, and X₇ is glycine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is serine, X₃ is threonine, X₄ is glycine, X₅ is threonine, X₆ is tyrosine, and X₇ is glycine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is histidine, X₃ is

aspartic acid, X₄ is lysine, X₅ is threonine, X₆ is serine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is proline, X₂ is histidine, X₃ is aspartic acid, X₄ is glycine, X₅ is threonine, X₆ is serine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is histidine, X₃ is aspartic acid, X₄ is glutamine, X₅ is threonine, X₆ is serine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₁ is glutamic acid, X₂ is histidine, X₃ is aspartic acid, X₄ is glycine, X₅ is threonine, X₆ is tyrosine, and X₇ is serine. The PSMA binding domain of the present disclosure may in some embodiments comprise CDR1, CDR2, and CDR3 sequences wherein X₂ is histidine, and X₇ is serine. Exemplary framework sequences are disclosed as SEQ ID NO: 165-168.

[0070] In some embodiments, PSMA binding domains described herein comprise a polypeptide having a sequence described in Table 9 (SEQ ID NO: 113-140) and subsequences thereof. In some embodiments, the HSA binding domain comprises a polypeptide having at least 70%-95% or more homology to a sequence described in Table 9 (SEQ ID NO: 113-140). In some embodiments, the HSA binding domain comprises a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, or more homology to a sequence described in Table 9 (SEQ ID NO: 113-140). In some embodiments, the HSA binding domain has a sequence comprising at least a portion of a sequence described in Table 9 (SEQ ID NO: 113-140). In some embodiments, the HSA binding domain comprises a polypeptide comprising one or more of the sequences described in Table 9 (SEQ ID NO: 113-140).

[0071] In some embodiments, PSMA binding domains described herein comprise a single domain antibody with a CDR1 comprising SE ID NO: 114-116, and 125. In some embodiments, PSMA binding domains described herein comprise a single domain antibody with a CDR1 comprising SEQ ID NO: 117-123, and 126. In some embodiments, PSMA binding domains described herein comprise a single domain antibody with a CDR1 comprising SE ID NO: 124 and 127.

PSMA Trispecific Protein Modifications

[0072] The PSMA targeting trispecific proteins described herein encompass derivatives or analogs in which (i) an amino acid is substituted with an amino acid residue that is not one encoded by the genetic code, (ii) the mature polypeptide is fused with another compound such as polyethylene glycol, or (iii) additional amino acids are fused to the protein, such as a leader or secretory sequence or a sequence for purification of the protein.

[0073] Typical modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0074] Modifications are made anywhere in PSMA targeting trispecific proteins described herein, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Certain common peptide modifications that are useful for modification of PSMA targeting trispecific proteins include glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, and ADP-ribosylation.

Polynucleotides Encoding PSMA targeting trispecific proteins

[0075] Also provided, in some embodiments, are polynucleotide molecules encoding a PSMA trispecific antigen-binding protein described herein. In some embodiments, the polynucleotide molecules are provided as a DNA construct. In other embodiments, the polynucleotide molecules are provided as a messenger RNA transcript.

[0076] The polynucleotide molecules are constructed by known methods such as by combining the genes encoding the three binding domains either separated by peptide linkers or, in other embodiments, directly linked by a peptide bond, into a single genetic construct operably linked to a suitable promoter, and optionally a suitable transcription terminator, and expressing it in bacteria or other appropriate expression system such as, for example CHO cells. In the embodiments where the PSMA binding domain is a small molecule, the polynucleotides contain genes encoding the CD3 binding domain and the half-life extension domain. In the embodiments where the half-life extension domain is a small molecule, the polynucleotides contain genes encoding the domains that bind to CD3 and PSMA. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. The promoter is selected such that it drives the expression of the polynucleotide in the respective host cell.

[0077] In some embodiments, the polynucleotide is inserted into a vector, preferably an expression vector, which represents a further embodiment. This recombinant vector can be constructed according to known methods. Vectors of particular interest include plasmids,

phagemids, phage derivatives, virii (e.g., retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, lentiviruses, and the like), and cosmids.

[0078] A variety of expression vector/host systems may be utilized to contain and express the polynucleotide encoding the polypeptide of the described trispecific antigen-binding protein. Examples of expression vectors for expression in *E.coli* are pSKK (Le Gall et al., J Immunol Methods. (2004) 285(1):111-27) or pcDNA5 (Invitrogen) for expression in mammalian cells.

[0079] Thus, the PSMA targeting trispecific proteins as described herein, in some embodiments, are produced by introducing a vector encoding the protein as described above into a host cell and culturing said host cell under conditions whereby the protein domains are expressed, may be isolated and, optionally, further purified.

Pharmaceutical Compositions

[0080] Also provided, in some embodiments, are pharmaceutical compositions comprising a PSMA trispecific antigen-binding protein described herein, a vector comprising the polynucleotide encoding the polypeptide of the PSMA targeting trispecific proteins or a host cell transformed by this vector and at least one pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" includes, but is not limited to, any carrier that does not interfere with the effectiveness of the biological activity of the ingredients and that is not toxic to the patient to whom it is administered. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Preferably, the compositions are sterile. These compositions may also contain adjuvants such as preservative, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents.

[0081] In some embodiments of the pharmaceutical compositions, the PSMA targeting trispecific proteins described herein are encapsulated in nanoparticles. In some embodiments, the nanoparticles are fullerenes, liquid crystals, liposome, quantum dots, superparamagnetic nanoparticles, dendrimers, or nanorods. In other embodiments of the pharmaceutical compositions, the PSMA trispecific antigen-binding protein is attached to liposomes. In some instances, the PSMA trispecific antigen-binding protein are conjugated to the surface of liposomes. In some instances, the PSMA trispecific antigen-binding protein are encapsulated within the shell of a liposome. In some instances, the liposome is a cationic liposome.

[0082] The PSMA targeting trispecific proteins described herein are contemplated for use as a medicament. Administration is effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. In some embodiments, the

route of administration depends on the kind of therapy and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. Dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind of therapy, general health and other drugs being administered concurrently. An "effective dose" refers to amounts of the active ingredient that are sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology and may be determined using known methods.

Methods of treatment

[0083] Also provided herein, in some embodiments, are methods and uses for stimulating the immune system of an individual in need thereof comprising administration of a PSMA targeting trispecific protein described herein. In some instances, the administration of a PSMA targeting trispecific protein described herein induces and/or sustains cytotoxicity towards a cell expressing PSMA. In some instances, the cell expressing PSMA is a cancer cell.

[0084] Also provided herein are methods and uses for a treatment of a disease, disorder or condition associated with PSMA comprising administering to an individual in need thereof a PSMA targeting trispecific protein described herein. Diseases, disorders or conditions associated with PSMA include, but are not limited to, a proliferative disease or a tumorous disease. In one embodiment, the disease, disorder or condition associated with PSMA is prostate cancer. In another embodiment, the disease, disorder, or condition associated with PSMA is renal cancer.

[0085] In some embodiments, the prostate cancer is an advanced stage prostate cancer. In some embodiments, the prostate cancer is drug resistant. In some embodiments, the prostate cancer is anti-androgen drug resistant. In some embodiments, the prostate cancer is metastatic. In some embodiments, the prostate cancer is metastatic and drug resistant (*e.g.*, anti-androgen drug resistant). In some embodiments, the prostate cancer is castration resistant. In some embodiments, the prostate cancer is metastatic and castration resistant. In some embodiments, the prostate cancer is enzalutamide resistant. In some embodiments, the prostate cancer is enzalutamide and abiraterone resistant. In some embodiments, the prostate cancer is enzalutamide, abiraterone, and bicalutamide resistant. In some embodiments, the prostate cancer is docetaxel resistant. In some of these embodiments, the prostate cancer is enzalutamide, abiraterone, bicalutamide, and docetaxel resistant.

[0086] In some embodiments, administering a PSMA targeting trispecific protein described herein inhibits prostate cancer cell growth; inhibits prostate cancer cell migration; inhibits prostate cancer cell invasion; ameliorates the symptoms of prostate cancer; reduces the size of a

prostate cancer tumor; reduces the number of prostate cancer tumors; reduces the number of prostate cancer cells; induces prostate cancer cell necrosis, pyroptosis, oncosis, apoptosis, autophagy, or other cell death; or enhances the therapeutic effects of a compound selected from the group consisting of enzalutamide, abiraterone, docetaxel, bicalutamide, and any combinations thereof.

[0087] In some embodiments, the method comprises inhibiting prostate cancer cell growth by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises inhibiting prostate cancer cell migration by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises inhibiting prostate cancer cell invasion by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises ameliorating the symptoms of prostate cancer by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises reducing the size of a prostate cancer tumor by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises reducing the number of prostate cancer tumors by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises reducing the number of prostate cancer cells by administering a PSMA targeting trispecific protein described herein. In some embodiments, the method comprises inducing prostate cancer cell necrosis, pyroptosis, oncosis, apoptosis, autophagy, or other cell death by administering a PSMA targeting trispecific protein described herein.

