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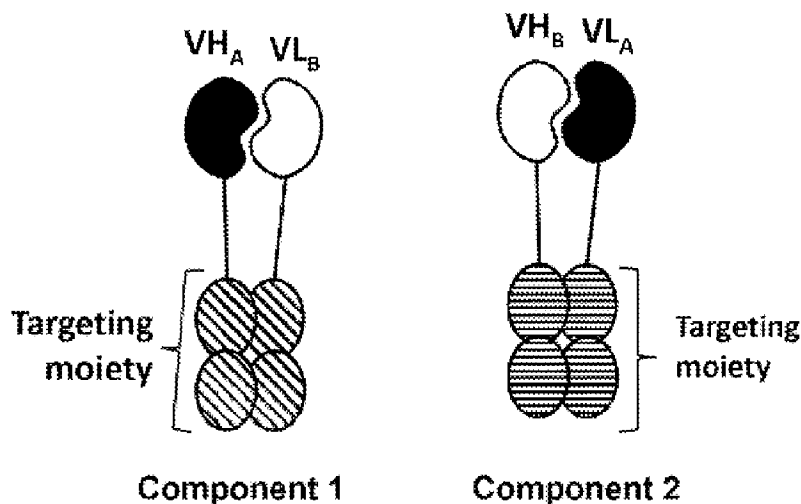


Fig. 1A

(57) **Abrégé/Abstract:**

A Twin Immune Cell Engager (TWICE) is a kit or composition for treating cancer comprising a first component and second component. Each component comprises a targeting moiety that binds a tumor antigen expressed by the cancer or an antigen expressed by a non-cancer cell in the tumor microenvironment. In some embodiments, the first and second components each comprise an immune cell binding domain capable of immune cell binding activity when binding the immune cell binding domain in the other component, and a complementary binding domain capable of binding to a complementary antigen when binding the complementary binding domain in the other component. In some embodiments, the first and/or second components comprise a complementary functional domain with activity when targeted to the cancer cell or its microenvironment. The present TWICE complexes offer a unique ability to combine multiple functions into a two-component complex that becomes activated in the tumor microenvironment. The present complexes, thus, provide meaningful advantages in having a single approach to administering a two-component complex that is localized to the tumor microenvironment and has the ability to effect two different signals to benefit patients. This unique construct offers benefits that were not present in prior art constructs.

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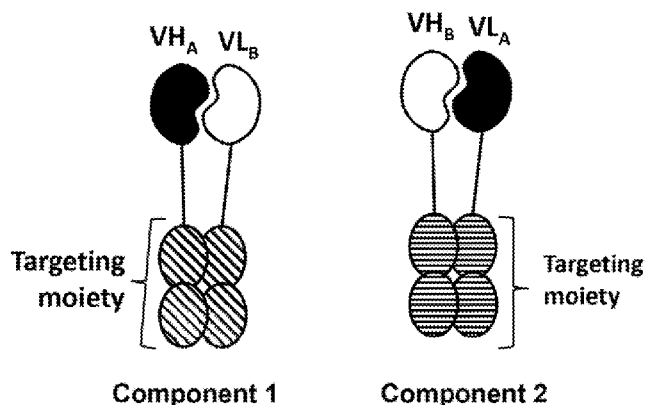


Fig. 1A

(57) Abstract: A Twin Immune Cell Engager (TWICE) is a kit or composition for treating cancer comprising a first component and second component. Each component comprises a targeting moiety that binds a tumor antigen expressed by the cancer or an antigen expressed by a non-cancer cell in the tumor microenvironment. In some embodiments, the first and second components each comprise an immune cell binding domain capable of immune cell binding activity when binding the immune cell binding domain in the other component, and a complementary binding domain capable of binding to a complementary antigen when binding the complementary binding domain in the other component. In some embodiments, the first and/or second components comprise a complementary functional domain with activity when targeted to the cancer cell or its microenvironment. The present TWICE complexes offer a unique ability to combine multiple functions into a two-component complex that becomes activated in the tumor microenvironment. The present complexes, thus, provide meaningful advantages in having a single approach to administering a two-component complex that is localized to the tumor microenvironment and has the ability to effect two different signals to benefit patients. This unique construct offers benefits that were not present in prior art constructs.

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TWIN IMMUNE CELL ENGAGER

DESCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of US Provisional Application No. 62/780,770, filed December 17, 2018, which is incorporated by reference herein in its entirety for any purpose.

SEQUENCE LISTING

[002] The present application is filed with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled “2019-12-12_01131-0023-00PCT_ST25.txt” created on December 12, 2019, which is 212,992 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD

[003] This application relates to improved targeted immune cell engaging antibody complexes for treating a condition characterized by the presence of cancer cells. In particular, it relates to agents that can be used to treat a condition characterized by the presence of cancer cells.

BACKGROUND

[004] Cancer creates significant loss of life, suffering, and economic impact. Immunotherapeutic strategies for targeting cancer have been an active area of translational clinical research.

[005] Various immunooncology approaches have been developed to address cancer and other diseases caused by the presence of unwanted cells. In one context, targeted T-cell engaging antibody complexes have shown promising data. *See* US 10,035,856. These masked compositions comprise a first component and a second component that both bind the unwanted cell and that each become available to bind to the other after the release of an inert binding partner from each component in the tumor microenvironment. After the first component and second component bind to each other, they form an active T-cell binding domain.

[006] Other developments have shown that other immune interventions (in addition to activating T cells) have benefitted patients with cancer and other diseases.

For example, PD-L1 pathway inhibition can block immune evasion and reverse T-cell exhaustion of some tumors (*see* Chen DS et al. *Clin Cancer Res.* 18:6580-6587 (2012)). Simultaneous activation of T cells and binding of tumor-associated antigens, such as checkpoint molecules, has been proposed as a more robust and selective approach to activate T cells against cancer cells (*see* Harris et. al *Cancer Biol Med* 13(2):171-193 (2016) and Kobold et al. *Front Oncol* 8:285 (2018)). No single therapeutic approach, however, has encompassed all of these features in a way that the various functions only become active at the desired site of treatment.

[007] Thus, depending on the disease and patient, further immunooncology or other biological interventions could also benefit the patient. Some prior constructs had the ability to activate T cells in the tumor microenvironment but did not have any other attributes. Specifically, the prior constructs that restricted activity to the desired site of treatment did not have any way to incorporate additional functional domains. Therefore, additional developments in this field of re-directed immunotherapy are required.

SUMMARY

[008] The present TWICE complexes offer a unique ability to combine multiple functions into a two-component complex that becomes activated in the tumor microenvironment. The present complexes, thus, provide meaningful advantages in having a single approach to administering a two-component complex that is localized to the tumor microenvironment and has the ability to effect two different signals to benefit patients. This unique construct offers benefits that were not present in prior art constructs.

[009] In accordance with the description, this application describes a kit or composition for treating cancer in a patient comprising a first component comprising a targeted immune cell binding agent comprising a first targeting moiety that binds a tumor antigen expressed by the cancer; a first immune cell binding domain capable of immune cell binding activity when binding a second immune cell binding domain, wherein the second immune cell binding domain is not part of the first component, and wherein the first immune cell binding domain is either a VH domain or VL domain; and a first complementary binding domain capable of binding to a complementary antigen when binding a second complementary binding domain, wherein the second complementary binding domain is not part of the first component,

when the first immune cell binding domain is a VH domain the first complementary binding domain is a VL domain, when the first immune cell binding domain is a VL domain, the first complementary binding domain is a VH domain, and wherein the first complementary binding domain is a binding partner for the first immune cell binding domain, such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the first immune cell binding domain is not bound to the first complementary domain; and a second component comprising a targeted immune cell binding agent comprising a second targeting moiety; a second immune cell binding domain capable of immune cell binding activity when binding a first immune cell binding domain, wherein the second immune cell binding domain is a VH if the first immune cell binding domain is a VL and wherein the second immune cell binding domain is a VL if the first immune cell binding domain is a VH; and a second complementary binding domain capable of binding to a complementary antigen when binding the first complementary binding domain, wherein when the second immune cell binding domain is a VH domain the second complementary binding domain is a VL domain, when the second immune cell binding domain is a VL domain, the second complementary binding domain is a VH domain, and wherein the second complementary binding domain is a binding partner for the second immune cell binding domain, such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the second immune cell binding domain is not bound to the second complementary domain.

[0010] In some embodiments, the first immune cell binding domain is bound to the first complementary binding domain by a first dimerization domain and a second dimerization domain, wherein the first dimerization domain is attached to the first immune cell binding domain by a first linker; the second dimerization domain is attached to the first complementary binding domain by a second linker; and the first and/or second linker is a cleavable linker. In some embodiments, the first and second linkers are cleavable linkers.

[0011] In some embodiments, the second T-cell binding domain is bound to the second complementary binding domain by a first dimerization domain and a second dimerization domain, wherein the first dimerization domain is attached to the second T-cell binding domain by a first linker; the second dimerization domain is attached to the second complementary binding domain by a second linker; and the first and/or second linker is a cleavable linker.

[0012] In some embodiments, the first and second linkers are cleavable linkers. In some embodiments, the first and second linkers are the same. In some embodiments, the first and second linkers are different. In some embodiments, the first and second linkers are from 5 to 30 amino acids in length. In some embodiments, the first and second linkers are from 8 to 16 amino acids in length. In some embodiments, the protease cleavage sites of the first and/or second cleavable linkers are cleaved by a protease expressed by the cancer or tumor microenvironment cell. In some embodiments, the protease cleavage sites of the first and/or second cleavable linkers are cleaved by a protease that is colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds a tumor antigen expressed by the cancer and that is the same or different from the targeting moiety in at least one of the first components or the second component.

[0013] In some embodiments, the first and second dimerization domains are both leucine zippers; immunoglobulin domains; or T-cell receptor (TCR) domains. In some embodiments, the immunoglobulin domains comprise immunoglobulin variable domains or immunoglobulin constant domains. In some embodiments, the immunoglobulin constant domains comprise CH1/CL, CH2, CH3, or CH4. In some embodiments, the TCR domains comprise TCR constant domains. In some embodiments, the dimerization domains in the first component are the same as the dimerization domains in the second component. In some embodiments, the dimerization domains in the first component are different than the dimerization domains in the second component.

[0014] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding the cancer. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding an immune checkpoint molecule, RANK or RANKL, or a cell-death-inducing antigen.

[0015] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding an immune checkpoint molecule. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding PD-L1. In some embodiments, the first

and second complementary binding domains comprise all or part of a VH and/or VL of atezolizumab, durvalumab, or avelumab.

[0016] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding RANK. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding RANKL. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of denosumab.

[0017] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a cell-death-inducing antigen. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding Fas/CD95/Apo1, TNFR1/p55/CD120a, DR3/Apo3/WSL-1/TRAMP/LARD, TRAIL-R1/DR4, DR5/Apo2/TRAIL-R2/TRICK2/KILLER, DR6, or CAR1. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding TRAIL-R1/DR4. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of mapatumumab. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding DR5/Apo2/TRAIL-R2/TRICK2/KILLER. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of conatumumab (AMG655), lexatumumab, tigatuzumab (CS1008), or drozitumab (PRO95780).

[0018] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a molecule associated with the extracellular matrix.

[0019] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T cell, a macrophage, or a natural killer cell.

[0020] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T cell. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of

binding CD3, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, glucocorticoid induced TNF receptor (GITR or TNFRSF18), T cell immune receptor with Ig and ITIM domains (TIGIT), or inducible T-cell costimulatory (ICOS).

[0021] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding CD3. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of muromonab, orelizumab, teplizumab, visilizumab, foralumab, SP34, or blinatumomab.

[0022] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding PD-1. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of pembrolizumab or nivolumab.

[0023] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding CTLA-4. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of ipilimumab.

[0024] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding TIM-3. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of TSR-022 or Sym023.

[0025] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding LAG-3. In some embodiments, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of BMS-986016.

[0026] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding KIR. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of lirilumab.

[0027] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding CD28. In some embodiments,

the first and second complementary binding domains comprise all or part of a VH and/or VL of theralizumab.

[0028] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding CD137. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of utomilumab or urelumab.

[0029] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding OX40. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of PF-04518600 or BMS 986178.

[0030] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding CD27. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of varlilumab.

[0031] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding GITR (TNFRSF18). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of GWN323 or BMS-986156.

[0032] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding TIGIT. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of OMP-313M32, MTIG7192A, BMS-986207, or MK-7684.

[0033] In some embodiments, the first and second complementary binding domains are, when bound together, capable of binding ICOS. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of JTX-2011.

[0034] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a macrophage.

[0035] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CSF1R. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of emactuzumab or IMC-CS4.

[0036] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD40. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of CP-870,893.

[0037] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a natural killer cell.

[0038] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD16A. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of NTM-1633 or AFM13.

[0039] In some embodiments, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding the same antigen.

[0040] In some embodiments, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding different antigens on the same cell.

[0041] In some embodiments, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding different cells.

[0042] In some embodiments, the first and second immune cell binding domains are capable of forming a Fv when not bound to the first and second complementary binding domains. In some embodiments, the first and second complementary binding domains are capable of forming a Fv when not bound to the first and second immune cell binding domains.

[0043] This application also describes a kit or composition for treating cancer in a patient comprising a first component comprising a targeted immune cell binding agent comprising a first targeting moiety that binds a tumor antigen expressed by the cancer; a first immune cell binding domain capable of immune cell binding activity when binding a second immune cell binding domain, wherein the second immune cell binding domain is not part of the first component, and wherein the first immune cell

binding domain is either a VH domain or VL domain; a first inert binding partner for the first immune cell binding domain, wherein the first inert binding partner binds to the first immune cell binding domain such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the inert binding partner is removed, wherein if the first immune cell binding domain is a VH domain, the inert binding partner is a VL domain and if the first immune cell binding domain is a VL domain, the inert binding partner is a VH domain; a protease cleavage site separating the first immune cell binding domain and the first inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease expressed by the cancer or a tumor microenvironment cell; or colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment; and a first complementary functional domain capable of immune cell binding, and a second component comprising a targeted immune cell binding agent comprising a second targeting moiety; a second immune cell binding domain; and optionally a second complementary functional domain capable of immune cell binding.

[0044] In some embodiments, the second component comprises a complementary functional domain. In some embodiments, the complementary functional domain of the first and/or second component comprises a ligand for a receptor. In some embodiments, the complementary functional domain is a latent form of a member of the TGF-beta family. In some embodiments, the complementary functional domain of the first and/or second component comprises a cytokine. In some embodiments, the cytokine is IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[0045] In some embodiments, the complementary functional domain of the first and/or second component comprises an attenuated cytokine. In some embodiments, the attenuated cytokine is a variant of IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[0046] In some embodiments, the second component further comprises a second inert binding partner for the second immune cell binding domain, wherein the second inert binding partner binds to the second immune cell binding domain such

that the second immune cell binding domain does not bind to the first immune cell binding domain unless the inert binding partner is removed, wherein if the second immune cell binding domain is a VH domain, the inert binding partner is a VL domain and if the second immune cell binding domain is a VL domain, the inert binding partner is a VH domain; and further wherein a protease cleavage site separating the second immune cell binding domain and the second inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease expressed by the cancer; or colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment.

[0047] In some embodiments, the first component is not covalently bound to the second component. In some embodiments, the first component is covalently bound to the second component. In some embodiments, the first component is covalently bound to the second component via a cleavable linker.

[0048] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a T cell, a macrophage, or a natural killer cell.

[0049] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a T cell. In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD3, the T-cell receptor, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, GITR (TNFRSF18), TIGIT, or inducible T-cell costimulatory (ICOS).

[0050] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD3. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of muromonab, oteelixizumab, teplizumab, visilizumab, foralumab, SP34, or blinatumomab.

[0051] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding the T-cell receptor.

[0052] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding PD-1. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of pembrolizumab or nivolumab.

[0053] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding CTLA-4. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of ipilimumab.

[0054] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding TIM-3. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of TSR-022 or Sym023.

[0055] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding LAG-3. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of BMS-986016.

[0056] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding KIR. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of lirilumab.

[0057] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding CD28. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of theralizumab.

[0058] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding CD137. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of utomilumab or urelumab.

[0059] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding OX40. In some embodiments,

the first and second immune cell binding domains comprise all or part of a VH and/or VL of PF-04518600 or BMS 986178.

[0060] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding CD27. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of varlilumab.

[0061] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding GITR (TNFRSF18). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of GWN323 or BMS-986156.

[0062] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding TIGIT. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of OMP-313M32, MTIG7192A, BMS-986207, or MK-7684.

[0063] In some embodiments, the first and second immune cell binding domains are, when bound together, capable of binding ICOS. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of JTX-2011.

[0064] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a macrophage.

[0065] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CSF1R. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of emactuzumab or IMC-CS4.

[0066] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD40. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of CP-870,893.

[0067] In some embodiments, the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a natural killer cell. In some embodiments, the first immune cell binding domain and the second immune binding domain are, when bound to each other, capable of binding CD16A.

[0068] In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of NTM-1633 or AFM13. In some embodiments, the first and the second targeting moieties are different.

[0069] In some embodiments, the first and the second targeting moieties are the same.

[0070] In some embodiments, the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof. In some embodiments, the first and/or second targeting moiety comprises a DNA aptamer, RNA aptamer, albumin, lipocalin, fibronectin, ankyrin, fynomer, Obody, DARPIn, knotin, avimer, atrimer, anti-callin, affilin, affibody, bicyclic peptide, cys-knot, FN3 (adnectins, centryrins, pronectins, or TN3), or Kunitz domain.

[0071] In some embodiments, the second targeting moiety binds a tumor antigen expressed by the cancer. In some embodiments, the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof that binds α 4-integrin; A33; ACVRL 1/ALK1; ADAM17; ALK; APRIL; BCMA; C242; CA125; Cadherin-19; CAIX; CanAg; Carbonic Anhydrase IX; CCN1; CCR4; CD123; CD133; CD137 (4-1BB); CD138/Syndecan1; CD19; CD2; CD20; CD22; CD30; CD33; CD37; CD38; CD4; CD40; CD44; CD45; CD48; CD5; CD52; CD56; CD59; CD70; CD70b; CD71; CD74; CD79b; CD80; CD86; CD98; CEA; CEACAM; CEACAM1; CK8; c-Kit; Claudin-1 (CLDN1); CLDN18 (including CLDN18.2); CLDN6; c-met/HGFR; c-RET; Cripto; CTLA-4; CXCR4; DKK-1; DLL3; DLL4; TRAIL-R2/DR5; DRS; EGFL7; EGFR; EGFRvIII; endoglin; ENPP3; EpCAM; EphA2; Episialin; FAP; FGFR1; FGFR2; FGFR3; FGFR4; fibronectin extra-domain B; FLT-3; flt4; folate receptor 1; Guanylyl Cyclase C (GCC); GD2; GD3; Glypican-3; Glypicans; GM3; GPNMB; GPR49; GRP78; Her2/Neu; HER3/ERBB3; HLA-DR; ICAM-1; IGF-1R; IGFR; IL-3Ra; Integrin α 5 β 1; Integrin α 6 β 4; Integrin α V; Integrin α V β 3; Lewis Y; Lewis y/b antigen; LFL2; LIV-1; Ly6E; MCP-1; Mesothelin; MMP-9; MUC1; MUC18; MUC5A; MUC5AC; Myostatin; NaPi2b; Neuropilin 1; NGcGM3; NRP1; P-cadherin; PCLA; PD-1; PDGFRa; PD-L1; PD-L2; Phosphatidylserine; PIVKA-II; PLVAP; PRLR; Progastrin; PSCA; PSMA; RANKL; RG1; Siglec-15; SLAMF6; SLAMF7; SLC44A4; STEAP-1; TACSTD-2; Tenascin C; TPBG; TRAIL-R1/DR4; TROP-2; TWEAKR; TYRP1; VANGL2; VEGF; VEGF-C; VEGFR-2; or VEGF-R2.

[0072] In some embodiments, the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof is an anti α 4-integrin antibody; an anti-CD137 antibody; an anti-CCR4 antibody; an anti-CD123 antibody; an anti-CD133 antibody; an anti-CD138 antibody; an anti-CD19 antibody; an anti-CD20 antibody; an anti-CD22 antibody; an anti-CD33 antibody; an anti-CD38 antibody; an anti-CD40 antibody; an anti-CD49d antibody; an anti-CD52 antibody; an anti-CD70 antibody; an anti-CD74 antibody; an anti-CD79b antibody; an anti-CD80 antibody; an anti-CEA antibody; an anti-cMet antibody; an anti-Cripto antibody; an anti-CTLA-4 antibody; an anti-DLL3 antibody; an anti-TRAIL-2/DR5 antibody; an anti-E-cadherin antibody; an anti-endoglin antibody; an anti-EpCAM antibody; an anti-epidermal growth factor receptor antibody; an anti-FGFR3 antibody; an anti-fibronectin extra-domain B antibody; an anti-folate receptor 1 antibody; an anti-glypican 3 antibody; an anti-gp95/97 antibody; an anti-Her2 antibody; an anti-IGF-1R antibody; an anti-IL-13R antibody; an anti-IL-4 antibody; an anti-IL-6 antibody; an anti-MMP-9 antibody; an anti-MUC1 antibody; an anti-mucin core protein antibody; an anti-NGcGM3 antibody; an anti-P-cadherin antibody; an anti-PD-L1 antibody; an anti-p-glycoprotein antibody; an anti-PSCA antibody; an anti-PSMA antibody; an anti-SLAMF7 antibody; an anti-TRAIL-R1/DR4 antibody; an anti-transferrin antibody; an anti-TROP-2 antibody; or an anti-VEGF antibody.

[0073] In some embodiments, the first and/or second targeting moiety comprises Alemtuzumab, Andecaliximab, Atezolizumab, Avelumab, BCD-100, Bevacizumab, BGB-A317, Blinatumomab, Brentuximab, BU59, Camrelizumab, Carotuximab, Catumaxomab, Cemiplimab, Cetuximab, Daratumumab, Depatuxizumab, Dinutuximab, DS-8201, Durvalumab, Edrecolomab, Elotuzumab, G544, Gemtuzumab, Glembatumumab, GP1.4, hp67.6, IBI308, Ibritumomab, Inotuzumab, Ipilimumab, Isatuximab, L19IL2, L19TNF, Margetuximab, Mirvetuximab, Mogamulizumab, Moxetumomab, Natalizumab, Necitumumab, Nivolumab, Obinutuzumab, Ofatumumab, Olaratumab, Oportuzumab, Panitumumab, PDR001, Pembrolizumab, Pertuzumab, Polatuzumab, Racotumomab, Ramucirumab, Rituximab, Rovalpituzumab, Sacituzumab, SM3, TAK-164, Tositumomab, Trastuzumab, Tremelimumab, Ublituximab, Urelumab, Utomilumab, XMAB-5574, or Zolbetuximab.

[0074] In some embodiments, the first and/or second targeting moiety comprises IL-2, IL-4, IL-6, α -MSH, transferrin, folic acid, EGF, TGF, PD-1, IL-13, stem cell factor, insulin-like growth factor (IGF), or CD40. In some embodiments, the first and/or second targeting moiety comprises a full-length sequence of IL-2, IL-4, IL-6, α -MSH, transferrin, folic acid, EGF, TGF, PD-1, IL-13, stem cell factor, insulin-like growth factor (IGF), or CD40. In some embodiments, the first and/or second targeting moiety comprises a truncated form, analog, variant, or derivative of IL-2, IL-4, IL-6, α -MSH, transferrin, folic acid, EGF, TGF, PD-1, IL-13, stem cell factor, insulin-like growth factor (IGF), or CD40.

[0075] In some embodiments, the first and/or second targeting moiety binds the IL-2 receptor, IL-4, IL-6, melanocyte stimulating hormone receptor (MSH receptor), transferrin receptor (TR), folate receptor 1 (FOLR), folate hydroxylase (FOLH1), EGF receptor, PD-L1, PD-L2, IL-13R, CXCR4, IGFR, or CD40L.

[0076] In some embodiments, the second targeting moiety binds an antigen expressed by a tumor microenvironment cell. In some embodiments, the tumor microenvironment cell is a fibroblast or macrophage. In some embodiments, the antigen expressed by a fibroblast is fibroblast activation protein. In some embodiments, the antigen expressed by a macrophage is MAC-1/CD11b or sideroflexin 3.

[0077] This application also describes a method of treating cancer expressing a tumor antigen that binds the first targeting moiety and/or second targeting moiety in a patient comprising administering a composition to the patient. In some embodiments, the cancer is evaluated for the presence of infiltrating immune cells before administering the composition. In some embodiments, the cancer is evaluated for the presence of tumor antigens before administering the composition. In some embodiments, the first targeting moiety and second targeting moiety bind the same antigen. In some embodiments, the first targeting moiety and second targeting moiety bind different antigens.

[0078] In some embodiments, the cancer expressing a tumor antigen that binds the first and/or second targeting moiety is any one of breast cancer, ovarian cancer, endometrial cancer, cervical cancer, bladder cancer, renal cancer, melanoma, lung cancer, prostate cancer, testicular cancer, thyroid cancer, brain cancer, esophageal cancer, gastric cancer, pancreatic cancer, colorectal cancer, liver cancer, leukemia, myeloma, nonHodgkin lymphoma, Hodgkin lymphoma, acute myeloid

leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, lymphoproliferative disorder, myelodysplastic disorder, myeloproliferative disease or premalignant disease.

[0079] In some embodiments, a method of targeting immune cells to cancer expressing a tumor antigen that binds both the first and/or second targeting moiety in a patient comprises administering a composition to the patient. In some embodiments, a method of targeting immune cells to cancer expressing two tumor antigens, wherein one tumor antigen binds the first targeting moiety and one tumor antigen binds the second targeting moiety, in a patient comprises administering a composition to the patient.

[0080] In some embodiments, a method of delivering a cytokine to an immune cell of a patient comprises administering a composition to the patient, wherein the first and/or second complementary functional domain of the composition comprise IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[0081] Additional objects and advantages will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice. The objects and advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[0082] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

[0083] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) embodiment(s) and together with the description, serve to explain the principles described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0084] **Figures 1A-1B** shows a Twin Immune Cell Engager (“TWICE”). Figure 1A shows the two constructs before administration to a patient. Figure 1B shows the TWICE binding and engaging two signaling molecules on one effector (immune) cell after targeting of both components of the TWICE to a cancer cell. The hexagon and diamond represent two different antigens on a cancer cell bound by the

targeting moieties (two sets each of four striped ovals) of the first and second components. Each component comprises an immune cell binding domain (black domains of each component) and a complementary binding domain (white domains of each component). Linkers between the targeting moiety and the immune cell binding domain and the complementary binding domain are also shown. When the two components are in close proximity, domain swapping can occur such the two immune cell binding domains (one from each component) and the two complementary binding domains (one from each component) can pair with each other. The triangle and circle represent different antigens on the effector cell that can be bound by either the paired immune cell binding domains (binding to the antigen represented by the triangle) or the paired complementary binding domains (binding to the antigen represented by the circle). In the absence of domain swapping and pairing, the immune cell binding domains and the complementary binding domains of the two components do not modulate the effector cell.

[0085] **Figure 2** shows a TWICE that engages two signaling molecules on one effector (immune) cell after targeting of the first component to a cancer cell and targeting of the second component to a non-cancer cell. The hexagon represents an antigen on a cancer cell bound by the targeting moiety (four striped ovals) of the first component. The square represents an antigen on a non-cancer cell in the tumor micro-environment (such as a tumor-associated macrophage or fibroblast) bound by the targeting moiety (four striped ovals) of the second component. Each component comprises an immune cell binding domain (black domains of each component) and a complementary binding domain (white domains of each component). When the two components are in close proximity, domain swapping can occur such the two immune cell binding domains (one from each component) and the two complementary binding domains (one from each component) can pair with each other. The triangle and circle represent different antigens on the effector cell that can be bound by the paired immune cell binding domains (antigen represented by the triangle) and the paired complementary binding domains (antigen represented by the circle). In the absence of domain swapping and pairing, the immune cell binding domains and the complementary binding domains of the two components do not modulate the effector cell.

[0086] **Figure 3** shows a TWICE that binds and engages signaling molecules on two different effector (immune) cells after targeting of both components of the

TWICE to a cancer cell. Various targeting strategies may be employed. If a TWICE has two components that bind a cancer cell, it may employ three different configurations: (a) the two components of a TWICE may either bind the same antigen (single antigen targeting) using two copies of the same targeting moiety; (b) the TWICE may use a single antigen strategy using different targeting moieties that target different epitopes on the same antigen; or (c) the two components of a TWICE may bind two different antigens (dual antigen targeting) when both targeting moieties bind a cancer cell. This Figure represents single antigen targeting using different targeting moieties that target different epitopes on the same antigen. Turning back to the figure, the diamonds represent two molecules of the same antigen on a cancer cell bound by the targeting moieties (two sets of four striped ovals) of the first and second components. Each component comprises an immune cell binding domain (black domains of each component) and a complementary binding domain (white domains of each component). When the two components are in close proximity, domain swapping can occur such the two immune cell binding domains (one from each component) and the two complementary binding domains (one from each component) can pair with each other. The triangle and circle represent antigens on two different effector cells that can be bound by either the paired immune cell binding domains (binding to the antigen represented by the triangle on effector cell #1) or the paired complementary binding domains (binding to the antigen represented by the circle on effector cell #2). In the absence of domain swapping and pairing, the immune cell binding domains and the complementary binding domains of the two components do not modulate the effector cells.

[0087] **Figure 4** shows a TWICE that binds and engages a signaling molecule on an effector (immune) cell and binds a signaling molecule on the cancer cell after targeting of both components of the TWICE to a cancer cell. The diamonds represent two molecules of the same antigen on a cancer cell bound by the targeting moieties (two sets of four striped ovals) of the first and second components. Each component comprises an immune cell binding domain (black domains of each component) and a complementary binding domain (white domains of each component). When the two components are in close proximity, domain swapping can occur such the two immune cell binding domains (one from each component) and the two complementary binding domains (one from each component) can pair with each other. The triangle represents an antigen on an effector cell that can be bound by the paired immune cell binding

domains. The striped rectangle represents an antigen on the cancer cell that can be bound by the paired complementary binding domains. In the absence of domain swapping and pairing, the immune cell binding domains and the complementary binding domains do not modulate the effector cell or the cancer cell.

[0088] **Figure 5** shows a TWICE comprising a functional domain before administration to a patient. The targeting moieties (two sets each of four striped ovals) of the first and second components are shown. Each component comprises an immune cell binding domain (black curved domains of each component). Each component also comprises an inert binding domain (white curved domains of each component, also known as an inert binding partner) attached to the immune cell binding domains. One component also comprises a complementary functional domain (black triangle). Linkers between the targeting moiety and the immune cell binding domain or the complementary functional domain are also shown. In addition, a linker is shown between the immune cell binding domains and the inert binding partners of each component. The linker attaching the inert binding partner may be a cleavable linker that is cleaved in the tumor microenvironment. This cleavage releases the inert binding partners.

[0089] When the two components are in close proximity and the inert binding partners have been released, domain swapping can occur such the two immune cell binding domains (one from each component) can pair with each other. In addition, the complementary functional domain can bind and engage a target cell based on the targeting moiety localizing it to a target cell (such as a cancer cell or an effector cell). Activity of the complementary functional domain occurs without the need for pairing with another domain, but is enhanced when the complementary functional domain is localized to a target cell by the targeting moiety. In the absence of domain swapping and pairing, the immune cell binding domains of the two components do not modulate the target cell. In some embodiments, both components may comprise a complementary functional domain. In some embodiments, only one component comprises an inert binding partner.

[0090] **Figure 6** shows a TWICE comprising dimerization domains before administration to a patient. The targeting moieties (two sets each of four striped ovals) of the first and second components are shown. Each component comprises an immune cell binding domain (black domains of each component) and a complementary binding domain (white domains of each component). Linkers between the targeting

moiety and the immune cell binding domain and the complementary binding domain are also shown. A dimerization domain is attached to the immune cell binding domain and to the complementary binding domain of each component via a linker (such that each component has a dimerization domain A and dimerization domain B that can associate). Association of a pair of dimerization domains (pairing of dimerization domain A and dimerization domain B) enhances association of the VH and VL domains of each component. The dimerization domain linker may be cleavable, such that the linker is cleaved and the dimerization domain is lost following cleavage in the tumor microenvironment. After removal of the dimerization domains and when the two components are in close proximity, domain swapping can occur such the two immune cell binding domains (one from each component) and the two complementary binding domains (one from each component) can pair with each other. In the absence of domain swapping and pairing, the immune cell binding domains and complementary binding domains of the two components do not modulate the target cell. In the absence of cleavage of the dimerization domain linker, the VH and VL domains of each component remain associated together without a domain swap. In some embodiments, only one component comprises a pair of dimerization domains, and the other component does not comprise dimerization domains.

[0091] **Figures 7A-7C** show expression and cleavage of TWICE molecules listed in Table 12 with various dimerization domain linkers and dimerization domains. Figure 7A shows TWICE with various dimerization domain linkers and dimerization domains. TWICE were analysed by SDS-PAGE (Figure 7B) and cleavage products were also analysed (Figure 7C).

[0092] **Figures 8A-8B** show target binding by TWICE containing Fab or dsFv binding moieties over a range of concentrations (Figure 8A) or with an EC50 measurement (Figure 8B).

[0093] **Figures 9A-9C** show bridging ELISA of CD3/CTLA4 TWICE with hetero-Fc dimerization domain. Figure 9A shows the assay design, while Figure 9B shows results with CD3 bridging and Figure 9C shows results with CTLA-4 bridging.

[0094] **Figures 10A-10B** shows results for CD3/CTLA4 TWICE with coiled-coil dimerization domain with CD3 bridging ELISA (Figure 10A) or CTLA4 bridging ELISA (Figure 10B).

[0095] **Figures 11A-11B** show results with a CD28/CD3 TWICE in a CD3 bridging ELISA (Figure 11A) or a CD28 bridging ELISA (Figure 11B).

[0096] **Figures 12A-12B** show a bridging ELISA with Theralizumab (anti-CD28) and Urelumab (anti-4-1BB) TWICE. Specifically, this shows results with a CD28/4-1BB (CD137) TWICE in a CD28 bridging ELISA (Figure 12A) or a 4-1BB bridging ELISA (Figure 12B).

[0097] **Figure 13** shows PD-L1 binding of Atezolizumab/Ipilimumab TWICE. Specifically, this shows results with a PD-L1/CTLA-4 TWICE in a PD-L1 bridging ELISA.

[0098] **Figures 14A-14B** show T-cell redirection by a CD3/CTLA4 TWICE with or without pre-cleavage on HCT-15 cells. Figure 14A shows activation by pre-cleavage. Figure 14B shows experimental results with and without pre-cleavage.

[0099] **Figures 15A-15D** show T cell redirection and killing of HCT15 cells by a CD3/CTLA-4 TWICE. Figure 15A shows results of cleavage analysis. Figure 15B shows interferon gamma release. Figures 15C and 15D show results for LDH release measuring specific lysis of HCT15 cells under different conditions.

[00100] **Figure 16** shows T-cell redirection by CD3/CD28 TWICE on HCT-15. This figure shows HCT-15 cell killing as measured by LDH release with a CD3/CD28 TWICE.

[00101] **Figures 17A-17B** show redirection of pre-activated/exhausted peripheral blood mononuclear cells (PMBCs) against HCT15 cells as measured by flow cytometry (Figure 17A) or interferon gamma release (Figure 17B).

[00102] **Figures 18A-18B** show redirection of NK cells against HCT15 cells as measured by a CD16a bridging ELISA (Figure 18A) or LDH release (Figure 18B).

DESCRIPTION OF THE SEQUENCES

[00103] Table 1 provides a listing of certain sequences referenced herein.

Table 1: Description of the Sequences and SEQ ID NOs		
Description	Sequence	#
ADAM28 cleavage site	KPAKFFRL	1
ADAM28 cleavage site	DPAKFFRL	2
ADAM28 cleavage site	KPMKFFRL	3
ADAM28 cleavage site	LPAKFFRL	4
ADAM28 cleavage site	LPMKFFRL	5
ADAM28 cleavage site	KPAMFFRL	6
ADAM28 cleavage site	YPAKFFRL	7

ADAM28 cleavage site	KWAKFFRL	8
ADAM28 cleavage site	DPMKFFRL	9
ADAM28 cleavage site	DPAMFFRL	10
ADAM28 cleavage site	DPMFFRL	11
ADAM28 cleavage site	KMAMFFRL	12
ADAM28 cleavage site	KMAMFFIM	13
ADAM28 cleavage site	KPAMFFIM	14
ADAM28 cleavage site	LPAMFFRL	15
ADAM28 cleavage site	LPMMFFRL	16
ADAM28 cleavage site	LMAMFFRL	17
ADAM28 cleavage site	LMAMFFIM	18
ADAM28 cleavage site	LPAMFFIM	19
ADAM28 cleavage site	LPAMFFYM	20
ADAM28 cleavage site	KPMMFFRL	21
ADAM28 cleavage site	KPAKFFYM	22
ADAM28 cleavage site	KPAKFFIM	23
ADAM28 cleavage site	IPMKFFRL	24
ADAM28 cleavage site	IPAMFFRL	25
ADAM28 cleavage site	IPMMFFRL	26
ADAM28 cleavage site	IMAMFFRL	27
ADAM28 cleavage site	IMAMFFIM	28
ADAM28 cleavage site	IPAMFFIM	29
ADAM28 cleavage site	IPAMFFYM	30
cathepsin B cleavage site	FR	31
cathepsin B cleavage site	FK	32
cathepsin B cleavage site	VA	33
cathepsin B cleavage site	VR	34
cathepsin B cleavage site {Cit}= citrulline	V{Cit}	35
cathepsin B cleavage site	HLVEALYL	36
cathepsin B cleavage site	SLLKSRMVPNFN	37
cathepsin B cleavage site	SLLIARRMPNFN	38
cathepsin B cleavage site	KKFA	39
cathepsin B cleavage site	AFKK	40
cathepsin B cleavage site	QQQ	41
cathepsin D cleavage site	PRSFFRLGK	42
cathepsin D cleavage site	SGVVIATVIVIT	43
cathepsin K cleavage site	GGP	44
MMP1 cleavage site	SLGPQGIWGQFN	45
MMP2 cleavage site	AIPVSLR	46
MMP2 cleavage site	SLPLGLWAPNFN	47
MMP2 cleavage site	HPVGLLAR	48
MMP2 cleavage site	GPLGVRGK	49
MMP2 cleavage site	GPLGLWAQ	50
MMP3 cleavage site	STAVIVSA	51

MMP7 cleavage site	GPLGLARK	52
MMP7 cleavage site	RPLALWRS	53
MMP7 cleavage site	SLRPLALWRSFN	54
MMP2/9 cleavage site	GILGVP	55
MMP2/9 cleavage site	GPLGIAGQ	56
MMP9 cleavage site	AVRWLLTA	57
MMP9 cleavage site	PLGLYAL	58
MMP9 cleavage site	GPQGIAGQR	59
MMP9 cleavage site	KPVSLSYR	60
MMP11 cleavage site	AAATSIAM	61
MMP11 cleavage site	AAGAMFLE	62
MMP13 cleavage site	GPQGLAGQRGIV	63
MMP14 cleavage site	PRHLR	64
MMP14 cleavage site	PQGLLGAPGILG	65
MMP14 cleavage site	PRSAKELR	66
PSA / KLK3	HSSKLQ	67
PSA / KLK3	SSKLQ	68
KLK4	RQQR	69
TMPRSS2	GGR	70
Legumain	AAN	71
ST14 (Matriptase)	QAR	72
C1s cleavage site	YLGRSYKV	73
C1s cleavage site	MQLGRX	74
MASP2 cleavage site	SLGRKIQI	75
C2a and Bb cleavage site	GLARSNLDE	76
uPa cleavage site	TYSRSRYL	77
uPa cleavage site	KKSPGRVVGGSV	78
uPa cleavage site	NSGRAVTY	79
uPa cleavage site	AFK	80
tissue-type plasminogen activator (tPA)	GGSGQRGRKALE	81
ADAM10	PRYEAYKMGK	82
ADAM12	LAQAF	83
ADAM17	EHADLLAVVAK	84
flexible amino acid linker (may be presented in repeating fashion)	GGGS	85
flexible amino acid linker (may be presented in repeating fashion)	GGGS	86
flexible amino acid linker (may be presented in repeating fashion)	GS	87
flexible amino acid linker (may be presented in repeating fashion)	GSGGS	88
flexible amino acid linker (may be presented in repeating fashion)	GGSG	89

flexible amino acid linker (may be presented in repeating fashion)	GGSGG	90
flexible amino acid linker (may be presented in repeating fashion)	GSGSG	91
flexible amino acid linker (may be presented in repeating fashion)	GSGGG	92
flexible amino acid linker (may be presented in repeating fashion)	GGGSG	93
flexible amino acid linker (may be presented in repeating fashion)	GSSSG	94
Anti-EGFR aptamer (tight binder with $K_d=2.4$ nM)	UGCCGCUAUAUAUGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	95
Anti-EGFR aptamer	UGGCGCUAAAUAAGCACGGAAUAUAUCGCCG UAGAAAAGCAUGUCAAGCCG	96
Anti-EGFR aptamer	UGCUGUAUAUCGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	97
Anti-EGFR aptamer	UGCCGCCAUUAUCACACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	98
Anti-EGFR aptamer	UUCGCGUGUAUAACACGGACUUAUAUCGCCG UAGUAAAGCAUGUCAAGCCG	99
Anti-EGFR aptamer	UGUCGCUCUAUUGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	100
Anti-EGFR aptamer	UGCUGCUUUAUCCCAUAUUUUUUCUUUU CAUAACAAUAUUUCUUUUUU	101
Anti-EGFR aptamer	UGCNGCUAUAUCGCNCGUAUUUAAUCGCCG UAGAAAAGCAUGUCNANGCCG	102
Anti-EGFR aptamer	UGCAAAGAAAACGCACGUAUUUAAUCGCCG UAGUAAAGCAUGUCAAGCCG	103
Anti-EGFR aptamer	UGCAUCACUAUCGAACCUAUUUAAUCCACC AAAUAUAUUGCAAGUCCAUCUC	104
Anti-EGFR aptamer	UGCCNNAUAUAACACACNUAUUAUAUCGCCG UACAAAUAUGUCAANCCG	105
Anti-EGFR aptamer	UGCAGCUGUAUUGCACGUAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	106
Anti-EGFR aptamer	UUCGGAUAAUCCCGCGUACUAAAUACCAU AGUCAACAAUUUCCAACCUC	107
Anti-EGFR aptamer	UCCACUAUAUCACACGUAUUUAAUCGCCG AGAAAAGCAUGUCAAGCCG	108
Anti-EGFR aptamer	UCCCUCAACCUCGCUACUAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCU	109
Anti-EGFR aptamer	UGCCGCUAUAUCACACGAAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	110

Anti-EGFR aptamer	AGCCCCUAGAACACACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	111
Anti-EGFR aptamer	UGCCAAUAUAUAACACGGAAUUAUCGCCG UAGAAAAGCAUGUCAAGCCG	112
Anti-EGFR aptamer	UGCCGCUAUAGCGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	113
Anti-EGFR aptamer	UGCAGAUUAUUGUCACUCAUUAUCCCCGU AUAAAAACAUAACUAAGCUC	114
Anti-EGFR aptamer	UGUAGCUGUAUUGCACACAUUAAAUCGCCG UAGUAAAGCAUGUCAAGCCG	115
Anti-EGFR aptamer	UACCAAUAUAUCGCCACACAUAUUCGCCGU AGAAAAGCAUGUCAAGCCG	116
Anti-EGFR aptamer	UGCCGCUAUGCCCACGGAAUUUAAUCGCCG UAGAAAAACAUGUCAAGUCG	117
Anti-EGFR aptamer	UGCCGCUAUUUAGCACGGAUUAAAUCGCCG UAGAAAAGCAUGUCAAGCCG	118
Anti-EGFR aptamer	UGCCGCUAUUUAGCACGGAUUAAAUCGCCG UAGAAAAGCAUGUCNAAGCCG	119
Anti-EGFR aptamer	UGUAGUAUAUUGACACGGAUUUAAUCGCCG UAGAAAAGCANGUCAAGCCU	120
Anti-EGFR aptamer	UGUCGCCAUUACGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	121
Anti-EGFR aptamer	UGCCCCCAAACUACACAAAUUUAAUCGCCG UAUAAAAGCAUGUCAAGCCG	122
Anti-EGFR aptamer	UGCACUAUCUCACACGUACUAAUCGCCGUA UAAAAGCAUGUCAAGCCG	123
Anti-EGFR aptamer	UGUCGCAAUAUACACUAAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	124
Anti-EGFR aptamer	UGCAACAAUAUAGCACGUUUUAAUCGCCG UAGUAAAGCAUGUCAAGG	125
Anti-EGFR aptamer	CUACCACAAAUCCCACAUUUUAAUCUCCC AAUCAAAUCUUGUCCAUCUCCC	126
Anti-EGFR aptamer	UGCCCUAAACUCACACGGAUAUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	127
Anti-EGFR aptamer	UUGUCGUAUGUCACACGUUUUAAUCGCCG UAUAAAAGCAUGUCAAGCCG	128
Anti-EGFR aptamer	UCCGCUAUAACACACGGAGAAAUCGCCG UAGUAAAGCAUGUCAAGCCG	129
Anti-EGFR aptamer	UGCCGAUAUAACGCACGGAUAUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	130
Anti-EGFR aptamer	UGCCAUUAUACAGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	131
Anti-EGFR aptamer	UCCAGAAUAUUGCACACAUUUAAUCGCCGU AGAAAAGCAUGUCAAGCCG	132
Anti-EGFR aptamer	UCCGCUAAACAACACGGAUACAAUCGCCGU AGAAAAGCAUGUCCAAGCCG	133
Anti-EGFR aptamer	UGCACUAUCUCACACGUACUAAUCGCCGUA UAAAAGCAUGUCAANNNG	134

Anti-EGFR aptamer	AUNGCNANNNUACACGUAUUNAAUCGCCGU AGAAAAGCAUGUCANAGCCG	135
Anti-EGFR aptamer	UGCUGCUAUAAUUGCAAUUUUUAAACUAAG UAGAAAACCAUGUACAAGUCG	136
Anti-EGFR aptamer	UGUCGCCAUAAUUGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCCAAGCCG	137
Anti-EGFR aptamer	UGCCGUUAUAACCCACGGAAUUUAACCUCC GUAGAAAAGCAUGUCAAGCCG	138
Anti-EGFR aptamer	UGUGAAUAUAUAUCACGGAUUUAAUCGCCG UAUAAAAGCNAUGUCAAGCCG	139
Anti-EGFR aptamer	UGCCGAUAUNNANCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCCAAGCCG	140
Anti-EGFR aptamer	UGUCACUAAAUUGCACGUAUAUAAUCGCCG UAGUAAGCAUGUCAAGCCG	141
Anti-EGFR aptamer	UGCAACCAUAAAGCACGUAUAAAUCGCCG UAUAUAAGCAUGUCaAAGCCG	142
Anti-EGFR aptamer	UGCCGCUAUUAAGCACGUAUUAAUCGCCGU AGUAAAGCAUGUCaAAGCCG	143
Anti-EGFR aptamer	UGCCGCUAUAGCACACGGAAUUUAAUCGCC GUAGUAAAGCAUGUCAAGCCG	144
Anti-EGFR aptamer	UGCAGGUUAUAACNCGGAUUUAAUCGCCG UAGAAAAGCAUGUCNAAGCCG	145
Anti-EGFR aptamer	UGCUCCUAUAACACACGGAUUUAAUCGCCG UAGAAAAGCAUGUCCAAGCCG	146
Anti-EGFR aptamer	UGCCCGUAAUUGCACGGAUUUAAUCGCCGU AGAAAAGCAUGUCCAAGCCGG	147
Anti-EGFR aptamer	ACUCCCUAUAUNGCAACUACAUAUAAUCGCCG UAAAUAAAGCAUGUNCAAGCCG	148
Anti-EGFR aptamer	UGAAGCUAGAUCACACUAAAUUAUAAUCGCCG UAGAAAAGCAUGUCAAAAAGCCG	149
Anti-EGFR aptamer	UGACUCUUUAUCCCCCGUACAUAUUcACC GAACCAAGCAUUAACCAUCCCC	150
Anti-EGFR aptamer	UGACGCCCUAACACACGUAUAUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	151
Anti-EGFR aptamer	UGUCGCAAAUAGCACGUAUUUAAUCGCCG UAGAAAAGCAUGUCCAAGCCG	152
Anti-EGFR aptamer	UGAGUGUAUAAUUCACGUAUUUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	153
Anti-EGFR aptamer	UGCACUAUAUCGUAGGUAAUAAUCGCCC UACAAACUCACUCUAAAACCG	154
Anti-EGFR aptamer	UUACGCUAUAUCACACGGAUUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	155
Anti-EGFR aptamer	CCCAUCUGUACUACAGGAUUUAAUCGCCG UAGAAAAGCAUGUCCAAGCCG	156
Anti-EGFR aptamer	UGCCCAUAAAUAGCACGGAUUUAAUCGCCG UAGAAAAGCAUGUCCAAGCCG	157
Anti-EGFR aptamer	UGCCGCAUAACAUAACACAUAUAAUCGCCG UAGAAAAGCAUGUCAAGCCG	158

Anti-EGFR aptamer	UGCAACUAUAUCGCACGUAUGUAAUCGCCG UAGAAAAAGCAUGUCAAGCC	159
Anti-EGFR aptamer	UCCCGCUAUUAGCACGGAAUUAUCGCCG UAGAAAAAGCAUGUCCAAGCCG	160
Anti-EGFR aptamer	UCCCGCUAAGUCACACGAAAUUAAUCGCCG UAGAAAAAGCAUGUCCAAGCCG	161
Anti-EGFR aptamer	UGUAGCAAUAUCACACGUAUUAUCGCCG UAUAUAAGCAUGUCAAGCCG	162
Anti-EGFR aptamer	UGCCGUUAUAUAUCACGGAUUUAUCGCCG UAGAAAAAGCAUGUCCAAGCCG	163
Anti-EGFR aptamer	UACACAUAUAUCAAGUAACUUAUCUCCUU AGUAACCAUCUCCAAGCCG	164
Anti-EpCAM VH-CH1, SP34 VH, FXa, basic coil	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVKPGGSLRL SCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKGRFTISRDDSKNSLYL QMNSLKTEDTAVYYCVRHGNFGNSYVSWFAY WGQGTLLTVSSGGGGSGIEGRGGGGSKNAQCK KKLQALKKKNAQLKWKLQALKKKLAQGH HHH	165
Anti-EpCAM VL-CL, Atezolizumab VL, MMP2, acid coil	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKSTYSLSSTLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQTQSPSSLSASVGDRVTITCRA SQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISLQPEDFATYYCQQY LYHPATFGQGTKVEIKGGPLGVRGKGGGSENA QCEKELQALEKENAQLEWELQALEKELAQ	166
Anti-EpCAM VH-CH1, SP34 VH, FXa, IgE CH2	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVKPGGSLRL SCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKGRFTISRDDSKNSLYL QMNSLKTEDTAVYYCVRHGNFGNSYVSWFAY WGQGTLLTVSSGIEGRGPPTVKILQSSCDGGGH	167

	FPPTIQLLCLVSGYTPGTIQITWLEDGQVMDVD LSTASTTQEGELASTQSEL TLSQKHWLSDRTYT CQV TYQGHTFEDSTKKCAHHHHHH	
Anti-EpCAM VL-CL, Atezolizumab VL, FXa, IgE CH2	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQMTQSPSSLASVGDRVTITCRA SQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISLQPEDFATYYCQQY LYHPATFGQGTKVEIKGGGIEGRGPPTVKILQS SCDGGGHFPPTIQLLCLVSGYTPGTIQITWLEDG QVMDVDLSTASTTQEGELASTQSEL TLSQKHW LSDRTYTCQV TYQGHTFEDSTKKCA	168
Anti-EpCAM VH-CH1, SP34 VH, FXa, IgE CH2	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSW NSGALTSKVHTFPAVLQSSGLYSLSVTVTPSS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVKPGGSLRL SCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKGRFTISRDDSKNSLYL QMNSLKTEDTAVYYCVRHGNFGNSYVSWFAY WGQGTLLTVSSGGGIEGRGPPTVKILQSSCDGG GHFPPTIQLLCLVSGYTPGTIQITWLEDGQVMD VDLSTASTTQEGELASTQSEL TLSQKHWLSDRT YTCQV TYQGHTFEDSTKKCAHHHHHH	169
Anti-EpCAM VL-CL, Atezolizumab VL, FXa, IgE CH2	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQMTQSPSSLASVGDRVTITCRA SQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISLQPEDFATYYCQQY LYHPATFGQGTKVEIKGGGIEGRGGGDKTHC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYQSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTIKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVSGFYPSDIAVEWESNGQPEN NYKTTTPVLDSDGSFKLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	170

Anti-EpCAM VH-CH1, SP34 VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVKPGGSLRL SCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKGRFTISRDDSKNSLYL QMNSLKTEDTAVYYCVRHGNFGNSYVSWFAY WGQGTLLTVSSGGGIEGRGGGDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YQSTYRVVSVLTVLHQDWLNGKEYKCKVSNK ALPAPIEK TISKAKGQPREPQVYTLPPSRDELTK NQVKLTCLVKGFIYPSDIAVEWESNGQPENNYK TPPVLDSDGSFFLYSLLTVDKSRWQQGNVFC SVMHEALHNHYTQKSLSLSPG	171
Anti-EpCAM VL-CL, Atezolizumab VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNLSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVCLLNFFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTSLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQMTQSPSSLSASVGDRVTITCRA SQDVSTAVAWYQQKPGKAPKLLIYSASFYSG VPSRFSGSGSGTDFTLTISLQPEDFATYYCQQY LYHPATFGQGTKVEIKGGGIEGRGGGDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYQSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEK TISKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVSGFIYPSDIAVEWESNGQPE NYKTPPVLDSDGSFKLISKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	172
Anti-EpCAM VH-CH1, SP34 VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVKPGGSLRL SCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKGRFTISRDDSKNSLYL QMNSLKTEDTAVYYCVRHGNFGNSYVSWFAY WGQGTLLTVSSGGGGGIEGRGGGDKTHTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV	173

	VDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYQSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDEL T KNQVKL TCL VKGFYPSDIAVEWESNGQPENNY KTTTPVLDSGDSFLLYSLLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPG	
Anti-EpCAM VL-CL, Atezolizumab VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSTLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQTQSPSSLSASVGDRVTITCRA SQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISLQPEDFATYYCQQY LYHPATFGQGTKVEIKGGGGSGIEGRGGGDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAK T KPREEQYQSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS RDELTKNQVSLTCLVSGFYPSDIAVEWESNGQP ENNYKTTTPVLDSGDSFKLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPG	174
Anti-EpCAM VH-CH1, SP34 VH, FXa, basic coil	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGQTTVTVSSASTKGPSV FPLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVKPGGSLRL SCAASGFTFNTYAMNWVRQAPGKGLEWVARI RSKYNNYATYYADSVKGRFTISRDDSKNSLYL QMNSLKTEDTAVYYCVRHGNFGNSYVSWFAY WGQGTLLTVSSGGGGSGIEGRGGSGGGSKNAQ CKKKLQALKKKNAQLKWKLQALKKKLAQGH HHHHH	175
Anti-EpCAM VL-CL, Atezolizumab VL, FXa, acid coil	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSTLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQTQSPSSLSASVGDRVTITCRA SQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISLQPEDFATYYCQQY LYHPATFGQGTKVEIKGGGGSGIEGRGGSGGGSE NAQCEKELQALEKENAQLEWELQALEKELAQ	176

Anti-EpCAM VL-CL, SP34 VH, FXa, Ckappa	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPRKAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSSTLTLSKADYEKHK KYYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSEVQLVESGGGLVKPGGSLRLSCAA SGFTFNTYAMNWVRQAPGKGLEWVARIRSKY NNYATYYADSVKGRFTISRDDSKNSLYLQMNS LKTEDTAVYYCVRHGNFGNSYVSWFAYWGQ GTLVTVSSIEGRGPSVFIFPPSDEQLKSGTASVV CLLNNFYPRKAKVQWKVDNALQSGNSQESVT EQDSKDYSLSSSTLTLSKADYEKHKKYYACEV THQGLSSPVTKSFNRGEC	177
Anti-EpCAM VH-CH1, Atezolizumab VL, FXa, CH1, 6His	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGQGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSDIQMTQSPSSLSASVGDRVTI TCRASQDVSTAVAWYQQKPGKAPKLLIYSASF LYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQYLYHPATFGQGTKVEIKGGIEGRGPSVFPL APSSKSTSGGTAAALGCLVKDYFPEPVTVSWNS GALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCHHHHH H	178
Anti-EpCAM VL-CL, Atezolizumab VH, FXa, Ckappa	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPRKAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSSTLTLSKADYEKHK KYYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSEVQLVESGGGLVQPGGSLRLSCAA SGFTFSDSWIHWVRQAPGKGLEWVAWISPYGG STYYADSVKGRFTISADTSKNTAYLQMNSLRA EDTAVYYCARRHWPGGFDYWGQGTTLVTVSSI EGRGPSVFIFPPSDEQLKSGTASVVCLLNNFYPR EAKVQWKVDNALQSGNSQESVTEQDSKDYSL SSSTLTLSKADYEKHKKYYACEVTHQGLSSPV TKSFNRGEC	179
Anti-EpCAM VH-CH1, SP34 VL, FXa, CH1, 6His	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGQGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSW	180

	NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS SLGTQTYICNVNHNKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSQAVVTQEPSLTVSPGGTVTL TCRSSTGAVTASNYANWVQQKPGQAPRGLIGG TNKRAPWTPARFSGSLLGGKAALTITGAQAED EADYYCALWYSNLWVFGGGTKLTVLGGIEGR GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHNKPSNTKVDKKVEPKSC HHHHHH	
Anti-EpCAM VL-CL, Ipilimumab VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSEIVLTQSPGTLSPGERATLSCRAS QSVGSSYLAWYQQKPGQAPRLLIYGAFSRATG IPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQY GSSPWTFGGTKVEIKGGGIEGRGGGDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYQSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVSGFYPSDIAVEWESNGQPN NYKTTTPVLDSGSKFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	181
Anti-EpCAM VH-CH1, Ipilimumab VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS SLGTQTYICNVNHNKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSQVQLVESGGGVVQPGRSLR LSCAASGFTFSSYTMHWVRQAPGKGLEWVTFI SYDGNNKYYADSVKGRFTISRDNSTNTLYLQM NSLRAEDTAIYYCARTGWLGPFDYWGGGTLV TVSSGGGIEGRGGGDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYQSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPSRDELTKNQVKLTCL VKGFIYPSDIAVEWESNGQPNENNYKTTTPVLDS DGSFFLYSLLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPG	182
Anti-EpCAM VL-CL, SP34 VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND	183

	YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVCLLNFFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSSTLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GSGGGGSQAVVTQEPSLTVSPGGTVTLTCRSS TGAVTASNYANWVQQKPGQAPRGLIGGTNKR APWTPARFSGSLLGGKAALTITGAQAEDEADY YCALWYSNLWVFGGGTKLTVLGGGIEGRGGG DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYQSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVY TLPPSRDELTKNQVSLTCLVSGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFKL YSKLTVDK SRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG	
Anti-EpCAM VL-CL, Ipilimumab VL, MMP2, acid coil	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVCLLNFFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSSTLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GSGGGGSEIVLTQSPGTLSPGERATLSCRAS QSVGSSYLAWYQQKPGQAPRLLIYGAFSRATG IPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQY GSSPWTFGGQTKVEIKGGPLGVRGKGGGSENA QCEKELQALEKENAQLEWELQALEKELAQ	184
Anti-EpCAM VH-CH1, Ipilimumab VH, FXa, basic coil	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSQVQLVESGGGVVQPGRSLR LSCAASGFTFSSYTMHWVRQAPGKGLEWVTFI SYDGNNKYYADSVKGRFTISRDN SKNTLYLQM NSLRAEDTAIYYCARTGWLGPFDYWGGGTLV TVSSGGGGSGIEGRGGGGSKNAQCKKKLQALKK KNAQLKWKLQALKKKLAQGHHHHHH	185
Anti-EpCAM VL-CL, SP34 VL, MMP2, acid coil	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVCLLNFFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSSTLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GSGGGGSQAVVTQEPSLTVSPGGTVTLTCRSS TGAVTASNYANWVQQKPGQAPRGLIGGTNKR APWTPARFSGSLLGGKAALTITGAQAEDEADY	186

	YCALWYSNLWVFGGGTKLTVLGGPLGVRGKG GGSENAQCEKELQALEKENAQLEWELQALEK ELAQ	
Anti-EpCAM VH-CH1, Atezolizumab VH, FXa, basic coil	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVQPGGSLRL SCAASGFTFSDSWIHWVRQAPGKGLEWVAWIS PYGGSTYYADSVKGRFTISADTSKNTAYLQMN SLRAEDTAVYYCARRHWPGGFDYWGGGTLVT VSSGGGGSGIEGRGGGGSKNAQCKKKLQALKK KNAQLKWKLQALKKKLAQGHHHHHH	187
Anti-EpCAM VL-CL, Ipilimumab VL, FXa, acid coil	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSEIVLTQSPGTLSPGERATLSCRAS QSVGSSYLAWYQQKPGQAPRLLIYGAFSRATG IPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQY GSSPWTFGGGTKVEIKGGGGSGIEGRGGSGGGSE NAQCEKELQALEKENAQLEWELQALEKELAQ	188
Anti-EpCAM VH-CH1, Ipilimumab VH, FXa, basic coil	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSQVQLVESGGGVVQPGRSLR LSCAASGFTFSSYTMHWVRQAPGKGLEWVTFI SYDGNNKYYADSVKGRFTISRDNSTNTLYLQM NSLRAEDTAIYYCARTGWLGPFDYWGGGTLV TVSSGGGGSGIEGRGGSGGGSKNAQCKKKLQAL KKKNAQLKWKLQALKKKLAQGHHHHHH	189
Anti-EpCAM VL-CL, SP34 VL, FXa, acid coil	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSQAVVTQEPSLTVSPGGTVTLTCRSS TGAVTASNYANWVQQKPGQAPRGLIGGTNKR	190

	APWTPARFSGSLLGGKAALTITGAQAEDEADY YCALWYSNLWVFGGGTKLTVLGGGGSIEGRG GSGGGSENAQCEKELQALEKENAQLEWELQA LEKELAQ	
Anti-EpCAM VH 44Cys, Sp34 VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHCLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSGGGSGGG GSEVQLVESGGGLVKPGGSLRLSCAASGFTFNT YAMNWRQAPGKGLEWVARIRSKYNNYATY YADSVKGRFTISRDDSKNSLYLQMNSLKTEDT AVYYCVRHGNFGNSYVSWFAYWGQGTLLTV SSGGGIEGRGGGDKTHTCPPCPAPELLGGPSVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYQSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVKLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSD GSFFLYSLLTVDKSRWQQGNVFSVMSHEALH NHYTQKSLSLSPG	191
Anti-EpCAM VL 100 Cys, Ipilimumab VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNLSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGCGTKLEIKGGGGSGGGGSEIVLTQS PGTSLSPGERATLSCRASQSVGSSYLAWYQQ KPGQAPRLLIYGAFSRATGIPDRFSGSGSGTDFT LTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVE IKGGGIEGRGGGDKTHTCPPCPAPELLGGPSVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYQSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVS GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FKLYSKLTVDKSRWQQGNVFSVMSHEALHN HYTQKSLSLSPG	192
Anti-EpCAM VH 44 Cys, Ipilimumab VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHCLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSGGGSGGG GSQVQLVESGGGVVQPGRSLRLSCAASGFTFSS YTMHWVRQAPGKGLEWVTFISYDGNNKYA DSVKGRFTISRDNKNTLYLQMNSLRAEDTAIY YCARTGWLGPFDYWGGGTLTVVSSGGGIEGR GGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYQSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSRDELTKNQVKLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSDGSFFLYSLLTV	193

	DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG	
Anti-EpCAM VL 100 Cys, SP34 VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGCGTKLEIKGGGGSGGGGSQAVVTQ EPSLTVSPGGTVTLTCRSSTGAVTASNYANWV QQKPGQAPRGLIGGTNKRAPWTPARFSGSLLG GKAALTITGAQAEDADYYCALWYSNLWVFG GGTKLTVLGGGIEGRGGGDKTHTCPPCPAPELL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYQST YRVVSVLTVLHQDWLNGKEYKCKVSNKALPA PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVS LTCLVSGFYPSDIAVEWESNGQPENNYKTTPPV LDSGDSFKLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG	194
Anti-EpCAM VH-CH1, Therelizumab VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSW NSGALTSKVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSEVQLVESGGGLVQPGGSLRL SCAASGFTFSDSWIHWVRQAPGKGLEWVAWIS PYGGSTYYADSVKGRFTISADTSKNTAYLQMN SLRAEDTAVYYCARRHWPGGFDYWGQGTLLV VSSGGGGSGIEGRGGSGGGSKNAQCKKKLQALK KKNAQLKWKLLQALKKKLLAQGHHHHHH	195
Anti-EpCAM VL-CL, Therelizumab VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNFFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIQMTQSPSSLSASVGDRVTITCHA SQNIYVWLNWYQQKPGKAPKLLIYKASNLHT GVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQ GQTPYPTFGGGTKVEIKGGGIEGRGGGDKTHT CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYQSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVSGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGDSFKLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPG	196
Anti-EpCAM VH-CH1, Urelumab VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF	197

	KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGSGGGGSQVQLQQWGAGLLKPSETLS LTCAVYGGSFSGYYWSWIRQSPEKGLEWIGEI NHGGYVTYNPSLESRTISVDTSKNQFSLKLSS VTAADTAVYYCARDYGPGNYDWYFDLWGRG TLVTVSSGGGIEGRGGGDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYQSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVKL TCLVKGFYPSDIAVEWESNGQPENNYKTPPV LDDSGSFFLYSLLTVDKSRWQQGNVFCFSVMH EALHNHYTQKSLSLSPG	
Anti-EpCAM VL-CL, Urelumab VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GSGGGGSDIQMTQSPSSLSASVGDRVTITCHA SQNIYVWLNWYQQKPGKAPKLLIYKASNLHT GVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQ GQTPPYTFGGGTKVEIKGGGIEGRGGGDKTHT CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV TCVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYQSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVSGFYPSDIAVEWESNGQPE NNYKTPPVLDSDGSFGLYSKLTVDKSRWQQG NVFCFSVMHEALHNHYTQKSLSLSPG	198
Anti-EpCAM VL-CL, Dummy VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDYSLSSLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GSGGGGSAVVTQEPSTVSPGGTVTLTCRSS TGAVTTSNYANWVQQKPGQAPRGLIGGTNKR APWTPARFSGSLLGGKAALTITGAQAEDADY YCALWYSNLA VFGGGTKLTVLGGGIEGRGGG DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYQSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVY	199

	TLPPSRDELTKNQVSLTCLVSGFYPSDIAVEWE SNGQPENNYKTPPVLDSDGSFKLYSKLTVDK SRWQQGNVFSVMSHEALHNHYTQKSLSLSPG	
Anti-EpCAM VH-CH1, Dummy VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGSGGGGSEVQLVESGGGLVQPGGSLRL SCAASGFTFSSYAMNWVRQAPGKGLEWVARIS GSGGSTYYADSVKDRFTISRDDSKNSLYLQMN SLKTEDTAVYYCVRGKGNTHKPYGYVRYFDV WGQGTLLTVSSGGGIEGRGGGDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YQSTYRVVSVLTVLHQDWLNGKEYKCKVSNK ALPAPIEKISKAKGQPREPQVYTLPPSRDELTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYK TPPVLDSDGSFFLYSLLTVDKSRWQQGNVFSV MSHEALHNHYTQKSLSLSPG	200
Basic coil	KNAQCKKKLQALKKKNAQLKWKLQALKKKL AQ	201
Acid coil	ENACEKELQALEKENAQLEWELQALEKELA Q	202
Leucine zipper sequence	GGGGSIEGRGGGGS	203
Leucine zipper sequence	GGPLGVRGKGGGS	204
Leucine zipper sequence	GGGGSIEGRGGSGGGS	205
Hetero Fc sequence	GGGIEGRGGG	206
Hetero Fc sequence	GGGGSIEGRGGG	207
CH1 sequence	IEGRG	208
CL sequence	GGIEGRG	209
IgE CH2 sequence	GIEGRG	210
IgE CH2 sequence	GGGIEGRG	211
Anti-EpCAM VH-CH1, OKT3 VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF KGKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGGGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGSGGGGSDVQLVQSGAEVKKPGASVK VSKASGYTFTRYTMHWVRQAPGQGLEWIGY INPSRGYTNADSVKGRFTITTDKSTSTAYMEL SSLRSEDATYYCARYYDDHYCLDYWGQGT TVVSSGGGIEGRGGGDKTHTCPPCPPELLGGP SVFLFPPKPKDTLMISRTPEVTCVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYQSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK	212

	TISKAKGQPREPQVYTLPPSRDELTKNQVKLTC LVKGFYPSDIAVEWESNGQPENNYKTPPVLD SDGSFFLYSLLTVDKSRWQQGNVFCSCVMHEA LHNHYTQKSLSLSPG	
Anti-EpCAM VL-CL, OKT3 VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYLSSTLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSDIVLTQSPATLSLSPGERATLSCRA SQSVSYMNWYQQKPGKAPKRWIYDTSKVASG VPARFSGSGSGTDYSLTINSLEAEDAATYYCQQ WSSNPLTFGGGKVEIKGGGIEGRGGGDKTHT CPPCPAPPELLGPSVFLFPPKPKDTLMISRTPEV TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYQSTYRVVSVLTVLHQDWLNGKEYKC KVSNAKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVSGFYPSDIAVEWESNGQPE NNYKTPPVLDSDGSFKLYSKLTVDKSRWQQG NVFCSCVMHEALHNHYTQKSLSLSPG	213
Anti-EpCAM VH-CH1, anti-CD16A VH, FXa, Fc	EVQLLEQSGAELVRPGTSVKISCKASGYAFTNY WLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKF K GKATLTADKSSSTAYMQLSSLTFEDSAVYFC ARLRNWDEPMDYWGQGTTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTGGGGSGGGGSQVQLVQSGAEVKKPGESLK VSCKASGYTFTSYMHWRQAPGQGLEWMGI INPSGGSTSYAQKFQGRVTMTRDTSTSTVYME LSSLRSEDYAVYYCARGSAYYYDFADYWGQG TLVTVSSGGGIEGRGGGDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH DPEVKFNWYVDGVEVHNAKTKPREEQYQSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVKL TCLVKGFYPSDIAVEWESNGQPENNYKTPPV LDSDGSFFLYSLLTVDKSRWQQGNVFCSCVMH EALHNHYTQKSLSLSPG	214
Anti-EpCAM VL-CL, anti-CD16A VL, FXa, Fc	ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSG NQKNYLTWYQQKPGQPPKLLIYWASTRESGVP DRFTGSGSGTDFTLTISSVQAEDLAVYYCQND YSYPLTFGAGTKLEIKRTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKDSTYLSSTLTLSKADYEKH KVVACEVTHQGLSSPVTKSFNRGECGGGGSGG GGSGGGGSSYVLTQPSSVSVAPGQTATISCGGH NIGSKNVHWYQQRPQGSPVLVIYQDNKRPSGIP	215

	ERFSGSNSGNTATLTISGTQAMDEADYYCQVW DNYSVLFGGGTKLTVLGGGIEGRGGGDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYQSTYRVVSVLTVLHQDWLNGKEYKCK VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVSGFYPSDIAVEWESNGQPEN NYKTTTPVLDSGSKLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG	
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DESCRIPTION OF THE EMBODIMENTS

I. Twin Immune Cell Engager

[00104] A Twin Immune Cell Engager (“TWICE”) refers to a two-component complexes wherein domains of the two components can pair and bind and engage immune cells. As such, a single TWICE comprises two components, which may be present in two separate polypeptides or in a single polypeptide. Thus, a TWICE may be a single construct or it may be a kit of two components. The use of the term TWICE throughout the application and claims encompasses both embodiments, unless specifically discussed otherwise. This application describes a range of TWICE. TWICE can activate different pathways to mediate a variety of anti-cancer effects.