[0088] As used herein, in some embodiments, “treatment” or “treating” or “treated” refers to therapeutic treatment wherein the object is to slow (lessen) an undesired physiological condition, disorder or disease, or to obtain beneficial or desired clinical results. For the purposes described herein, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms; diminishment of the extent of the condition, disorder or disease; stabilization (*i.e.*, not worsening) of the state of the condition, disorder or disease; delay in onset or slowing of the progression of the condition, disorder or disease; amelioration of the condition, disorder or disease state; and remission (whether partial or total), whether detectable or undetectable, or enhancement or improvement of the condition, disorder or disease. Treatment includes eliciting a clinically significant response without excessive levels of side effects. Treatment also includes prolonging survival as compared to expected survival if not receiving treatment. In other embodiments, “treatment” or “treating” or “treated” refers to prophylactic measures, wherein the object is to delay onset of or reduce severity of an undesired physiological condition, disorder or disease, such as, for example is a person who is predisposed to a disease (e.g., an individual who carries a genetic marker for a disease such as prostate cancer).

[0089] In some embodiments of the methods described herein, the PSMA targeting trispecific proteins are administered in combination with an agent for treatment of the particular disease, disorder or condition. Agents include but are not limited to, therapies involving antibodies, small molecules (e.g., chemotherapeutics), hormones (steroidal, peptide, and the like), radiotherapies (γ -rays, X-rays, and/or the directed delivery of radioisotopes, microwaves, UV radiation and the like), gene therapies (e.g., antisense, retroviral therapy and the like) and other immunotherapies. In some embodiments, the PSMA targeting trispecific proteins are administered in combination with anti-diarrheal agents, anti-emetic agents, analgesics, opioids and/or non-steroidal anti-inflammatory agents. In some embodiments, the PSMA targeting trispecific proteins are administered before, during, or after surgery.

Certain Definitions

[0090] As used herein, “elimination half-time” is used in its ordinary sense, as is described in *Goodman and Gilman's The Pharmaceutical Basis of Therapeutics* 21-25 (Alfred Goodman Gilman, Louis S. Goodman, and Alfred Gilman, eds., 6th ed. 1980). Briefly, the term is meant to encompass a quantitative measure of the time course of drug elimination. The elimination of most drugs is exponential (i.e., follows first-order kinetics), since drug concentrations usually do not approach those required for saturation of the elimination process. The rate of an exponential process may be expressed by its rate constant, k , which expresses the fractional change per unit of time, or by its half-time, $t_{1/2}$ the time required for 50% completion of the process. The units of these two constants are time^{-1} and time, respectively. A first-order rate constant and the half-time of the reaction are simply related ($k \times t_{1/2} = 0.693$) and may be interchanged accordingly. Since first-order elimination kinetics dictates that a constant fraction of drug is lost per unit time, a plot of the log of drug concentration versus time is linear at all times following the initial distribution phase (i.e. after drug absorption and distribution are complete). The half-time for drug elimination can be accurately determined from such a graph.

[0091] As used herein, the phrase “prostate cancer” or “advanced stage prostate cancer” includes a class of prostate cancers that has progressed beyond early stages of the disease. Typically, advanced stage prostate cancers are associated with a poor prognosis. Types of advanced stage prostate cancers include, but are not limited to, metastatic prostate cancer, drug-resistant prostate cancer such as anti-androgen-resistant prostate cancer (e.g., enzalutamide-resistant prostate cancer, abiraterone-resistant prostate cancer, bicalutamide-resistant prostate cancer, and the like), hormone refractory prostate cancer, castration-resistant prostate cancer, metastatic castration-resistant prostate cancer, docetaxel-resistant prostate cancer, androgen receptor splice variant-7 (AR-V7)-induced drug-resistant prostate cancer such as AR-V7-induced anti-androgen-resistant prostate cancer (e.g., AR-V7-induced enzalutamide-resistant

prostate cancer), aldo-keto reductase family 1 member C3 (AKR1C3)-induced drug-resistant prostate cancer such as AKR1C3-induced anti-androgen-resistant prostate cancer (*e.g.*, AKR1C3-induced enzalutamide-resistant prostate cancer), and combinations thereof. In some instances, the advanced stage prostate cancers do not generally respond, or are resistant, to treatment with one or more of the following conventional prostate cancer therapies: enzalutamide, arbiraterone, bicalutamide, and docetaxel. Compounds, compositions, and methods of the present disclosure are provided for treating prostate cancer, such as advanced stage prostate cancer, including any one or more (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, or more) of the types of advanced stage prostate cancers disclosed herein.

EXAMPLES

Example 1: Methods to assess binding and cytotoxic activities of trispecific antigen binding molecules

[0092] Protein Production

[0093] Sequences of trispecific molecules were cloned into mammalian expression vector pcDNA 3.4 (Invitrogen) preceded by a leader sequence and followed by a 6x Histidine Tag (SEQ ID NO: 161). Expi293F cells (Life Technologies A14527) were maintained in suspension in Optimum Growth Flasks (Thomson) between 0.2 to 8 x 1e6 cells/ml in Expi293 media. Purified plasmid DNA was transfected into Expi293 cells in accordance with Expi293 Expression System Kit (Life Technologies, A14635) protocols, and maintained for 4-6 days post transfection. Conditioned media was partially purified by affinity and desalting chromatography. Trispecific proteins were subsequently polished by ion exchange or, alternatively, concentrated with Amicon Ultra centrifugal filtration units (EMD Millipore), applied to Superdex 200 size exclusion media (GE Healthcare) and resolved in a neutral buffer containing excipients. Fraction pooling and final purity were assessed by SDS-PAGE and analytical SEC.

[0094] Affinity Measurements

[0095] The affinities of the all binding domains molecules were measured by biolayer interferometry using an Octet instrument.

[0096] PSMA affinities were measured by loading human PSMA-Fc protein (100 nM) onto anti-human IgG Fc biosensors for 120 seconds, followed by a 60 second baseline, after which associations were measured by incubating the sensor tip in a dilution series of the trispecific molecules for 180 seconds, followed by dissociation for 50 seconds. EGFR and CD3 affinities were measured by loading human EGFR-Fc protein or human CD3-Flag-Fc protein, respectively, (100 nM) onto anti-human IgG Fc biosensors for 120 seconds, followed by a 60

second baseline, after which associations were measured by incubating the sensor tip in a dilution series of the trispecific molecules for 180 seconds, followed by dissociation for 300 seconds. Affinities to human serum albumin (HSA) were measured by loading biotinylated albumin onto streptavidin biosensors, then following the same kinetic parameters as for CD3 affinity measurements. All steps were performed at 30°C in 0.25% casein in phosphate-buffered saline.

[0097] Cytotoxicity assays

[0098] A human T-cell dependent cellular cytotoxicity (TDCC) assay was used to measure the ability of T cell engagers, including trispecific molecules, to direct T cells to kill tumor cells (Nazarian et al. 2015. J Biomol Screen. 20:519-27). In this assay, T cells and target cancer cell line cells are mixed together at a 10:1 ratio in a 384 wells plate, and varying amounts of T cell engager are added. After 48 hours, the T cells are washed away leaving attached to the plate target cells that were not killed by the T cells. To quantitate the remaining viable cells, CellTiter-Glo® Luminescent Cell Viability Assay (Promega) is used. In some cases, the target cells are engineered to express luciferase. In these cases, viability of the target cells is assessed by performing a luminescent luciferase assay with STEADYGLO® reagent (Promega), where viability is directly proportional to the amount of luciferase activity.

[0099] Stability assays

[00100] The stability of the trispecific binding proteins was assessed at low concentrations in the presence of non-human primate serum. TriTACs were diluted to 33 µg/ml in Cynomolgus serum (BioReclamationIVT) and either incubated for 2 d at 37°C or subjected to five freeze/thaw cycles. Following the treatment, the samples were assessed in cytotoxicity (TDCC) assays and their remaining activity was compared to untreated stock solutions.

[00101] Xenograft assays

[00102] The in vivo efficacy of trispecific binding proteins was assessed in xenograft experiments (Crown Bioscience, Taicang). NOD/SCID mice deficient in the common gamma chain (NCG, Model Animal Research Center of Nanjing University) were inoculated on day 0 with a mixture of 5e6 22Rv1 human prostate cancer cells and 5e6 resting, human T cells that were isolated from a healthy, human donor. The mice were randomized into three groups, and treated with vehicle, 0.5 mg/kg PSMA TriTAC C324 or 0.5 mg/kg PSMA BiTE. Treatments were administered daily for 10 days via i.v. bolus injection. Animals were checked daily for morbidity and mortality. Tumor volumes were determined twice weekly with a caliper. The study was terminated after 30 days.

[00103] PK assays

[00104] The purpose of this study was to evaluate the single dose pharmacokinetics of trispecific binding proteins following intravenous injection. 2 experimentally naïve cynomolgus monkeys per group (1 male and 1 female) were given compound via a slow IV bolus injection administered over approximately 1 minute. Following dose administration, cage side observations were performed once daily and body weights were recorded weekly. Blood samples were collected and processed to serum for pharmacokinetic analysis through 21 days post dose administration.

[00105] Concentrations of test articles were determined from monkey serum with an electroluminescent readout (Meso Scale Diagnostics, Rockville). 96 well plates with immobilized, recombinant CD3 were used to capture the analyte. Detection was performed with sulfo-tagged, recombinant PSMA on a MSD reader according to the manufacturer's instructions.

Example 2: Assessing the impact of CD3 affinity on the properties of trispecific molecules

[00106] PSMA targeting trispecific molecules with distinct CD3 binding domains were studied to demonstrate the effects of altering CD3 affinity. An exemplary PSMA targeting trispecific molecule is illustrated in Figure 1. Table 1 lists the affinity of each molecule for the three binding partners (PSMA, CD3, HSA). Affinities were measured by biolayer interferometry using an Octet instrument (Pall Forté Bio). Reduced CD3 affinity leads to a loss in potency in terms of T cell mediated cellular toxicity (Figures 2A-C). The pharmacokinetic properties of these trispecific molecules was assessed in cynomolgus monkeys. Molecules with high affinity for CD3 like TriTAC C236 have a terminal half-life of approx. 90 h (Figure 3). Despite the altered ability to bind CD3 on T cells, the terminal half-life of two molecules with different CD3 affinities shown in Figure 4 is very similar. However, the reduced CD3 affinity appears to lead to a larger volume of distribution, which is consistent with reduced sequestration of trispecific molecule by T cells. There were no adverse clinical observations or body weight changes noted during the study period.

Table 1: Binding Affinities for Human and Cynomolgus Antigens

	anti-PSMA KD value (nM)			anti-Albumin KD value (nM)			anti-CD3e KD value (nM)		
	human	cyno	ratio cyno/hum	pHSA	CSA	ratio cyno/hum	human	cyno	ratio cyno/hum
Tool TriTAC high aff. - C236	16.3	0	0	22.7	25.4	1.1	6.0	4.7	0.8
TriTAC CD3 high aff. - C324	17.9	0	0	9.8	9.7	1	7.4	5.8	0.8
TriTAC CD3 med aff. - C339	13.6	0	0	8.8	8.3	0.9	40.6	33.6	0.8
TriTAC CD3 low aff. - C325	15.3	0	0	10.1	9.7	1	217	160	0.7

Example 3: Assessing the impact of PSMA affinity on the properties of trispecific molecules

[00107] PSMA targeting trispecific molecules with distinct PSMA binding domains were studied to demonstrate the effects of altering PSMA affinity. Table 2 lists the affinity of each molecule for the three binding partners (PSMA, CD3, HSA). Reduced PSMA affinity leads to a loss in potency in terms of T cell mediated cellular toxicity (Figures 5A-C).

Table 2: Binding Affinities for Human and Cynomolgus Antigens

	anti-PSMA KD value (nM)			anti-Albumin KD value (nM)			anti-CD3e KD value (nM)		
	human	cyno	ratio cyno/hum	pHSA	CSA	ratio cyno/hum	human	cyno	ratio cyno/hum
PSMA-TriTAC (p8)-C362	22.0	0	n/a	6.6	6.6	1.0	8.3	4.3	0.52
PSMA TriTAC (HDS) – C363	3.7	540	146	7.6	8.4	1.1	8.0	5.2	0.65
PSMA TriTAC (HTS)- C364	0.15	663	4423	8.4	8.6	1.0	7.7	3.8	0.49

Example 4: In vivo efficacy of PSMA targeting trispecific molecules

[00108] The PSMA targeting trispecific molecule C324 was assessed for its ability to inhibit the growth of tumors in mice. For this experiment, immunocompromised mice reconstituted with human T cells were subcutaneously inoculated with PSMA expressing human prostate tumor cells (22Rv1) and treated daily for 10 days with 0.5 mg/kg i.v. of either PSMA targeting BiTE or TriTAC molecules. Tumor growth was measured for 30. Over the course of the experiment, the trispecific molecule was able to inhibit tumor growth with an efficacy comparable to a BiTE molecule (Figure 6).

Example 5: Specificity of trispecific molecules

[00109] In order to assess the specificity of PSMA targeting TriTAC molecules, their ability to induce T cells to kill tumor cells was tested with tumor cells that are negative for PSMA (Figure 7A). An EGFR targeting TriTAC molecule served as positive control, a GFP targeting TriTAC molecule as negative control. All three TriTACs with distinct PSMA binding domains showed the expected activity against the PSMA positive cell line LNCaP (Figure 7B), but did not reach EC50s in the PSMA negative tumor cell lines KMS12BM and OVCAR8 (Figures 7C and 7D). The EC50s are summarized in Table 3. At very high TriTAC concentrations (> 1 nM), some limited off-target cell killing could be observed for TriTACs C362 and C363, while C364 did not show significant cell killing under any of the tested conditions.

Table 3: Cell killing activity of TriTAC molecules in with antigen positive and negative tumor cell lines (EC50 [pM])

TriTAC	LNCaP	KMS12BM	OVCAR8
PSMA p8 TriTAC C362	13.0	>10,000	>10,000
PSMA HDS TriTAC C363	6.2	>10,000	>10,000
PSMA HTS TriTAC C364	0.8	>10,000	>10,000
EGFR TriTAC C131	9.4	>10,000	6
GFP TriTAC C	>10,000	>10,000	>10,000

Example 6: Stress tests and protein stability

[00110] Four PSMA targeting trispecific molecules were either incubated for 48 h in Cynomolgus serum at low concentrations (33.3 µg/ml) or subjected to five freeze thaw cycles in Cynomolgus serum. After the treatment, the bio-activity of the TriTAC molecules was assessed in cell killing assays and compared to unstressed samples (“positive control”, Figure 8A-D). All molecules maintained the majority of their cell killing activity. TriTAC C362 was the most stress resistant and did not appear to lose any activity under the conditions tested here.

Example 7: Xenograft Tumor Model

[00111] The PSMA targeting trispecific proteins of the previous examples are evaluated in a xenograft model.

[00112] Male immune-deficient NCG mice are subcutaneously inoculated with 5×10^6 22Rv1 cells into their the right dorsal flank. When tumors reach 100 to 200 mm³, animals are allocated into 3 treatment groups. Groups 2 and 3 (8 animals each) are intraperitoneally injected with 1.5×10^7 activated human T-cells. Three days later, animals from Group 3 are subsequently treated with a total of 9 intravenous doses of 50 µg PSMA trispecific antigen-binding protein of Example 1 (qdx9d). Groups 1 and 2 are only treated with vehicle. Body weight and tumor volume are determined for 30 days.

[00113] It is expected that tumor growth in mice treated with the PSMA trispecific antigen-binding protein have a significantly reduced growth in comparison to the tumor growth in respective vehicle-treated control group.

Example 8: Proof-of-Concept Clinical Trial Protocol for Administration of the PSMA trispecific antigen-binding protein of Example 1 to Prostate Cancer Patients

[00114] This is a Phase I/II clinical trial for studying the PSMA trispecific antigen-binding protein of Example 1 as a treatment for Prostate Cancer.

[00115] Study Outcomes:

[00116] *Primary*: Maximum tolerated dose of PSMA targeting trispecific proteins of the previous examples

[00117] *Secondary*: To determine whether in vitro response of PSMA targeting trispecific proteins of the previous examples are associated with clinical response

[00118] Phase I

[00119] The maximum tolerated dose (MTD) will be determined in the phase I section of the trial.

1.1 The maximum tolerated dose (MTD) will be determined in the phase I section of the trial.

1.2 Patients who fulfill eligibility criteria will be entered into the trial to PSMA targeting trispecific proteins of the previous examples.

1.3 The goal is to identify the highest dose of PSMA targeting trispecific proteins of the previous examples that can be administered safely without severe or unmanageable side effects in participants. The dose given will depend on the number of participants who have been enrolled in the study prior and how well the dose was tolerated. Not all participants will receive the same dose.

[00120] Phase II

2.1 A subsequent phase II section will be treated at the MTD with a goal of determining if therapy with therapy of PSMA targeting trispecific proteins of the previous examples results in at least a 20% response rate.

Primary Outcome for the Phase II ---To determine if therapy of PSMA targeting trispecific proteins of the previous examples results in at least 20% of patients achieving a clinical response (blast response, minor response, partial response, or complete response)

[00121] Eligibility:

Histologically confirmed newly diagnosed aggressive prostate cancer according to the current World Health Organisation Classification, from 2001 to 2007

Any stage of disease.

Treatment with docetaxel and prednisone (+/- surgery).

Age $\geq$ 18 years

Karnofsky performance status $\geq$ 50% or ECOG performance status 0-2

Life expectancy $\geq$ 6 weeks

Example 9: Activity of an exemplary PSMA antigen-binding protein (PSMA targeting TriTAC molecule) in redirected T cell killing assays using a panel of PSMA expressing cell lines and T cells from different donors

[00122] This study was carried out to demonstrate that the activity of the exemplary PSMA trispecific antigen-binding protein is not limited to LNCaP cells or a single cell donor.

[00123] Redirected T cell killing assays were performed using T cells from four different donors and the human PSMA-expressing prostate cancer cell lines VCaP, LNCaP, MDAPCa2b, and 22Rv1. With one exception, the PSMA trispecific antigen-binding protein was able to direct killing of these cancer cell lines using T cells from all donors with EC₅₀ values of 0.2 to 1.5 pM, as shown in Table 4. With the prostate cancer cell line 22 Rv1 and Donor 24, little to no killing was observed (data not shown). Donor 24 also only resulted approximately 50% killing of the MDAPCa2b cell line whereas T cells from the other 3 donors resulted in almost complete killing of this cell line (data not shown). Control assays demonstrated that killing by the PSMA trispecific antigen-binding protein was PSMA specific. No killing was observed when PSMA-expressing cells were treated with a control trispecific protein targeting green fluorescent protein (GFP) instead of PSMA (data not shown). Similarly, the PSMA trispecific antigen-binding protein was inactive with cell lines that lack PSMA expression, NCI-1563 and HCT116, also shown in Table 4.