[00105] As this application will describe, a TWICE can comprise a first and second component that each comprise a targeting moiety, an immune cell binding domain, and a complementary binding domain. The targeting moieties of the first and second components function to target the TWICE to cancer cells or cells in the tumor microenvironment. When the immune cell binding domain from both components pair together and/or the complementary binding domain from both components pair together, these paired domains can function to modulate immune cell activity or serve other (i.e., complementary) functions. For example, paired complementary binding domains may block immune checkpoints or induce death cell death of cancer cells.

[00106] The presence of complementary binding domains in a TWICE is expected to improve their efficacy in treating cancer compared to complexes not comprising complementary domains (i.e., compared to complexes that only bind a single antigen on immune cells).

[00107] As this application will also describe, the first component and optionally the second component can comprise a complementary functional domain

capable of immune cell binding. In some embodiments with complementary functional domains, the immune cell binding domain is masked. The complementary functional domain of a TWICE can modulate immune function without needing to pair with a different domain.

[00108] A major advantage of the present TWICE complexes described herein is that the immune cell binding domains, and in some embodiments the complementary binding domains, will only be paired when both the first and second components of the TWICE are in close proximity (here when in the tumor microenvironment because they are both targeted to a cancer). The pairing of domains between different components of the TWICE at the cancer site may improve specificity and reduce off-target effects, because the immune cell binding domains and optionally the complementary binding domains only function after pairing of domains from both components at the cancer cell or its microenvironment.

[00109] After describing some representative types of TWICE, the application will then describe components that comprise different types of TWICE. The TWICE is not limited to the representative descriptions herein, as a TWICE could modulate many pathways related to cancer survival and the anti-cancer immune responses.

[00110] The present TWICE complexes offer a unique ability to combine multiple functions into a two-component complex that becomes activated in the tumor microenvironment. The present complexes, thus, provide meaningful advantages in having a single approach to administering a two-component complex that is localized to the tumor microenvironment and has the ability to effect two different signals to benefit patients. In some embodiments, one of those signals (from the paired immune cell binding domains) is activated only when the two-component complex comes together. In some embodiments, both of those signals (from the paired immune cell binding domains and from the paired complementary binding domains) are activated only when the two-component complex comes together. This unique construct offers many benefits that were not present in prior art constructs.

1. TWICE that activates two signaling pathways on one type of immune cell

[00111] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, bind to the same type of immune cell.

[00112] As used herein, immune cells may also be termed “effector cells.” The term “effector cell” mean an immune cell that mediates activity against the cancer.

[00113] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, bind to T cells.

[00114] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, bind to the same antigen on an immune cell. In this embodiment, the two signaling pathways are the same and the impact is amplified. In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, bind to different antigens on an immune cell. In this embodiment, the two signaling pathways are different and two different signals are transmitted to the immune cell.

[00115] In general, the process of T-cell activation involves multiple signals. The primary signal comes from the binding of the T-cell receptor (TCR) to the major histocompatibility complex (MHC) molecule presented by an antigen presenting cell (APC). A costimulatory signal may arise from one of several distinct T-cell and APC interactions (*see* Buchbinder EI, Desai A *Am J Clin Oncol.* 39(1):98-106 (2016)). The activation of T cells is enhanced by several co-stimulatory receptors, such as CD28, CD137 (4-1BB), OX40, CD27, GITR (TNFRSF18), and ICOS. The interactions between T cell and APC or cancer cells can also involve several inhibitory/checkpoint molecules that reduce T cell activation, such as PD-1, CTLA-4, TIM-3, LAG-3, and KIR (Torphy RJ et al. *Int J Mol Sci.* 18:2642 (2017)). Thus, combining the effects of two antibodies against costimulatory or coinhibitory receptors can enhance an anti-tumor T-cell response.

[00116] The TWICE can comprise (after pairing) two functional antibodies against co-stimulatory or checkpoint molecules on T cells, leading to robust T-cell activation (Figure 1B, showing pairing of the black immune cell binding domains of the two components and pairing of the white complementary binding domains of the two components). Robust T-cell activation may be useful in patients when T cells are present in the tumor but are inactive due to immunosuppressive signals from the cancer cells (Wu AA et al. *OncoImmunology* 4:7 (2015)).

[00117] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, can engage an immune cell through agonistic or antagonistic binding to T cells.

[00118] By “agonistic,” it is meant that binding of paired immune cell binding or paired complementary binding domains to an antigen activates a signaling pathway. Agonistic binding may be of interest when binding to an antigen leads to immune cell activation.

[00119] By “antagonistic,” it is meant that binding of paired immune cell binding or paired complementary binding domains to an antigen blocks or inhibits a signaling pathway. Antagonistic binding may be of interest when binding of natural ligands to an antigen normally leads to immune cell deactivation. In this manner, binding of an “antagonistic” set of paired domains of the present TWICE can activate immune cells by blocking or inhibiting a pathway that normally mediates immune cell anergy or deactivation.

[00120] Antibodies known to have antagonist or agonist effects on immune cells can be used for generating paired binding domains of the present TWICE. If a signaling pathway promotes T-cell activation, an agonistic approach will increase T-cell activation. If a signaling pathway inhibits T-cell activation, an antagonistic approach will increase T-cell activation. Thus, many different types of pathways can be employed in this approach.

[00121] One example of an agonistic antibody is a CD3 epsilon antibody that stimulates the TCR complex (e.g. SP34, OKT3). In another example, agonistic antibodies against GITR may activate CD8⁺ T effector cells, while inhibiting regulatory T cell function (*see* Knee et al., *European Journal of Cancer* 67:1e10 (2016)). Examples of antagonistic antibodies that lead to the stimulation of immune cells are PD-1 antibodies (e.g., Pembrolizumab or Nivolumab). PD-1 inhibits the immune cell and thus these antibodies block the interaction of PD-1 with its ligands and thereby antagonize an inhibitory signal.

[00122] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, can generate two agonistic antibodies and provide two positive stimuli to the immune system (e.g., an anti-CD3 epsilon with an anti-CD137 antibody). Both CD3 and CD137 promote T-cell activation.

[00123] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, can generate one agonist antibody and one antagonist antibody (e.g., an anti-CD3 epsilon antibody and an anti-CTLA4 antibody). CD3, as discussed above, stimulates the TCR complex, while CTLA4 provides an inhibitory signal. Antagonizing the inhibitory signal of CTLA4 will promote T-cell activation.

[00124] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, can generate two antagonistic antibodies that block two inhibitory signals (e.g. anti-CTLA4 antibody and anti-PD-1 antibody). As discussed above, both of these signals inhibit T-cell activation and antagonizing them will promote T-cell activation.

[00125] Another exemplary TWICE combines a CD3 epsilon (CD3e) antibody with another antibody that promotes T-cell activation. Current evidence suggests that CD3 bispecific antibodies are further regulated through diverse checkpoint molecules (*see* Kobold S et al. *Front Oncol.* 8: 285 (2018)). In addition, the therapeutic effect of T-cell-activating bispecific antibodies may, in some patients or settings, also be restricted by induced T-cell anergy (the absence of normal immune response to a particular antigen). Thus, combining an anti-CD3 epsilon antibody that activates T cells with an antibody that potentiates T-cell activation and prevents anergy may generate an enhanced and/or more durable anti-tumor T-cell response.

2. TWICE that modulates two immune cells

[00126] In some embodiments, the immune cell binding domains, when paired together, and the complementary binding domains, when paired together, bind to two different immune cells. In this way, a TWICE may modulate two different types of immune cells. A wide range of different immune cells could be modulated by a TWICE, including the following representative examples. Figure 2 shows a representative model of activation of two immune cells (effector cell #1 and effector cell #2) via a TWICE.

a) TWICE that stimulates T cells and inhibit tumor-associated macrophages

[00127] Binding of two different immune cells may be useful in a tumor microenvironment, where T cells are excluded from the tumor and tumor-associated macrophages (TAM) can be present. TAMs represent a class of alternatively activated macrophages that facilitate tumor growth by promoting angiogenesis,

immunosuppression, and inflammation (Poh and Ernst, *Front Oncol.* 12(8):49 (2018)). In some embodiments, the TWICE, when the complementary binding domain and the immune cell binding domains are paired, combines activities by blocking TAM activity and activating T cells.

[00128] In some embodiments, the TWICE comprises immune cell binding domains, where paired together, that mediate T-cell activation and complementary binding domains, when paired together, that inhibit TAMs.

[00129] For example, targeting TAMs with an anti-CSF1R blocking antibody reduces TAM infiltration and promotes CD8⁺ T-cell expansion (Ries CH et al. *Cancer Cell.* 25(6):846-59 (2014)). Activation of the CD40 pathway can also interfere with the immunosuppressive effect of TAMs. A CD40 agonistic antibody can lead to the reprogramming or recruitment of classically activated macrophages, which may be used trigger effective immune responses against tumors. Representative TWICE that combine a T-cell activating antibody and a CD40 agonistic antibody or an anti-CSF1R blocking antibody, after pairing of the immune binding domains and the complementary binding domains, could provide additive efficacy compared to any of these antibodies alone.

b) TWICE that stimulates T cells and NK cells

[00130] A TWICE may be used to activate both T cells and NK cells to kill cancer cells. The coactivation of T cells and NK cells may lead to a more robust immune response to the cancer than activation of either cell alone.

[00131] In some embodiments, the TWICE comprises immune cell binding domains, where paired together, that mediate T-cell activation and complementary binding domains, when paired together, that mediate NK-cell activation such as with an anti-CD16A antibody. For example, in a TWICE comprising TWICE440 and TWICE441, the anti-CD16A antibody was combined with the anti-CD3 antibody OKT3 (See SEQ ID NOs: 212-215 and Figures 18A-18B).

3. TWICE that stimulates immune cells and mediate effects on cancer cells

[00132] A TWICE can also bind an immune cell with the paired immune cell binding domains and bind the cancer cell with the paired complementary binding domains. This therapeutic strategy can induce a biological function directly on the targeted cancer cell, as well as mediating anti-cancer immune cell activation.

The biological function induced in the cancer cell could be, for example, the induction of cell death via a death receptor or the inhibition of an immunosuppressive signal.

[00133] Paired immune cell binding domains that can activate immune cells have already been described, and TWICE components can also comprise complementary binding domains that bind cancer.

a) TWICE that stimulates immune cells and block immune cell deactivation by the cancer

[00134] The present TWICE may be used to both activate immune cells and block inhibitory signals from cancer cells to immune cells. In some embodiments, the complementary binding domains, when paired together, may block a signal from the cancer cell that inhibits immune cell activation.

[00135] For example, some cancers express PD-L1, which can bind PD-1 or B7.1 on T cells and lead to T-cell inactivation. In some embodiments, the complementary binding domains, when paired together, bind to PD-L1 on cancer cells. In some embodiments, binding to PD-L1 blocks interaction with PD-1 on T cells. Blocking the interaction of PD-1 and PD-L1 has been shown to improve anti-tumor responses. PD-L1 antibodies (such as Atezolizumab, Durvalumab, and Avelumab) are approved to treat several malignant diseases, including non-small cell lung cancer and melanoma.

[00136] In another example, some cancers can express tumor necrosis factor Receptor (TNFR) family members, which downregulate anti-tumor immune response. An example of such a molecule is receptor activator of nuclear factor kappa-B (RANK), which binds to its ligand, RANKL, on regulatory T cells to generate an immunosuppressive environment (*see* Wu et al. *Oncoimmunology*. 4(7) (2015)). In some embodiments, the complementary binding domains, when paired together, bind to RANK on cancer cells. In some embodiments, binding to RANK blocks interaction with RANKL on T cells. Blockade of RANK signaling by an antagonistic antibody against RANKL enhances the anti-tumor effect of an anti-CTLA4 antibody, which can activate T cells, in a preclinical mouse model of cancer (Ahern et al *Clin Cancer Res*. 23(19):5789-5801 (2017)).

b) TWICE that stimulates immune cells and trigger cell death of cancer cells

[00137] The present TWICE may also be used to both activate immune cells and trigger cell death of cancer cells. In some embodiments, the complementary

binding domains, when paired together, may trigger death of cancer cells by binding to receptors expressed by cancer cells.

[00138] Examples of receptors that can be stimulated to trigger cell death include members of the TNFR family, such as Fas/CD95/Apo1, TNFR1/p55/CD120a, DR3/Apo3/WSL-1/TRAMP/LARD, TRAIL-R1/DR4, DR5/Apo2/TRAIL-R2/TRICK2/KILLER, or CAR1. A TNFR family agonist antibody in clinical development that induces cell death in cancer cells is mapatumumab (NCT01258608). A TWICE that combines activity to directly kill cancer cells and activate an anti-cancer immune response could have additive efficacy.

4. TWICE comprising complementary functional domains

[00139] The first and/or second component of a TWICE can also comprise complementary functional domains. “Complementary functional domains,” as used herein, refers to molecules that modulate immune function when targeted to the appropriate immune cell, without needing to pair with another domain. Thus, a TWICE can target complementary functional domains to immune cells and facilitate activity of complementary functional domains that lack activity without targeting or may be too toxic to deliver systemically. Exemplary complementary functional domains are attenuated cytokines that must be targeted to appropriate immune cells to have activity. This embodiment is shown in Figure 5. Attenuated cytokines can preferentially activate target cells, as shown by data with bempegaldesleukin (NKTR-214), a CD-122-preferential IL-2 pathway agonist.

[00140] In this way, a TWICE can mediate additive anti-cancer immune responses by the activity of paired immune cell binding domains, as well activity from complementary functional domains—in effect delivering a “care-package” for effector cells that is only activated in the effector cell/cancer cell engagement synapse.

B. Single Polypeptide Chain or Two Components

[00141] A TWICE may comprise a single polypeptide chain or two separate components.

[00142] In some embodiments, the first component is not covalently bound to the second component. In some embodiments, the first component is covalently bound to the second component.

[00143] In some embodiments, the TWICE is comprised of two separate components. In other words, the TWICE can be comprised of a first and second component that are separate polypeptides.

[00144] In some components, the TWICE is comprised of a single polypeptide chain. In some embodiments, the first and second components are contained within a single amino acid sequence.

[00145] When the TWICE is comprised of a single polypeptide chain, the first and second components may be separated by a linker. In some embodiments, this linker covalently binds the first and second components. In some embodiments, this linker comprises a cleavable linker. In some embodiments, the cleavable linker between the first and second components comprises a protease cleavage site.

[00146] In some embodiments, a cleavage site comprised within a linker covalently binding a first component and the second component is a protease cleavage site. SEQ ID NOs: 1-84 list some exemplary protease cleavage sites that may be used, but the TWICE is not limited to this set of proteases cleavage sites and other protease cleavage sites may be employed.

[00147] In some embodiments, a cleavage site comprised within a linker covalently binding a first component and the second component is a tumor-associated protease cleavage site. A tumor associated-protease is one that is associated with a tumor. In some embodiments, a tumor-associated protease has higher expression in the tumor versus other regions of the body. Table 2 (shown in Section IV below) provides examples of tumor-associated proteases, although any protease with expression in a tumor may be used to select a tumor-associated protease cleavage site for the TWICE. In some embodiments, the protease is expressed by a non-cancer cell in the tumor microenvironment, such as a tumor-associated macrophage or fibroblast.

[00148] In some embodiments, a cleavage site comprised within a linker covalently binding a first component and the second component is a cleavage site for a protease found in the blood. Exemplary proteases found in the blood include thrombin, neutrophil elastase, and furin.

C. Targeting Moieties

[00149] The targeting moieties of the first components function by delivering the agent to the local environment of the cancer cells, enabling a localized treatment strategy. In some embodiments, the first targeting moiety targets the cancer cells by specifically binding to the cancer cells.

[00150] In some embodiments, both the first and second targeting moieties binds a tumor antigen expressed by the cancer.

[00151] In some embodiments, the first targeting moiety binds a tumor antigen expressed by the cancer and the second targeting moiety binds an antigen expressed by a tumor microenvironment cell.

[00152] In some embodiments, a targeting moiety is an antibody or antigen-binding fragment thereof. By antigen-binding fragment, we mean any antibody fragment that retains its binding activity to the target, such as an scFv or other functional fragment including an immunoglobulin devoid of light chains, VHH, VNAR, Fab, Fab', F(ab')₂, Fv, antibody fragment, diabody, scAB, single-domain heavy chain antibody, single-domain light chain antibody, Fd, CDR regions, or any portion or peptide sequence of the antibody that is capable of binding antigen or epitope. VHH and VNAR are alternatives to classical antibodies and even though they are produced in different species (camelids and sharks, respectively), we will also include them in antigen-binding fragments of antibodies. In some embodiments, an Fv domain is a disulfide-stabilized Fv (dsFv). Unless specifically noted as “full length antibody,” when the application refers to antibody it inherently includes a reference to an antigen-binding fragment thereof.

[00153] In some embodiments, a targeting moiety is not an antibody, but is another type of targeting moiety. A wide range of targeting moieties capable of targeting cancer are known, including DNA aptamers, RNA aptamers, albumins, lipocalins, fibronectins, ankyrins, fynomers, Obodies, DARPins, knotins, avimers, atrimers, anticallins, affilins, affibodies, bicyclic peptides, cys-knots, FN3 (adnectins, centryrins, pronectins, TN3), and Kunitz domains. These and other non-antibody scaffold structures may be used for targeting to a cancer cell. Smaller non-antibody scaffolds are rapidly removed from the bloodstream and have a shorter half-life than monoclonal antibodies. They also show faster tissue penetration owing to fast extravasation from the capillary lumen through the vascular endothelium and basement membrane. *See Vazquez-Lombardi et al., Drug Discovery Today* 20(1):1271-1283 (2015). A number of non-antibody scaffolds targeting cancer are already under clinical development, with other candidates in the preclinical stage. *See Vazquez-Lombardi, Table 1.*

1. Targeting Moiety Capable of Targeting the Cancer

[00154] In some embodiments, only the first targeting moiety binds an antigen expressed by the cancer, and the second targeting moiety does not. In some embodiments, both the first and second targeting moieties bind an antigen expressed by the cancer.

[00155] In some embodiments, the first and second targeting moieties are the same and bind the same antigen. In some embodiments, the first and second targeting moieties bind the same antigen expressed by the cancer. In some embodiments, the first and second targeting moieties bind different epitopes on the same antigen expressed by the cancer. In some embodiments, the first and second targeting moieties bind different antigens expressed by the cancer.

[00156] Certain tumor antigens that may be used for targeting (with examples of cancer cell types in parentheses) include: Her2/Neu (epithelial malignancies); CD22 (B cell malignancies); EpCAM (CD326) (epithelial malignancies); EGFR (epithelial malignancies); PSMA (prostate carcinoma); CD30 (B cell malignancies); CD20 (B cell malignancies); CD33 (myeloid malignancies); CD80 (B cell malignancies); CD86 (B cell malignancies); CD2 (T-cell or NK-cell lymphomas); CA125 (multiple cancers including ovarian carcinoma); Carbonic Anhydrase IX (multiple cancers including renal cell carcinoma); CD70 (B-cell malignancies); CD74 (B-cell malignancies); CD56 (T-cell or NK-cell lymphomas); CD40 (B-cell malignancies); CD19 (B-cell malignancies); c-met/HGFR (gastrointestinal tract and hepatic malignancies); TRAIL-R1/DR4 (multiple malignancies including ovarian and colorectal carcinoma); DRS (multiple malignancies including ovarian and colorectal carcinoma); PD-1 (B-cell malignancies); PD-L1 (multiple malignancies including epithelial adenocarcinoma); IGF-1R (most malignancies including epithelial adenocarcinoma); VEGF-R2 (the vasculature associated with the majority of malignancies including epithelial adenocarcinomas; prostate stem cell antigen (PSCA) (prostate adenocarcinoma); MUC1 (epithelial malignancies); CanAg (tumors such as carcinomas of the colon and pancreas); Mesothelin (many tumors including mesothelioma and ovarian and pancreatic adenocarcinoma); P-cadherin (epithelial malignancies, including breast adenocarcinoma); Myostatin (GDF8) (many tumors including sarcoma and ovarian and pancreatic adenocarcinoma); Cripto (TDGF1) (epithelial malignancies including colon, breast, lung, ovarian, and pancreatic cancers); ACVRL 1/ALK1 (multiple

malignancies including leukemias and lymphomas); MUC5AC (epithelial malignancies, including breast adenocarcinoma); CEACAM (epithelial malignancies, including breast adenocarcinoma); CD137 (B-cell or T-cell malignancies); CXCR4 (B-cell or T-cell malignancies); Neuropilin 1 (epithelial malignancies, including lung cancer); Glypicans (multiple cancers including liver, brain and breast cancers); HER3/ERBB3 (epithelial malignancies); PDGFRa (epithelial malignancies); EphA2 (multiple cancers including neuroblastoma, melanoma, breast cancer, and small cell lung carcinoma); CD38 (myeloma); CD138 (myeloma); α 4-integrin (AML, myeloma, CLL, and most lymphomas); CCR4 antibody (lymphoma); CD52 (leukemia); CD79b (lymphoma); CTLA-4 (multiple cancers including non-small cell lung, head and neck, urothelial cancer, and hepatocellular carcinoma); DLL3 (lung cancer); endoglin (multiple cancers including soft tissue sarcoma, angiosarcoma, and renal cell carcinoma); fibronectin extra-domain B (melanoma); folate receptor 1 (multiple cancers including epithelial ovarian cancer, peritoneal carcinoma, and fallopian tube cancer); MMP-9 (gastric cancer or gastroesophageal junction adenocarcinoma); NGcGM3 (lung cancer); SLAMF7 (multiple myeloma); TROP-2 (breast cancer); or VEGF (colorectal cancer).

[00157] In some embodiments, the targeting moiety binds α 4-integrin; A33; ACVRL 1/ALK1; ADAM17; ALK; APRIL; BCMA; C242; CA125; Cadherin-19; CAIX; CanAg; Carbonic Anhydrase IX; CCN1; CCR4; CD123; CD133; CD137 (4-1BB); CD138/Syndecan1; CD19; CD2; CD20; CD22; CD30; CD33; CD37; CD38; CD4; CD40; CD44; CD45; CD48; CD5; CD52; CD56; CD59; CD70; CD70b; CD71; CD74; CD79b; CD80; CD86; CD98; CEA; CEACAM; CEACAM1; CK8; c-Kit; CLDN1; CLDN18; CLDN18.2; CLDN6; c-met/HGFR; c-RET; Cripto; CTLA-4; CXCR4; DKK-1; DLL3; DLL4; TRAIL-R2/DR5; DRS; EGFL7; EGFR; EGFRvIII; endoglin; ENPP3; EpCAM; EphA2; Episialin; FAP; FGFR1; FGFR2; FGFR3; FGFR4; fibronectin extra-domain B; FLT-3; flt4; folate receptor 1; GCC; GD2; GD3; Glypican-3; Glypicans; GM3; GPNMB; GPR49; GRP78; Her2/Neu; HER3/ERBB3; HLA-DR; ICAM-1; IGF-1R; IGFR; IL-3Ra; Integrin α 5 β 1; Integrin α 6 β 4; Integrin α V; Integrin α V β 3; Lewis Y; Lewis y/b antigen; LFL2; LIV-1; Ly6E; MCP-1; Mesothelin; MMP-9; MUC1; MUC18; MUC5A; MUC5AC; Myostatin; NaPi2b; Neuropilin 1; NGcGM3; NRP1; P-cadherin; PCLA; PD-1; PDGFRa; PD-L1; PD-L2; Phosphatidylserine; PIVKA-II; PLVAP; PRLR; Progastrin; PSCA; PSMA; RANKL; RG1; Siglec-15; SLAMF6; SLAMF7; SLC44A4; STEAP-1; TACSTD-2; Tenascin C;

TPBG; TRAIL-R1/DR4; TROP-2; TWEAKR; TYRP1; VANGL2; VEGF; VEGF-C; VEGFR-2; or VEGF-R2.

[00158] Table 2 (shown in Section IV below) provides nonlimiting examples of cancer types, possible targets for targeting moieties, and proteases that are expressed by those cancer types. A protease associated with a cancer may be termed a tumor-associated protease. In order to prepare a TWICE that needs cleavage at the site of the tumor, the cancer may be identified and a target chosen for the targeting moiety (as desired), and one or two proteases chosen for the cancer type or the desired tumor microenvironment cell (such as a TAM or TAF). Not every TWICE requires protease cleavage, but protease cleavage may be beneficial for a TWICE that is generated as single molecules with a cleavage site or for a TWICE comprising inert binding partners.

[00159] Table 3 (shown in Section IV below) provides additional information about cancers that may be targeting with different targeting moieties, including the fact that some targeting moieties may be able to target a number of different types of cancer. In a TWICE, both the first component and the second component may comprise a targeting moiety capable of targeting a cancer. The targeting moiety of the first component and the second component may bind to the same antigen or to different antigens.

[00160] Antibodies that bind tumor antigens and that have specificity for tumor cells are well-known in the art.

[00161] The FDA maintains listings of approved antibody drugs for treating cancer, many of which bind to cancer antigens and can be employed in this context. *See* The Orange Book Online or Drugs@FDA on the FDA website. The FDA also maintains listings of clinical trials in progress in the clinicaltrials.gov database, which may be searched by disease names. Table 4 (shown in Section IV below) provides a representative list of approved antibodies with specificity for tumor cells. Table 5 (shown in Section IV below) provides a representative list of antibodies in development with specificity for tumor cells.

[00162] Table 6 (shown in Section IV below) summarizes selected publications on exemplary antibodies that bind tumor antigens and that could be used as targeting moieties in the TWICE. These publications show that targeting moieties capable of binding tumor antigens were well within both the skill in the art and the possession of the inventors at the time of filing of the present application.

[00163] Other antibodies well-known in the art may be used as targeting moieties to target to a given cancer. The antibodies and their respective antigens include nivolumab (anti-PD-1 Ab), TA99 (anti-gp75), 3F8 (anti-GD2), 8H9 (anti-B7-H3), abagovomab (anti-CA-125 (imitation)), adecatumumab (anti-EpCAM), afutuzumab (anti-CD20), alacizumab pegol (anti-VEGFR2), altumomab pentetate (anti-CEA), amatuximab (anti-mesothelin), AME-133 (anti-CD20), anatumomab mafenatox (anti-TAG-72), apolizumab (anti-HLA-DR), arcitumomab (anti-CEA), bavituximab (anti-phosphatidylserine), bectumomab (anti-CD22), belimumab (anti-BAFF), besilesomab (anti-CEA-related antigen), bevacizumab (anti-VEGF-A), bivatumumab mertansine (anti-CD44 v6), blinatumomab (anti-CD19), BMS-663513 (anti-CD137), brentuximab vedotin (anti-CD30 (TNFRSF8)), cantuzumab mertansine (anti-mucin CanAg), cantuzumab raptansine (anti-MUC1), capromab pendetide (anti-prostatic carcinoma cells), carlumab (anti-MCP-1), catumaxomab (anti-EpCAM, CD3), cBR96-doxorubicin immunoconjugate (anti-Lewis-Y antigen), CC49 (anti-TAG-72), cedelizumab (anti-CD4), Ch.14.18 (anti-GD2), ch-TNT (anti-DNA associated antigens), citatuzumab bogatox (anti-EpCAM), cixutumumab (anti-IGF-1 receptor), clivatuzumab tetraxetan (anti-MUC1), conatumumab (anti-TRAIL-R2), CP-870893 (anti-CD40), dacetuzumab (anti-CD40), daclizumab (anti-CD25), dalotuzumab (anti-insulin-like growth factor I receptor), daratumumab (anti-CD38 (cyclic ADP ribose hydrolase)), demcizumab (anti-DLL4), detumomab (anti-B-lymphoma cell), drozitumab (anti-DR5), duligotumab (anti-HER3), dusigitumab (anti-ILGF2), ecromeximab (anti-GD3 ganglioside), edrecolomab (anti-EpCAM), elotuzumab (anti-SLAMF7), elsilimomab (anti-IL-6), enavatuzumab (anti-TWEAK receptor), enoticumab (anti-DLL4), ensituximab (anti-5AC), epitumomab cituxetan (anti-episialin), epratuzumab (anti-CD22), ertumaxomab (anti-HER2/neu, CD3), etaracizumab (anti-integrin $\alpha v \beta 3$), faralimomab (anti-Interferon receptor), farletuzumab (anti-folate receptor 1), FBTA05 (anti-CD20), ficlatuzumab (anti-HGF), figitumumab (anti-IGF-1 receptor), flanvotumab (anti-TYRP1(glycoprotein 75)), fresolimumab (anti-TGF β), futuximab (anti-EGFR), galiximab (anti-CD80), ganitumab (anti-IGF-I), gemtuzumab ozogamicin (anti-CD33), girentuximab (anti-carbonic anhydrase 9 (CA-IX)), glembatumumab vedotin (anti-GPNMB), guselkumab (anti-IL13), ibalizumab (anti-CD4), ibritumomab tiuxetan (anti-CD20), icrucumab (anti-VEGFR-1), igovomab (anti-CA-125), Zolbetuximab (IMAB362, an

anti-CLDN18.2), IMC-CS4 (anti-CSF1R), IMC-TR1 (TGF β R2), imgatuzumab (anti-EGFR), inclacumab (anti-selectin P), indatuximab ravtansine (anti-SDC1), inotuzumab ozogamicin (anti-CD22), intetumumab (anti-CD51), ipilimumab (anti-CD152), iratumumab (anti-CD30 (TNFRSF8)), KM3065 (anti-CD20), KW-0761 (anti-CD194), LY2875358 (anti-MET) labetuzumab (anti-CEA), lambrolizumab (anti-PDCD1), lexatumumab (anti-TRAIL-R2), lintuzumab (anti-CD33), lirilumab (anti-KIR2D), lorvotuzumab mertansine (anti-CD56), lucatumumab (anti-CD40), lumiliximab (anti-CD23 (IgE receptor)), mapatumumab (anti-TRAIL-R1), margetuximab (anti-ch4D5), matuzumab (anti-EGFR), mavrilimumab (anti-GMCSF receptor α -chain), milatuzumab (anti-CD74), minretumomab (anti-TAG-72), mitumomab (anti-GD3 ganglioside), mogamulizumab (anti-CCR4), moxetumomab pasudotox (anti-CD22), nacolomab tafenatox (anti-C242 antigen), naptumomab estafenatox (anti-5T4), narnatumab (anti-RON), necitumumab (anti-EGFR), nesvacumab (anti-angiopoietin 2), nimotuzumab (anti-EGFR), nivolumab (anti-IgG4), nofetumomab merpentan, ocrelizumab (anti-CD20), ocaratuzumab (anti-CD20), olaratumab (anti-PDGF-R α), onartuzumab (anti-c-MET), ontuxizumab (anti-TEM1), oportuzumab monatox (anti-EpCAM), oregovomab (anti-CA-125), otlertuzumab (anti-CD37), pankomab (anti-tumor specific glycosylation of MUC1), parsatuzumab (anti-EGFL7), pascolizumab (anti-IL-4), patritumab (anti-HER3), pemtumomab (anti-MUC1), pertuzumab (anti-HER2/neu), pidilizumab (anti-PD-1), pinatuzumab vedotin (anti-CD22), pintumomab (anti-adenocarcinoma antigen), polatuzumab vedotin (anti-CD79B), pritumumab (anti-vimentin), PRO131921 (anti-CD20), quilizumab (anti-IGHE), racotumomab (anti-N-glycolylneuraminic acid), radretumab (anti-fibronectin extra domain-B), ramucirumab (anti-VEGFR2), rilotumumab (anti-HGF), robatumumab (anti-IGF-1 receptor), roledumab (anti-RHD), rovelizumab (anti-CD11 & CD18), samalizumab (anti-CD200), satumomab pendetide (anti-TAG-72), seribantumab (anti-ERBB3), SGN-CD19A (anti-CD19), SGN-CD33A (anti-CD33), sibrotuzumab (anti-FAP), siltuximab (anti-IL-6), solitomab (anti-EpCAM), sontuzumab (anti-episialin), tabalumab (anti-BAFF), tacatuzumab tetraxetan (anti- α -fetoprotein), taplitumomab paptox (anti-CD19), telimomab aritox, tenatumomab (anti-tenascin C), teneliximab (anti-CD40), teprotumumab (anti-CD221), TGN1412 (anti-CD28), ticilimumab (anti-CTLA-4), tigatuzumab (anti-TRAIL-R2), TNX-650 (anti-IL-13), tositumomab (anti-CS20), tovetumab (anti-CD140a), TRBS07 (anti-GD2), tregalizumab (anti-CD4), tremelimumab (anti-CTLA-

4), TRU-016 (anti-CD37), tucotuzumab celmoleukin (anti-EpCAM), ublituximab (anti-CD20), urelumab (anti-4-1BB), vantictumab (anti-Frizzled receptor), vapaliximab (anti-AOC3 (VAP-1)), vatelizumab (anti-ITGA2), veltuzumab (anti-CD20), vesencumab (anti-NRP1), visilizumab (anti-CD3), volociximab (anti-integrin $\alpha 5\beta 1$), vorsetuzumab mafodotin (anti-CD70), votumumab (anti-tumor antigen CTAA16.88), zalutumumab (anti-EGFR), zanolimumab (anti-CD4), zatuximab (anti-HER1), ziralimumab (anti-CD147 (basigin)), RG7636 (anti-ETBR), RG7458 (anti-MUC16), RG7599 (anti-NaPi2b), MPDL3280A (anti-PD-L1), RG7450 (anti-STEAP1), TAK-164 (anti-GCC), and GDC-0199 (anti-Bcl-2).

[00164] Antibodies that bind these antigens may also be used in the TWICE, especially for the types of cancers noted: aminopeptidase N (CD13), annexin A1, B7-H3 (CD276, various cancers), CA125 (ovarian cancers), CA15-3 (carcinomas), CA19-9 (carcinomas), L6 (carcinomas), Lewis Y (carcinomas), Lewis X (carcinomas), alpha fetoprotein (carcinomas), CA242 (colorectal cancers), placental alkaline phosphatase (carcinomas), prostate specific antigen (prostate), prostatic acid phosphatase (prostate), epidermal growth factor (carcinomas), CD2 (Hodgkin's disease, NHL lymphoma, multiple myeloma), CD3 epsilon (T-cell lymphoma, lung, breast, gastric, ovarian cancers, autoimmune diseases, malignant ascites), CD19 (B cell malignancies), CD20 (non-Hodgkin's lymphoma, B-cell neoplasmas, autoimmune diseases), CD21 (B-cell lymphoma), CD22 (leukemia, lymphoma, multiple myeloma, SLE), CD30 (Hodgkin's lymphoma), CD33 (leukemia, autoimmune diseases), CD38 (multiple myeloma), CD40 (lymphoma, multiple myeloma, leukemia (CLL)), CD51 (metastatic melanoma, sarcoma), CD52 (leukemia), CD56 (small cell lung cancers, ovarian cancer, Merkel cell carcinoma, and the liquid tumor, multiple myeloma), CD66e (carcinomas), CD70 (metastatic renal cell carcinoma and non-Hodgkin lymphoma), CD74 (multiple myeloma), CD80 (lymphoma), CD98 (carcinomas), CD123 (leukemia), mucin (carcinomas), CD221 (solid tumors), CD22 (breast, ovarian cancers), CD262 (NSCLC and other cancers), CD309 (ovarian cancers), CD326 (solid tumors), CEACAM3 (colorectal, gastric cancers), CEACAM5 (CEA, CD66e) (breast, colorectal and lung cancers), DLL4 (A-like-4), EGFR (various cancers), CTLA4 (melanoma), CXCR4 (CD 184, heme-oncology, solid tumors), Endoglin (CD 105, solid tumors), EPCAM (epithelial cell adhesion molecule, bladder, head, neck, colon, NHL prostate, and ovarian cancers), ERBB2 (lung, breast, prostate cancers), FCGR1 (autoimmune diseases), FOLR (folate receptor, ovarian cancers), FGFR (carcinomas),

GD2 ganglioside (carcinomas), G-28 (a cell surface antigen glycolipid, melanoma), GD3 idiotype (carcinomas), heat shock proteins (carcinomas), HER1 (lung, stomach cancers), HER2 (breast, lung and ovarian cancers), HLA-DR10 (NHL), HLA-DRB (NHL, B cell leukemia), human chorionic gonadotropin (carcinomas), IGF1R (solid tumors, blood cancers), IL-2 receptor (T-cell leukemia and lymphomas), IL-6R (multiple myeloma, RA, Castleman's disease, IL6 dependent tumors), integrins ($\alpha\beta3$, $\alpha5\beta1$, $\alpha6\beta4$, $\alpha11\beta3$, $\alpha5\beta5$, $\alpha\nu\beta5$, for various cancers), MAGE-1 (carcinomas), MAGE-2 (carcinomas), MAGE-3 (carcinomas), MAGE 4 (carcinomas), anti-transferrin receptor (carcinomas), p97 (melanoma), MS4A1 (membrane-spanning 4-domains subfamily A member 1, Non-Hodgkin's B cell lymphoma, leukemia), MUC1 (breast, ovarian, cervix, bronchus and gastrointestinal cancer), MUC16 (CA125) (ovarian cancers), CEA (colorectal cancer), gp100 (melanoma), MART1 (melanoma), MPG (melanoma), MS4A1 (membrane-spanning 4-domains subfamily A, small cell lung cancers, NHL), nucleolin, Neu oncogene product (carcinomas), P21 (carcinomas), nectin-4 (carcinomas), paratope of anti-(N-glycolylneuraminic acid, breast, melanoma cancers), PLAP-like testicular alkaline phosphatase (ovarian, testicular cancers), PSMA (prostate tumors), PSA (prostate), ROB04, TAG 72 (tumour associated glycoprotein 72, AML, gastric, colorectal, ovarian cancers), T-cell transmembrane protein (cancers), Tie (CD202b), tissue factor, TNFRSF10B (tumor necrosis factor receptor superfamily member 10B, carcinomas), TNFRSF13B (tumor necrosis factor receptor superfamily member 13B, multiple myeloma, NHL, other cancers, RA and SLE), TPBG (trophoblast glycoprotein, renal cell carcinoma), TRAIL-R1 (tumor necrosis apoptosis inducing ligand receptor 1, lymphoma, NHL, colorectal, lung cancers), VCAM-1 (CD106, Melanoma), VEGF, VEGF-A, VEGF-2 (CD309) (various cancers). Some other tumor associated antigen targets have been reviewed (Gerber, et al, mAbs 2009 1:247-253; Novellino et al, Cancer Immunol Immunother. 2005 54:187-207, Franke, et al, Cancer Biother Radiopharm. 2000, 15:459-76, Guo, et al., Adv Cancer Res. 2013; 119: 421-475, Parmiani et al. J Immunol. 2007 178:1975-9). Examples of these antigens include Cluster of Differentiations (CD4, CDS5, CD6, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CD12w, CD14, CD15, CD16, CDw17, CD18, CD21, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD31, CD32, CD34, CD35, CD36, CD37, CD41, CD42, CD43, CD44, CD45, CD46, CD47, CD48, CD49b, CD49c, CD53, CD54, CD55, CD58, CD59, CD61, CD62E, CD62L, CD62P, CD63, CD68, CD69, CD71, CD72, CD79,

CD81, CD82, CD83, CD86, CD87, CD88, CD89, CD90, CD91, CD95, CD96, CD100, CD103, CD105, CD106, CD109, CD117, CD120, CD127, CD133, CD134, CD135, CD138, CD141, CD142, CD143, CD144, CD147, CD151, CD152, CD154, CD156, CD158, CD163, CD166, .CD168, CD184, CDw186, CD195, CD202 (a, b), CD209, CD235a, CD271, CD303, CD304), annexin A1, nucleolin, endoglin (CD105), ROB04, amino-peptidase N, -like-4 (DLL4), VEGFR-2 (CD309), CXCR4 (CD184), Tie2, B7-H3, WT1, MUC1, LMP2, HPV E6 E7, EGFRvIII, HER-2/neu, idiotype, MAGE A3, p53 nonmutant, NY-ESO-1, GD2, CEA, MelanA/MART1, Ras mutant, gp100, p53 mutant, proteinase3 (PR1), bcr-abl, tyrosinase, survivin, hTERT, sarcoma translocation breakpoints, EphA2, PAP, ML-IAP, AFP, EpCAM, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, ALK, androgen receptor, cyclin B 1, polysialic acid, MYCN, RhoC, TRP-2, GD3, fucosyl GM1 , mesothelin, PSCA, MAGE A1, sLe(a), CYPIB I, PLAC1, GM3, BORIS, Tn, GloboH, ETV6-AML, NY-BR-1, RGS5, SART3, STn, carbonic anhydrase IX, PAXS, OY-TES1, sperm protein 17, LCK, HMWMAA, AKAP-4, SSX2, XAGE 1, B7H3, legumain, Tie 2, Page4, VEGFR2, MAD-CT-1, FAP, PDGFR- β , MAD-CT-2, and Fos-related antigen 1.

[00165] In some embodiments, the targeting moiety capable of targeting a cancer is not an antibody, but is another type of targeting moiety. Table 7 (shown in Section IV below) presents some representative non-antibody targeting moieties and their corresponding targets.

[00166] In another embodiment, a targeting moiety may be a binding partner for a protein known to be expressed on the cancer cell. Such expression levels may include overexpression. For example, the binding partners described in Table 8 (shown in Section IV below) may bind to the following targets on a cancer cell.

[00167] The binding partner need not comprise the full length or wildtype sequence for the binding partners listed in Table 8. All that is required is that the binding partner bind to the target on the cancer cell and can thus include truncated forms, analogs, variants, and derivatives that are well known in the art.

[00168] Additionally, in some embodiments, the binding partner may be an aptamer that is capable of binding to a protein known to be expressed on the cancer cell. Aptamers that bind cancer cells are well known and methods for designing them are known.

[00169] Cell-based SELEX systems may be used to select a panel of target cell-specific aptamers from a random candidate library. A ssDNA or ssRNA pool may be

dissolved in binding buffer and denatured and then incubated with target cells. After washing the bound DNAs or RNAs may be eluted by heating and then incubated with negative cells (if desired), centrifuged, and the supernatant removed. The supernatant may be amplified by PCR with biotin labeled primers. The selected sense ssDNA or ssRNA may be separated from the antisense biotinylated strand using streptavidin coated beads. To increase affinity, washing strength may be increased through increasing washing time, volume of buffer, and number of washes. After the desired rounds of selection, the selected ssDNA or ssRNA pool may be PCR amplified and cloned into *E. coli* and sequenced. *See* Shangguan et al., Aptamers evolved from live cells as effective molecular probes for cancer study, *PNAS* 103(32):11838-11843 (2006); Lyu et al, Generating Cell Targeting Aptamers for Nanotherapeutics Using Cell-SELEX, *Theranostics* 6(9):1440-1452 (2016); *see also* Li et al., Inhibition of Cell Proliferation by an Anti-EGFR Aptamer, *PLoS One* 6(6):e20229 (2011). The specific approaches for designing aptamers and specific aptamers binding to cancer cells in these references are hereby incorporated by reference.

[00170] For example, an aptamer may comprise SEQ ID NO: 94 to 164. In some embodiments, an aptamer may comprise SEQ ID NO: 95. These aptamers are directed to EGFR and are provided only as representative of the aptamers that can bind to targets presented on cancer cells. Other aptamers against other targets on cancer cells are equally part of the description herein and incorporated by reference as described in Zhu et al., *Theranostics* 4(9):931-944 (2014).

[00171] In some embodiments, aptamers for use herein bind to the target on the cancer cell with a K_d in the nanomolar to picomolar range (such as 1 picomolar to 500 nanomolar or 1 picomolar to 100 nanomolar).

2. Targeting Moiety Capable of Targeting a Tumor Microenvironment Cell

[00172] Current cancer immunotherapeutic antibodies require effector cells to be present within the tumor. However, tumors can generate an immunosuppressive environment that excludes effector cells, rendering immunotherapy less effective (*see* Herbst et al *Nature*. 515(7528):563-7 (2014)). Tumors devoid of effector cells may be termed “cold tumors” (*see* Whiteside TL et al. *Clin Cancer Res*. 22(8):1845-55 (2016)).

[00173] Effector cells may be excluded from the tumors by the tumor stroma, in particular by non-cancer cells in the tumor microenvironment, such as

tumor-associated fibroblasts (TAFs) (*see* Ziani et al, *Front Immunol.* 9:414 (2018)). A non-cancer cell in the tumor microenvironment may be termed a “tumor microenvironment cell.” Since T cells in cold tumors can be restricted to the stroma, immunotherapeutics targeted to the interface of malignant cells and the stroma could potentially overcome immune exclusion. Targeting the tumor microenvironment cell with the second target moiety may therefore provide additional specificity for the present kit or composition.

[00174] In some embodiments, the first targeting moiety binds an antigen expressed by the cancer, and the second targeting moiety binds an antigen expressed by a tumor microenvironment cell. In some embodiments, the tumor microenvironment cell is a fibroblast or macrophage. These cells may be termed tumor-associated fibroblasts or cancer-associated fibroblasts (TAFs or CAFs) or tumor-associated macrophages or cancer-associated macrophages (TAMs or CAMs). As used herein, a “tumor-associated fibroblast” refers to a fibroblast found in close proximity to or within a tumor mass. As used herein, a “tumor-associated macrophage” refers to a macrophage found in close proximity to or with a tumor mass.

[00175] Figure 2 shows targeting of one component of a TWICE to a cancer cell and targeting of the other component to a non-cancer cell in the tumor microenvironment. With this type of TWICE, the immune binding domains of the two components and/or the complementary binding domains of the two components would only pair when the cancer cells and the non-cancer tumor microenvironment cell were in close proximity.

[00176] TAFs are characterized by the expression of activation markers such as fibroblast activation protein alpha (FAP) and alpha-smooth muscle actin (*see* Barnett and Vilar, *J Natl Cancer Inst* 110(1):11-13 (2018)) and may create an immunosuppressive environment by direct or indirect mechanisms. Direct immunosuppressive effects may be mediated by growth factors such as TGF-beta or cytokines such as CXCL12, and the indirect immunosuppressive effects may be mediated by remodeling of the extracellular matrix in the tumor microenvironment (*see* Ziani et al, 2018).

[00177] In some embodiments, the targeting moiety of the second component binds to an antigen expressed by TAFs. In some embodiments, the targeting moiety targeting to TAFs comprises an antibody that binds FAP such as

sibrotuzumab (*see* US20120258119A1). FAP is expressed mostly expressed on tumor stroma and not on normal fibroblasts (Brennen et al. *Mol Cancer Ther.* 11(2): 257–266 (2012)).

[00178] In some embodiments, the targeting moiety of the second component binds to an antigen expressed by TAMs. In some embodiments, the targeting moiety targeting to TAMs comprises an antibody that binds MAC-1/CD11b (*see* EP0488061A2) or sideroflexin 3 (*see* WO2018020000).

D. Immune cell binding domains

[00179] The first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding an antigen on an immune cell. As used herein, an “immune cell” can be any cell comprised in the immune system. The immune cell may be a cell involved in the innate or adaptive immune response.

[00180] In some embodiments, the immune cell is a natural killer cell (NK), macrophage, or T cell. In some embodiments, the T cell is a $\gamma\delta$ T cell or a natural killer T cell (NKT cells).

1. T-cell binding domains

[00181] In some embodiments, the first T-cell binding domain and the second T-cell binding domain are, when bound to each other, capable of binding a T cell. In some embodiments, the first T-cell binding domain and the second T-cell binding domain are, when bound to each other, capable of binding CD3 or the T-cell receptor (TCR).

[00182] CD3 is present on all T cells and consists of subunits designated γ , δ , ϵ , ζ , and η . TCR is another molecule present on T cells that consists of different subunits designated α , β , γ , and δ . The cytoplasmic tail of CD3 is sufficient to transduce the signals necessary for T-cell activation in the absence of the other components of the TCR receptor complex. Normally, activation of T-cell cytotoxicity depends first on binding of the TCR with a major histocompatibility complex (MHC) protein, itself bound to a foreign antigen, located on a separate cell. Without outside intervention, only when this initial TCR-MHC binding has taken place can the CD3 dependent signally cascade responsible for T-cell clonal expansion and, ultimately, T-cell cytotoxicity ensue. In some of the present embodiments, however, when the first T-cell binding domain and the second T-cell binding domain bind to CD3 and/or the TCR, activation of cytotoxic T cells in the absence of independent TCR-MHC can

take place by virtue of the crosslinking of the CD3 and/or TCR molecules mimicking an immune synapse formation. This means that T cells may be cytotoxically activated in a clonally independent fashion, i.e. in a manner that is independent of the specific TCR clone carried by the T cell. This allows for activation of the entire T-cell compartment rather than only specific T cells of a certain clonal identity.

[00183] In some embodiments, the first T-cell binding domain is a VH domain and the second T-cell binding domain is a VL domain. In some embodiments, the first T-cell binding domain is a VL domain and the second T-cell binding domain is a VH domain. In some embodiments, the first and second T-cell binding domains when paired together may comprise an Fv. As used herein, an “Fv” refers to a VH and VL associated together. In other words, when paired together the first and second T-cell binding domains may comprise an “scFv,” except for the fact that the VH and VL are not in a single-chain configuration with a linker between the VH and VL.

[00184] If the first and second T-cell binding domains are a pair of VH and VL domains, the VH and VL domains may be specific for an antigen expressed on the surface of a T cell, such as CD3 or TCR. In some embodiments, the anti-CD3 or anti-TCR antibody binds to a subunit of CD3 or TCR. If the antigen is CD3, one potential T-cell binding domain may be derived from muromonab (muromonab-CD3 or OKT3), oteelixizumab, teplizumab, visilizumab, foralumab, SP34, or blinatumomab. One skilled in the art would be aware of a wide range of anti-CD3 antibodies, some of which are approved therapies or have been clinically tested in human patients (*see* Kuhn and Weiner *Immunotherapy* 8(8):889-906 (2016)). Table 9 (shown in Section IV below) presents selected publications on exemplary anti-CD3 antibodies. Antibodies with specificity to the TCR, including the $\alpha\beta$ and $\gamma\delta$ TCRs, are also well-known. Table 10 (shown in Section IV below) presents selected publications on exemplary anti-TCR antibodies.

[00185] T-cell binding domains can also include any antibodies listed as potential complementary binding domains, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, GITR (TNFRSF18), TIGIT, or inducible T-cell costimulatory (ICOS).

2. Natural killer cell binding domains

[00186] In some embodiments, the first and second immune cell binding domains, when paired together, are capable of activating natural killer (NK) cells. In some embodiments, the first and second immune cell binding domains, when paired together, are capable of binding CD16A. In some embodiments, binding of paired immune cell binding domains to CD16A on NK cells activates NK cells against CD33-positive leukemia cells. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of NTM-1633 (*see* NCT03603665) or AFM13 (*see* NCT01221571).

3. Macrophage binding domains

[00187] In some embodiments, the first and second immune cell binding domains, when paired together, are capable of binding macrophages. In some embodiments, the macrophages are activated macrophages.

[00188] In some embodiments, the first and second immune cell binding domains, when paired together, are capable of binding CSF1R. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of Emactuzumab/RG7155 (NCT01494688) or IMC-CS4 (NCT01346358).

[00189] In some embodiments, the first and second immune cell binding domains, when paired together, are capable of binding CD40. In some embodiments, the first and second immune cell binding domains comprise all or part of a VH and/or VL of CP-870,893.

E. Complementary binding domains

[00190] In some embodiments, the first and second components comprise complementary binding domains. When the complementary binding domains of the two components are paired, this leads to an additional function. This additional function may be, for example, to induce death of cancer cells or increase the anti-tumor immune response. The additional function may also serve to make the tumor microenvironment more hospitable to immune cells.

[00191] Because the two components of the TWICE are targeted to the cancer or to the tumor microenvironment, the additional function mediated by pairing of the complementary binding domains would happen in the tumor or its microenvironment. In some embodiments, the additional function mediated by the paired complementary binding domains is limited to the tumor and its

microenvironment, thereby reducing off-targets effects. For example, this ability to limit activity to the tumor and its microenvironment may be an advantage for activating or inhibiting a target molecule with widespread expression and/or a range of physiologic functions, such as TGF-beta.

[00192] In some embodiments, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding the same antigen. For example, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, may bind CD3 on T cells. Binding of both the paired complementary binding domains and the paired immune cell binding domains to CD3 may lead to more robust activation of an anti-cancer immune response.

[00193] In some embodiments, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding different antigens on the same cell. For example, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, may bind different antigens on T cells. Binding of both the paired complementary binding domains and the paired immune cell binding domains to two different antigens on T cells may lead to robust activation of an anti-cancer immune response.

[00194] In some embodiments, the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding different cells. For example, the first complementary binding domain and the second complementary binding domain, when bound to each other, may bind a cancer cell to lead to cell death and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, may bind a T cell to mediate an anti-cancer immune response. Activation of a cell death pathways in the tumor cell *and* activation of an anti-cancer immune response may increase tumor regression.

[00195] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of modulating T-cell, macrophage, or NK-cell activity. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a costimulatory antigen or a coinhibitory antigen on T cells.

[00196] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of cell-death-inducing activity directed towards the cancer. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a cell-death-inducing antigen.

[00197] In some embodiments, the first complementary binding domain is a VH domain and the second complementary binding domain is a VL domain. In some embodiments, the first complementary binding domain is a VL domain and the second complementary binding domain is a VH domain. In some embodiments, the first and second complementary binding domains when paired together may comprise an Fv. In other words, when paired together the first and second complementary binding domains may comprise an “scFv,” except for the fact that the VH and VL are not in a single-chain configuration.

[00198] In some embodiments, the VH domain of a known antibody can be used as the first complementary binding domain, while the VL domain can be used as the second complementary binding domain.

[00199] In some embodiments, the VL domain of a known antibody can be used as the first complementary binding domain, while the VH domain can be used as the second complementary binding domain.

1. Complementary binding domains that modulate T-cell activity

[00200] In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of modulating TCR activity. In some embodiments, the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T-cell co-signaling molecule. T-cell co-signaling molecules have been well characterized in the literature (see Chen L and Flies DB *Nat*

Rev Immunol 13(4): 227–242 (2013)) and include costimulatory and coinhibitory antigens. In some embodiments, the co-signaling molecules are members of the immunoglobulin superfamily (IgSF) or tumor necrosis factor receptor superfamily (TNFRSF). A coinhibitory antigen may also be termed an immune checkpoint molecule.

[00201] Table 11 (shown in Section IV below) provides examples of clinically studied antibodies that can induce anti-tumor responses by modulating T-cell function.

[00202] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD3e. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of muromonab, oteelixizumab, teplizumab, visilizumab, foralumab, SP34, or blinatumomab.

[00203] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding programmed cell death protein 1 (PD-1). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of pembrolizumab or nivolumab.

[00204] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of ipilimumab.

[00205] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding T-cell immunoglobulin and mucin-domain 3 (TIM-3). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of TSR-022 or Sym023.

[00206] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding lymphocyte-activation gene 3 (LAG-3). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of BMS-986016.

[00207] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding killer-cell

immunoglobulin-like receptor (KIR). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of lirilumab.

[00208] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD28. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of theralizumab.

[00209] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD137. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of utomilumab or urelumab.

[00210] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding OX40. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of PF-04518600 or BMS 986178.

[00211] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD27. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of varlilumab.

[00212] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding GITR. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of GWN323 or BMS-986156.

[00213] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding T cell immunoreceptor with Ig and ITIM domains (TIGIT). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of OMP-313M32, MTIG7192A, BMS-986207, or MK-7684.

[00214] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding inducible T-cell costimulatory (ICOS). In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of JTX-2011 (an anti-ICOS antibody in clinical development).

2. Complementary binding domains that target tumor-associated macrophages (TAMs)

[00215] In some embodiments, binding of the first and second complementary binding domains to antigens on TAMs mediates increased immune responses to tumors. For example, an anti-CSF1R blocking antibody reduces TAM infiltration and promotes CD8⁺ T-cell expansion (Ries CH et al. *Cancer Cell*. 25(6):846-59 (2014)).

[00216] In some embodiments, the first and second complementary binding domains, when paired together, are capable of inhibiting TAMs. In some embodiments, the first and second complementary binding domains, when paired together, are capable of reprogramming or recruitment of active macrophages.

[00217] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CSF1R. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of emactuzumab/RG7155 (NCT01494688) or IMC-CS4 (NCT01346358).

[00218] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD40. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of CP-870,893.

3. Complementary binding domains that stimulate NK cells

[00219] In some embodiments, the first and second complementary binding domains, when paired together, are capable of activating NK cells. In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD16A. In some embodiments, binding of paired complementary binding domains to CD16A on NK cells activates NK cells against CD33-positive leukemia cells. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of NTM-1633 (*see* NCT03603665) or AFM13 (*see* NCT01221571).

4. Complementary binding domains that inhibit checkpoint molecules expressed by cancer cells

[00220] In some embodiments, the first and second complementary binding domains, when paired together, are capable of inhibiting a checkpoint molecule expressed by cancer cells. In some embodiments, the first and second

complementary binding domains, when paired together, are capable of blocking a signal from the cancer cell that inhibits immune cell activation. A number of different immune checkpoint molecules have been described in the literature (*see* Park et al., *Experimental & Molecular Medicine* 50:109 (2018), which is incorporated by reference in its entirety for the disclosure of different immune checkpoint molecules).

[00221] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding PD-L1. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of Atezolizumab, Durvalumab, or Avelumab.

[00222] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding CD73.

[00223] In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of CPI-006 or MEDI9447.

5. Complementary binding domains that bind RANK

[00224] In some embodiments, the first and second complementary binding domains, when paired together, are capable of inhibiting activation of regulatory T cells.

[00225] In some embodiments, the complementary binding domains, when paired together, bind to RANK or RANKL. RANK is expressed on cancer cells and can bind to RANKL on regulatory T cells to generate an immunosuppressive environment (*see* de Groot et al., *Cancer Treatment Reviews* 62:18-28 (2018)). In some embodiments, binding to RANK or RANKL on cancer cells blocks regulatory T-cell activation and stimulates an immune response.

[00226] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding RANK.

[00227] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding RANKL.

[00228] In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of denosumab.

6. Complementary binding domains that bind cell-death-inducing antigens

[00229] As used herein, a “cell-death-inducing” antigen is an antigen that stimulates cell death after being bound by a ligand. All types of cell death are encompassed. The cell death may be programmed (e.g., apoptosis) or non-

programmed (e.g., necrosis). In some embodiments, the cell death is necrosis, apoptosis, or necroptosis.

[00230] In some embodiments, the cell-death-inducing antigen comprises a death receptor. Death receptors are members of the tumor necrosis factor receptor superfamily characterized by the presence of an intracellular “death domain.” In some embodiments, the death receptor is Fas/CD95/Apo1, TNFR1/p55/CD120a, DR3/Apo3/WSL-1/TRAMP/LARD, TRAIL-R1/DR4, DR5/Apo2/TRAIL-R2/TRICK2/KILLER, DR6, or CAR1.

[00231] In some embodiments, the first and second complementary binding domains, when paired together, are capable of binding TRAIL-R1/DR4. In some embodiments, the first and second complementary binding domains comprise all or part of a VH and/or VL of mapatumumab (anti-TRAIL-R1/DR4 death receptor antibody). In one embodiment, an anti-DR5/Apo2/TRAIL-R2/TRICK2/KILLER antibody comprises conatumumab (AMG655), lexatumumab, tigatuzumab (CS1008), or drozitumab (PRO95780).

F. Dimerization domains

[00232] In some embodiments, the first and second components further comprise dimerization domains, as shown in Figure 6. As used herein, “dimerization domains” refer to amino acid sequences that allow two protein monomers (i.e., single proteins) to bind together. As such, dimerization domains can comprise any sequences that cause two protein monomers to form a dimer. In some embodiments, this binding is covalent. In some embodiments, this binding is non-covalent.

[00233] A dimerization domain may be attached to a complementary binding domain or to an immune cell binding domain.

[00234] In some embodiments, dimerization domains promote association of the first complementary binding domain with the first immune cell binding domain and/or the second complementary binding domain with the second immune cell binding domain. In this way, dimerization can reduce the potential for the complementary binding domain to dissociate from the immune cell binding domain outside of the tumor site.

[00235] In some embodiments, a cleavable linker (i.e., a linker comprising a cleavage site) mediates dissociation of the first immune cell binding domain from the first complementary binding domain of the first component and/or the second immune cell binding domain from the second complementary binding

domain. In some embodiments, the first immune cell binding domain binds to the first complementary binding domain until at least one cleavable linker is cleaved so that either (or both) the first immune cell binding domain and the first complementary binding domain is released from its respective dimerization domain. In some embodiments, the second immune cell binding domain binds to the second complementary binding domain until at least one cleavable linker is cleaved so that either (or both) the second immune cell binding domain and the second complementary binding domain is released from its respective dimerization domain.

[00236] In some embodiments, the first immune cell binding domain is bound to the first complementary binding domain by a first dimerization domain and a second dimerization domain, wherein the first dimerization domain is attached to the first immune cell binding domain by a first linker; the second dimerization domain is attached to the first complementary binding domain by a second linker; and the first and/or second linker is a cleavable linker.

[00237] In some embodiments, the second immune cell binding domain is bound to the second complementary binding domain by a first dimerization domain and a second dimerization domain, wherein the first dimerization domain is attached to the second immune cell binding domain by a first linker; the second dimerization domain is attached to the second complementary binding domain by a second linker; and the first and/or second linker is a cleavable linker.

[00238] Dimerization domains may comprise all or part of known dimerization domain amino acid sequences. Dimerization domains may comprise sequences with homology to known dimerization amino acid sequences, but not match a known sequence. In some embodiments, dimerization domains may comprise amino acids that are optimized to improve binding.

[00239] In some embodiments, the dimerization domains comprise amino acid sequences from transcription factors or receptor/ligand pairs. Non-limiting examples of dimerization domains include sequences from receptor tyrosine kinases, transcription factors, 14-3-3 proteins, and G-protein coupled receptors.

[00240] In some embodiments, dimerization domains comprise leucine zippers (also known as coiled coils). As used herein, a “leucine zipper” refers to any amino acid sequence comprising periodic repetition of a leucine residue. A wide variety of leucine zippers have been described for use in dimerization, such as Patent US 9,994,646.

[00241] In some embodiments, dimerization domains comprise immunoglobulin domains.

[00242] In some embodiments, dimerization domains comprise immunoglobulin variable domains. In some embodiments, the immunoglobulin variable domains are VH or VL domains.

[00243] In some embodiments, dimerization domains comprise immunoglobulin constant domains. In some embodiments, the immunoglobulin constant domains are Fc domains. In some embodiments, the immunoglobulin constant domains are CH1/CL, CH2, CH3, or CH4.

[00244] In some embodiments, dimerization domains comprise IgE CH2 domains.

[00245] In some embodiments, dimerization domains comprise TCR constant domains. The TCR constant domains may be from any chain (e.g., α , β , γ , or δ).

[00246] In some embodiments, the dimerization domains in the first component are the same as the dimerization domains in the second component. For example, the dimerization domains in the first component and the second component may be leucine zippers. When the dimerization domains in the first and second component are the same, this may be termed “homodimerization.”

[00247] In some embodiments, the dimerization domains in the first component are different than the dimerization domains in the second component. For example, the dimerization domains in the first component and the second component may comprise a receptor/ligand pair. When the dimerization domains in the first and second component are different, this may be termed “heterodimerization.”

[00248] Dimerization domains may also be engineered to create a “knob” or a “hole” to dimerize two proteins. Such knob-into-hole dimerization sequences can include knobs-into-holes Fc or knobs-into-holes CH3 domains. A wide range of knobs-into-holes examples have been described for production of bispecific antibodies (*see* Xu et al., *mAbs* 7(1):231-242 (2015)).

[00249] In some embodiments, dimerization domains are engineered. By “engineered,” it is meant that a mutation is made to the amino acid sequence to change one or more properties of a protein. In some embodiments, engineering makes association of two protein domains electrostatically favorable, when these two domains do not generally associate in the absence of the engineering. In some

embodiments, two immunoglobulin constant domains are engineered and may be referred to as “engineered immunoglobulin constant domains.” In some embodiments, engineering of Fc regions leads to heterodimerization of Fc regions, such as CH domains.

[00250] In some embodiments, two Fc regions are engineered to comprise opposite charges and associate together (*see* WO2009089004). In some embodiments, two Fc regions are engineered to comprise two leucine zippers and associate together (*see* US 9,994,646). In some embodiments, two Fc regions are engineered by lysine repositioning (*see* WO2017106462).

[00251] The domains may be engineered so that each component of the TWICE is sufficiently stable during manufacturing, transport, and administration, but that when the two components of a TWICE were in close proximity, binding kinetics would still favor pairing of the immune binding domains to each other and, when applicable, the complementary functional domains to each other.

[00252] Several ways of generating a heterodimeric Fc may be employed to make the constructs described herein. In an effort to generate bispecific antibodies in the form of an asymmetric IgG, i.e. an IgG that is formed by two different heavy chains paired into a heterodimer, Ridgway et al. have engineered the CH3 domains of IgG1 by mutations such that one chain contains a “knob” and the other chain contains a corresponding “hole” (J.B. Ridgway, et al. *Protein Eng.* 9:617–621 (1996)). The two engineered heavy chains of this “knobs-into-holes” approach would preferentially form heterodimers when co-expressed from the same cells due to a steric clash in the homodimer. Several other mutations have since been published using similar strategies of steric clashes or repelling charges in engineered Fc domains to facilitate the formation of heterodimers between two different chains (Atwell et al. *Journal of Molecular Biology* 270:26-35 (1997); Gunasekaran et al., *Journal of Biological Chemistry* 285:19637-46 (2010); Moore et al. *mAbs* 3, 546–557 (2011); Strop P et al. *J. Mol. Biol.* 420, 204–219 (2012), Von Kreudenstein et al. 2013, *mAbs*; 5:646-54 (2013)) The combination of steric clashes and repelling charges has also been employed to engineer IgG heavy chains to efficiently form heterodimers (WO2017106462A1). Using a different approach, Davis et al. (Davis et al., *Protein Engineering, Design & Selection* 23(4):195–202 (2010)) have developed strand-exchange engineered domains (SEED) using IgG and IgA sequences to engineer an asymmetric CH3 domain that enables Fc heterodimerization.

G. Complementary functional domains

[00253] In some embodiments, the first or second component comprise a complementary functional domain. As used herein, “a complementary functional domain” refers to a domain that has function when binding a specific cell type. Complementary functional domains are different from complementary binding domains, in that complementary functional domains do not need to pair with another domain to have function.

[00254] In some embodiments, both the first and the second component comprise a complementary functional domain. In some embodiments, only the first component comprises a complementary functional domain.

[00255] In general, any extracellular domain of a cell surface protein that act as a ligand for a receptor may be used as a complementary functional domain. In some embodiments, the complementary functional domain is a costimulatory molecule for T cell activation. In some embodiments, the complementary functional domain is a ligand for binding to an integrin.

[00256] In some embodiments, the complementary functional domain is a latent form of a member of the TGF-beta family. In some embodiments, the latent form is activated in the tumor microenvironment to act locally at a tumor cell.

[00257] In some embodiments, the complementary functional domain is a cytokine. A wide range of cytokines have been tested clinically in cancer (see Vazquez-Lombardi et al, *Antibodies* 2:426-451 (2013)). Further, incorporating a cytokine as a complementary functional domain of a TWICE allows local delivery of a cytokine and thus may improve upon systemic administration of cytokines. In some embodiments, the cytokine is IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[00258] In some embodiments, the complementary functional domain is an attenuated cytokine.

[00259] As used herein, “an attenuated cytokine” is one with a mutation that decreases its activity compared to the wild-type cytokine or a cytokine that is “masked” with the mask linked to the cytokine by a linker that may be cleavable in the tumor or tumor stromal microenvironment. The attenuated cytokine may thus be a variant form of a naturally-occurring cytokine. In some embodiments, the attenuated cytokine lacks activity unless it is targeted to the target cell. Attenuated cytokines that are targeted to tumors may have robust anti-tumor effects with limited systemic

toxicity (*see* Pogue et al., *PLoS ONE* 11(9):e0162472 (2016) and Pogue et al., *Cytokine* 1:66 (2015)).

[00260] Attenuated cytokines have also been proposed to activate the immune system and induce proliferation of effector T cells, when the same cytokine without attenuation may lead to depression of the immune system and proliferation of regulatory T cells.

[00261] In some embodiments, an attenuated cytokine is a variant form of IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily. In some embodiments, the attenuated cytokine must be targeted to a cancer or its microenvironment to have function.

H. Linkers

[00262] By linker, we include any chemical moiety that attaches parts of the TWICE together.