Table 4: EC₅₀ Values from TDCC Assays with Six Human Cancer Cell Lines and Four Different T Cell Donors

Cell Line	TDCC EC ₅₀ Values (M)			
	Donor 24	Donor 8144	Donor 72	Donor 41
LNCaP	1.5E-12	2.2E-13	3.6E-13	4.3E-13
MDAPCa2b	4.8E-12	4.1E-13	4.9E-13	6.5E-13
VCaP	6.4E-13	1.6E-13	2.0E-13	3.5E-13
22Rv1	n/a	7.2E-13	1.4E-12	1.3E-12
HCT116	>1.0E-8	>1.0E-8	>1.0E-8	>1.0E-8
NCI-1563	>1.0E-8	>1.0E-8	>1.0E-8	>1.0E-8

Example 10: Stimulation of cytokine expression in by an exemplary PSMA trispecific antigen-binding protein (PSMA targeting TriTAC molecule) in redirected T cell killing assays

[00124] This study was carried out to demonstrate activation of T cells by the exemplary PSMA trispecific antigen-binding protein during redirected T cell killing assays by measuring secretion of cytokine into the assay medium by activated T cells.

[00125] Conditioned media collected from redirected T cell killing assays, as described above in Example 9, were analyzed for expression of the cytokines TNF α and IFN γ . Cytokines were measured using AlphaLISA assays (Perkin-Elmer). Adding a titration of the PSMA antigen-binding protein to T cells from four different donors and four PSMA-expressing cell lines,

LNCaP, VCaP, MDAPCa2b, and 22Rv1 resulted in increased levels of TNF α . The results for TNF α expression and IFN γ expression levels in the conditioned media are shown in Tables 5 and 6, respectively. The EC₅₀ values for the PSMA antigen-binding protein induced expression of these cytokines ranged from 3 to 15 pM. Increased cytokine levels were not observed with a control trispecific protein targeting GFP. Similarly, when assays were performed with two cell lines that lack PSMA expression, HCT116 and NCI-H1563, PSMA HTS TriTAC also did not increase TNF α or IFN γ expression.

Table 5: EC₅₀ Values for TNF α Expression in Media from PSMA Trispecific Antigen-Binding Protein TDCC Assays with Six Human Cancer Cell Lines and T Cells from Four Different Donors

Cell Line	Donor 24	Donor 8144	Donor 41	Donor72
LNCaP	4.9E-12	2.8E-12	4.0E-12	3.2E-12
VCaP	3.2E-12	2.9E-12	2.9E-12	2.9E-12
MDAPCa2b	2.1E-11	4.0E-12	5.5E-12	3.6E-12
22Rv1	8.9E-12	2.5E-12	4.0E-12	3.3E-12
HCT116	>1E-8	>1E-8	>1E-8	>1E-8
NCI-H1563	>1E-8	>1E-8	>1E-8	>1E-8

Table 6: EC₅₀ Values for IFN γ Expression in Media from PSMA Trispecific Antigen-Binding Protein TDCC Assays with Six Human Cancer Cell Lines and T Cells from Four Different Donors

Cell Line	Donor 24	Donor 8144	Donor 41	Donor72
LNCaP	4.2E-12	4.2E-12	4.2E-12	2.8E-12
VCaP	5.1E-12	1.5E-11	3.4E-12	4.9E-12
MDAPCa2b	1.5E-11	5.8E-12	9.7E-12	3.5E-12
22Rv1	7.8E-12	3.0E-12	9.1E-12	3.0E-12
HCT116	>1E-8	>1E-8	>1E-8	>1E-8
NCI-H1563	>1E-8	>1E-8	>1E-8	>1E-8

Example 11: Activity of an exemplary PSMA trispecific antigen-binding protein (PSMA targeting TriTAC) in redirected T cell killing assay (TDCC) using T cells from cynomolgus monkeys

[00126] This study was carried out to test the ability of the exemplary PSMA trispecific antigen-binding protein to direct T cells from cynomolgus monkeys to kill PSMA-expressing cell lines.

[00127] TDCC assays were set up using peripheral blood mononuclear cells (PBMCs) from cynomolgus monkeys. Cyno PBMCs were added to LNCaP cells at a 10:1 ratio. It was observed that the PSMA trispecific antigen-binding protein redirected killing of LNCaP by the

cyno PBMCs with an EC₅₀ value of 11 pM. The result is shown in Figure 9A. To confirm these results, a second cell line was used, MDAPCa2b, and PBMCs from a second cynomolgus monkey donor were tested. Redirected killing of the target cells was observed with an EC₅₀ value of 2.2 pM. The result is shown in Figure 9B. Killing was specific to the anti-PSMA arm of the PSMA trispecific antigen-binding protein as killing was not observed with a negative control trispecific protein targeting GFP. These data demonstrate that the PSMA antigen-binding trispecific protein can direct cynomolgus T cells to kill target cells expressing human PSMA.

Example 12: Expression of markers of T cell activation in redirect T cell killing assays with an exemplary PSMA trispecific antigen-binding protein (PSMA targeting TriTAC molecule)

[00128] This study was performed to assess whether T cells were activated when the exemplary PSMA trispecific antigen-binding protein directed the T cells to kill target cells.

[00129] The assays were set up using conditions for the redirected T cell killings assays described in the above example. T cell activation was assessed by measuring expression of CD25 and CD69 on the surface of the T cells using flow cytometry. The PSMA trispecific antigen-binding protein was added to a 10:1 mixture of purified human T cells and the prostate cancer cell line VCaP. Upon addition of increasing amounts of the PSMA trispecific antigen-binding protein, increased CD69 expression and CD25 expression was observed, as shown in Figure 10. EC₅₀ value was 0.3 pM for CD69 and 0.2 pM for CD25. A trispecific protein targeting GFP was included in these assays as negative control, and little to no increase in CD69 or CD25 expression is observed with the GFP targeting trispecific protein, also shown in Figure 10.

Example 13: Stimulation of T cell proliferation by an exemplary PSMA trispecific antigen-binding protein (PSMA targeting TriTAC molecule) in the presence of PSMA expressing target cells

[00130] This study was used as an additional method to demonstrate that the exemplary PSMA trispecific antigen-binding protein was able to activate T cells when it redirects them to kill target cells.

[00131] T cell proliferation assays were set up using the conditions of the T cell redirected killing assay using LNCaP target cells, as described above, and measuring the number of T cells present at 72 hours. The exemplary PSMA trispecific antigen-binding protein stimulated proliferation with an EC₅₀ value of 0.5 pM. As negative control, a trispecific protein targeting GFP was included in the assay, and no increased proliferation was observed with this protein. The results for the T cell proliferation assay are illustrated in Figure 11.

Example 14: Redirected T cell killing of LNCaP cells by three exemplary PSMA trispecific antigen-binding proteins (PSMA targeting TriTAC molecules PH1T, PH, and Z2)

[00132] This study was carried out to test the ability of three exemplary PSMA trispecific antigen-binding proteins, having the sequences as set forth in SEQ ID Nos: 150, 151, and 152, to redirect T cells to kill the LNCaP cell line.

[00133] In TDCC assays, set up as described in above examples, the PSMA PH1T TriTAC (SEQ ID No: 150) and PSMA PH1 TriTAC (SEQ ID NO: 151) proteins directed killing with EC₅₀ values of 25 and 20 pM, respectively, as shown in Figure 12A; and the PSMA Z2 TriTAC (SEQ ID NO: 152) protein directed killing with an EC₅₀ value of 0.8 pM, as shown in Figure 12B.

Table 7: CD3 Binding Domain Sequences

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
1	Anti-CD3, clone 2B2	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAINWVRQAPGKG LEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNTAYLQMNN LKTEDTAVYYCVRHANFGNSYISYWAYWGQGLTVTVSSGGGGS GGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCASSTGAVTSGNY PNWVQQKPGQAPRGLIGGTFKFLVPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCTLWYSNRWVFGGGTKLTVL
2	Anti-CD3, clone 9F2	EVQLVESGGGLVQPGGSLKLSAASGFEFNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSFSAVTSNGY PNWVQQKPGQAPRGLIGGTFKFLVPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCVLWYDNRWVFGGGTKLTVL
3	Anti-CD3, clone 5A2	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSHISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGYVTSGN YPNWVQQKPGQAPRGLIGGTSFLVPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCVLWYSNRWIFGGGTKLTVL
4	Anti-CD3, clone 6A2	EVQLVESGGGLVQPGGSLKLSAASGFMFNKYAMNWVRQAPGK GLEWVARIRSKNNYATYYADSVKDRFTISRDDSKNTAYLQMNN LKTEDTAVYYCVRHGNFGNSYISYWATWGQGLTVTVSSGGGGS GGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSFSAVTSNGY PNWVQQKPGQAPRGLIGGTFKLLVPGTPARFSGSLLGGKAALTLG VQPEDEAEYYCVLWYSNSWVFGGGTKLTVL
5	Anti-CD3, clone 2D2	EVQLVESGGGLVQPGGSLKLSAASGFTFNTYAMNWVRQAPGK