[00263] In some embodiments, linkers used in a TWICE may be flexible linkers. Linkers include peptides, polymers, nucleotides, nucleic acids, polysaccharides, and lipid organic species (such as polyethylene glycol). In some embodiments, the linker is a peptide linker. Peptide linkers may be from about 2-100, 10-50, or 15-30 amino acids long. In some embodiments, peptide linkers may be at least 10, at least 15, or at least 20 amino acids long and no more than 80, no more than 90, or no more than 100 amino acids long. In some embodiments, the linker is a peptide linker that has a single or repeating GGGGS (SEQ ID NO: 85), GGGS (SEQ ID NO: 86), GS (SEQ ID NO: 87), GSGGS (SEQ ID NO: 88), GGSG (SEQ ID NO: 89), GGSGG (SEQ ID NO: 90), GSGSG (SEQ ID NO: 91), GSGGG (SEQ ID NO: 92), GGGSG (SEQ ID NO: 93), and/or GSSSG (SEQ ID NO: 94) sequence(s).

[00264] In some embodiments, the linker does not comprise a cleavage site (i.e., non-cleavable linkers). Exemplary linkers not comprising a cleavage site include maleimide (MPA) or SMCC linkers.

[00265] In some embodiments, the linker comprises a cleavage site (i.e., a cleavable linker).

[00266] In some embodiments, linkers attach targeting moieties to immune cell binding domains, complementary binding domains, or complementary functional domains. In some embodiments, linkers attach dimerization domains to immune cell binding domains or complementary binding domains.

1. Linkers to attach targeting moieties

[00267] In some embodiments, a linker attaches a targeting moiety to the immune cell binding domain. In some embodiments, a linker attaches a targeting moiety to a complementary binding domain or complementary functional domain. In some embodiments, linkers attaching targeting moieties to complementary binding domains or to complementary functional domains are non-cleavable linkers. In some embodiments, linkers attaching targeting moieties to complementary binding domains or to complementary functional domains are flexible linkers.

2. Linkers to attach dimerization domains

[00268] In some embodiments, a linker attaches a dimerization domain to an immune cell binding domain. In some embodiments, a linker attaches a dimerization domain to a complementary binding domain.

[00269] A linker used to attach a dimerization domain to an immune cell binding domain or a complementary binding domain may be referred to as a “dimerization domain linker.” In some embodiments, the dimerization domain linker is a cleavable linker.

[00270] In some embodiments, a dimerization domain linker may comprise a cleavage site. In some embodiments, the dimerization domain linker comprises a protease cleavage site. In some embodiments, the dimerization domain linker comprises a protease cleavage site that can be cleaved in the tumor microenvironment. In some embodiments, the dimerization domain linker comprises a cleavage site for one or more matrix metalloproteases.

[00271] Depending on the dimerization domain employed, a range of dimerization domain linker lengths may be used in the TWICE. The length of a dimerization domain linker may need to be long enough to allow flexibility for protease cleavage. However, a dimerization domain linker that is too long could allow the two dimerization domains to be too spatially far apart to ensure dimerization.

[00272] In some embodiments, the first dimerization domain linker and the second dimerization domain linker are the same or similar lengths. In some embodiments, the first dimerization domain linker and second dimerization domain linker are different lengths.

[00273] In some embodiments, the dimerization domain linker length helps promote association of the two dimerization domains.

[00274] In some embodiments, the first and second dimerization domain linkers are from 5-30 amino acids in length. In some embodiments, the first and second dimerization domain linkers are from 8-16 amino acids in length.

[00275] In some embodiments, the first and second dimerization domain linkers are from 5-15 amino acids in length. For example, linkers of this length may be appropriate when the dimerization domains are CH1/CL.

[00276] In some embodiments, the first and second dimerization domain linkers are from 12-30 amino acids in length. For example, linkers of this length may be appropriate when the dimerization domains are leucine zippers (coiled coils). In some embodiments, the first and second dimerization domain linkers are from 15-20 amino acids in length. For example, linkers of this length may be appropriate for certain leucine zipper dimerization domains.

[00277] Examples of representative dimerization domain linkers include SEQ ID NOs: 203-211.

I. Cleavage sites and cleavable linkers

[00278] In some embodiments, cleavage sites allow specific cleavage of constructs at certain locations. A linker comprising a cleavage site may be referred to as a cleavable linker.

[00279] For example, a TWICE that is a single polypeptide construct may comprise a cleavable linker between the first and second components. Other embodiments of this TWICE may also employ cleavable linkers, such as dimerization domain linkers.

[00280] In some embodiments, cleavage can occur outside the unwanted cell, without first being internalized into a cell and being engaged in the classical antigen-processing pathways.

[00281] In certain embodiments, at least one cleavage site may be cleaved by an enzyme expressed by the cancer cells. Cancer cells, for instance, are known to express certain enzymes, such as proteases. By way of nonlimiting example, cathepsin B cleaves FR, FK, VA and VR amongst others; cathepsin D cleaves PRSFFRLGK (SEQ ID NO: 45), ADAM28 cleaves KPAKFFRL (SEQ ID NO: 1), DPAKFFRL (SEQ ID NO: 2), KPMKFFRL (SEQ ID NO: 3) and LPAKFFRL (SEQ ID NO: 4); and MMP2 cleaves AIPVSLR (SEQ ID NO: 46), SLPLGLWAPNFN (SEQ ID NO: 47), HPVGLLAR (SEQ ID NO: 48), GPLGVRGK (SEQ ID NO: 49), and GPLGLWAQ (SEQ ID NO: 50), for example. Other cleavage sites listed in Table

1 or 2 may also be employed. Protease cleavage sites and proteases associated with cancer are well known in the art. Oncomine (www.oncomine.org) is an online cancer gene expression database, so when the agent of the TWICE is for treating cancer, the skilled person may search the Oncomine database to identify a particular protease cleavage site (or two protease cleavage sites) that will be appropriate for treating a given cancer type. Alternative databases include the European Bioinformatic Institute (www.ebi.ac.uk), in particular (www.ebi.ac.uk/gxa). Protease databases include ExPASy Peptide Cutter (www.ca.expasy.org/tools/peptidecutter).

[00282] In some embodiments, the protease is expressed by a non-cancer cell in the tumor microenvironment, such as a tumor-associated macrophage of fibroblast.

[00283] In some embodiments, the protease cleavage sites of one or more cleavable linkers are cleaved by a protease that is colocalized. In some embodiments, the protease is colocalized to the cancer by a targeting moiety that binds a tumor antigen expressed by the cancer and that is the same or different from the targeting moiety in the first component or the second component. In some embodiments, the protease is colocalized to the cancer by a targeting moiety that binds an antigen expressed by a cell in the tumor microenvironment.

[00284] Cleavage sites in a cleavable linker can function to release the dimerization domain from the complementary binding domain and/or immune cell binding domain. The cleavage sites can function to release the complementary binding domain and/or immune cell binding domain from the first and/or immune cell engaging domain in the microenvironment of the unwanted cells.

[00285] In some embodiments, the protease cleavage sites in the first and/or second cleavable linkers that attach dimerization domains to immune cell binding domains or complementary binding domains are cleaved by a protease expressed by the cancer or colocalized to the cancer by a targeting moiety that binds a tumor antigen expressed by the cancer.

J. Binding of a complementary binding domain to an immune cell binding domain

[00286] In some embodiments, the first complementary binding domain is a binding partner for the first immune cell binding domain, such that the first immune cell binding domain does not bind to the second immune cell binding domain

unless the first immune cell binding domain is not bound to the first complementary domain.

[00287] In some embodiments, the second complementary binding domain is a binding partner for the second immune cell binding domain, such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the second immune cell binding domain is not bound to the second complementary domain.

[00288] When a complementary binding domain dissociates from an immune cell binding domain and associates with a paired complementary domain or when an immune cell binding domain dissociates from a complementary binding domain and associates with a paired immune cell binding domain, this can be referred to as a domain swap. In this domain swap event, the domains move from a mismatched configuration to an active and paired configuration.

[00289] In some embodiments, the first and second immune cell binding domains are capable of forming a Fv when not bound to the first and second complementary binding domains. In some embodiments, the first and second complementary binding domains are capable of forming a Fv when not bound to the first and second immune cell binding domains.

[00290] In some embodiments, the first and second components comprise dimerization domains to promote association of the immune cell binding domain and the complementary binding domain of a single component. In some embodiments, cleavage of a cleavable linker in a dimerization domain linker allows dissociation of the immune cell binding domain and the complementary binding domain of a single component at the cancer cell or its microenvironment. In some embodiments, the first and second components do not comprise dimerization domains.

K. Masking by inert binding partners

[00291] In some embodiments, the TWICE comprises an inert binding partner to mask the first and/or second immune cell binding domain. In some embodiments, only the first immune cell binding domain is masked. In some embodiments, both the first and second immune cell binding domain are masked.

[00292] For example, In some embodiments, inert binding partners are a component of those TWICE that comprise complementary functional domains, as those described in Figure 5 and Section Complementary functional domainsG.

[00293] In some embodiments, first and/or second components that comprise a complementary functional domain comprise an inert binding partner.

[00294] In some embodiments, a first inert binding partner binds to the first immune cell binding domain such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the inert binding partner is removed.

[00295] In some embodiments, the first immune cell binding domain is a VH domain, and the inert binding partner is a VL domain. In some embodiments, the first immune cell binding domain is a VL domain, and the inert binding partner is a VH domain.

[00296] In some embodiments, a protease cleavage site separates the first immune cell binding domain and the first inert binding partner. In some embodiments, the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease.

[00297] In some embodiments, a second inert binding partner binds to the second immune cell binding domain such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the inert binding partner is removed.

[00298] In some embodiments, the second immune cell binding domain is a VH domain, and the inert binding partner is a VL domain. In some embodiments, the second immune cell binding domain is a VL domain, and the inert binding partner is a VH domain.

[00299] In some embodiments, a protease cleavage site separates the second immune cell binding domain and the second inert binding partner. In some embodiments, protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease.

[00300] In some embodiments, the protease is expressed by the cancer. In some embodiments, the protease is expressed by a cell in the tumor microenvironment, such as a TAF or TAM.

[00301] In some embodiments, the protease colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment.

L. Methods of Making

[00302] The different TWICE as described herein can be made using genetic engineering techniques. Specifically, one or two nucleic acids may be expressed in a suitable host to produce a TWICE or its components. For example, a vector may be prepared comprising a nucleic acid sequence that encodes the TWICE including all of its component parts and linkers and that vector may be used to transform an appropriate host cell (or individual vectors may be used to individually express each component).

[00303] Various regulatory elements may be used in the vector as well, depending on the nature of the host and the manner of introduction of the nucleic acid into the host, and whether episomal maintenance or integration is desired.

[00304] Chemical linkage techniques, such as using maleimide or SMCC linkers, may also be employed.

[00305] In instances where the targeting moiety is an aptamer, a person of ordinary skill in the art would appreciate how to conjugate an aptamer to a protein, namely the immune cell binding domain or the complementary binding domain. Aptamers may be conjugated using a thiol linkage or other standard conjugation chemistries. A maleimide, succinimide, or SH group may be affixed to the aptamer to attach it to the immune cell binding domain or the complementary binding domain.

II. Pharmaceutical Compositions

[00306] The TWICE may be employed as pharmaceutical compositions. As such, they may be prepared along with a pharmaceutically acceptable carrier. If parenteral administration is desired, for instance, the TWICE may be provided in sterile, pyrogen-free water for injection or sterile, pyrogen-free saline or other form that is acceptable for parenteral administration. Alternatively, the TWICE may be provided in lyophilized form for resuspension with the addition of a sterile liquid carrier. If in separate form, the TWICE may be provided in a single pharmaceutical composition or in two pharmaceutical compositions.

III. Methods of Using TWICE

[00307] The TWICE described herein may be used in a method of treating cancer comprising administering a TWICE comprising at least a first and a second component to the patient, as each of the components have been described in detail in various embodiments above.

[00308] Additionally, the agents described herein may also be used in a method of targeting a patient's own immune response to cancer cells comprising administering a TWICE to the patient.

[00309] In some embodiments, the TWICE described herein may be used in a method of targeting immune cells to cancer expressing a tumor antigen that binds both the first targeting moiety and the second targeting moiety. In some embodiments, the immune cells are T cells expressing CD3 or TCR.

[00310] In some embodiments, the TWICE described herein may be used in a method of targeting immune cells to cancer expressing two tumor antigens, wherein one tumor antigen binds the first targeting moiety and one tumor antigen binds the second targeting moiety. In some embodiments, the immune cells are T cells expressing CD3 or TCR.

[00311] In some embodiments, the TWICE described herein may be used in a method of delivering a cytokine to an immune cell of a patient. In some embodiments, the first and/or second complementary functional domain of the TWICE comprise IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[00312] In some embodiments, the patient has cancer or a recognized pre-malignant state. In some embodiments, the patient has undetectable cancer, but is at high risk of developing cancer, including having a mutation associated with an increased risk of cancer. In some embodiments, the patient at high risk of developing cancer has a premalignant tumor with a high risk of transformation. In some embodiments, the patient at high risk of developing cancer has a genetic profile associated with high risk. In some embodiments, the presence of cancer or a pre-malignant state in a patient is determined based on the presence of circulating tumor DNA (ctDNA) or circulating tumor cells. In some embodiments, treatment is pre-emptive or prophylactic. In some embodiments, treatment slow or blocks the occurrence or reoccurrence of cancer.

[00313] The amount of the agent administered to the patient may be chosen by the patient's physician so as to provide an effective amount to treat the condition in question. The first component and the second component of the TWICE may be administered in the same formulation or two different formulations within a sufficiently close period of time to be active in the patient.

[00314] The patient receiving treatment may be a human. The patient may be a primate or any mammal. Alternatively, the patient may be an animal, such as a domesticated animal (for example, a dog or cat), a laboratory animal (for example, a laboratory rodent, such as a mouse, rat, or rabbit), or an animal important in agriculture (such as horses, cattle, sheep, or goats).

[00315] The cancer may be a solid or non-solid malignancy. In some embodiments, the cancer may be a solid tumor wherein the solid tumor is not a lymphoma. The cancer may be any cancer such as breast cancer, ovarian cancer, endometrial cancer, cervical cancer, bladder cancer, renal cancer, melanoma, lung cancer, prostate cancer, testicular cancer, thyroid cancer, brain cancer, esophageal cancer, gastric cancer, pancreatic cancer, colorectal cancer, liver cancer, leukemia, myeloma, nonHodgkin lymphoma, Hodgkin lymphoma, acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, lymphoproliferative disorder, myelodysplastic disorder, myeloproliferative disease, and premalignant disease or any other cancer that is suitable for treatment.

[00316] In some embodiments, one skilled in the art may evaluate a tumor prior to initiating treatment.

[00317] For example, one skilled in the art may evaluate a tumor for the presence of infiltrating T cells. In some embodiments, a method of treatment comprises confirming the presence of T cells in the tumor before initiating treatment. In some embodiments, the patient will have a solid tumor with T-cell infiltration. A tumor sample may be stained for T-cell markers and demonstrate the presence and optionally concentration of T-cell infiltration in the tumor using imaging techniques.

[00318] In some embodiments, a method of treatment comprises confirming the presence of tumor antigens before initiating treatment. In some embodiments, a method of treatment comprises evaluating the concentration of tumor antigens before initiating treatment. For example, one skilled in the art could select tumor antigens or select patients for treatment based on a profile of antigens overexpressed by the tumor. One skilled in the art could also select patients for treatment with a TWICE based on their antigen expression profiles by select patients overexpressing particular antigens. In some embodiments, these tumor antigens are cell-surface tumor antigens. A tumor sample may be stained for antigens of interest to demonstrate the presence and optionally concentration of tumor antigens. The lower limit of expression can also be determined using *in vitro* T-cell activation assays with

a specific TWICE. These methods allow an investigator to confirm that a patient's tumor has appropriate antigens for targeting of the TWICE to the tumor microenvironment.

[00319] In some embodiments, a method of treatment comprises (i) confirming the presence of T cells in the tumor; (ii) confirming the presence of tumor antigen(s), and/or (iii) evaluating the concentration of tumor antigens. In some embodiments, the expression of tumor antigen(s) that bind the first and second targeting moieties are expressed at a high enough level to bring the two components of the TWICE into close proximity.

[00320] In some embodiments, the presence of a biomarker is used to select patients for receiving the TWICE. A wide variety of tumor markers are known in the art, such as those described at www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-fact-sheet. In some embodiments, the tumor marker is ALK gene rearrangement or overexpression; alpha-fetoprotein; beta-2-microglobulin; beta-human chorionic gonadotropin; BRCA1 or BRCA2 gene mutations; BCR-ABL fusion genes (Philadelphia chromosome); BRAF V600 mutations; C-kit/CD117; CA15-3/CA27.29; CA19-9; CA-125; calcitonin; carcinoembryonic antigen (CEA); CD20; chromogranin A (CgA); chromosomes 3, 7, 17, or 9p21; circulating tumor cells of epithelial origin (CELLSEARCH®); cytokeratin fragment 21-1; EGFR gene mutation analysis; estrogen receptor (ER)/progesterone receptor (PR); fibrin/fibrinogen; HE4; HER2/neu gene amplification or protein overexpression; immunoglobulins; KRAS gene mutation analysis; lactate dehydrogenase; neuron-specific enolase (NSE); nuclear matrix protein 22; programmed death ligand 1 (PD-L1); prostate-specific antigen (PSA); thyroglobulin; urokinase plasminogen activator (uPA); plasminogen activator inhibitor (PAI-1); 5-protein signature (OVA1®); 21-gene signature (Oncotype DX®); or 70-gene signature (Mammaprint®).

[00321] The TWICE may be administered alone or in conjunction with other forms of therapy, including surgery, radiation, traditional chemotherapy, or other immunotherapy.

[00322] In some embodiments, the other immunotherapy may include separate treatment with immune cytokines or cytokine fusions. Cytokines refer to cell-signaling proteins naturally made by the body to activate and regulate the immune system. Cytokine fusions refer to engineered molecules comprising all or part of a

cytokine. For example, a cytokine fusion may comprise all or part of a cytokine attached to an antibody that allows targeting to a tumor such as Darleukin (*see* Zegers et al. (2015) Clin. Cancer Res., 21, 1151-60), Teleukin (*see* WO2018087172).

[00323] In some embodiments, the other immunotherapy is cancer treatment vaccination. In some embodiments, cancer treatment vaccination boosts the body's natural defenses to fight cancer. These can either be against shared tumor antigens (such as E6, E7, NY-ESO, MUC1, or HER2) or against personalized mutational neoantigens.

IV. Embodiments

[00324] The following numbered items provide embodiments as described herein, though the embodiments recited here are not limiting.

[00325] Item 1. A kit or composition for treating cancer in a patient comprising a first component comprising a targeted immune cell binding agent comprising a first targeting moiety that binds a tumor antigen expressed by the cancer; a first immune cell binding domain capable of immune cell binding activity when binding a second immune cell binding domain, wherein the second immune cell binding domain is not part of the first component, and wherein the first immune cell binding domain is either a VH domain or VL domain; and a first complementary binding domain capable of binding to a complementary antigen when binding a second complementary binding domain, wherein the second complementary binding domain is not part of the first component, when the first immune cell binding domain is a VH domain the first complementary binding domain is a VL domain, when the first immune cell binding domain is a VL domain, the first complementary binding domain is a VH domain, and wherein the first complementary binding domain is a binding partner for the first immune cell binding domain, such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the first immune cell binding domain is not bound to the first complementary domain; and a second component comprising a targeted immune cell binding agent comprising a second targeting moiety; a second immune cell binding domain capable of immune cell binding activity when binding a first immune cell binding domain, wherein the second immune cell binding domain is a VH if the first immune cell binding domain is a VL and wherein the second immune cell binding domain is a VL if the first immune cell binding domain is a VH; and a second complementary binding domain capable of binding to a complementary antigen when binding the first complementary

binding domain, wherein when the second immune cell binding domain is a VH domain the second complementary binding domain is a VL domain, when the second immune cell binding domain is a VL domain, the second complementary binding domain is a VH domain, and wherein the second complementary binding domain is a binding partner for the second immune cell binding domain, such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the second immune cell binding domain is not bound to the second complementary domain.

[00326] Item 2. The kit or composition of item 1, wherein the first immune cell binding domain is bound to the first complementary binding domain by a first dimerization domain and a second dimerization domain, wherein the first dimerization domain is attached to the first immune cell binding domain by a first linker; the second dimerization domain is attached to the first complementary binding domain by a second linker; and the first and/or second linker is a cleavable linker.

[00327] Item 3. The kit or composition of any one of items 1-2, wherein the second T-cell binding domain is bound to the second complementary binding domain by a first dimerization domain and a second dimerization domain, wherein the first dimerization domain is attached to the second T-cell binding domain by a first linker; the second dimerization domain is attached to the second complementary binding domain by a second linker; and the first and/or second linker is a cleavable linker.

[00328] Item 4. The kit or composition of any one of items 2-3, wherein the first and second linkers are cleavable linkers.

[00329] Item 5. The kit or composition of any one of items 2-4, wherein the first and second linkers are the same.

[00330] Item 6. The kit or composition of any one of items 2-4, wherein the first and second linkers are different.

[00331] Item 7. The kit or composition of any one of items 2-6, wherein the first and second linkers are from 5 to 30 amino acids in length.

[00332] Item 8. The kit or composition of any one of items 2-6, wherein the first and second linkers are from 8 to 16 amino acids in length.

[00333] Item 9. The kit or composition of any one of items 2-8, wherein the protease cleavage sites of the first and/or second cleavable linkers are cleaved by a protease expressed by the cancer or tumor microenvironment cell.

[00334] Item 10. The kit or composition of any one of items 2-9, wherein the protease cleavage sites of the first and/or second cleavable linkers are cleaved by a protease that is colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds a tumor antigen expressed by the cancer and that is the same or different from the targeting moiety in at least one of the first components or the second component.

[00335] Item 11. The kit or composition of any one of items 1-10, wherein the first and second dimerization domains are both leucine zippers; immunoglobulin domains; or T-cell receptor (TCR) domains.

[00336] Item 12. The kit or composition of item 11, wherein the immunoglobulin domains comprise immunoglobulin variable domains or immunoglobulin constant domains.

[00337] Item 13. The kit or composition of item 12, wherein the immunoglobulin constant domains comprise CH1/CL, CH2, CH3, or CH4.

[00338] Item 14. The kit or composition of item 11, wherein the TCR domains comprise TCR constant domains.

[00339] Item 15. The kit or composition of any one of items 1-14, wherein the dimerization domains in the first component are the same as the dimerization domains in the second component.

[00340] Item 16. The kit or composition of any one of items 1-14, wherein the dimerization domains in the first component are different than the dimerization domains in the second component.

[00341] Item 17. The kit or composition of any one of items 1-16, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding the cancer.

[00342] Item 18. The kit or composition of item 17, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding an immune checkpoint molecule, RANK or RANKL, or a cell-death-inducing antigen.

[00343] Item 19. The kit or composition of item 18, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding an immune checkpoint molecule.

[00344] Item 20. The kit or composition of item 19, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding PD-L1.

[00345] Item 21. The kit or composition of item 20, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of atezolizumab, durvalumab, or avelumab.

[00346] Item 22. The kit or composition of item 19, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD73.

[00347] Item 23. The kit or composition of item 22, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of CPI-006 or MEDI9447.

[00348] Item 24. The kit or composition of item 18, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding RANK.

[00349] Item 25. The kit or composition of item 18, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding RANKL.

[00350] Item 26. The kit or composition of item 25, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of denosumab.

[00351] Item 27. The kit or composition of item 18, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a cell-death-inducing antigen.

[00352] Item 28. The kit or composition of item 27, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding Fas/CD95/Apo1, TNFR1/p55/CD120a, DR3/Apo3/WSL-1/TRAMP/LARD, TRAIL-R1/DR4, DR5/Apo2/TRAIL-R2/TRICK2/KILLER, DR6, or CAR1.

[00353] Item 29. The kit or composition of item 28, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding TRAIL-R1/DR4.

[00354] Item 30. The kit or composition of item 29, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of mapatumumab.

[00355] Item 31. The kit or composition of item 28, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding DR5/Apo2/TRAIL-R2/TRICK2/KILLER.

[00356] Item 32. The kit or composition of item 31, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of conatumumab (AMG655), lexatumumab, tigatuzumab (CS1008), or drozitumab (PRO95780).

[00357] Item 33. The kit or composition of any one of items 1-16, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a molecule associated with the extracellular matrix.

[00358] Item 34. The kit or composition of any one of items 1-16, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T cell, a macrophage, or a natural killer cell.

[00359] Item 35. The kit or composition of item 34, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T cell.

[00360] Item 36. The kit or composition of item 35, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD3, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, GITR (TNFRSF18), TIGIT, or inducible T-cell costimulatory (ICOS).

[00361] Item 37. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding CD3.

[00362] Item 38. The kit or composition of item 37, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL

of muromonab, otelexizumab, teplizumab, visilizumab, foralumab, SP34, or blinatumomab.

[00363] Item 39. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding PD-1.

[00364] Item 40. The kit or composition of item 39, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of pembrolizumab or nivolumab.

[00365] Item 41. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding CTLA-4.

[00366] Item 42. The kit or composition of item 41, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of ipilimumab.

[00367] Item 43. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding TIM-3.

[00368] Item 44. The kit or composition of item 43, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of TSR-022 or Sym023.

[00369] Item 45. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding LAG-3.

[00370] Item 46. The kit or composition of item 45, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of BMS-986016.

[00371] Item 47. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding KIR.

[00372] Item 48. The kit or composition of item 47, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of lirilumab.

[00373] Item 49. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding CD28.

[00374] Item 50. The kit or composition of item 49, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of theralizumab.

[00375] Item 51. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding CD137.

[00376] Item 52. The kit or composition of item 51, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of utomilumab or urelumab.

[00377] Item 53. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding OX40.

[00378] Item 54. The kit or composition of item 53, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of PF-04518600 or BMS 986178.

[00379] Item 55. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding CD27.

[00380] Item 56. The kit or composition of item 55, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of varlilumab.

[00381] Item 57. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding GITR (TNFRSF18).

[00382] Item 58. The kit or composition of item 57, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of GWN323 or BMS-986156.

[00383] Item 59. The kit or composition of item 36, wherein the first and second complementary binding domains, when paired together, are capable of binding TIGIT.

[00384] Item 60. The kit or composition of item 59, wherein first and second complementary binding domains comprise all or part of a VH and/or VL of OMP-313M32, MTIG7192A, BMS-986207, or MK-7684.

[00385] Item 61. The kit or composition of item 36, wherein the first and second complementary binding domains are, when bound together, capable of binding ICOS.

[00386] Item 62. The kit or composition of item 61, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of JTX-2011.

[00387] Item 63. The kit or composition of item 34, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a macrophage.

[00388] Item 64. The kit or composition of item 63, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CSF1R.

[00389] Item 65. The kit or composition of item 65, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of emactuzumab or IMC-CS4.

[00390] Item 66. The kit or composition of item 63, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD40.

[00391] Item 67. The kit or composition of item 66, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of CP-870,893.

[00392] Item 68. The kit or composition of item 34, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a natural killer cell.

[00393] Item 69. The kit or composition of item 68, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD16A.

[00394] Item 70. The kit or composition of item 69, wherein the first and second complementary binding domains comprise all or part of a VH and/or VL of NTM-1633 or AFM13.

[00395] Item 71. The kit or composition of any one of items 1-70, wherein the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding the same antigen.

[00396] Item 72. The kit or composition of any one of items 1-70, wherein the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding different antigens on the same cell.

[00397] Item 73. The kit or composition of any one of items 1-70, wherein the first complementary binding domain and the second complementary binding domain, when bound to each other, and the first immune cell binding domain and the second immune cell binding domain, when bound to each other, are capable of binding different cells.

[00398] Item 74. The kit or composition of any one of items 1-73, wherein the first and second immune cell binding domains are capable of forming a Fv when not bound to the first and second complementary binding domains.

[00399] Item 75. The kit or composition of any one of items 1-74, wherein the first and second complementary binding domains are capable of forming a Fv when not bound to the first and second immune cell binding domains.

[00400] Item 76. A kit or composition for treating cancer in a patient comprising a first component comprising a targeted immune cell binding agent comprising a first targeting moiety that binds a tumor antigen expressed by the cancer; a first immune cell binding domain capable of immune cell binding activity when binding a second immune cell binding domain, wherein the second immune cell binding domain is not part of the first component, and wherein the first immune cell binding domain is either a VH domain or VL domain; a first inert binding partner for the first immune cell binding domain, wherein the first inert binding partner binds to the first immune cell binding domain such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the inert binding partner is removed, wherein if the first immune cell binding domain is a VH domain, the inert binding partner is a VL domain and if the first immune cell binding domain is a VL domain, the inert binding partner is a VH domain; a protease cleavage site

separating the first immune cell binding domain and the first inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease expressed by the cancer or a tumor microenvironment cell; or colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment; and a first complementary functional domain capable of immune cell binding, and a second component comprising a targeted immune cell binding agent comprising a second targeting moiety; a second immune cell binding domain; and optionally a second complementary functional domain capable of immune cell binding.

[00401] Item 77. The kit or composition of item 76, wherein the second component comprises a complementary functional domain.

[00402] Item 78. The kit or composition of any one of items 76-77, wherein the complementary functional domain of the first and/or second component comprises a ligand for a receptor.

[00403] Item 79. The kit or composition of item 78, wherein the complementary functional domain is a latent form of a member of the TGF-beta family.

[00404] Item 80. The kit or composition of item 78, wherein the complementary functional domain of the first and/or second component comprises a cytokine.

[00405] Item 81. The kit or composition of item 78, wherein the cytokine is IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[00406] Item 82. The kit or composition of item 80, wherein the complementary functional domain of the first and/or second component comprises an attenuated cytokine.

[00407] Item 83. The kit or composition of item 82, wherein the attenuated cytokine is a variant of IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

[00408] Item 84. The kit or composition of any one of items 76-83, wherein the second component further comprises a second inert binding partner for

the second immune cell binding domain, wherein the second inert binding partner binds to the second immune cell binding domain such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the inert binding partner is removed, wherein if the second immune cell binding domain is a VH domain, the inert binding partner is a VL domain and if the second immune cell binding domain is a VL domain, the inert binding partner is a VH domain; and further wherein a protease cleavage site separating the second immune cell binding domain and the second inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease expressed by the cancer; or colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment.

[00409] Item 85. The kit or composition of any one of items 1-84, wherein the first component is not covalently bound to the second component.

[00410] Item 86. A molecule comprising the kit or composition of any one of items 1-85, wherein the first component is covalently bound to the second component.

[00411] Item 87. The single molecule of item 86, wherein the first component is covalently bound to the second component via a cleavable linker.

[00412] Item 88. The kit or composition of any one of items 1-87, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a T cell, a macrophage, or a natural killer cell.

[00413] Item 89. The kit or composition of item 88, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a T cell.

[00414] Item 90. The kit or composition of item 89, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD3, the T-cell receptor, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain 3 (TIM-3), lymphocyte-activation gene 3

(LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, GITR (TNFRSF18), TIGIT, or inducible T-cell costimulatory (ICOS).

[00415] Item 91. The kit or composition of item 90, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD3.

[00416] Item 92. The kit or composition of item 91, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of muromonab, oteixizumab, teplizumab, visilizumab, foralumab, SP34, or blinatumomab.

[00417] Item 93. The kit or composition of item 90, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding the T-cell receptor.

[00418] Item 94. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding PD-1.

[00419] Item 95. The kit or composition of item 94, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of pembrolizumab or nivolumab.

[00420] Item 96. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding CTLA-4.

[00421] Item 97. The kit or composition of item 96, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of ipilimumab.

[00422] Item 98. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding TIM-3.

[00423] Item 99. The kit or composition of item 98, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of TSR-022 or Sym023.

[00424] Item 100. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding LAG-3.

[00425] Item 101. The kit or composition of item 100, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of BMS-986016.

[00426] Item 102. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding KIR.

[00427] Item 103. The kit or composition of item 102, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of lirilumab.

[00428] Item 104. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding CD28.

[00429] Item 105. The kit or composition of item 104, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of theralizumab.

[00430] Item 106. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding CD137.

[00431] Item 107. The kit or composition of item 106, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of utomilumab or urelumab.

[00432] Item 108. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding OX40.

[00433] Item 109. The kit or composition of item 108, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of PF-04518600 or BMS 986178.

[00434] Item 110. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding CD27.

[00435] Item 111. The kit or composition of item 110, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of varlilumab.

[00436] Item 112. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding GITR (TNFRSF18).

[00437] Item 113. The kit or composition of item 112, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of GWN323 or BMS-986156.

[00438] Item 114. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding TIGIT.

[00439] Item 115. The kit or composition of item 114, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of OMP-313M32, MTIG7192A, BMS-986207, or MK-7684.

[00440] Item 116. The kit or composition of item 90, wherein the first and second immune cell binding domains are, when bound together, capable of binding ICOS.

[00441] Item 117. The kit or composition of item 116, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of JTX-2011.

[00442] Item 118. The kit or composition of item 88, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a macrophage.

[00443] Item 119. The kit or composition of item 118, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CSF1R.

[00444] Item 120. The kit or composition of item 119, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of emactuzumab or IMC-CS4.

[00445] Item 121. The kit or composition of item 120, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD40.

[00446] Item 122. The kit or composition of item 121, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of CP-870,893.

[00447] Item 123. The kit or composition of item 88, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a natural killer cell.

[00448] Item 124. The kit or composition of item 123, wherein the first immune cell binding domain and the second immune binding domain are, when bound to each other, capable of binding CD16A.

[00449] Item 125. The kit or composition of item 124, wherein the first and second immune cell binding domains comprise all or part of a VH and/or VL of NTM-1633 or AFM13.

[00450] Item 126. The kit or composition of any one of items 1-125, wherein the first and the second targeting moieties are different.

[00451] Item 127. The kit or composition of any one of items 1-125, wherein the first and the second targeting moieties are the same.

[00452] Item 128. The kit or composition of any one of items 1-127, wherein the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof.

[00453] Item 129. The kit or composition of any one of items 1-128, wherein the first and/or second targeting moiety comprises a DNA aptamer, RNA aptamer, albumin, lipocalin, fibronectin, ankyrin, fynomer, Obody, DARPin, knotin, avimer, atrimer, anti-callin, affilin, affibody, bicyclic peptide, cys-knot, FN3 (adnectins, centryrins, pronectins, or TN3), or Kunitz domain.

[00454] Item 130. The kit of composition of any one of items 1-129, wherein the second targeting moiety binds a tumor antigen expressed by the cancer.

[00455] Item 131. The kit of composition of item 128, wherein the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof that binds $\alpha 4$ -integrin; A33; ACVRL 1/ALK1; ADAM17; ALK; APRIL; BCMA; C242; CA125; Cadherin-19; CAIX; CanAg; Carbonic Anhydrase IX; CCN1; CCR4; CD123; CD133; CD137 (4-1BB); CD138/Syndecan1; CD19; CD2; CD20; CD22; CD30; CD33; CD37; CD38; CD4; CD40; CD44; CD45; CD48; CD5; CD52; CD56; CD59; CD70; CD70b; CD71; CD74; CD79b; CD80; CD86; CD98; CEA; CEACAM; CEACAM1; CK8; c-Kit; CLDN1; CLDN18; CLDN18.2; CLDN6; c-met/HGFR; c-RET; Cripto; CTLA-4; CXCR4; DKK-1; DLL3; DLL4; TRAIL-R2/DR5; DRS; EGFL7; EGFR; EGFRvIII; endoglin; ENPP3; EpCAM; EphA2; Episialin; FAP; FGFR1; FGFR2; FGFR3; FGFR4; fibronectin extra-domain B; FLT-

3; flt4; folate receptor 1; GCC; GD2; GD3; Glypican-3; Glypicans; GM3; GPNMB; GPR49; GRP78; Her2/Neu; HER3/ERBB3; HLA-DR; ICAM-1; IGF-1R; IGFR; IL-3Ra; Integrin $\alpha 5\beta 1$; Integrin $\alpha 6\beta 4$; Integrin αV ; Integrin $\alpha V\beta 3$; Lewis Y; Lewis y/b antigen; LFL2; LIV-1; Ly6E; MCP-1; Mesothelin; MMP-9; MUC1; MUC18; MUC5A; MUC5AC; Myostatin; NaPi2b; Neuropilin 1; NGcGM3; NRP1; P-cadherin; PCLA; PD-1; PDGFRa; PD-L1; PD-L2; Phosphatidylserine; PIVKA-II; PLVAP; PRLR; Progastrin; PSCA; PSMA; RANKL; RG1; Siglec-15; SLAMF6; SLAMF7; SLC44A4, STEAP-1; TACSTD-2; Tenascin C; TPBG; TRAIL-R1/DR4; TROP-2; TWEAKR; TYRP1; VANG2; VEGF; VEGF-C; VEGFR-2; or VEGF-R2.

[00456] Item 132. The kit or composition of item 128, wherein the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof is an anti $\alpha 4$ -integrin antibody; an anti-CD137 antibody; an anti-CCR4 antibody; an anti-CD123 antibody; an anti-CD133 antibody; an anti-CD138 antibody; an anti-CD19 antibody; an anti-CD20 antibody; an anti-CD22 antibody; an anti-CD33 antibody; an anti-CD38 antibody; an anti-CD40 antibody; an anti-CD49d antibody; an anti-CD52 antibody; an anti-CD70 antibody; an anti-CD74 antibody; an anti-CD79b antibody; an anti-CD80 antibody; an anti-CEA antibody; an anti-cMet antibody; an anti-Cripto antibody; an anti-CTLA-4 antibody; an anti-DLL3 antibody; an anti-TRAIL-2/DR5 antibody; an anti-E-cadherin antibody; an anti-endoglin antibody; an anti-EpCAM antibody; an anti-epidermal growth factor receptor antibody; an anti-FGFR3 antibody; an anti-fibronectin extra-domain B antibody; an anti-folate receptor 1 antibody; an anti-glypican 3 antibody; an anti-gp95/97 antibody; an anti-Her2 antibody; an anti-IGF-1R antibody; an anti-IL-13R antibody; an anti-IL-4 antibody; an anti-IL-6 antibody; an anti-MMP-9 antibody; an anti-MUC1 antibody; an anti-mucin core protein antibody; an anti-NGcGM3 antibody; an anti-P-cadherin antibody; an anti-PD-L1 antibody; an anti-p-glycoprotein antibody; an anti-PSCA antibody; an anti-PSMA antibody; an anti-SLAMF7 antibody; an anti-TRAIL-R1/DR4 antibody; an anti-transferrin antibody; an anti-TROP-2 antibody; or an anti-VEGF antibody.

[00457] Item 133. The kit or composition of item 128, wherein the first and/or second targeting moiety comprises Alemtuzumab, Andecaliximab, Atezolizumab, Avelumab, BCD-100, Bevacizumab, BGB-A317, Blinatumomab, Brentuximab, BU59, Camrelizumab, Carotuximab, Catumaxomab, Cemiplimab, Cetuximab, Daratumumab, Depatuxizumab, Dinutuximab, DS-8201, Durvalumab, Edrecolomab, Elotuzumab, G544, Gemtuzumab, Glembatumumab, GP1.4, hp67.6,

IBI308, Ibritumomab, Inotuzumab, Ipilimumab, Isatuximab, L19IL2, L19TNF, Margetuximab, Mirvetuximab, Mogamuizumab, Moxetumomab, Natalizumab, Necitumumab, Nivolumab, Obinutuzumab, Ofatumumab, Olaratumab, Oportuzumab, Panitumumab, PDR001, Pembrolizumab, Pertuzumab, Polatuzumab, Racotumomab, Ramucirumab, Rituximab, Rovalpituzumab, Sacituzumab, SM3, TAK-164, Tositumomab, Trastuzumab, Tremelimumab, Ublituximab, Urelumab, Utomilumab, XMAB-5574, or Zolbetuximab.

[00458] Item 134. The kit or composition of any one of items 1-127, wherein the first and/or second targeting moiety comprises IL-2, IL-4, IL-6, α -MSH, transferrin, folic acid, EGF, TGF, PD-1, IL-13, stem cell factor, insulin-like growth factor (IGF), or CD40.

[00459] Item 135. The kit or composition of item 134, wherein the first and/or second targeting moiety comprises a full-length sequence of IL-2, IL-4, IL-6, α -MSH, transferrin, folic acid, EGF, TGF, PD-1, IL-13, stem cell factor, insulin-like growth factor (IGF), or CD40.

[00460] Item 136. The kit or composition of item 134, wherein the first and/or second targeting moiety comprises a truncated form, analog, variant, or derivative of IL-2, IL-4, IL-6, α -MSH, transferrin, folic acid, EGF, TGF, PD-1, IL-13, stem cell factor, insulin-like growth factor (IGF), or CD40.

[00461] Item 137. The kit or composition of any one of items 1-134, wherein the first and/or second targeting moiety binds the IL-2 receptor, IL-4, IL-6, melanocyte stimulating hormone receptor (MSH receptor), transferrin receptor (TR), folate receptor 1 (FOLR), folate hydroxylase (FOLH1), EGF receptor, PD-L1, PD-L2, IL-13R, CXCR4, IGFR, or CD40L.

[00462] Item 138. The kit or composition of any one of items 1-129, wherein the second targeting moiety binds an antigen expressed by a tumor microenvironment cell.

[00463] Item 139. The kit or composition of item 138, wherein the tumor microenvironment cell is a fibroblast or macrophage.

[00464] Item 140. The kit or composition of item 139, wherein the antigen expressed by a fibroblast is fibroblast activation protein.

[00465] Item 141. The kit or composition of item 139, wherein the antigen expressed by a macrophage is MAC-1/CD11b or sideroflexin 3.

[00466] Item 142. A method of treating cancer expressing a tumor antigen that binds the first targeting moiety and/or second targeting moiety in a patient comprising administering the composition of any one of items 1-141 to the patient.

[00467] Item 143. The method of item 142, wherein the cancer is evaluated for the presence of infiltrating immune cells before administering the composition.

[00468] Item 144. The method of any one of items 142-143, wherein the cancer is evaluated for the presence of tumor antigens before administering the composition.

[00469] Item 145. The method of any one of items 142-144, wherein the first targeting moiety and second targeting moiety bind the same antigen.

[00470] Item 146. The method of any one of items 142-144, wherein the first targeting moiety and second targeting moiety bind different antigens.

[00471] Item 147. The method of any one of items 142-146, wherein the cancer expressing a tumor antigen that binds the first and/or second targeting moiety is any one of breast cancer, ovarian cancer, endometrial cancer, cervical cancer, bladder cancer, renal cancer, melanoma, lung cancer, prostate cancer, testicular cancer, thyroid cancer, brain cancer, esophageal cancer, gastric cancer, pancreatic cancer, colorectal cancer, liver cancer, leukemia, myeloma, nonHodgkin lymphoma, Hodgkin lymphoma, acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, lymphoproliferative disorder, myelodysplastic disorder, myeloproliferative disease or premalignant disease.

[00472] Item 148. A method of targeting immune cells to cancer expressing a tumor antigen that binds both the first and/or second targeting moiety in a patient comprising administering the composition of any one of items 1-141 to the patient.

[00473] Item 149. A method of targeting immune cells to cancer expressing two tumor antigens, wherein one tumor antigen binds the first targeting moiety and one tumor antigen binds the second targeting moiety, in a patient comprising administering the composition of any one of items 1-141 to the patient.

[00474] Item 150. A method of delivering a cytokine to an immune cell of a patient comprising administering the composition of any one of items 76-147 to the patient, wherein the first and/or second complementary functional domain of the

TWICE comprise IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

V. Supporting Tables

[00475] The following supporting tables have been referred to in the application above.

Table 2: Coordination of Cancer Type, Targets for Targeting Moiety, and Optional Proteases that Can Cleave Cleavage Sites		
Cancer	Targets for Targeting Moiety	Optional Proteases that can Cleave Cleavage Site if TWICE Presented as a Cleavable Single Polypeptide Chain or if TWICE comprises an inert binding partner
Prostate Cancer	ADAM17, CD59, EpCAM, HER2, Integrin α V, Integrin α V β 3, MCP-1, PCLA, PSCA, PSMA, RANKL, RG1, SLC44A4 STEAP-1, VEGF-C	KLK2, KLK3 (PSA), KLK4, ADAM17, Cathepsin B, uPA, uPAR, HPN, ST14, TMPRSS2
Breast Cancer	CA125, CCN1, CD44, CD98, c-RET, DLL4, EpCAM, Episialin, GPNMB, HER2/neu, HER3, IGF-1R, Integrin α 6 β 4, LFL2, LIV-1, Ly6E, MUC1, MUC18, NRP1, Phosphatidylserine, PRLR, TACSTD-2, Tenascin C, TWEAKR, VANGL2, PD-L1, PD-L2	MMP2, MMP9, Cathepsin L, Cathepsin K, Cathepsin B, MMP11, HPN, ST14, ADAM28
Myeloma	BCMA, IGF-1R, DKK-1, ICAM-1, CD138/Syndecan1, CD38, GRP78, FGFR3, SLAMF6, CD48, Tfr(CD71) APRIL, CD40, CD19, TRAIL-R2/DR5, CXCR4	MMP2, MMP9, MMP1, MMP7, TMPRSS2, PRSS22, KLK11
B-cell Lymphoma	CD20, CD22, CD19, CD37, CD70, HLA-DR, CD70b	ADAM28, Cathepsin B, MMP9
Renal Cell carcinoma	PD-L1, PD-L2, CAIX, TPBG, CD70, ENPP3, FGFR1	ST14, MMP9
Gastric Carcinoma	VEGFR-2, CLDN18, GCC, C242, HER2/neu, FGFR2, EpCAM, GPR49, HER3, IGFR	MMP2, MMP9, Cathepsin B, uPA, uPAR
Glioblastoma	HER2/neu, EGFR, ALK, EphA2, GD2, EGFRvIII, ALK	MMP2, MMP9,
T-cell lymphoma	CD2, CD4, CD5, CD71, CD30	Cathepsin B, Cathepsin D, MMP9
Hodgkin Lymphoma	CD30, CD40, IL-3Ra, CD30	Cathepsin B

Lung Cancer	EGFR, IGF-1R, HER3, Integrin $\alpha 5\beta 1$, Lewis y/b antigen, EGFL7, TPBG, DKK-1, NaPi2b, flt4, cMet, CD71	Cathepsin B, MMP2, MMP9, ST14, ADAM17
Pancreatic Carcinoma	SLC44A4, uPAR, MUC1, MUC16, TACSTD-2, CEA, EphA4, mesothelin, EGFR, MUC13, MU5AC, AGF-1R, HER3, CD71	Cathepsin B, ST14, ADAM28
Head and Neck cancer	EGFR, EpCAM, HER2	Cathepsin B, ST14, ADAM17
Acute myeloid leukemia	CD33, CD133, CD123, CD45, CD98, c-Kit, Lewis Y, Siglec-15, FLT-3	ADAM17, Cathepsin B, uPA, uPAR
Melanoma	MUC18, CD40, GD2, CEACAM1, Cadherin-19, GM3, Integrin $\alpha 5\beta 1$, TYRP1, GD3, Integrin αV	Cathepsin B, MMP9
Ovarian Cancer	HER2/neu, EpCAM, CA125, DLL4, Integrin $\alpha V\beta 3$, MUC5A, NaPi2B, Mesothelin, CLDN6	Cathepsin B, MMP2, MMP9
Liver Cancer	Glypican-3, FGFR4, ENPP3, PIVKA-II, PLVAP, cMet, EpCAM	Cathepsin B, MMP9
Colorectal Carcinoma	EGFR, Lewis y/b, Progastrin, GPR49, CEA, CLDN1, A33, CK8, Integrin αV , EpCAM, DLL4, EGFL7, FAP,	Cathepsin S, Cathepsin L, Cathepsin B, uPA, uPAR, MMP2, MMP9, ST14

Table 3: Potential Targeting Moieties

Targeting Moiety	Cancer Type
Antibody against CD20 (such as Rituximab)	Lymphoma
Antibody against CD80	Lymphoma
Antibody against CD22 (such as Inotuzumab)	Lymphoma
Antibody against CD70	Lymphoma
Antibody against CD30	Lymphoma (Hodgkin, T-cell, and B-cell)
Antibody against CD19	Lymphoma
Antibody against CD74	Lymphoma
Antibody against CD40	Lymphoma
Antibody against HER2	Epithelial malignancies, breast cancer, sarcoma
Antibody against EpCAM	Epithelial malignancies, hepatocellular carcinoma, lung cancer, pancreatic cancer, colorectal carcinoma
Antibody against EGFR (such as Cetuximab)	Breast cancer, epithelial malignancies, gliomas, lung cancer, colorectal carcinoma, ovarian carcinoma, brain cancer
Antibody against mucin protein core	Breast cancer
Antibody against transferrin receptor	Gliomas
Antibody against gp95/gp97	Drug-resistant melanomas

Antibody against p-glycoprotein	Drug-resistant melanomas
Antibody against TRAIL-R1/DR4	Multiple malignancies, including ovarian and colorectal carcinoma
Antibody against TRAIL-R2/DR5	Multiple malignancies, including ovarian and colorectal carcinoma
Antibody against IL-4	Lymphomas and leukemias
Antibody against IL-6	Lymphomas and leukemias
Antibody against PSMA	Prostate carcinoma
Antibody against PSCA	Prostate carcinoma
Antibody against P-cadherin (CDH3)	Epithelial malignancies
Antibody against LI-cadherin (CDH17)	Gastrointestinal malignancies
Antibody against CEACAM5	Epithelial malignancies
Antibody against CEACAM6	Epithelial malignancies
Antibody against CEACAM7	Epithelial malignancies
Antibody against Tmprss4	Epithelial malignancies
Antibody against CA9	Epithelial malignancies
Antibody against GPA33	Epithelial malignancies
Antibody against STEAP1	Epithelial malignancies, particularly prostate
Antibody against CLDN6	Epithelial malignancies, particularly ovarian
Antibody against CLDN16	Epithelial malignancies, particularly ovarian
Antibody against LRRC15	Epithelial malignancies
Antibody against TREM2	Epithelial malignancies
Antibody against CLDN18	Epithelial malignancies, particularly pancreatic
Antibody against Cripto (TDGF1)	Epithelial malignancies
Antibody against PD-L1	Epithelial adenocarcinoma
Antibody against IGF-1R	Epithelial adenocarcinoma
Antibody against CD38	Myeloma
Antibody against BCMA	Myeloma
Antibody against CD138	Myeloma
Antibody against CD33	Myeloid malignancies, such as AML
Antibody against CD37	B-cell malignancies
Antibody against CD123	Myeloid malignancies such as AML
Antibody against CD133	Myeloid malignancies such as AML
Antibody against CD49d	Myeloid malignancies such as AML
Antibody against Glypican 3	Hepatocellular carcinoma
Antibody against TM4SF5	Hepatocellular carcinoma, pancreatic cancer
Antibody against cMet	Hepatocellular carcinoma
Antibody against MUC1	Pancreatic cancer, ovarian carcinoma
Antibodies against mesothelin (MSLN)	Pancreatic, ovarian and epithelial cancers and mesothelioma

Antibody against GD2	Sarcoma, brain cancers
Antibody against HER3	Breast cancer
Antibody against IL-13R	Brain cancer
Antibody against DLL3	Small-cell carcinoma, brain cancer
Antibody against MUC16	Ovarian cancer
Antibodies against TFR2	Liver cancer
Antibodies against TCR B1 or TCRB2 constant region	T-cell malignancies
Antibodies against TSHR	Thyroid malignancies

Table 4: Representative antibodies approved for cancer indications

International Nonproprietary Name	Target; Format	1st indication approved / reviewed
Ado-trastuzumab emtansine	HER2; Humanized IgG1, ADC	Breast cancer
Alemtuzumab	CD52; Humanized IgG1	Chronic myeloid leukemia; multiple sclerosis
Atezolizumab	PD-L1; Humanized IgG1	Bladder cancer
Avelumab	PD-L1; Human IgG1	Merkel cell carcinoma
Bevacizumab	VEGF; Humanized IgG1	Colorectal cancer
Blinatumomab	CD19, CD3; Murine bispecific tandem scFv	Acute lymphoblastic leukemia
Brentuximab vedotin	CD30; Chimeric IgG1, ADC	Hodgkin lymphoma, systemic anaplastic large cell lymphoma
Catumaxomab	EPCAM/CD3; Rat/mouse bispecific mAb	Malignant ascites
Cemiplimab	PD-1; Human mAb	Cutaneous squamous cell carcinoma
Cetuximab	EGFR; Chimeric IgG1	Colorectal cancer
Daratumumab	CD38; Human IgG1	Multiple myeloma
Dinutuximab	GD2; Chimeric IgG1	Neuroblastoma
Durvalumab	PD-L1; Human IgG1	Bladder cancer
Edrecolomab	EpCAM; Murine IgG2a	Colorectal cancer
Elotuzumab	SLAMF7; Humanized IgG1	Multiple myeloma
Gemtuzumab	CD33; Humanized IgG4, ADC	Acute myeloid leukemia
Ibritumomab tiuxetan	CD20; Murine IgG1	Non-Hodgkin lymphoma
Inotuzumab	CD22; Humanized IgG4, ADC	Hematological malignancy
Ipilimumab	CTLA-4; Human IgG1	Metastatic melanoma

Mogamuizumab	CCR4; Humanized IgG1	Cutaneous T-cell lymphoma
Moxetumomab pasudotox	CD22; Murine IgG1 dsFv immunotoxin	Hairy cell leukemia
Necitumumab	EGFR; Human IgG1	Non-small cell lung cancer
Nivolumab	PD-1; Human IgG4	Melanoma, non-small cell lung cancer
Obinutuzumab	CD20; Humanized IgG1; Glycoengineered	Chronic lymphocytic leukemia
Ofatumumab	CD20; Human IgG1	Chronic lymphocytic leukemia
Olaratumab	PDGFR α ; Human IgG1	Soft tissue sarcoma
Panitumumab	EGFR; Human IgG2	Colorectal cancer
Pembrolizumab	PD-1; Humanized IgG4	Melanoma
Pertuzumab	HER2; Humanized IgG1	Breast Cancer
Ramucirumab	VEGFR2; Human IgG1	Gastric cancer
Rituximab	CD20; Chimeric IgG1	Non-Hodgkin lymphoma
Sacituzumab govitecan	TROP-2; Humanized IgG1 ADC	Triple-negative breast cancer
Tositumomab-I131	CD20; Murine IgG2a	Non-Hodgkin lymphoma
Trastuzumab	HER2; Humanized IgG1	Breast cancer

Table 5: Antibodies in development for cancer indications			
INN or code name	Molecular format	Target	Late-stage study indication(s)
Utomilumab	Human IgG2	CD137 (4-1BB)	Diffuse large B-cell lymphoma
XMAB-5574, MOR208	Humanized IgG1	CD19	Diffuse large B-cell lymphoma
Ublituximab	Chimeric IgG1	CD20	Chronic lymphocytic Leukemia, non-Hodgkin lymphoma, multiple sclerosis
Moxetumomab pasudotox	Murine IgG1 dsFv immunotoxin	CD22	Hairy cell leukemia
Isatuximab	Humanized IgG1	CD38	Multiple myeloma
Polatuzumab vedotin	Humanized IgG1 ADC	CD79b	Diffuse large B-cell lymphoma
Tremelimumab	Human IgG2	CTLA-4	Non-small cell lung, head & neck, urothelial cancer, hepatocellular carcinoma
Rovalpituzumab tesirine	Humanized IgG1 ADC	DLL3	Small cell lung cancer
Depatuxizumab mafodotin	IgG1 ADC	EGFR	Glioblastoma
Carotuximab	Chimeric IgG1	Endoglin	Soft tissue sarcoma, angiosarcoma, renal cell carcinoma, wet age-related macular degeneration
Oportuzumab monatox	Humanized scFv immunotoxin	EpCAM	Bladder cancer
L19IL2/L19TNF	scFv immuno-conjugates	Fibronectin extra-domain B	Melanoma
Mirvetuximab soravtansine	IgG1 ADC	Folate receptor 1	Epithelial ovarian cancer, peritoneal carcinoma, fallopian tube cancer
Glembatumumab vedotin	Human IgG2 ADC	gpNMB	gpNMB+ breast cancer, melanoma
Margetuximab	Chimeric IgG1	HER2	Breast cancer
(vic-)trastuzumab duocarmazine	Humanized IgG1 ADC	HER2	Breast cancer
DS-8201	Humanized ADC	HER2	HER2+ gastric or gastroesophageal junction adenocarcinoma
Andecaliximab	Humanized IgG4	MMP-9	Gastric cancer or gastroesophageal junction adenocarcinoma
Racotumomab	Murine IgG1	NGcGM3	Non-small cell lung cancer
Camrelizumab	Humanized IgG4	PD-1	Hepatocellular carcinoma, esophageal carcinoma
Cemiplimab	Human mAb	PD-1	Cutaneous squamous cell carcinoma; non-small cell lung cancer, cervical cancer

IBI308	Human mAb	PD-1	Squamous cell non-small cell lung cancer
BGB-A317	Humanized mAb	PD-1	Non-small cell lung cancer
BCD-100	Human mAb	PD-1	Melanoma
PDR001	Humanized IgG4	PD-1	Melanoma
Sacituzumab govitecan	IgG1 ADC	TROP-2 (epithelial glyco- protein-1)	Triple-neg. breast cancer

Table 6: Selected publications on antibodies that bind tumor antigens

Antigen	Publications
Her2/Neu	• Carter P et al., <i>Humanization of an anti-p185HER2 antibody for human cancer therapy</i>, Proc Natl Acad Sci U S A 89(10):4285-9 (1992). This paper discloses the heavy and light chain sequences in its Figure 1B. • US20090202546 (<i>Composition comprising antibody that binds to domain II of her2 and acidic variants thereof</i>). This application discloses the variable light and variable heavy chain sequences in its claim 8. • Olafsen T et al., <i>Characterization of engineered anti-p185HER-2 (scFv-CH3)₂ antibody fragments (minibodies) for tumor targeting</i>, Protein Eng Des Sel (4):315-23 (2004). This paper discloses light and heavy chain variable region sequences in its Figure 1.
EpCAM/CD326	• WO2008122551 (<i>Anti-epcam antibody and uses thereof</i>). This application discloses CDR sequences in claims 1-7. • WO2010142990 A1 (<i>Anti-EpCAM Antibodies</i>). This application discloses CDR sequences in its claims 1-5 and 7. • US6969517 (<i>Recombinant tumor specific antibody and use thereof</i>). This application discloses light and heavy chain sequences in its claims 1-4.
EGFR	• Garrett J et al., <i>Antibodies specifically targeting a locally misfolded region of tumor associated EGFR</i>, Proc Natl Acad Sci U S A 106(13): 5082-5087 and pages 1-7 of Supporting Information including Figures S1-S5 (2009). This paper discloses CDR sequences in its Supplemental Information Figure S1. (A). • US5844093 (<i>Anti-egfr single-chain fws and anti egfr antibodies</i>). This patent discloses CDR sequences in its Figure 1.
PSMA	• US20110028696 A1 (<i>Monoclonal antibodies against prostate specific membrane antigen (psma) lacking in fucosyl residues</i>). This application discloses CDR sequences in claims 3-4. • WO2003064606 (<i>Human monoclonal antibodies to prostate specific membrane antigen (psma)</i>). This application discloses CDR sequences in its claim 1.

CA125	• WO2011119979 A2 (<i>Antibodies to muc16 and methods of use thereof</i>). This application discloses VH and VL sequences in its claim 6. • US20080311134 A1 (<i>Cysteine engineered anti-muc16 antibodies and antibody drug conjugates</i>). Figures 1-4 of this application show heavy and light chain sequences.
Carbonic Anhydrase IX	• WO2007065027 A2 (<i>Carbonic anhydrase ix (g250) antibodies and methods of use thereof</i>). This application discloses CDR sequences in its claims 4-10. • US7378091B2 (<i>Antibodies against carbonic anhydrase IX (CA IX) tumor antigen</i>). This application discloses CDR sequences in its Figures 26-29.
c-met/HGFR	• US20050054019 A1 (<i>Antibodies to c-met</i>). This application discloses heavy and light chain sequences in its claim 6 and CDR sequences in its claim 7. • US 20090175860 A1 (<i>Compositions and methods of use for antibodies of c-Met</i>). This application discloses CDRs in its Figures 1-3 and heavy and light chain sequences in its claims 12-13.
TRAIL-R1/DR4	• US20040214235 A1 (<i>Anti-trail-r antibodies</i>). This application discloses heavy and light chain sequences in its claims 54-55. • US20060062786 A1 (<i>Antibodies that immunospecifically bind to TRAIL receptors</i>). This application discloses VH and VL sequences in its claims 1-2.
TRAIL-R2/DR5	• US20070031414A1 (<i>DR5 antibodies and uses thereof</i>). This application discloses heavy and light chain sequences in its claim 1. • US7790165B2 (<i>Antibody selective for a tumor necrosis factor-related apoptosis-inducing ligand receptor and uses thereof</i>). This application discloses heavy and light chains sequences in its claims 1-5.
IGF-1R	• US 20040086503 A1 (<i>Antibodies to insulin-like growth factor receptor</i>). This application discloses light and heavy chain variable region sequences and CDR sequences in its claims 11-14. • US 20070196376 A1 (<i>Binding proteins specific for insulin-like growth factors and uses thereof</i>). This application discloses CDR sequence data in its claims 46-47. • WHO Drug Information Vol. 24, No. 2, 2010 INN_PL103. This document discloses the sequence of ganitumab on pages 144-145.
VEGF-R2	• Rinderknecht M et al., <i>Phage-Derived Fully Human Monoclonal Antibody Fragments to Human Vascular Endothelial Growth Factor-C Block Its Interaction with VEGF Receptor-2 and 3</i>, PLoS One 5(8): e11941 (2010). This paper discloses CDR sequences in its Table 2.

	WO1998045331 A2 (<i>Anti-VEGF antibodies</i>). This application discloses CDR sequences in its claims 6, 8, and 9.
Prostate stem cell antigen (PSCA)	- US20090181034 A1 (<i>Antibodies and related molecules that bind to psca proteins</i>). This application discloses VH and VL sequences in its claim 17. - US6,790,939 B2 (<i>Anti-PSCA antibodies</i>). This application discloses CDR sequences in its Figure 61. - WO2009032949 A2 (<i>High affinity anti-prostate stem cell antigen (psca) antibodies for cancer targeting and detection</i>). This application discloses CDR sequences in its Figure 2.
MUC1	- Thie H et al., <i>Rise and Fall of an Anti-MUC1 Specific Antibody</i>, PLoS One Jan 14;6(1): e15921 (2011). This paper discloses CDR sequences in its Figure 1. - Henderikx H et al., <i>Human Single-Chain Fv Antibodies to MUC1 Core Peptide Selected from Phage Display Libraries Recognize Unique Epitopes and Predominantly Bind Adenocarcinoma</i>, Cancer Res. 58(19):4324-32 (1998). This paper discloses CDR sequences in its Table 2.
CanAg	US20080138898 A1 (<i>Methods for improving antibody production</i>). This application discloses CDR sequences in its Figure 5.
Mesothelin	WO2009068204 A1 (<i>Anti-mesothelin antibodies and uses therefor</i>). This application discloses CDR sequences in its Table 7.
P-cadherin	WO2010001585 A1 (<i>Anti-CDH3 antibodies labeled with radioisotope label and uses thereof</i>). This application discloses VH and VL variable region sequences disclosed in its paragraph [0033] and CDR sequences in claim 2-7.
Myostatin/GDF8	- US7,632,499 B2 (<i>Anti-myostatin antibodies</i>). This application discloses CDR sequences in its claim 1. - US 20090148436 A1 (<i>Antibody to GDF8 and uses thereof</i>). This application discloses CDR, VH, and VL sequences in its claims 2-8.
Cripto/TDGF1	- US20100008906 A1 (<i>Cripto binding molecules</i>). This application discloses light and heavy chain sequences in its paragraph [0491] and CDR sequences in its paragraph [0492]. - US7,531,174 B2 (<i>Cripto blocking antibodies and uses thereof</i>). This application discloses a list of hybridomas that secrete anti-Cripto antibodies in its Tables 1 and 2. These hybridomas were available for purchase from the ATCC.
MUC5AC	Chung WC et al., <i>CREB mediates prostaglandin F2alpha-induced MUC5AC overexpression</i>, J Immunol 182(4):2349-56 (2009) at page 3, second paragraph discloses that clone 45M1 was an anti-MUC5AC antibody available for purchase.

CEACAM	• Pavoni E. et al., <i>Selection, affinity maturation, and characterization of a human scFv antibody against CEA protein</i>, BMC Cancer 6:41 (2006). This paper discloses CDR sequences of clone E8 in its Figure 3. Reactivity of E8 with CEACAM is shown in its Figure 6.
SLC44A4 (formerly known as protein 24P4C12 which was renamed SLC44A4 by the Hugo Convention (see US8039497 at 114:56-62))	• US20090175796 A1 (<i>Antibodies and related molecules that bind to 24p4c12 Proteins</i>). This application discloses light and heavy chain variable domain sequences in its Figures 2 and 3. • US8,039,597 B2 (<i>Antibodies and related molecules that bind to 24p4c12 Proteins</i>). This application discloses light and heavy chain variable domain sequences in its claim 1 and in its Figures 2 and 3. • US8,309,093 B2 (<i>Antibody drug conjugates (ADC) that bind to 24P4C12 proteins</i>). This application discloses light and heavy chain variable domain sequences in its claim 1 and in its Figures 2 and 3. • US20100330107 A1 (<i>Antibody drug conjugates (ADC) that bind to 24P4C12 proteins</i>). This application discloses light and heavy chain variable domain sequences in its claims 1 and 2, and in its Figures 2 and 3. • WO2010111018 A1 (<i>Antibody drug conjugates (ADC) that bind to 24P4C12 proteins</i>). This application discloses light and heavy chain variable domain sequences in its claims 1 and 2, and in its Figures 2 and 3.
Neuropilin 1	• US8,318,163 B2 (<i>Anti-pan neuropilin antibody and binding fragments thereof</i>). This application discloses light and heavy chain variable domain sequences in its claim 1 and in its Figures 7 and 8. • WO 2008/143666 (<i>Crystal structures of neuropilin fragments and neuropilin-antibody complexes</i>). This application discloses light and heavy chain variable domain sequences in its claim 8 and in its Figures 7 and 8.
Glypican	• US7,867,734 B2 (<i>Anti-glypican 3 antibody having modified sugar chain</i>). This application discloses the heavy chain variable region in its claim 1. CDR sequences are disclosed in Table 1 of this application. • US7,871,613 B2 (<i>Adjuvant therapy with the use of anti-glypican 3 antibody</i>). This application discloses the heavy chain sequence in its claim 6 and the light chain sequence in its claim 7.
EphA2	• US20100298545 A1. (<i>Epha2 agonistic monoclonal antibodies and methods of use thereof</i>). This application discloses CDR sequences in its claim 50. • US20100278838 A1. (<i>Epha2 monoclonal antibodies and methods of use thereof</i>). This application discloses VH/VL and CDR sequences in its claim 101. • US20100183618 A1 (<i>Anti-epha2 antibody</i>). This application discloses CDR sequences in its claim 11.

E-cadherin	US5,610,281 (<i>Antibodies for modulating heterotypic E-cadherin interactions with human T lymphocytes</i>). This application discloses that anti- E-cadherin clone E4.6 is available for the ATCC (HB 11996) in its claim 4.
CEA	- WO2004032962 A1 (<i>Combination therapy with class iii anti-cea monoclonal antibodies and therapeutic agents</i>). This application discloses CDR sequences in its claim 6 and its claim 14. - US5,877,293 (<i>CDR grafted anti-CEA antibodies and their production</i>). This application discloses antibody sequences in its claims 1-5. - US20080069816 A1 (<i>Humanized anti-cea t84.66 antibody and uses thereof</i>). This application discloses antibody sequence in its claims 22-23.
FGFR3	- US20080044419 A1 (<i>Treatment of T Cell Mediated Diseases by Inhibition of Fgfr3</i>). This application discloses scFv sequences in claim 6 and VH/VL sequences in its claims 7-10. - US20090175866 A1 (<i>Treatment of B-cell malignancies</i>). This application discloses Vh, Vl and CDR sequences in its claims 11-12. - Martínez-Torrecuadrada J et al. <i>Targeting the extracellular domain of fibroblast growth factor receptor 3 with human single-chain Fv antibodies inhibits bladder carcinoma cell line proliferation</i>. Clin Cancer Res 11(17):6280-90 (2005). This publication shows VH and VL sequences of a scFv in its Figure 2.
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Table 7: Non-Antibody Scaffolds and Corresponding Targets

Scaffold	Demonstrated Targets
Adnectin	EGFR, IGF-1R
Affibodies	HER2, EGFR, IGF-1R, HER3
Affinlins	CTLA-4
Anticalins	CD137/HER2 (a bispecific)
Atrimers	TRAIL-R1/DR4
Avimers	IL6 (could be used in oncology to block growth)
Bicyclic peptides	HER2
Cys-knots	NaV1.7 (proof of concept)
DARPin	VEGF-a, HER2, VEGF/HGF (bispecific)
Fynomers	HER2
Pronectins	VEGFR2
TN3	TRAIL-R2

Table 8: Non-Antibody Binding Partners and Corresponding Targets

Binding Partner	Target on Cancer Cell
IL-2	IL-2 receptor
IL-4	IL-4 receptor
IL-6	IL-6 receptor

α -MSH	MSH receptor (melanocyte stimulating hormone receptor)
Transferrin	TR (transferrin receptor)
Folic acid	FOLR (folate receptor 1) and/or FOLH1 (folate hydroxylase)
EGF and/or TGF α	EGFR (EGF receptor)
PD-1	PD-L1 and/or PD-L2
IL13	IL-13R (Glioblastoma)
Stem cell factor	CXCR4
Insulin-like growth factor (IGF)	IGFR
CD40	CD40L

Table 9: Selected References Showing Specificity of Exemplary Anti-CD3 Antibodies

Muromonab/OKT3	- Herold KC et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. <i>Diabetes</i>. 54(6):1763-9 (2005). - Richards J et al. Phase I evaluation of humanized OKT3: toxicity and immunomodulatory effects of hOKT3gamma4. <i>Cancer Res</i>. 59(9):2096-10 (1999). - Kuhn C and Weiner HL. Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. <i>Immunotherapy</i> 8(8):889-906 (2016).
Otelixizumab	- Kuhn C et al. Human CD3 transgenic mice: preclinical testing of antibodies promoting immune tolerance. <i>Sci Transl Med</i>. 3(68):68ra10 (2011). - Kuhn C and Weiner HL. Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. <i>Immunotherapy</i> 8(8):889-906 (2016). - Dean Y et al. Combination therapies in the context of anti-CD3 antibodies for the treatment of autoimmune diseases. <i>Swiss Med Wkly</i>. 142:w13711 (2012). - Daifotis AG et al. Anti-CD3 clinical trials in type 1 diabetes mellitus. <i>Clin Immunol</i>. 149(3):268-78 (2013) (abstract). - Chatenoud L and Waldmann H. CD3 monoclonal antibodies: a first step towards operational immune tolerance in the clinic. <i>Rev Diabet Stud</i>. 9(4):372-81. (2012).
Teplizumab	- Masharani UB and Becker J. Teplizumab therapy for type 1 diabetes. <i>Expert Opin Biol Ther</i>. 10(3):459-65 (2010). - Herold KC et al. Treatment of patients with new onset Type 1 diabetes with a single course of anti-CD3 mAb Teplizumab preserves insulin production for up to 5 years. <i>Clin Immunol</i>. 132(2):166-73 (2009). - Kuhn C and Weiner HL. Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. <i>Immunotherapy</i> 8(8):889-906 (2016).