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
		GLEWVARIRSKYNNYATYYKDSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSPISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVVSGN YPNWVQKPGQAPRGLIGGTEFLAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCWLWYSNRWVFGGGTKLTVL
6	Anti-CD3, clone 3F2	EVQLVESGGGLVQPGGSLKLSAASGFTYNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADEVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSPISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVTSGN YPNWVQKPGQAPRGLIGGTEFLAPGTPARFSGSLLGGKAALTL SGVQPEDEAEYYCTLWYSNRWVFGGGTKLTVL
7	Anti-CD3, clone 1A2	EVQLVESGGGLVQPGGSLKLSAASGNTFNKYAMNWVRQAPGK GLEWVARIRSKYNNYETYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHTNFGNSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVTSGY YPNWVQKPGQAPRGLIGGTYFLAPGTPARFSGSLLGGKAALTL SGVQPEDEAEYYCWLWYSNRWVFGGGTKLTVL
8	Anti-CD3, clone 1C2	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADAVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSQISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVTDGN YPNWVQKPGQAPRGLIGGIKFLAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCWLWYSNRWVFGGGTKLTVL
9	Anti-CD3, clone 2E4	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAVNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGESTGAVTSGN YPNWVQKPGQAPRGLIGGKILAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCWLWYSNRWVFGGGTKLTVL
10	Anti-CD3, clone 10E4	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYPMNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKNEDTAVYYCVRHGNFNNSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVTKGN YPNWVQKPGQAPRGLIGGKMLAPGTPARFSGSLLGGKAALTL SGVQPEDEAEYYCALWYSNRWVFGGGTKLTVL
11	Anti-CD3, clone 2H2	EVQLVESGGGLVQPGGSLKLSAASGFTFNGYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADEVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSPISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPLTVSPGGTVTLTCGSSTGAVVSGN YPNWVQKPGQAPRGLIGGTEFLAPGTPARFSGSLLGGKAALTLS

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
		GVQPEDEAEYYCVLWYSNRWVFGGGTKLTVL
12	Anti-CD3, clone 2A4	EVQLVESGGGLVQPGGSLKLSAASGNTFNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGDSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVTHGN YPNWVQKPGQAPRGLIGGTKVLAPGTPARFSGSLLGGKAALTL SGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVL
13	Anti-CD3, clone 10B2	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGK GLEWVARIRSGYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSYTGAVTSGN YPNWVQKPGQAPRGLIGGTKFNAPGTPARFSGSLLGGKAALTL SGVQPEDEAEYYCVLWYANRWVFGGGTKLTVL
14	Anti-CD3, clone 1G4	EVQLVESGGGLVQPGGSLKLSAASGFENKYAMNWVRQAPGK GLEWVARIRSKYNNYETYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSLISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSGAVTSGNY PNWVQKPGQAPRGLIGGTKFGAPGTPARFSGSLLGGKAALTL GVQPEDEAEYYCVLWYSNRWVFGGGTKLTVL
15	wt anti-CD3	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGLTVTVSSGGGG SGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGN YPNWVQKPGQAPRGLIGGTKFLAPGTPARFSGSLLGGKAALTL SGVQPEDEAEYYCVLWYSNRWVFGGGTKLTVL
16	wt anti-CD3 HC CDR1	GFTFNKYAMN
17	wt anti-CD3 HC CDR2	RIRSKYNNYATYYADSVK
18	wt anti-CD3 HC CDR3	HGNFGNSYISYWAY
19	wt anti-CD3 LC CDR1	GSSTGAVTSGNYPN
20	wt anti-CD3 LC CDR2	GTKFLAP
21	wt anti-CD3 LC CDR3	VLWYSNRWV
22	HC CDR1 variant 1	GNTFNKYAMN
23	HC CDR1 variant 2	GFEFNKYAMN
24	HC CDR1 variant 3	GFMFNKYAMN
25	HC CDR1 variant 4	GFTYNKYAMN
26	HC CDR1 variant 5	GFTFNKYAMN
27	HC CDR1 variant 6	GFTFNGYAMN

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
28	HC CDR1 variant 7	GFTFNTYAMN
29	HC CDR1 variant 8	GFTFNEYAMN
30	HC CDR1 variant 9	GFTFNKYPMN
31	HC CDR1 variant 10	GFTFNKYAVN
32	HC CDR1 variant 11	GFTFNKYAIN
33	HC CDR1 variant 12	GFTFNKYALN
34	HC CDR2 variant 1	RIRSGYNNTYATYYADSVK
35	HC CDR2 variant 2	RIRSKSNNTYATYYADSVK
36	HC CDR2 variant 3	RIRSKYNKYATYYADSVK
37	HC CDR2 variant 4	RIRSKYNNTYETYYADSVK
38	HC CDR2 variant 5	RIRSKYNNTYATEYADSVK
39	HC CDR2 variant 6	RIRSKYNNTYATYYKDSVK
40	HC CDR2 variant 7	RIRSKYNNTYATYYADEVK
41	HC CDR2 variant 8	RIRSKYNNTYATYYADAVK
42	HC CDR2 variant 9	RIRSKYNNTYATYYADQVK
43	HC CDR2 variant 10	RIRSKYNNTYATYYADDVK
44	HC CDR3 variant 1	HANFGNSYISYWAY
45	HC CDR3 variant 2	HTNFGNSYISYWAY
46	HC CDR3 variant 3	HGNFNNSYISYWAY
47	HC CDR3 variant 4	HGNFGDSYISYWAY
48	HC CDR3 variant 5	HGNFGNSHISYWAY
49	HC CDR3 variant 6	HGNFGNSPISYWAY
50	HC CDR3 variant 7	HGNFGNSQISYWAY
51	HC CDR3 variant 8	HGNFGNSLISYWAY
52	HC CDR3 variant 9	HGNFGNSGISYWAY
53	HC CDR3 variant 10	HGNFGNSYISYWAT
54	LC CDR1 variant 1	ASSTGAVTSGNYPN
55	LC CDR1 variant 2	GESTGAVTSGNYPN
56	LC CDR1 variant 3	GSYTGAVTSGNYPN
57	LC CDR1 variant 4	GSSFAGVTSNYPN
58	LC CDR1 variant 5	GSSKGAVTSGNYPN
59	LC CDR1 variant 6	GSSSGAVTSGNYPN
60	LC CDR1 variant 7	GSSTGYVTSGNYPN
61	LC CDR1 variant 8	GSSTGAVVSGNYPN
62	LC CDR1 variant 9	GSSTGAVTDGNYPN
63	LC CDR1 variant 10	GSSTGAVTKGNYPN
64	LC CDR1 variant 11	GSSTGAVTHGNYPN

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
65	LC CDR1 variant 12	GSSTGAVTVGNYPN
66	LC CDR1 variant 13	GSSTGAVTSGYYPN
67	LC CDR2 variant 1	GIKFLAP
68	LC CDR2 variant 2	GTEFLAP
69	LC CDR2 variant 3	GTYFLAP
70	LC CDR2 variant 4	GTSFLAP
71	LC CDR2 variant 5	GTNFLAP
72	LC CDR2 variant 6	GTKLLAP
73	LC CDR2 variant 7	GTKELAP
74	LC CDR2 variant 8	GTKILAP
75	LC CDR2 variant 9	GTKMLAP
76	LC CDR2 variant 10	GTKVLAP
77	LC CDR2 variant 11	GTKFNAP
78	LC CDR2 variant 12	GTKFGAP
79	LC CDR2 variant 13	GTKFLVP
80	LC CDR3 variant 1	TLWYSNRWV
81	LC CDR3 variant 2	ALWYSNRWV
82	LC CDR3 variant 3	VLWYDNRWV
83	LC CDR3 variant 4	VLWYANRWV
84	LC CDR3 variant 5	VLWYSNSWV
85	LC CDR3 variant 6	VLWYSNRWI
86	LC CDR3 variant 7	VLWYSNRWA
87	Anti-CD3, clone 2G5	EVQLVESGGGLVQPGGSLKLSAASGFTFNKYALNWVRQAPGK GLEWVARIRSKYNNYATEYADSVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSPISYWAYWGQGTSLTVSSGGGG SGGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVTSGN YPNWWVQKPGQAPRGLIGGTNFLAPGTPERFSGSLLGGKAALTLS GVQPEDEAEYYCVLWYSNRWAFGGGTKLTVL
88	Anti-CD3, clone 8A5	EVQLVESGGGLVQPGGSLKLSAASGFTFNEYAMNWVRQAPGK GLEWVARIRSKYNNYATYYADDVKDRFTISRDDSKNTAYLQMN NLKTEDTAVYYCVRHGNFGNSGISYWAYWGQGTSLTVSSGGGG SGGGGSGGGGSQTVVTQEPSLTVSPGGTVTLTCGSSTGAVTVGN YPNWWVQKPGQAPRGLIGGTEFLAPGTPARFSGSLLGGKAALTLS GVQPEDEAEYYCVLWYSNRWVFGGGTKLTVL

Table 8: HSA Binding Domain Sequences

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
89	Anti-HSA sdAb clone 6C	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSRFGMSWVRQAPGKGL EWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDT AVYYCTIGGSLSRSSQGTLVTVSS
90	Anti-HSA sdAb clone 7A	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSKFGMSWVRQAPGKG LEWVSSISGSGADTLYADSLKGRFTISRDN AKTTLYLQMNSLRPED TAVYYCTIGGSLSKSSQGTLVTVSS
91	Anti-HSA sdAb clone 7G	EVQLVESGGGLVQPGNSLRRLSCAASGFTYSSFGMSWVRQAPGKG LEWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TAVYYCTIGGSLSKSSQGTLVTVSS
92	Anti-HSA sdAb clone 8H	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSKFGMSWVRQAPGKG LEWVSSISGSGTDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TAVYYCTIGGSLSRSSQGTLVTVSS
93	Anti-HSA sdAb clone 9A	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSRFGMSWVRQAPGKGL EWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDT AVYYCTIGGSLSKSSQGTLVTVSS
94	Anti-HSA sdAb clone 10G	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSKFGMSWVRQAPGKG LEWVSSISGSGRDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPED TAVYYCTIGGSLSVSSQGTLVTVSS
95	wt anti-HSA	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSFGMSWVRQAPGKGL EWVSSISGSGSDTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDT AVYYCTIGGSLSRSSQGTLVTVSS
96	wt anti-HSA CDR1	GFTFSFGMS
97	wt anti-HSA CDR2	SISGSGSDTLYADSVK
98	wt anti-HSACDR3	GGSLSR
99	CDR1 variant 1	GFTFSRFGMS
100	CDR1 variant 2	GFTFSKFGMS
101	CDR1 variant 3	GFTYSSFGMS
102	CDR2 variant 1	SISGSGADTLYADSLK
103	CDR2 variant 2	SISGSGTDTLYADSVK
104	CDR2 variant 3	SISGSGRDTLYADSVK
105	CDR2 variant 4	SISGSGSDTLYAESVK
106	CDR2 variant 5	SISGSGTDTLYAESVK
107	CDR2 variant 6	SISGSGRDTLYAESVK
108	CDR3 variant 1	GGSLSK
109	CDR3 variant 2	GGSLSV
110	Anti-HSA sdAb clone 6CE	EVQLVESGGGLVQPGNSLRRLSCAASGFTFSRFGMSWVRQAPGKGL EWVSSISGSGSDTLYAESVKGRFTISRDN AKTTLYLQMNSLRPEDT

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
		AVYYCTIGGSLRSSQGTLVTVSS
111	Anti-HSA sdAb clone 8HE	EVQLVESGGGLVQPGNSLRSLCAASGFTFSKFGMSWVRQAPGKG LEWVSSISGSGTDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPED TAVYYCTIGGSLRSSQGTLVTVSS
112	Anti-HSA sdAb clone 10GE	EVQLVESGGGLVQPGNSLRSLCAASGFTFSKFGMSWVRQAPGKG LEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPED TAVYYCTIGGSLSVSSQGTLVTVSS

Table 9: PSMA Binding Domain Sequences

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
113	wt anti-PSMA	EVQLVESGGGLVQPGGSLTLSCAASRFMISEYSMHVVRQAPGKG LEWVSTINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLKPED TAVYYCDGYGYRGQGTQVTVSS
114	CDR1 variant 1	RFMISEYHMH
115	CDR1 variant 2	RFMISPYSMH
116	CDR1 variant 3	RFMISPYHMH
117	CDR2 variant 1	DINPAGTTDYAESVKG
118	CDR2 variant 2	TINPAKTTDYAESVKG
119	CDR2 variant 3	TINPAGQTDYAESVKG
120	CDR2 variant 4	TINPAGTTDYAEYVKG
121	CDR2 variant 5	DINPAKTTDYAESVKG
122	CDR2 variant 6	DINPAGQTDYAESVKG
123	CDR2 variant 7	DINPAGTTDYAEYVKG
124	CDR3 variant 1	DSYGY
125	CDR1 variant 4	RFMISEYSMH
126	CDR2 variant 8	TINPAGTTDYAESVKG
127	CDR3 variant 2	DGYGY
128	Anti-PSMA clone 1	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYSMHVVRQAPGKG LEWVSTINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLRAED TAVYYCDGYGYRGQGTQVTVSS
129	Anti-PSMA clone 2	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYHMHVVRQAPGKG LEWVSDINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLRAED TAVYYCDSYGYRGQGTQVTVSS
130	Anti-PSMA clone 3	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYHMHVVRQAPGKG LEWVSTINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLRAED TAVYYCDSYGYRGQGTQVTVSS

<u>SEQ ID NO:</u>	<u>Description</u>	<u>AA Sequence</u>
131	Anti-PSMA clone 4	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYSMHWVRQAPGKG LEWVSTINPAKTTDYAESVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDSYGYRGQGT LVT VSS
132	Anti-PSMA clone 5	EVQLVESGGGLVQPGGSLRLSCAASRFMISPYSMHWVRQAPGKG LEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDGYGYRGQGT LVT VSS
133	Anti-PSMA clone 6	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYSMHWVRQAPGKG LEWVSTINPAGQTDYAESVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDGYGYRGQGT LVT VSS
134	Anti-PSMA clone 7	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYSMHWVRQAPGKG LEWVSTINPAGTTDYAEYVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDGYGYRGQGT LVT VSS
135	Anti-PSMA clone 8	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYHMHWRQAPGKG LEWVSDINPAKTTDYAESVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDSYGYRGQGT LVT VSS
136	Anti-PSMA clone 9	EVQLVESGGGLVQPGGSLRLSCAASRFMISPYHMHWRQAPGKG LEWVSDINPAGTTDYAESVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDSYGYRGQGT LVT VSS
137	Anti-PSMA clone 10	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYHMHWRQAPGKG LEWVSDINPAGQTDYAESVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDSYGYRGQGT LVT VSS
138	Anti-PSMA clone 11	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYHMHWRQAPGKG LEWVSDINPAGTTDYAEYVKGRFTISRDNKNTLYLQMNSLRAED TAVYYCDSYGYRGQGT LVT VSS
139	Anti-PSMA clone 12	EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWRQAPGKG LEWVSDINPAGTTDYAESVKGRFTISRDNKNTLYLQMNSLKPED TAVYYCDSYGYRGQGTQVT VSS
140	Anti-PSMA clone 13	EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWRQAPGKG LEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQMNSLKPED TAVYYCDSYGYRGQGTQVT VSS

Table 10: PSMA Targeting Trispecific Protein Sequences

SEQ ID NO:	C-Number	Construct	Sequence
141	C00324	PSMA TriTAC CD3 high aff.	EVQLVESGGGLVQPGGSLTSCAASRFMISEYSMHWVRQAP GKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQM NSLKPEDTAVYYCDGYGYRGQGTQVTVSSGGGSGGGSEV QLVESGGGLVQPGNSLRSLSCAASGFTFSKFGMSWVRQAPG KGLEWVSSISGSGRDTLYADSVKGRFTISRDNKNTLYLQM NSLRPEDTAVYYCTIGGSLSVSSQGTTLTVSSGGGSGGGSE VQLVESGGGLVQPGGSLKLSAASGFTFNKYAINWVRQAP GKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGT LTVSSGGGSGGGGSGGGGSQT VVTQEPSLTVSPGGTVTLT CASSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLVPGTPA RFGSLLGGKAAALTLSGVQPEDEAEYYCTLWYSNRWVFGG GTKLTVLHHHHHH
142	C00339	PSMA TriTAC CD3 med. aff.	EVQLVESGGGLVQPGGSLTSCAASRFMISEYSMHWVRQAP GKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQM NSLKPEDTAVYYCDGYGYRGQGTQVTVSSGGGSGGGSEV QLVESGGGLVQPGNSLRSLSCAASGFTFSKFGMSWVRQAPG KGLEWVSSISGSGRDTLYADSVKGRFTISRDNKNTLYLQM NSLRPEDTAVYYCTIGGSLSVSSQGTTLTVSSGGGSGGGSE VQLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAP GKGLEWVARIRSGYNNYATYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGT LTVSSGGGSGGGGSGGGGSQT VVTQEPSLTVSPGGTVTLT CGSYTGAVTSGNYPNWVQKPGQAPRGLIGGTKFNAPGTP ARFGSLLGGKAAALTLSGVQPEDEAEYYCVLWYANRWVFG GGTKLTVLHHHHHH
143	C00325	PSMA TriTAC CD3 low aff.	EVQLVESGGGLVQPGGSLTSCAASRFMISEYSMHWVRQAP GKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQM NSLKPEDTAVYYCDGYGYRGQGTQVTVSSGGGSGGGSEV QLVESGGGLVQPGNSLRSLSCAASGFTFSKFGMSWVRQAPG KGLEWVSSISGSGRDTLYADSVKGRFTISRDNKNTLYLQM NSLRPEDTAVYYCTIGGSLSVSSQGTTLTVSSGGGSGGGSE VQLVESGGGLVQPGGSLKLSAASGFENKYAMNWVRQAP GKGLEWVARIRSKYNNYETYYADSVKDRFTISRDDSKNTA YLQMNNLKTEDTAVYYCVRHGNFGNSLISYWAYWGQGT LTVSSGGGSGGGGSGGGGSQT VVTQEPSLTVSPGGTVTLT CGSSSGAVTSGNYPNWVQKPGQAPRGLIGGTKFGAPGTPA RFGSLLGGKAAALTLSGVQPEDEAEYYCVLWYSNRWVFGG GTKLTVLHHHHHH
144	C00236	Tool PSMA TriTAC	EVQLVESGGGLVQPGGSLTSCAASRFMISEYSMHWVRQAP GKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQM NSLKPEDTAVYYCDGYGYRGQGTQVTVSSGGGSGGGSEV QLVESGGGLVQPGNSLRSLSCAASGFTFSFGMSWVRQAPGK GLEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMN SLRPEDTAVYYCTIGGSLSRSSQGTTLTVSSGGGSGGGSEV QLVESGGGLVQPGGSLKLSAASGFTFNKYAMNWVRQAPG KGLEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAY LQMNNLKTEDTAVYYCVRHGNFGNSYISYWAYWGQGT LTVSSGGGSGGGGSGGGGSQT VVTQEPSLTVSPGGTVTLT C GSSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLAPGTPAR FSGSLLGGKAAALTLSGVQPEDEAEYYCVLWYSNRWVFGG TKLTVLHHHHHH