	• Dean Y et al. Combination therapies in the context of anti-CD3 antibodies for the treatment of autoimmune diseases. <i>Swiss Med Wkly.</i> 142:w13711 (2012). • Daifotis AG et al. Anti-CD3 clinical trials in type 1 diabetes mellitus. <i>Clin Immunol.</i> 149(3):268-78 (2013) (abstract). • Chatenoud L and Waldmann H. CD3 monoclonal antibodies: a first step towards operational immune tolerance in the clinic. <i>Rev Diabet Stud.</i> 9(4):372-81 (2012).
Visilizumab	• Kuhn C and Weiner HL. Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. <i>Immunotherapy</i> 8(8):889-906 (2016). • Shan L. ^{99m}Tc-Labeled succinimidyl-6-hydrazinonicotinate hydrochloride (SHNH)-conjugated visilizumab. Molecular Imaging and Contrast Agent Database (MICAD) [Internet]. Created: December 7, 2009; Last Update: January 12, 2010; Downloaded May 3, 2018. • Dean Y et al. Combination therapies in the context of anti-CD3 antibodies for the treatment of autoimmune diseases. <i>Swiss Med Wkly.</i> 142:w13711 (2012).
Foralumab	• Kuhn C and Weiner HL. Therapeutic anti-CD3 monoclonal antibodies: from bench to bedside. <i>Immunotherapy</i> 8(8):889-906 (2016). • Dean Y et al. Combination therapies in the context of anti-CD3 antibodies for the treatment of autoimmune diseases. <i>Swiss Med Wkly.</i> 142:w13711 (2012).
SP34	• Pessano S et al. The T3/T cell receptor complex: antigenic distinction between the two 20-kd T3 (T3-6 and T3-E) subunits. <i>EMBO Journal</i> 4(2):337-344 (1985).

Table 10: Selected References Showing Specificity of Exemplary Anti-TCR Antibodies

• Verma-B. et al. TCR Mimic Monoclonal Antibody Targets a Specific Peptide/HLA Class I Complex and Significantly Impedes Tumor Growth In Vivo Using Breast Cancer Models <i>J Immunol.</i> 184: 2156–2165 (2010). • Conrad ML et al. TCR and CD3 antibody cross-reactivity in 44 species. <i>Cytometry A.</i> 71(11):925-33 (2007). • Koenecke C et al. In vivo application of mAb directed against the gammadelta TCR does not deplete but generates "invisible" gammadelta T cells. <i>Eur J Immunol.</i> 39(2):372-9 (2009). • Exley MA et al. Selective activation, expansion, and monitoring of human iNKT cells with a monoclonal antibody specific for the TCR alpha-chain CDR3 loop. <i>Eur J Immunol.</i> 38(6):1756-66 (2008). • Deetz CO et al. Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the Toll-Like receptor 2 agonist Pam3Cys. <i>Infection and Immunity.</i> 74(8):4505-4511 (2006). • Tang X et al. Anti-TCR antibody treatment activates a novel population of nonintestinal CD8 alpha alpha+ TCR alpha beta+ regulatory T cells and prevents

- experimental autoimmune encephalomyelitis. *J Immunol.* 178(10):6043-50 (2007).
- Lavasani S et al. Monoclonal antibody against T-cell receptor alphabeta induces self-tolerance in chronic experimental autoimmune encephalomyelitis. *Scand J Immunol.* 65(1):39-47 (2007).
 - Nasreen M et al. In vivo treatment of class II MHC-deficient mice with anti-TCR antibody restores the generation of circulating CD4 T cells and optimal architecture of thymic medulla. *J Immunol.* 171(7):3394-400 (2003).

Table 11: Antibodies that modulate T-cell function		
Antigen	Antibody Examples	Function
CD3e	Blinatumomab (Blincyto®)	co-stimulatory
PD-1	Pembrolizumab (Keytruda®), Nivolumab (Opdivo®)	antagonist
CTLA-4	Ipilimumab (Yervoy®)	antagonist
TIM-3	TSR-022 (NCT02817633), Sym023 (NCT03489343)	antagonist
LAG-3	BMS-986016 (NCT01968109)	antagonist
KIR	BMS-986015/Lirilumab (NCT01714739)	antagonist
CD28	Theralizumab/TGN1412 (NCT03006029)	co-stimulatory
41BB/CD137/ TNFRSF9	Utomilumab/PF-05082566 (NCT03364348) or Urelumab	co-stimulatory
OX40/CD134	PF-04518600 (NCT02315066), BMS 986178 (NCT03410901)	co-stimulatory
CD27	Varlilumab/CDX-1127 (NCT01460134)	co-stimulatory
ICOS	JTX-2011 (NCT02904226)	co-stimulatory

EXAMPLES

Example 1. TWICE that block two inhibitory pathways in an immune cell

[00476] A TWICE can be developed to act on a range of cancer and immune cells. These examples serve to show how one skilled in the art could generate a TWICE with a particular function.

[00477] For example, a TWICE may be generated with a synergistic combination of two checkpoint inhibitory antibodies.

[00478] As a first step, one skilled in the art would first choose targeting moieties appropriate for the given cancer. The targeting moieties may be chosen based on literature on a given type of cancer and the prevalence of certain antigens. Alternatively, one could evaluate antigens that are expressed by the cancer in a specific patient. For example, rituximab (anti-CD20 antibody) can be used to target to CD20-positive lymphomas, and cetuximab (anti-EGFR antibody) can be used to target certain solid tumors.

[00479] A TWICE may allow use of two targeting moieties. One skilled in the art could choose two targeting moieties to target both the first component and the second component to a given cancer. Ovarian cancer may be targeted using CA125 for targeting one component and using EpCAM to target the other component.

[00480] After choosing appropriate targeting moieties, one skilled in the art could determine what type of immune cell binding domains and complementary binding domains, when paired, might have the greatest therapeutic efficacy.

[00481] **Figure 1** shows how a TWICE could employ two targeting domains to a cancer cell to allow activation through two signaling pathways on an immune cell.

[00482] To enhance an anti-tumor immune response, one may want to generate a TWICE that inhibits two checkpoint molecules, and therefore boost an anti-cancer immune response compared to inhibition of either checkpoint molecule alone.

[00483] For example, a TWICE could be generated where the first component comprises the VH of ipilimumab (anti-CTLA4 antibody) as the immune cell binding domain and the VL of nivolumab (an anti-PD-1 antibody) as the complementary binding domain. Then, the second component of this TWICE could comprise the VL of ipilimumab (anti-CTLA4 antibody) as the immune cell binding domain and the VH of nivolumab (an anti-PD-1 antibody) as the complementary binding domain. The function of the TWICE is not impacted by which component has the VH or VL of the given antibody, as long as one component has a VH and the other component has a VL.

[00484] By generating a TWICE in this manner, both the first and second components of the TWICE could be targeted to the cancer. At the cancer site, the VH and VL of ipilimumab and the VH and VL of nivolumab could pair based on the close proximity of the first and second components. This would allow the TWICE, after domain pairing, to block two immune checkpoints and lead to robust immune cell activation.

Example 2. TWICE that stimulates two pathways in an immune cell

[00485] A TWICE may be developed that (when the immune cell binding domains and the complementary binding domains are paired) stimulates two pathways in an immune cell to mediate a robust anticancer effect. For example, the cancer might be breast cancer that expresses EGFR.

[00486] For any of these Examples, the second targeting moiety may be selected to target to tumor microenvironment cells. In this Example, the second targeting moiety may be chosen to target to tumor-associated macrophages (TAMs). Thus, while the first targeting moiety (in the first component) may bind an antigen expressed by the tumor, the second targeting moiety (in the second component) may bind an antigen expressed by a TAM. For targeting breast cancer expressing EGFR, the first targeting moiety may be Cetuximab and the second targeting moiety may be MAC-1/CD11b.

[00487] **Figure 2** outlines a TWICE where the first component is targeted to a cancer cell and the second component is targeted to a non-cancer cell (in this Example a TAM). This type of TWICE will also pairing of domains only when the cancer cell is in close proximity to the TAM. Pairing of the immune cell binding domains and pairing of the complementary binding domains can stimulate 2 pathways on an immune effector cell.

[00488] For example, one skilled in the art may choose to activate both the CD3e and CD137 pathways. To do this, he/she could generate a TWICE wherein the first component comprises a VH for SP34 (anti-CD3 antibody) and a VL for utomilumab (anti-CD137 antibody) and the second component comprises a VL for SP34 (anti-CD3 antibody) and a VH for utomilumab (anti-CD137 antibody).

[00489] By generating a TWICE in this manner, both the first and second components of the TWICE could be targeted to a cancer cell that is in close proximity to a TAM, for example at the tumor stroma. The VH and VL of SP34 and the VH and VL of utomilumab could then pair based on the close proximity of the first and second components. This would allow the TWICE, after domain pairing, to provide two positive stimuli to T cells.

[00490] In a similar fashion to that described in this Example or Example 1, a TWICE could be generated to both block an inhibitory checkpoint molecule (e.g., by paired immune cell binding domains) and to provide a positive stimulus to the immune system (e.g. by paired complementary binding domains). These types of construct may also robustly activate an anti-cancer immune response.

Example 3. TWICE that activates two different immune cells at the cancer site

[00491] A TWICE could combine activity at two different immune effector cells. For example, a TWICE could be generated to mediate T-cell activation and macrophage activation.

[00492] A targeting moiety to an antigen expressed by the cancer cell could be chosen as outlined in Example 1. In this particular example, both targeting moieties can bind HER2 on the breast cancer cell. The two targeting moieties may either be identical or bind to different epitopes on HER2. Then, as shown in **Figure 3**, the paired immune cell binding domains of the TWICE could bind to a first effector cell (in this case a T cell) and the paired complementary binding domains could bind to a second effector cell (in this case a macrophage).

[00493] One skilled in the art may choose to activate both CD3 in a T cell and the CD40 pathway in a macrophage. To do this, he/she could generate a TWICE wherein the first component comprises a VH for SP34 (anti-CD3 antibody) and a VL for CP-870,893 (anti-CD40 antibody) and the second component comprises a VL for SP34 (anti-CD3 antibody) and a VH for CP-870,893 (anti-CD40 antibody).

[00494] By generating a TWICE in this manner, the first and second component would be targeted to the cancer cell. At the cancer cell, the VH and VL of SP34 and the VH and VL of CP-870,893 could pair based on the close proximity of the first and second components. This would allow the TWICE, after domain pairing, to stimulate both T cells and macrophages at the site of the cancer.

[00495] In an analogous fashion, a TWICE could be designed to activate combinations of T cells and NK cells at the site of the cancer.

Example 4. TWICE that modulates immune cells and cancer cells

[00496] A TWICE could also combine activity at both an immune cell and directly at the cancer cell. For example, a TWICE could be generated to mediate T-cell activation and to block an immune checkpoint molecule expressed by the cancer cell.

[00497] Two targeting moieties to a single antigen expressed by the cancer cell could be chosen as outlined in Example 3, such as HER2 on a breast cancer cell. Then, as shown in **Figure 4**, the paired immune cell binding domains of the TWICE could bind to an effector cell (in this case a T cell) and the paired complementary binding domains could bind to the cancer.

[00498] One skilled in the art may choose to activate CD3 in a T cell and block PD-L1 in the cancer cell. To do this, he/she could generate a TWICE wherein the first component comprises a VH for SP34 (anti-CD3 antibody) and a VL for avelumab (anti-PD-L1 antibody) and the second component comprises a VL for SP34 (anti-CD3 antibody) and a VH for avelumab (anti-PD-L1 antibody).

[00499] By generating a TWICE in this manner, both components would be targeted to the cancer cell. At the cancer cell, the VH and VL of SP34 and the VH and VL of avelumab could pair based on the close proximity of the first and second components. This would allow the TWICE, after domain pairing, to stimulate T cells in the tumor microenvironment and block immune inhibition mediated by PD-L1 expressed by the cancer cell.

[00500] In an analogous fashion, a TWICE could be designed that bind RANK or cell-death-inducing antigens on the cancer cell.

[00501] For example, one skilled in the art may choose to activate CD3 on a T cell and directly induce cancer cell death. To do this, he/she could generate a TWICE wherein the first component comprises a VH for SP34 (anti-CD3 antibody) and a VL for mapatumumab (anti-TRAIL-R1/DR4 death receptor antibody) and the second component comprises a VL for SP34 (anti-CD3 antibody) and a VH for mapatumumab (anti-TRAIL-R1/DR4). This would allow the TWICE, after domain pairing at the cancer cell, to stimulate T cells and directly trigger cancer cell death.

[00502] Thus, a TWICE could mediate a direct anti-cancer effect *and* stimulate an anti-cancer immune response.

Example 5. TWICE comprising dimerization domains

[00503] In any of constructs described in Examples 1-4, the TWICE may also comprise a dimerization domain, as shown in Figure 6. One dimerization domain of a pair could attach to the immune cell binding domain, while the other dimerization domain of the pair could attach to the complementary binding domain. For example, each component may comprise a pair of knob-into-hole dimerization sequences. One or both of the dimerization domains could be attached via a cleavable linker (termed a dimerization domain linker), such as a linker comprising an ADAM28 cleavage site (SEQ ID NO: 1).

[00504] In this way, the knob-into-hole dimerization domains of each component force pairing of the immune cell binding domain and the complementary binding domain of an individual component. After targeting to the tumor microenvironment by the targeting moieties, the cleavable linker attaching one or both dimerization domain could be cleaved, and the dimerization domain released. In this way, the immune cell binding domain and/or the complementary binding domain would become available to pair with their corresponding domain in the other component only in the tumor microenvironment.

[00505] Thus, dimerization domains could be used to force binding of the immune cell binding domain and complementary binding domain of each component outside of the desired site of action at the tumor microenvironment and to block potential pairing of domains between components outside of the tumor microenvironment. Cleavage of the dimerization domain linker by a protease in the tumor microenvironment, in contrast, could allow domain swapping and activity only at the tumor.

[00506] A TWICE could be designed where both components comprise dimerization domains or where only one component comprises dimerization domains.

Example 6. TWICE comprising a complementary functional domain

[00507] Other TWICE may comprise a complementary functional domain that has activity when targeted to a cancer cell, as shown in Figure 5. In this Example, the first component comprises an attenuated IFN-alpha cytokine (see Pogue et al., *PLoS ONE* 11(9): e0162472 (2016)).

[00508] Both the first and second components may comprise an anti-EPCAM scFv. The first component may comprise an anti-CD3E VH domain such as that of SP34 (i.e., the immune cell binding domain) linked through a 25-mer linker having an MMP2 cleavage site (AIPVSLR; SEQ ID NO: 46) to an inert binding partner VL domain from gantenerumab. The second component may comprise an anti-CD3E VL domain such as that of SP34 (i.e., the immune cell binding domain) linked through a 25-mer linker having an MMP2 cleavage site (AIPVSLR; SEQ ID NO: 46) to an inert binding partner VH domain from clone alpha-MUC1-1 antibody.

[00509] In this way, the immune cell binding domains of the first and second components are masked unless cleaved by the tumor-associated protease MMP2. When the first and second components are targeted to the EPCAM-positive tumor, cleavage by MMP2 can mediate release of the inert binding domains (i.e., inert binding partners). In this way, the VH and VL of SP34 could bind and activate a T cell at the site of the cancer. Further, targeting of the attenuated IFN-alpha to the cancer cell by the EPCAM antibody could lead to direct anti-cancer effects. This type of TWICE could mediate anti-cancer effects by an attenuated cytokine *and* stimulate an anti-cancer immune response.

[00510] Similarly, a TWICE could employ IL-15 or an attenuated IL-2 to modulate T-cell activation.

[00511] In an analogous fashion, a TWICE could be designed that comprise other attenuated cytokines as complementary functional domains in the first component. A TWICE could also be designed that comprise a complementary functional domain in *both* the first and second components for an additive effect.

Example 7. TWICE comprising various dimerization domain linkers and various dimerization domains

[00512] To construct examples of TWICE molecules, a well-established anti-EpCAM antibody targeting cancer cells was used. The EpCAM antibody Solitomab (Brischwein K, et al *Mol Immunol.* 43(8):1129-43 (2006)) was chosen, and this antibody was used in the format of an antigen binding fragment (Fab) comprising two chains, a heavy chain consisting of a heavy chain variable plus a CH1 domain, and a light chain consisting of a light chain variable and kappa constant domain.

[00513] For initial testing of these TWICE molecules, the VH of the anti-CD3epsilon antibody SP34 was fused to the C-terminus of the heavy chain of the Solitomab Fab, and the VL of the anti-PDL1 antibody Atezolizumab was fused to the C-terminus of the light chain of the Solitomab Fab using flexible linkers of 15 amino acids in length.

[00514] As shown in Figure 6, TWICE molecules may contain a dimerization domain that links the mispaired variable domains and prevents domain swap unless the TWICE are activated proteolytically in the tumor microenvironment. This dimerization domain may be constructed as a heterodimerization to enforce the efficient formation of heterodimers between the VH and VL domains and avoid the formation of homodimers. Several heterodimerization domains have been described in the literature. To test which heterodimerization domains are suitable for engineering TWICE, several TWICE were constructed and expressed using various heterodimerization domains.

[00515] First, a TWICE was constructed using a coiled-coil heterodimerization domain as shown in Figure 7A. Coiled-coils have also been called leucine zippers. Sequences of coiled-coil domains that have been engineered as acid and base coil to form heterodimers are listed in US 2016 0002356 A1 and in US 8877893 B2. In the present example, the heavy chain of the TWICE molecule was fused to a basic coil (SEQ ID NO: 201) and the light chain was fused to an acid coil (SEQ ID NO: 202). One of ordinary skill in the art will know that the heavy chain might also be fused to an acid coil and the light chain fused to a basic coil.

[00516] Table 12 lists the dimerization domain linkers included in the coiled-coil TWICE constructs. These dimerization domain linkers may contain a sequence that can be cleaved by a protease. Protease cleavage will lead to separation of the dimerization domain from the rest of the TWICE molecule, which allows the complementation of the immune cell binding VH or VL domain between two TWICE by domain swap. In the examples listed in Table 12, several examples of dimerization domain linkers are listed. In some of the examples, the linker contains a sequence that can be cleaved by a matrix metalloprotease in a tumor microenvironment. In other examples, the dimerization domain linker contains a protease site that can be cleaved by Factor Xa (FXa), which can be used to artificially activate TWICE molecules *in vitro*. In other words, a Factor Xa cleavage site was employed in the examples as a proxy for a protease cleavage site because Factor Xa is convenient to use in the laboratory setting. In some of the examples listed in Table 12, the heavy chain and the light chain of one TWICE molecule comprise the same protease cleavage sequence in their dimerization domain linker, and in others the heavy chain and the light chain of one TWICE molecule comprise different protease cleavage sequences in their dimerization domain linker.

[00517] In some of the examples listed in Table 12, the TWICE contains a heterodimeric Fc as a dimerization domain. Here, the lysine repositioning strategy disclosed in WO2017106462A1 was used to construct TWICE with a hetero-Fc dimerization domain. Table 12 shows the dimerization domain linker that were used in the TWICE molecules TWICE191 and TWICE192. The TWICE191 comprises the heavy chain of SEQ ID NO: 171 and the light chain of SEQ ID NO: 172, and the TWICE192 comprises the heavy chain of SEQ ID NO: 173 and the light chain of SEQ ID NO: 174. These TWICE contain the mutations S364K/K409L in the Fc of the heavy chain and K370S/F405K in the Fc of the light chain to promote the formation of a hetero-Fc between the heavy and light chain. Engineering TWICE with a hetero-Fc dimerization domain enables binding to FcRn, the neonatal Fc receptor, which mediates recycling of the molecules and extends their serum half-life. These TWICE examples further contain the N297Q mutation in the Fc, which produces an aglycosylated molecule with impaired effector function.

[00518] In another example the Fab constant domains (CH1 and CL) were used as dimerization domains to construct a TWICE molecule. In order to allow for efficient assembly of the heavy and light chain of a TWICE molecule, which also

contains a Fab as a cancer targeting moiety, the dimerization domain CH1 and CL were constructed as a CrossFab (Figure 7A). A CrossFab is a strategy that enables efficient assembly of the two light chains of a bispecific antibody construct by crossover of the constant domains in one of the Fabs such that the VH is linked to the CL and the VL is linked to the CH1 (Schaefer W et al., *Proc Natl Acad Sci U S A*. 108(27):11187-92 (2011)). In the TWICE constructs containing a CrossFab dimerization domain (TWICE200 and TWICE204, see Table 13), the VH of the immune cell engager is linked to a CL domain with a dimerization domain linker sequence, and the VL of the complementary engager is linked to a CH1 domain with the dimerization domain linker sequence. A Factor Xa protease cleavage sequence (Ile-Glu-Gly-Arg) was included in all of the dimerization domain linkers. The full sequence of the TWICE200 comprises the heavy chain of SEQ ID NO: 177 and the light chain of SEQ ID NO: 178, and the construct TWICE204 comprises the heavy chain of SEQ ID NO: 179 and the light chain of SEQ ID NO: 180.

[00519] In another example, the TWICE was constructed using the IgE CH2 domain as a dimerization domain. The IgE CH2 normally functions as a hinge domain in IgE, but this domain has been used to construct Fab-like molecules with similar binding properties by domain substitution (Cooke et al, *mAbs*. 10(8):1248-1259 (2018)). In the TWICE189 and TWICE190, the IgE CH2 domain was fused to the immune cell engager VH and VL domains using the dimerization domain linker sequences given in Table 12. The dimerization domain linkers contain a Factor Xa cleavage sequence (Ile-Glu-Gly-Arg).

Table 12. TWICE constructs encompassing various dimerization domains and linkers			
Construct	Dimerization domain linker (VH)	Dimerization domain linker (VL)	Dimerization domain
TWICE117	GGGGSIEGRGGGS (SEQ ID NO: 203)	GGPLGVRGKGGGS (SEQ ID NO: 204)	leucine zipper
TWICE193	GGGGSIEGRGGSGGGS (SEQ ID NO: 205)	GGGGSIEGRGGSGGGS (SEQ ID NO: 205)	leucine zipper
TWICE191	GGGIEGRGGG (SEQ ID NO: 206)	GGGIEGRGGG (SEQ ID NO: 206)	Hetero-Fc
TWICE192	GGGGSIEGRGGG (SEQ ID NO: 207)	GGGGSIEGRGGG (SEQ ID NO: 207)	Hetero-Fc
TWICE200	IEGRG (SEQ ID NO: 208)	GGIEGRG (SEQ ID NO: 209)	CH1/CL
TWICE204	IEGRG (SEQ ID NO: 208)	GGIEGRG (SEQ ID NO: 209)	CH1/CL
TWICE189	GIEGRG (SEQ ID NO: 210)	GIEGRG (SEQ ID NO: 210)	IgE CH2
TWICE190	GGGIEGRG (SEQ ID NO: 211)	GGGIEGRG (SEQ ID NO: 211)	IgE CH2

Table 13. Exemplary TWICE			
	Molecule	Heavy chain SEQ ID NO	Light chain SEQ ID NO
TWICE117	SP34 VH Atezolizumab VL coiled-coil	165	166
TWICE189	SP34 VH Atezolizumab VL EDH2	167	168
TWICE190	SP34 VH Atezolizumab VL 8-mer linker EDH2	169	170
TWICE191	SP34 VH Atezolizumab VL Fc	171	172
TWICE192	SP34 VH Atezolizumab VL G4S-linker Fc	173	174
TWICE193	SP34 VH Atezolizumab VL 20-mer linker coiled-coil	175	176
TWICE200	SP34 VH FXa CL Atezolizumab VL FXa CH1	177	178
TWICE204	Atezolizumab VH FXa CL SP34VL FXa CH1	179	180
TWICE277	SP34 VH Ipilimumab VL Fc	171	181
TWICE278	Ipilimumab VH SP34 VL Fc	182	183
TWICE281	SP34 VH Ipilimumab VL coiled-coil	165	184
TWICE282	Ipilimumab VH SP34 VL coiled-coil	185	186
TWICE283	Ipilimumab VH Atezolizumab VL coiled-coil	185	166
TWICE284	Atezolizumab VH Ipilimumab VL coiled-coil	187	184
TWICE285	SP34 VH Ipilimumab VL 20-mer linker coiled-coil	175	188
TWICE286	Ipilimumab VH -SP34 VL 20-mer linker coiled-coil	189	190
TWICE332	dsFv SP34 VH Ipilimumab VL Fc	191	192
TWICE335	dsFv Ipilimumab VH SP34 VL Fc	193	194
CTRL351	Epcam/Ipilimumab bsAb	185	184
CTRL352	EpCAM/SP34 bsAb	175	190
CTRL353	EpCAM/Ipilimumab bsAb	195	196
CTRL354	EpCAM/Atezolizumab bsAb	189	188
TWICE394	Theralizumab VH SP34 VL Fc	195	183
TWICE395	SP34 VH Theralizumab VL Fc	171	196
CTRL396	EpCAM/Theralizumab bsAb	195	196
CTRL399	EpCAM/Urelumab bsAb	197	198
TWICE400	Theralizumab VH Urelumab VL Fc	195	198
TWICE401	Urelumab VH Theralizumab VL Fc	197	198
CTRL426	EpCAM/SP34 Fc bsAb	171	183
TWICE431	SP34 VH dummy VL Fc	171	199
TWICE432	dummy VH SP34 VL Fc	200	183
CTRL406	EpCAM/CD16A Fc bsAb	212	213
CTRL437	EpCAM/OKT3 Fc bsAb	214	215
TWICE440	OKT3 VH/CD16A VL Fc	214	213
TWICE441	CD16A VH/OKT3 VL Fc	212	215

[00520] The TWICE proteins listed in Table 13 were expressed in transient HEK293 cultures (30-50 ml) in shake-flasks by co-transfection of the respective heavy chains and light chains. The expressed proteins were purified from

supernatant by FPLC using IMAC columns (Histrap Excel, GE) or in the case of Fc-containing TWICE proteins by protein A columns (MabSelect PrismaA, GE). The purified proteins were analyzed by SDS-PAGE and showed bands at the expected molecular weight for all the linkers and dimerization domains tested (Figure 7B). The expression levels were highest for TWICE with coiled-coil or hetero-Fc dimerization domains.

[00521] The TWICE were tested further by cleavage with recombinant FXa protease. In a reaction of 15 μ l approximately 1 μ g of purified TWICE and 0.1 μ g FXa (New England Biolabs #P8010) were incubated in a buffer containing 20 mM Tris-HCl (pH 8.0) with 100 mM NaCl and 2 mM CaCl_2 for 1 hour at ambient temperature. The reaction was stopped by addition of 5 μ l 4X LDS sample buffer (Thermo Fisher NP0007) and heating to 95°C for 2 minutes. The samples were then separated by SDS PAGE to analyze cleavage products (Figure 7C).

[00522] The samples TWICE193, TWICE191 and TWICE192, which contain FXa cleavage sequences in both dimerization domain linkers, i.e. in the heavy chain and in the light chain (Table 13), were cleaved completely by FXa (Figure 7C). The TWICE117, which has a FXa cleavage site in the heavy chain dimerization domain linker and an MMP2 cleavage site in the light chain dimerization domain linker, was cleaved only partially (Figure 7B). The constructs TWICE200, TWICE204, TWICE189 and TWICE190, which have FXa cleavage sites included in both dimerization domain linkers, were not cleaved by FXa protease under these conditions. This result suggests that the configuration of the dimerization domains (CH1/CL or IgE CH2) or the linker lengths in the constructs TWICE200, TWICE204, TWICE189 and TWICE190, which are 5 to 8 residues in length (Table 12), are not favorable for protease cleavage in these particular constructs. However, the coiled-coil and the hetero-Fc are dimerization domains that are included in the designs TWICE117, TWICE193, TWICE192, and TWICE193, are suitable for the construction of TWICE. The TWICE molecules containing these dimerization domains expressed well and could be activated by cleavage with recombinant protease.

Example 8. TWICE with various binding moieties: Fv vs Fab

[00523] TWICE molecules were engineered with the CD3 antibody SP34 and the CTLA4 antibody Ipilimumab and containing the hetero-Fc dimerization domain. In some examples, the anti-EpCAM antibody Solitomab was used as a Fab

(TWICE277 and TWICE278), in other examples this antibody was used as a disulfide-stabilized Fv (dsFv, TWICE332 and TWICE335) by engineering a disulfide between the residue 44 of the VH and residue 100 of the VL (referring to Kabat numbering). The TWICE molecules were expressed and purified by Protein A affinity chromatography as described above, and their ability to bind to EpCAM was tested in an ELISA binding assay. Recombinant soluble EpCAM-His6 protein (produced in-house) was coated on ELISA plates at 5 µg/ml at 4°C overnight and plates blocked with 1%BSA in PBS. Serial dilutions of TWICE were allowed to bind to EpCAM, and the bound TWICE were detected with a mouse anti-human Fc secondary (JDC-10 Abcam, ab99759). The results (Figures 8A-8B) showed that TWICE molecules with Fab or dsFv targeting moieties bound to the antigen with similar binding affinities, with KD ranging from 1.04 nM to 1.65 nM with the dsFv TWICEs, and 1.21 nM to 1.22 nM for the Fab TWICEs. Thus, TWICE molecules can be constructed with different targeting moieties, for example Fab or dsFv, to target cells in the tumor microenvironment.

Example 9. Testing of a CD3/CTLA-4 TWICE in a bridging ELISA

[00524] To test the domain swap and generation of functional paratopes (i.e., paired VH/VL domains) by TWICE pairs, a bridging ELISA assay was developed that measures the ability of TWICE pairs to bind target antigen and immune cell engaging molecules simultaneously. The schematic drawing in Figure 9A illustrates the principle of this bridging ELISA. Briefly, the ELISA plates are coated with target antigen (recombinant soluble EpCAM, black hexagon) at a concentration of 5 µg/ml overnight. TWICE molecules can be activated by cleavage with FXa as described above, and then added to the plate at a serial dilution. The TWICE can bind to the target antigen with their target binding moiety, which is drawn as Fab (4 grey ovals) in Figure 9A. Binding to the target antigen (EpCAM) on the plate brings the TWICEs in close proximity, which allows the immune cell binding domain and the complementary binding domain to undergo domain swap and form complete immune cell binding paratopes (black and white ovals in Figure 9A). The functional complementation is measured by incubation with an antigen that is bound by the immune cell binding domain, such as CD3 (striped triangle in Figure 9A). The antigen is provided as an Fc-fusion protein (murine IgG2a Fc), and can therefore be detected with an anti-mouse Fc secondary antibody. This detection step by a secondary antibody is omitted in the schematic drawing in Figure 9A for clarity.

[00525] Figure 9B and Figure 9C show examples of bridging ELISAs that measure the functional complementation of a TWICE pair by domain swap. In these examples, the TWICE277 and 278 (as described in Table 13) are used, which contain the immune cell binding antibody SP34 that binds CD3epsilon and the complementary antibody Ipilimumab that binds CTLA-4. The VH of the CD3 antibody and the VL of the CTLA-4 antibody are comprised in the TWICE277, and the VL of the anti-CD3 and the VH of the anti-CTLA-4 are comprised in the TWICE278. These examples of TWICE molecules further contain the hetero-Fc dimerization domains and FXa cleavage sites in both dimerization domain linkers. Figure 9B shows the result from a CD3 bridging ELISA, which measures the ability of TWICE to bind CD3, and Figure 9C shows the result of a CTLA-4 bridging ELISA, which measures the ability of TWICE to bind CTLA-4. In this experiment, the individual TWICE (277 or 278 alone) did not show binding to CD3 or to CTLA-4 even when activated by cleavage with FXa. Furthermore, when the TWICE 277 and 278 were combined but not activated by cleavage with FXa, the TWICE did not show binding to CD3 or CTLA-4. Only when the TWICE277 and 278 were combined and cleaved by FXa to remove the dimerization domain did they show binding to CD3 or CTLA-4 (diamond symbols in Figures 9B and 9C) in a dose-dependent response. These results demonstrate that the TWICE molecules can undergo domain swap and generate functional immune cell binding domains when both TWICE are bound to target antigen. Furthermore, the dimerization domain of these TWICE functions as intended as a locking mechanism that prevents domain swap of TWICE unless the dimerization domain is removed by protease cleavage.

[00526] Figures 10A-10B show another example of CD3/CTLA-4 TWICE molecules that were tested in a bridging ELISA experiment. The TWICE281 and 282 used in this experiment encompass the same EpCAM targeting and CD3 and CTLA-4 antibodies as TWICE277 and TWICE278 described above, but differ in that they contain a coiled-coil dimerization domain. Furthermore, TWICE281 and 282 contain a FXa cleavage site in the heavy chain and an MMP2 cleavage sequence in the light chain, and the molecules can therefore be activated by cleavage by FXa or MMP2. Similar to the previous experiment, the TWICE277 and 278 showed a dose-response binding curve only when activated by cleavage with FXa (Figure 10A and 10B). Without activation by FXa, the TWICE did not bind to either CD3 or CTLA-4. Thus, the hetero-Fc and coiled-coil dimerization domains are both suitable for

engineering TWICE. A hetero-Fc may be preferred when an extended serum half-life of the TWICE is desired.

Example 10. CD3/CD28 TWICE

[00527] Figures 11A-11B show an example of TWICE constructed from the CD3 antibody SP34 and the costimulatory anti-CD28 antibody Theralizumab that were tested in a bridging ELISA experiment (TWICE 394 and 395 from Table 13). These examples of TWICE were again engineered using the hetero-Fc dimerization domain and FXa cleavage sites in both the heavy and light chain linkers. Similar to the previous examples, the TWICE394 and TWICE395 showed binding to immune cell antigens CD3 and CD28 only when they were combined and activated by cleavage (Figure 11A and 11B). The individual TWICE did not show binding to either immune cell antigen. Cleavage was again necessary for domain swap and binding, as evidenced by the fact that the uncleaved TWICE did not show any bridging activity (grey squares in Fig 11A and 11B).