SEQ ID NO:	C-Number	Construct	Sequence
145	C00362	PSMA p8 TriTAC	EVQLVESGGGLVQPGGSLRLSCAASRFMISEYSMHVVRQA PGKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQ MNSLRAEDTAVYYCDGYGYRGQGTQVTVSSGGGGSGGG EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAP GKGLEWVSSISGSGRDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTAVYYCTIGGSLSVSSQGTQVTVSSGGGGSGGG SEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWVRQA PGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNT AYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGT LVTVSSGGGGSGGGSGGGGSQTVVTQEPSLTVSPGGTVTL TCASSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLVPGETP ARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFG GGTKLTVLHHHHHH
146	C00363	PSMA HDS TriTAC C363	EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHVVRQA PGKGLEWVSDINPAGTTDYAESVKGRFTISRDNKNTLYLQ MNSLKPEDTAVYYCDSYGYRGQGTQVTVSSGGGGSGGGSE VQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAP GKGLEWVSSISGSGRDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTAVYYCTIGGSLSVSSQGTQVTVSSGGGGSGGG SEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWVRQA PGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNT AYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGT LVTVSSGGGGSGGGSGGGGSQTVVTQEPSLTVSPGGTVTL TCASSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLVPGETP ARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFG GGTKLTVLHHHHHH
147	C00364	PSMA HTS TriTAC C364	EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHVVRQA PGKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQ MNSLKPEDTAVYYCDSYGYRGQGTQVTVSSGGGGSGGGSE VQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAP GKGLEWVSSISGSGRDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTAVYYCTIGGSLSVSSQGTQVTVSSGGGGSGGG SEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWVRQA PGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNT AYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGT LVTVSSGGGGSGGGSGGGGSQTVVTQEPSLTVSPGGTVTL TCASSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLVPGETP ARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFG GGTKLTVLHHHHHH
148	C00298	PSMA BiTE	QVQLVESGGGLVKPGESLRLSCAASGFTFSDYYMYWVRQA PGKGLEWVAISDGGYYTYYSDIKGRFTISRDNKNTLYLQ MNSLKAEDTAVYYCARGFPLLRHGAMDYWGQGTQVTVSS GGGGSGGGSGGGSDIQMTQSPSSLSASVGDRTITCKAS QNVDNTNVAWYQKPGQAPKSLIYSASYRSDVPSRFGSAS GTDFTLTISVQSEDFATYYCQYDSYPYTFGGGTKLEIKSG GGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNW VRQAPGKGLEWVARIRSKYNNYATYYADSVKDRFTISRDD SKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSYISYWAYW GQGTQVTVSSGGGGSGGGSGGGGSQTVVTQEPSLTVSPGG TVTLTCGSSTGAVTSGNYPNWVQKPGQAPRGLIGGTKFLA PGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNR WVFGGGTKLTVLHHHHHH

SEQ ID NO:	C-Number	Construct	Sequence
149	C00131	EGFR TrITAC	QVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMGWFRQA PGKEREFSVSGISWRGDSTGYADSVKGRFTISRDNKNTVDL QMNSLKPEDTAIYYCAAAAGSAWYGTLYEYDYWGQGTQV TVSSGGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFT FSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFT ISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGT TVSSGGGGSGGGSEVQLVESGGGLVQPGGSLKLSCAASGFT FNKYAMNWVRQAPGKGLEWVARIRSKYNNYATYYADSVK DRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNS YISYWAYWGQGTTLTVTVSSGGGGSGGGGSGGGGSQT VVTQEPSTVSPGGTVTLTCSSTGAVTSGNYPNWVQKPGQAPR GLIGGTFKFLAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYY CVLWYSNRWVFGGGTKLTVLHHHHHH
150	C00457	PSMA PH1 TrITAC	QVQLVESGGGVVQAGRSLTLSCAYSGVTNVYRMGWFRQ APGKEREFSVANINWSGNNRDYADSVRGRFTISRDNKNTLY LQMNSLRAEDTAVYYCASEKPGRLGEYDYGSQGTTLTVSS GGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKF GMSWVRQAPGKGLEWVSSISGSGRDTLYADSVKGRFTISR DNKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGT LTVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNK YAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVKDRF TISRDDSKNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISY WAYWGQGTTLTVTVSSGGGGSGGGGSGGGGSQT VVTQEPSTVSPGGTVTLTCSSTGAVTSGNYPNWVQKPGQAPRGLIG GTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCTL WYSNRWVFGGGTKLTVLHHHHHH
151	C00404	PSMA PH1 TrITAC	QVQLVESGGGVVQAGRSLRLSCAYSGVTNVYRMGWFRQ APGKEREFSVANINWSGNNRDYADSVRGRFTISRDNKNTLY LQMNSLRAEDTAVYYCASEKPGRLGEYDYGSQGTTLTVSS GGGGSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKF GMSWVRQAPGKGLEWVSSISGSGRDTLYADSVKGRFTISR DNKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGT LTVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNK YAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVKDRF TISRDDSKNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISY WAYWGQGTTLTVTVSSGGGGSGGGGSGGGGSQT VVTQEPSTVSPGGTVTLTCSSTGAVTSGNYPNWVQKPGQAPRGLIG GTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCTL WYSNRWVFGGGTKLTVLHHHHHH
152	C00410	PSMA Z2 TrITAC	EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHVVRQA PGKGLEWVSTINPAGTTDYAESVKGRFTISRDNKNTLYLQ MNSLRAEDTAVYYCDSYGYRGQGTTLTVTVSSGGGGSGGGSE VQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAP GKGLEWVSSISGSGRDTLYADSVKGRFTISRDNKTTLYLQ MNSLRPEDTAVYYCTIGGSLSVSSQGT LTVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWVRQA PGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNT AYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGT LTVTVSSGGGGSGGGGSGGGGSQT VVTQEPSTVSPGGTVTLTCSSTGAVTSGNYPNWVQKPGQAPRGLIGGTFKFLVPGTP ARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFG GGTKLTVLHHHHHH

Table 11: PSMA Binding Domain CDR sequences

<u>SEQ ID Nos.</u>	<u>Sequence</u>
SEQ ID No. 162	RFMISX ₁ YX ₂ MH
SEQ ID No. 163	X ₃ INPAX ₄ X ₅ TDYAEX ₆ VKG
SEQ ID No. 164	DX ₇ YGY

Table 12: Exemplary Framework Sequences

SEQ ID NO:	Description	Sequence
165	Framework (f1)	EVQLVESGGGLVQPGGSLTLSCAAS
166	Framework (f2)	WVRQAPGKGLEWVS
167	Framework (f3)	RFTISRDNAKNTLYLQMNSLRAEDTAVYYC
168	Framework (f4)	DGYGYRGQGLTVTVSS

[00134] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A prostate specific membrane antigen (PSMA) targeting trispecific protein, wherein said protein comprises
 - (a) a first domain (A) which specifically binds to human CD3;
 - (b) a second domain (B) which is a half-life extension domain; and
 - (c) a third domain (C) which specifically binds to PSMA,wherein the domains are linked in the order H₂N-(A)-(C)-(B)-COOH, H₂N-(B)-(A)-(C)-COOH, H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2.
2. The PSMA targeting trispecific protein of claim 1, wherein the first domain comprises a variable light chain and variable heavy chain each of which is capable of specifically binding to human CD3.
3. The PSMA targeting trispecific protein of claim 1, wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.
4. The PSMA targeting trispecific protein of claim 1, wherein the first domain is humanized or human.
5. The PSMA targeting trispecific protein of claim 1, wherein the first domain has a K_D binding of 150 nM or less to CD3 on CD3 expressing cells.
6. The PSMA targeting trispecific protein of claim 1, wherein the second domain binds human serum albumin.
7. The PSMA targeting trispecific protein of claim 1, wherein the second domain comprises a scFv, a variable heavy domain (VH), a variable light domain (VL), a peptide, a ligand, or a small molecule.
8. The PSMA targeting trispecific protein of claim 1, wherein the second domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 89-112.
9. The PSMA targeting trispecific protein of claim 1, wherein the third domain comprises a scFv, a VH domain, a VL domain, a non-Ig domain, a ligand, a knottin, or a small molecule entity that specifically binds to PSMA.
10. The PSMA targeting trispecific protein of claim 1, wherein the third domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 113-140.
11. The PSMA targeting trispecific protein of claim 1, wherein linkers L1 and L2 are each independently selected from (GS)_n (SEQ ID NO: 153), (GGS)_n (SEQ ID NO: 154), (GGGS)_n (SEQ ID NO: 155), (GGSG)_n (SEQ ID NO: 156), (GGSGG)_n (SEQ ID NO: 157), or (GGGGS)_n (SEQ ID NO: 158), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

12. The PSMA targeting trispecific protein of claim 1, wherein linkers L1 and L2 are each independently (GGGGS)₄ (SEQ ID NO: 159) or (GGGGS)₃ (SEQ ID NO: 160).
13. The PSMA targeting trispecific protein of claim 1, wherein the domains are linked in the order H₂N-(A)-(C)-(B)-COOH.
14. The PSMA targeting trispecific protein of claim 1, wherein the domains are linked in the order H₂N-(B)-(C)-(A)-COOH.
15. The PSMA targeting trispecific protein of claim 1, wherein the protein is less than about 80 kDa.
16. The PSMA targeting trispecific protein of claim 1, wherein the protein is about 50 to about 75 kDa.
17. The PSMA targeting trispecific protein of claim 1, wherein the protein is less than about 60 kDa.
18. The PSMA targeting trispecific protein of claim 1, wherein the protein has an elimination half-time of at least about 50 hours.
19. The PSMA targeting trispecific protein of claim 1, wherein the protein has an elimination half-time of at least about 100 hours.
20. The PSMA targeting trispecific protein of claim 1, wherein the protein has increased tissue penetration as compared to an IgG to the same PSMA.
21. The PSMA targeting trispecific protein of claim 1, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO: 140-152.
22. A pharmaceutical composition comprising (i) the PSMA targeting trispecific protein according to any one of claims 1 to 21 and (ii) a pharmaceutically acceptable carrier.
23. A method of treating an individual in need of treatment of cancer, the method comprising administration of an effective amount of the pharmaceutical composition of claim 22.
24. The method of claim 23, wherein the cancer is prostate cancer or renal cancer.
25. A PSMA targeting trispecific protein, wherein said protein comprises
 - (a) a first domain (A) which specifically binds to human CD3;
 - (b) a second domain (B) which is a half-life extension domain; and
 - (c) a third domain (C) which specifically binds to PSMA,wherein the second domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 113-140.
26. The PSMA targeting trispecific protein of claim 25, wherein the domains are linked in the order H₂N-(A)-(C)-(B)-COOH, H₂N-(B)-(A)-(C)-COOH, H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2.

27. The PSMA targeting trispecific protein of claim 25 or 26, wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.
28. The PSMA targeting trispecific protein of claim 25, 26, or 27, wherein the second domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 89-112.
29. A PSMA targeting trispecific protein, wherein said protein comprises a sequence selected from the group consisting of SEQ ID NO: 140-152.
30. The PSMA targeting trispecific protein of claim 29, wherein said protein comprises a sequence selected from the group consisting of SEQ ID NO: 150-152.
31. A prostate specific membrane antigen (PSMA) targeting trispecific protein, wherein said protein comprises
- (a) a first domain (A) which specifically binds to human CD3;
 - (b) a second domain (B) which is a half-life extension domain; and
 - (c) a third domain (C) which specifically binds to PSMA,
- wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2, and wherein the third domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 113-140.
32. The PSMA targeting trispecific protein of claim 31, wherein the first domain comprises a variable light chain and variable heavy chain each of which is capable of specifically binding to human CD3.
33. The PSMA targeting trispecific protein of claim 31, wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.
34. The PSMA targeting trispecific protein of claim 31, wherein the first domain is humanized or human.
35. The PSMA targeting trispecific protein of claim 31, wherein the first domain has a K_D binding of 150 nM or less to CD3 on CD3 expressing cells.
36. The PSMA targeting trispecific protein of claim 31, wherein the second domain binds human serum albumin.
37. The PSMA targeting trispecific protein of claim 31, wherein the second domain comprises a scFv, a variable heavy domain (VH), a variable light domain (VL), a peptide, a ligand, or a small molecule.
38. The PSMA targeting trispecific protein of claim 31, wherein the second domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 89-112.

39. The PSMA targeting trispecific protein of claim 31, wherein the third domain comprises a scFv, a VH domain, a VL domain, a non-Ig domain, a ligand, a knottin, or a small molecule entity that specifically binds to PSMA.
40. The PSMA targeting trispecific protein of claim 31, wherein linkers L1 and L2 are each independently selected from (GS)_n (SEQ ID NO: 153), (GGS)_n (SEQ ID NO: 154), (GGGS)_n (SEQ ID NO: 155), (GGSG)_n (SEQ ID NO: 156), (GGSGG)_n (SEQ ID NO: 157), or (GGGGS)_n (SEQ ID NO: 158), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.
41. The PSMA targeting trispecific protein of claim 31, wherein linkers L1 and L2 are each independently (GGGGS)₄ (SEQ ID NO: 159) or (GGGGS)₃ (SEQ ID NO: 160).
42. The PSMA targeting trispecific protein of claim 31, wherein the domains are linked in the order H₂N-(C)-L1-(B)-L2-(A)-COOH.
43. The PSMA targeting trispecific protein of claim 31, wherein the protein is less than about 80 kDa.
44. The PSMA targeting trispecific protein of claim 31, wherein the protein is about 50 to about 75 kDa.
45. The PSMA targeting trispecific protein of claim 31, wherein the protein is less than about 60 kDa.
46. The PSMA targeting trispecific protein of claim 31, wherein the protein has an elimination half-time of at least about 50 hours.
47. The PSMA targeting trispecific protein of claim 31, wherein the protein has an elimination half-time of at least about 100 hours.
48. The PSMA targeting trispecific protein of claim 31, wherein the protein has increased tissue penetration as compared to an IgG to the same PSMA.
49. The PSMA targeting trispecific protein of claim 31, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO: 140-152.
50. The PSMA targeting trispecific protein, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO: 150-152.
51. A pharmaceutical composition comprising (i) the PSMA targeting trispecific protein according to claim 31, and (ii) a pharmaceutically acceptable carrier.
52. A PSMA targeting trispecific protein, wherein said protein comprises
- (a) a first domain (A) which specifically binds to human CD3;
 - (b) a second domain (B) which is a half-life extension domain; and
 - (c) a third domain (C) which specifically binds to PSMA,
- wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2,

and wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.

53. The PSMA targeting trispecific protein of claim 52, wherein the first domain comprises a variable light chain and variable heavy chain each of which is capable of specifically binding to human CD3.

54. The PSMA targeting trispecific protein of claim 52, wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.

55. The PSMA targeting trispecific protein of claim 52, wherein the first domain is humanized or human.

56. The PSMA targeting trispecific protein of claim 52, wherein the first domain has a K_D binding of 150 nM or less to CD3 on CD3 expressing cells.

57. The PSMA targeting trispecific protein of claim 52, wherein the second domain binds human serum albumin.

58. The PSMA targeting trispecific protein of claim 52, wherein the second domain comprises a scFv, a variable heavy domain (VH), a variable light domain (VL), a peptide, a ligand, or a small molecule.

59. The PSMA targeting trispecific protein of claim 52, wherein the second domain comprises one or more sequences selected from the group consisting of SEQ ID NOs: 89-112.

60. The PSMA targeting trispecific protein of claim 52, wherein the third domain comprises a scFv, a VH domain, a VL domain, a non-Ig domain, a ligand, a knottin, or a small molecule entity that specifically binds to PSMA.

61. The PSMA targeting trispecific protein of claim 52, wherein linkers L1 and L2 are each independently selected from $(GS)_n$ (SEQ ID NO: 153), $(GGS)_n$ (SEQ ID NO: 154), $(GGGS)_n$ (SEQ ID NO: 155), $(GGSG)_n$ (SEQ ID NO: 156), $(GGSGG)_n$ (SEQ ID NO: 157), or $(GGGGS)_n$ (SEQ ID NO: 158), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

62. The PSMA targeting trispecific protein of claim 52, wherein linkers L1 and L2 are each independently $(GGGGS)_4$ (SEQ ID NO: 159) or $(GGGGS)_3$ (SEQ ID NO: 160).

63. The PSMA targeting trispecific protein of claim 31, wherein the domains are linked in the order $H_2N-(C)-L1-(B)-L2-(A)-COOH$.

64. The PSMA targeting trispecific protein of claim 52, wherein the protein is less than about 80 kDa.

65. The PSMA targeting trispecific protein of claim 52, wherein the protein is about 50 to about 75 kDa.

66. The PSMA targeting trispecific protein of claim 52, wherein the protein is less than about 60 kDa.

67. The PSMA targeting trispecific protein of claim 52, wherein the protein has an elimination half-time of at least about 50 hours.
68. The PSMA targeting trispecific protein of claim 52, wherein the protein has an elimination half-time of at least about 100 hours.
69. The PSMA targeting trispecific protein of claim 52, wherein the protein has increased tissue penetration as compared to an IgG to the same PSMA.
70. The PSMA targeting trispecific protein of claim 52, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO: 140-152.
71. The PSMA targeting trispecific protein of claim 52, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO: 150-152.
72. A pharmaceutical composition comprising (i) the PSMA targeting trispecific protein according to claim 52, and (ii) a pharmaceutically acceptable carrier.
73. A method of treating prostate cancer, the method comprising administration of an effective amount of a PSMA targeting trispecific protein, wherein said protein comprises
(a) a first domain (A) which specifically binds to human CD3;
(b) a second domain (B) which is a half-life extension domain; and
(c) a third domain (C) which specifically binds to PSMA,
wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2,
and wherein the third domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 113-140.
74. A method of treating prostate cancer, the method comprising administration of an effective amount of a PSMA targeting trispecific protein, wherein said protein comprises
(a) a first domain (A) which specifically binds to human CD3;
(b) a second domain (B) which is a half-life extension domain; and
(c) a third domain (C) which specifically binds to PSMA,
wherein the domains are linked in the order H₂N-(C)-(B)-(A)-COOH, or by linkers L1 and L2,
and wherein the first domain comprises one or more sequences selected from the group consisting of SEQ ID NO: 1-88.