Example 11. CD28/4-1BB TWICE

[00528] Figures 12A-12B show an example of TWICE constructed with the antibodies Theralizumab and Urelumab, which bind the co-stimulatory molecules CD28 and CD137 (4-1BB). The TWICE400 and 401 (as shown in Table 13) were constructed using the variable domains of Theralizumab and Urelumab and containing the hetero-Fc dimerization domain and FXa cleavage sites in both linkers. When these molecules were tested in a bridging ELISA format, the TWICE showed binding to CD28 (Figure 12A) and 4-1BB (Figure 12B), when the constructs were precleaved with FXa and both TWICE were present. Neither TWICE alone bound to CD28 or to 4-1BB.

Example 12. CTLA-4/PD-L1 TWICE

[00529] Figure 13 shows an example of TWICE constructed with the antibodies Ipilimumab and Atezolizumab, which bind to the checkpoint molecules CTLA-4 and PD-L1, respectively. The TWICE283 contains the VH of Ipilumumab and the VL of Atezolizumab, and the complementary TWICE284 contains the VH of Atezolizumab and the VL of Ipilumumab, as shown in Table 13. These TWICE were designed with a coiled-coil dimerization domain and a FXa cleavage site in the heavy chain. When these TWICE were tested in an EpCAM/PD-L1 bridging ELISA, the TWICE 284 exhibited significant binding to PD-L1 (Figure 13), even though this TWICE contains only the VH of Atezolizumab, but lacks the cognate Atezolizumab

VL. This result suggests that the VH of Atezolizumab is sufficient to mediate antigen binding.

[00530] This notion is consistent with the fact that a crystal structure of Atezolizumab in complex with PD-L1 (Lee HT et al. *Sci Rep* 7: 5532-5532 (2017)) reveals that the VL of the antibody makes no significant contact with the antigen and therefore may be dispensable or replaceable for antigen binding. Therefore, Atezolizumab is an example of an antibody that is not suitable for the construction of a TWICE, at least not in conjunction with Ipilimumab, due to the fact that it retains its binding properties in the VH domain when the paratope (i.e., VH/VL pair) is split in a TWICE.

[00531] However, as shown in Figures 9A-12B, Ipilimumab, SP34, Theralizumab, and Urelumab are exemplary antibodies that do not exhibit binding when the paratope is split, and such antibodies are therefore suitable for the construction of TWICE.

Example 13. Immune cell activation by TWICE

[00532] The redirection of T cells by TWICE constructs was tested in an *in vitro* T-cell activation assay. Briefly, colorectal cancer cells (HCT-15) were seeded in 96-well plates at a density of 5,000 cells/well and allowed to adhere overnight. Peripheral blood mononuclear cells (PBMCs) were added at an effector-to-target ratio of 10:1, and the cells were treated with serial dilutions of TWICE molecules. In some of the experiments the TWICE molecules were preactivated by cleavage with FXa protease (NEB # P8010L) prior to the addition to cells.

[00533] To quantify T cell activation, secreted interferon gamma was detected in the media 24 hours after addition of TWICE using an IFNgamma ELISA kit (Invitrogen 88-7316-88). The target cell killing was determined 48 hours after the start of treatment using a cytotoxicity assay that quantitatively measures lactate dehydrogenase (LDH) from lysed cancer cells (CytoTox96, Promega). The target cell killing is expressed as specific lysis (%) in the dose-response curves shown in Figures 14A-16, which corresponds to the fraction of LDH release compared to cells lysed with a lysis buffer, and subtracting the background LDH release from untreated cells.

[00534] Figures 14A-14B show the results of a cell killing assay with the TWICE277 and 278, and the TWICE281 and 282 that had been tested in bridging ELISAs before. As described above, these TWICE contain the anti-CD3epsilon engaging antibody SP34 and the CTLA-4 antibody Ipilimumab. TWICE277 and 278

are constructed with a hetero-Fc dimerization domain that is attached to VH or VL through the linker GGGIEGRGGG (SEQ ID NO: 206), which contains the FXa cleavage sequence Ile-Glu-Gly-Arg. In contrast, the TWICE281 and 282 contain coiled-coil dimerization domains with non-identical linkers in the heavy and light chain (Figure 14A). These TWICE are constructed with a FXa cleavage site in the heavy chain linker GGGGSIEGRGGGGS (SEQ ID NO: 203) and an MMP2 cleavage site in the light chain linker GGPLGVRGKGGGS (SEQ ID NO: 204). The LDH release data from this experiment (Figure 14B) demonstrated that both TWICE pairs induced a strong killing of cancer cells by PBMCs when the TWICEs were pre-activated by FXa prior to addition to the cells. The TWICE277 and 278 did not induce this killing response without precleavage, which confirms that cleavage is necessary for TWICE activation and domain swap, and is consistent with the data obtained in bridging ELISA experiments with these molecules (Figures 9B-9C). However, the TWICE281 and 282, which also required activation by FXa in the bridging ELISA (Figures 10A-10B), readily induced the killing of HCT15 cells by PBMCs in a dose dependent manner even when the TWICE were not preactivated.

[00535] These results show that the TWICE molecules can be activated by cancer cells, which can cleave the MMP2 site in the light chain linker. Altogether this experiment confirmed that: (1) TWICE can efficiently engage immune cells to kill cancer cells; (2) proteolytic cleavage is necessary for TWICE activation, and (3) a protease produced by cancer cells can activate TWICE molecules.

[00536] Figure 15A-15D show the results of a cleavage assay (Figure 15A), a T cell activation assay (Figure 15B), and killing assay (Figures 15C-15D) with TWICE 285 and TWICE286, which also contain the anti-CD3epsilon engaging antibody SP34 and the CTLA-4 antibody Ipilimumab. These examples of TWICE are designed with a FXa cleavage site in the dimerization domain linker GGGGSIEGRGGSGGGS (SEQ ID NO: 205) in both the heavy and the light chain, which is longer than the linkers of the examples shown in Figures 14A-14B above. These examples of TWICE also contain a coiled-coil dimerization domain.

[00537] When the TWICE285 was cleaved with FXa, the molecule produced the expected fragments on SDS-PAGE (Figure 15A), showing that the TWICE can be preactivated with FXa. The cytokine release and killing data shown in Figure 15B and Figure 15C confirmed that the TWICE molecules TWICE285 plus TWICE286 are able to engage and redirect PBMCs against HCT-15 cells when the

TWICE were activated by cleavage with FXa protease. Neither TWICE285 nor TWICE286 alone induced the release of interferon gamma by T cells or the killing of cancer cells.

[00538] However, when these TWICE were tested together without precleavage in an LDH release experiment, the molecules induced cancer cell lysis irrespective of preactivation by FXa (Figure 15D). This result is in contrast to the TWICE examples described above, which required cleavage for activation in the bridging ELISA and T cell activation assays. The data therefore establishes that the design of the dimerization domain and linker length are relevant to TWICE activation. The specific longer linker GGGGSIEGRGSGGGS (SEQ ID NO: 205) of TWICE285 and TWICE286 in conjunction with the coiled-coil dimerization domain present in these constructs may not prevent domain swap in the absence of cleavage.

Example 14. Costimulatory TWICE

[00539] Next, the TWICE394 and TWICE395, which engage CD3 and CD28 as shown in Table 13, were tested in a T cell redirection assay (Figure 16). As described above, these TWICE are constructed from the anti-CD28 antibody Theralizumab and the anti-CD3 epsilon antibody SP34. These molecules can therefore bind to CD3 and CD28 as shown in Figures 11A-11B, which provides costimulatory signals to trigger T cell activation. When these TWICE were tested in a T cell killing assay, the TWICE394 and 395 induced robust killing of HCT-15 cancer cells after preactivation with FXa, but showed no activity without prior cleavage.

[00540] In order to test the stimulatory signal from engagement of CD28 and CD3 by TWICE, a protocol was developed to exhaust T cells prior to testing TWICE activity. For T-cell activation and exhaustion, prostate cancer cells (PC-3) were seeded in a 6-well plate at a density of 150,000 cells/well and allowed to adhere overnight. Peripheral blood mononuclear cells (PBMCs) were added at an effector-to-target ratio of 10:1 and cells were treated with two-component T cell engagers based on SEQ ID NOs: 168 and 169 of US10035856B2 at a concentration of 100nM. This two-component system works similar to a CD3 bispecific antibody in that T cells are engaged to kill cancer cells. However, it has an advantage over a conventional bispecific antibody for pre-activation because the molecules cannot bind to CD3 in solution and can therefore easily be removed by washing the PBMCs. The PBMCs were analyzed 48 hours after the preactivation by flow cytometry, revealing the treatment induced robust upregulation of activation and exhaustion markers on

CD3+ cells (Figure 17A) demonstrating preactivation and exhaustion. Such preactivated immune cells may be more reflective of the immune cells in a tumor microenvironment than unstimulated PBMCs, because tumor-infiltrating lymphocytes are known to be exhausted and to display increased expression of exhaustion markers (Baitsch L et al. *Trends Immunol.* 33(7):364-72 (2012)).

[00541] The pre-activated PBMCs were subsequently used in a redirection assay against colorectal cancer cells (HCT-15) as described above to test the potency of TWICE molecules to trigger T cell activation, except the effector to target ratio was modified to 1:4 in this assay. Figure 17B shows the results of this T cell activation assay. The TWICE394 and TWICE395, which bind CD3 and CD28, induced robust activation of exhausted T cells as measured by the IFN gamma secretion. The TWICE277 and TWICE278, which bind CD3 and CTLA-4, also induced IFNgamma secretion, albeit to lower levels than TWICE394 and TWICE395. A pair of control TWICE, which contain the CD3 antibody VH and VL domains paired with non-functional VH and VL domains (SEQ ID NOs: 199 and 200), were also included in this assay. The control TWICE431 plus TWICE432 produced low levels of interferon gamma in this redirection experiment with exhausted T cells. Altogether, these results are consistent with a recent publication that showed co-stimulatory activation of CD28 together with CD3 by a trispecific antibody generates a superior T cell activation over either stimulus alone, and may amount to a stronger anti-tumor immune response (Wu, L., Seung, E., Xu, L. et al. Trispecific antibodies enhance the therapeutic efficacy of tumor-directed T cells through T cell receptor co-stimulation. *Nat Cancer* (published online November 18, 2019) doi:10.1038/s43018-019-0004-z). Consistent with this observation, the present data suggest that TWICE can potently stimulate exhausted T cells, and the additive effect of co-stimulation or checkpoint blockade can induce a stronger response than CD3 engagement alone. However, contrary to the published trispecific antibody, the TWICE can deliver these co-stimulatory signals in an activatable fashion.

Example 15. TWICE engaging NK cells through CD16A

[00542] In another example, TWICE were engineered to engage natural killer (NK) cells using an antibody against CD16A. The anti-CD16A antibody used to construct these TWICE molecules has been evaluated in clinical trials (NCT01221571) in the molecule AFM13, a bispecific antibody targeting CD30 and CD16A. For evaluating this antibody in a TWICE pair, the anti-CD16A antibody was

combined with the anti-CD3 antibody OKT3 to generate the TWICE molecules TWICE440 and TWICE441, which comprise the hetero-Fc dimerization domain and FXa cleavage sites in the dimerization domain linkers.

[00543] The TWICE molecules were first tested in a CD16A bridging ELISA, similar to the bridging ELISA experiments described above. The TWICE440 and 441 showed the expected binding to CD16A only when both TWICE were present and preactivated with FXa protease (Figure 18A). As seen with other TWICE molecules, precleavage of the TWICE was necessary, and the uncleaved TWICE did not exhibit binding to CD16A in this bridging ELISA assay. Furthermore, the individual TWICE molecules did not show binding to CD16A even when they were precleaved by FXa. The anti-CD16A antibody derived from AFM13 is therefore suitable for use in TWICE, as the halves of the split paratope (i.e. the VH and VL) are not binding to antigen when they are separated in TWICE.

[00544] The TWICE440 and 441 were subsequently tested in a redirection assay using HCT15 colon cancer cells and NK cells. As described above for redirection assays performed with PBMCs described above, the cancer cells were seeded in 96-well plates at 5000 cells/well and allowed to adhere overnight. Next, purified NK cells (Stem Cell Technologies, Cat#70036) were added to the cancer cells at an effector-to-target ratio of 20:1, and preactivated TWICE were added to the wells at various concentrations. Target cell killing was determined by measurement of LDH release 24 hours after addition of TWICE using a cytotoxicity assay that quantitatively measures LDH from lysed cancer cells (CytoTox96, Promega). The results of this NK cell redirection assay are shown in Figure 18B. In this experiment a control bispecific antibody targeting EpCAM and CD16a (CTRL406) induced the release of LDH from cancer cells demonstrating killing by NK cells.

[00545] A control bispecific antibody targeting EpCAM and CD3 also induced LDH release, although to a lesser extent than the CD16a/EpCAM bispecific antibody, possibly due to the presence of some NKT cells. The TWICE440 and 441 induced robust NK redirection and cancer cell killing, which was similar to the amount of killing observed with the CD16a/EpCAM bispecific antibody, demonstrating that TWICE molecules can be engineered to engage NK cells via a CD16a antibody. The engagement of NK cells via the anti-CD16 antibody can induce cancer cell killing.

EQUIVALENTS

[00546] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the embodiments. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiment may be practiced in many ways and should be construed in accordance with the appended claims and any equivalents thereof.

[00547] As used herein, the term about refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term about generally refers to a range of numerical values (e.g., +/-5-10% of the recited range) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). When terms such as at least and about precede a list of numerical values or ranges, the terms modify all of the values or ranges provided in the list. In some instances, the term about may include numerical values that are rounded to the nearest significant figure.

What is Claimed is:

1. A kit or composition for treating cancer in a patient comprising:
 - a. a first component comprising a targeted immune cell binding agent comprising:
 - i. a first targeting moiety that binds a tumor antigen expressed by the cancer;
 - ii. a first immune cell binding domain capable of immune cell binding activity when binding a second immune cell binding domain, wherein the second immune cell binding domain is not part of the first component, and wherein the first immune cell binding domain is either a VH domain or VL domain; and
 - iii. a first complementary binding domain capable of binding to a complementary antigen when binding a second complementary binding domain, wherein
 - (1) the second complementary binding domain is not part of the first component,
 - (2) when the first immune cell binding domain is a VH domain the first complementary binding domain is a VL domain,
 - (3) when the first immune cell binding domain is a VL domain, the first complementary binding domain is a VH domain, and
 - (4) wherein the first complementary binding domain is a binding partner for the first immune cell binding domain, such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the first immune cell binding domain is not bound to the first complementary domain; and
 - b. a second component comprising a targeted immune cell binding agent comprising:
 - i. a second targeting moiety;
 - ii. a second immune cell binding domain capable of immune cell binding activity when binding a first immune cell binding domain, wherein the second immune cell binding domain is a VH if the first immune cell binding domain is a VL and wherein the second immune

cell binding domain is a VL if the first immune cell binding domain is a VH; and

iii. a second complementary binding domain capable of binding to a complementary antigen when binding the first complementary binding domain, wherein

(1) when the second immune cell binding domain is a VH domain the second complementary binding domain is a VL domain,

(2) when the second immune cell binding domain is a VL domain, the second complementary binding domain is a VH domain, and

(3) wherein the second complementary binding domain is a binding partner for the second immune cell binding domain, such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the second immune cell binding domain is not bound to the second complementary domain.

2. The kit or composition of claim 1, wherein the first immune cell binding domain is bound to the first complementary binding domain by a first dimerization domain and a second dimerization domain, wherein:

- a. the first dimerization domain is attached to the first immune cell binding domain by a first linker;
- b. the second dimerization domain is attached to the first complementary binding domain by a second linker; and
- c. the first and/or second linker is a cleavable linker.

3. The kit or composition of claim 1, wherein the second T-cell binding domain is bound to the second complementary binding domain by a first dimerization domain and a second dimerization domain, wherein:

- a. the first dimerization domain is attached to the second T-cell binding domain by a first linker;
- b. the second dimerization domain is attached to the second complementary binding domain by a second linker; and
- c. the first and/or second linker is a cleavable linker.

4. The kit or composition of claim 2, wherein the first and second linkers are cleavable linkers; optionally wherein the protease cleavage sites of the first and/or second cleavable linkers are cleaved by a protease expressed by the cancer or tumor microenvironment cell.

5. The kit or composition of claim 2, wherein the first and second linkers are from 5 to 30 amino acids in length.
6. The kit or composition of claim 5, wherein the first and second linkers are from 8 to 16 amino acids in length.
7. The kit or composition of claim 2, wherein the first and second dimerization domains are both:
 - a. leucine zippers;
 - b. immunoglobulin domains; or
 - c. T-cell receptor (TCR) domains.
8. The kit or composition of claim 7, wherein the first and second dimerization domains are both immunoglobulin domains.
9. The kit or composition of claim 1, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding the cancer; optionally wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding an immune checkpoint molecule, RANK or RANKL, or a cell-death-inducing antigen.
10. The kit or composition of claim 1, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T cell, a macrophage, or a natural killer cell.
11. The kit or composition of claim 10, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a T cell; optionally wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD3, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and mucin-domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, GITR (TNFRSF18), TIGIT, or inducible T-cell costimulatory (ICOS).
12. The kit or composition of claim 10, wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding a natural killer cell; optionally wherein the first complementary binding domain and the second complementary binding domain are, when bound to each other, capable of binding CD16A.

13. A kit or composition for treating cancer in a patient comprising:
 - a. a first component comprising a targeted immune cell binding agent comprising:
 - i. a first targeting moiety that binds a tumor antigen expressed by the cancer;
 - ii. a first immune cell binding domain capable of immune cell binding activity when binding a second immune cell binding domain, wherein the second immune cell binding domain is not part of the first component, and wherein the first immune cell binding domain is either a VH domain or VL domain;
 - iii. a first inert binding partner for the first immune cell binding domain, wherein the first inert binding partner binds to the first immune cell binding domain such that the first immune cell binding domain does not bind to the second immune cell binding domain unless the inert binding partner is removed, wherein if the first immune cell binding domain is a VH domain, the inert binding partner is a VL domain and if the first immune cell binding domain is a VL domain, the inert binding partner is a VH domain;
 - iv. a protease cleavage site separating the first immune cell binding domain and the first inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease:
 - (1) expressed by the cancer or a tumor microenvironment cell; or
 - (2) colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment; and
 - v. a first complementary functional domain capable of immune cell binding, and;
 - b. a second component comprising a targeted immune cell binding agent comprising:
 - i. a second targeting moiety;
 - ii. a second immune cell binding domain; and

- iii. optionally a second complementary functional domain capable of immune cell binding.

14. The kit or composition of claim 13, wherein the second component comprises a complementary functional domain; optionally wherein the complementary functional domain of the first and/or second component comprises a ligand for a receptor; further optionally wherein the complementary functional domain comprises a latent form of a member of the TGF-beta family or a cytokine.

15. The kit or composition of claim 13, wherein the second component further comprises a second inert binding partner for the second immune cell binding domain, wherein the second inert binding partner binds to the second immune cell binding domain such that the second immune cell binding domain does not bind to the first immune cell binding domain unless the inert binding partner is removed, wherein if the second immune cell binding domain is a VH domain, the inert binding partner is a VL domain and if the second immune cell binding domain is a VL domain, the inert binding partner is a VH domain; and further wherein

a protease cleavage site separates the second immune cell binding domain and the second inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding partner from the immune cell binding domain in the presence of a protease:

- a. expressed by the cancer; or
- b. colocalized to the cancer by a targeting moiety that is an antibody or antigen binding fragment thereof that binds (a) a tumor antigen expressed by the cancer and that is the same or different from the first and/or second targeting moiety in the agent or (b) an antigen expressed by a cell in the tumor microenvironment.

16. The kit or composition of claim 1, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a T cell, a macrophage, or a natural killer cell.

17. The kit or composition of claim 16, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a T cell; optionally wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding CD3, the T-cell receptor, programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin and

mucin-domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), killer-cell immunoglobulin-like receptor (KIR), CD28, CD137, OX40, CD27, GITR (TNFRSF18), TIGIT, or inducible T-cell costimulatory (ICOS).

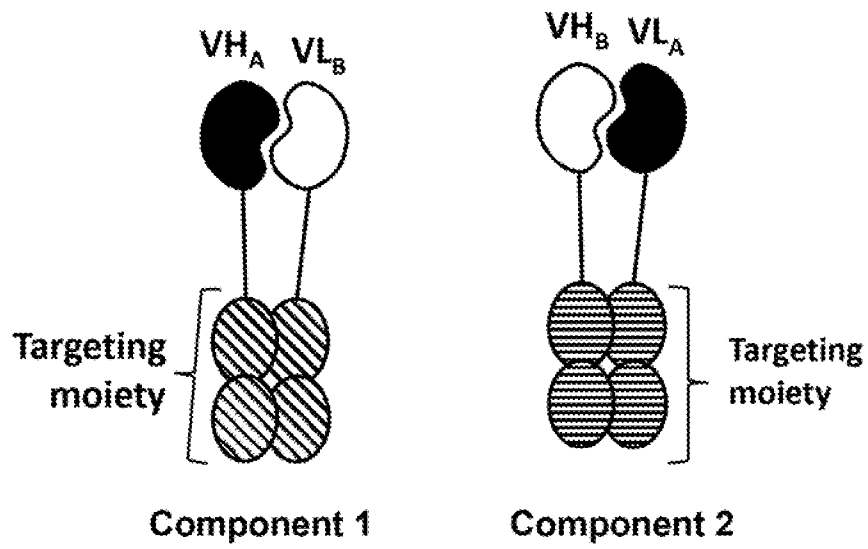
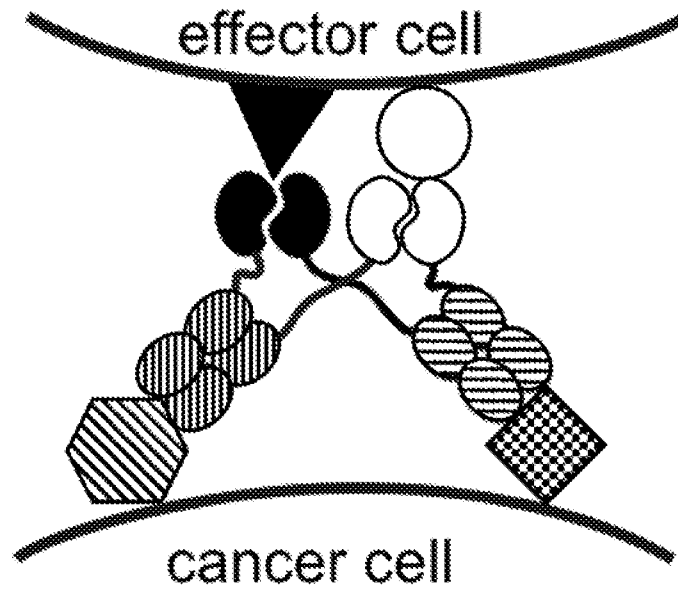
18. The kit or composition of claim 16, wherein the first immune cell binding domain and the second immune cell binding domain are, when bound to each other, capable of binding a natural killer cell; optionally wherein the first immune cell binding domain and the second immune binding domain are, when bound to each other, capable of binding CD16A.

19. The kit or composition of claim 1, wherein the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof; optionally wherein the first and/or second targeting moiety comprises an antibody or antigen binding fragment thereof that binds α 4-integrin; A33; ACVRL 1/ALK1; ADAM17; ALK; APRIL; BCMA; C242; CA125; Cadherin-19; CAIX; CanAg; Carbonic Anhydrase IX; CCN1; CCR4; CD123; CD133; CD137 (4-1BB); CD138/Syndecan1; CD19; CD2; CD20; CD22; CD30; CD33; CD37; CD38; CD4; CD40; CD44; CD45; CD48; CD5; CD52; CD56; CD59; CD70; CD70b; CD71; CD74; CD79b; CD80; CD86; CD98; CEA; CEACAM; CEACAM1; CK8; c-Kit; CLDN1; CLDN18; CLDN18.2; CLDN6; c-met/HGFR; c-RET; Cripto; CTLA-4; CXCR4; DKK-1; DLL3; DLL4; TRAIL-R2/DR5; DRS; EGFL7; EGFR; EGFRvIII; endoglin; ENPP3; EpCAM; EphA2; Episialin; FAP; FGFR1; FGFR2; FGFR3; FGFR4; fibronectin extra-domain B; FLT-3; flt4; folate receptor 1; GCC; GD2; GD3; Glypican-3; Glypicans; GM3; GPNMB; GPR49; GRP78; Her2/Neu; HER3/ERBB3; HLA-DR; ICAM-1; IGF-1R; IGF; IL-3Ra; Integrin α 5 β 1; Integrin α 6 β 4; Integrin α V; Integrin α V β 3; Lewis Y; Lewis y/b antigen; LFL2; LIV-1; Ly6E; MCP-1; Mesothelin; MMP-9; MUC1; MUC18; MUC5A; MUC5AC; Myostatin; NaPi2b; Neuropilin 1; NGcGM3; NRP1; P-cadherin; PCLA; PD-1; PDGFRa; PD-L1; PD-L2; Phosphatidylserine; PIVKA-II; PLVAP; PRLR; Progastrin; PSCA; PSMA; RANKL; RG1; Siglec-15; SLAMF6; SLAMF7; SLC44A4,STEAP-1; TACSTD-2; Tenascin C; TPBG; TRAIL-R1/DR4; TROP-2; TWEAKR; TYRP1; VANGL2; VEGF; VEGF-C; VEGFR-2; or VEGF-R2.

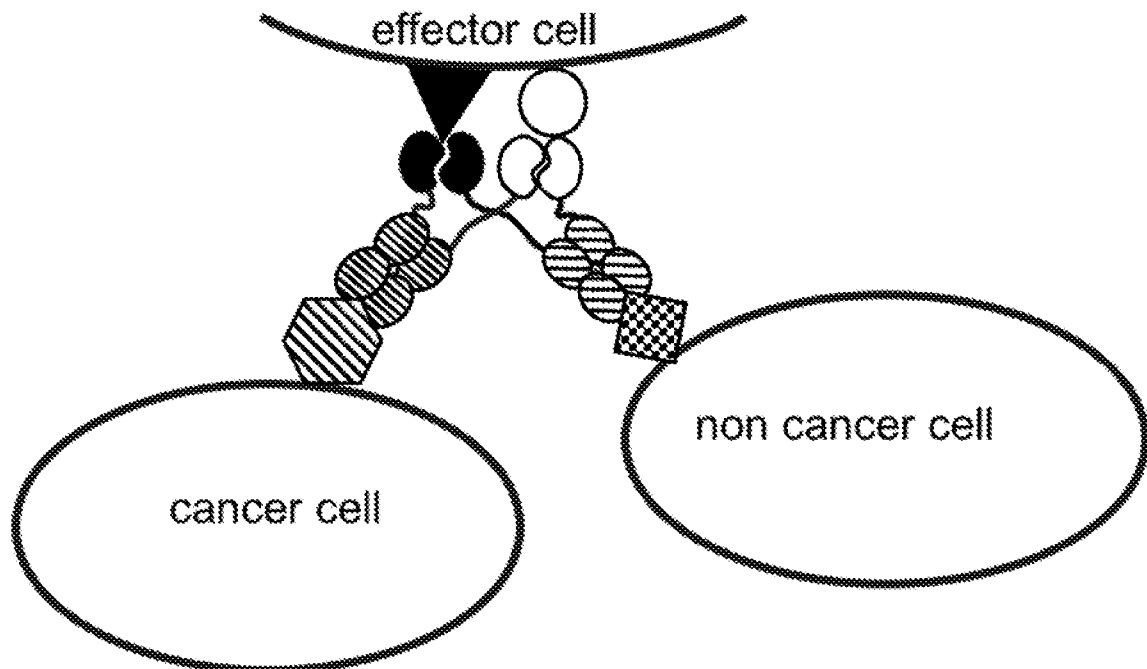
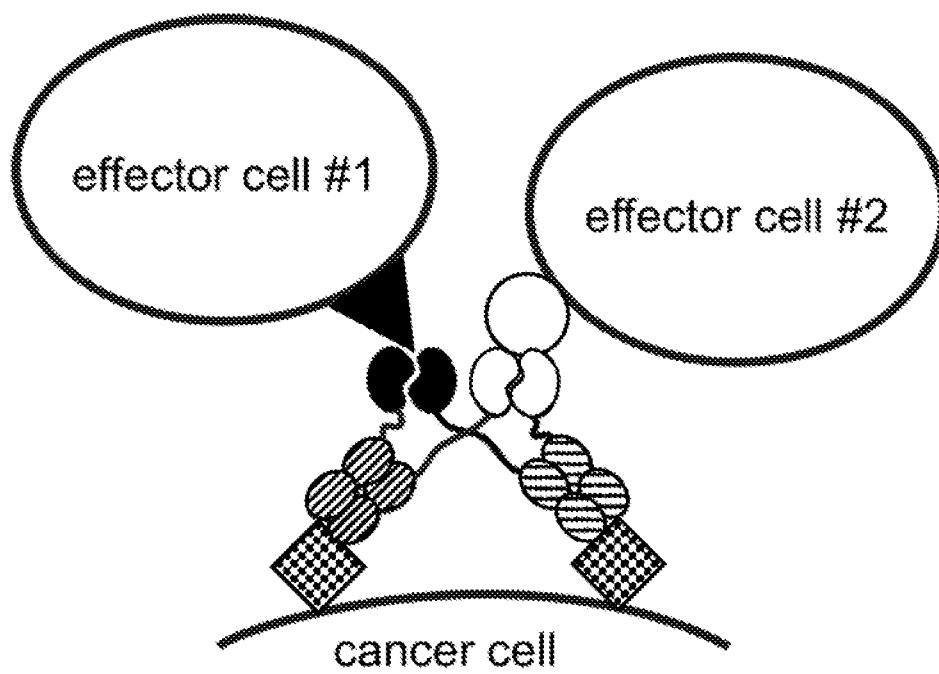
20. The kit or composition of claim 1, wherein the second targeting moiety binds an antigen expressed by a tumor microenvironment cell; optionally wherein the tumor microenvironment cell is a fibroblast or macrophage.

21. The kit or composition of claim 20, wherein the antigen expressed by a fibroblast is fibroblast activation protein or the antigen expressed by a macrophage is MAC-1/CD11b or sideroflexin 3.
22. A method of treating cancer expressing a tumor antigen that binds the first targeting moiety and/or second targeting moiety in a patient comprising administering the composition of claim 1 to the patient; optionally wherein the cancer expressing a tumor antigen that binds the first and/or second targeting moiety is any one of breast cancer, ovarian cancer, endometrial cancer, cervical cancer, bladder cancer, renal cancer, melanoma, lung cancer, prostate cancer, testicular cancer, thyroid cancer, brain cancer, esophageal cancer, gastric cancer, pancreatic cancer, colorectal cancer, liver cancer, leukemia, myeloma, nonHodgkin lymphoma, Hodgkin lymphoma, acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, lymphoproliferative disorder, myelodysplastic disorder, myeloproliferative disease or premalignant disease.
23. A method of targeting immune cells to cancer expressing a tumor antigen that binds both the first and/or second targeting moiety in a patient comprising administering the composition of claim 1 to the patient.
24. A method of targeting immune cells to cancer expressing two tumor antigens, wherein one tumor antigen binds the first targeting moiety and one tumor antigen binds the second targeting moiety, in a patient comprising administering the composition of claim 1 to the patient.
25. A method of delivering a cytokine to an immune cell of a patient comprising administering the composition of claim 13 to the patient, wherein the first and/or second complementary functional domain of the composition comprise IL-2, IL-7, IL-12, IL-15, GM-CSF, IFN- α , IFN- γ , or a member of the TNF-superfamily.

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*Fig. 1A**Fig. 1B*

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*Fig. 2**Fig. 3*

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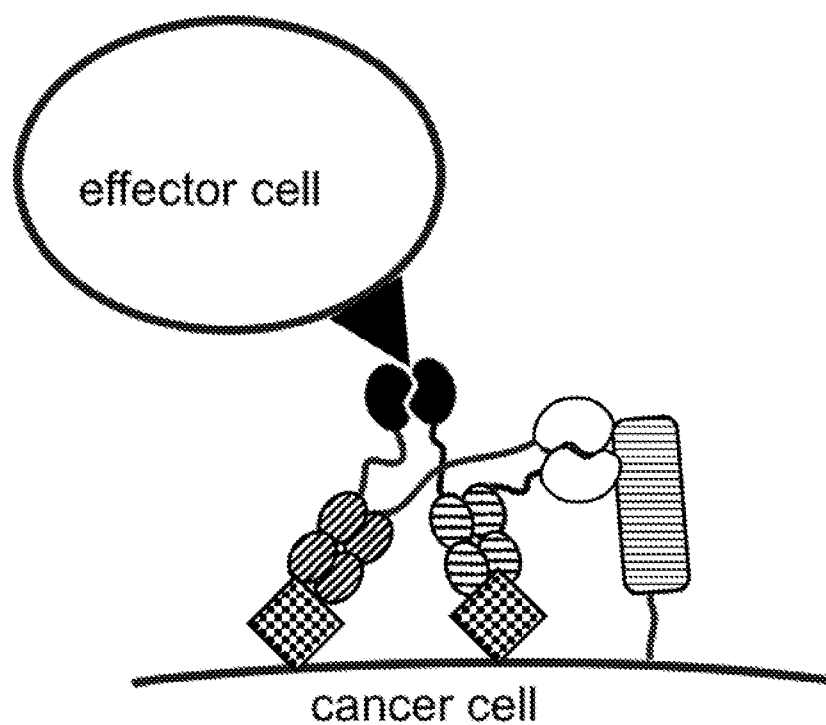


Fig. 4

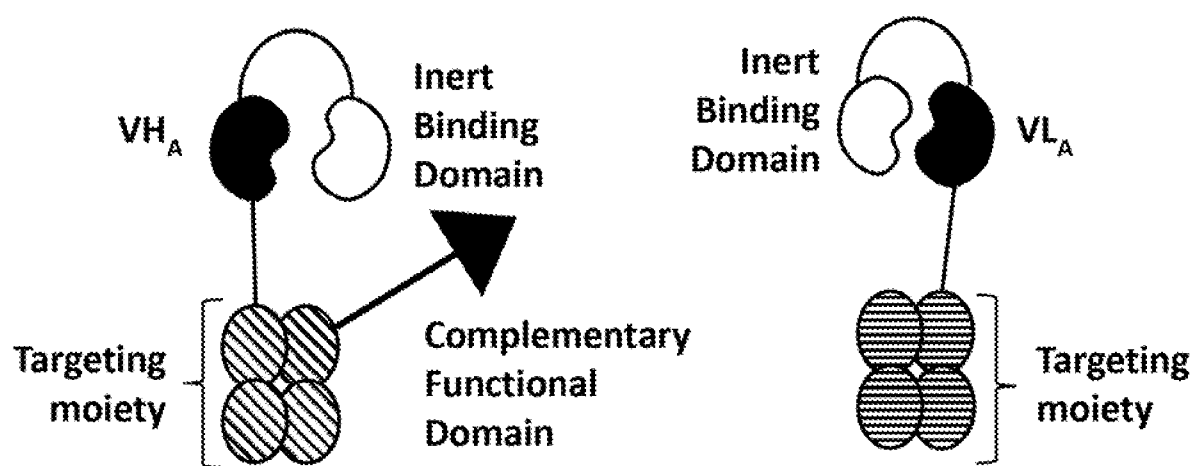


Fig. 5

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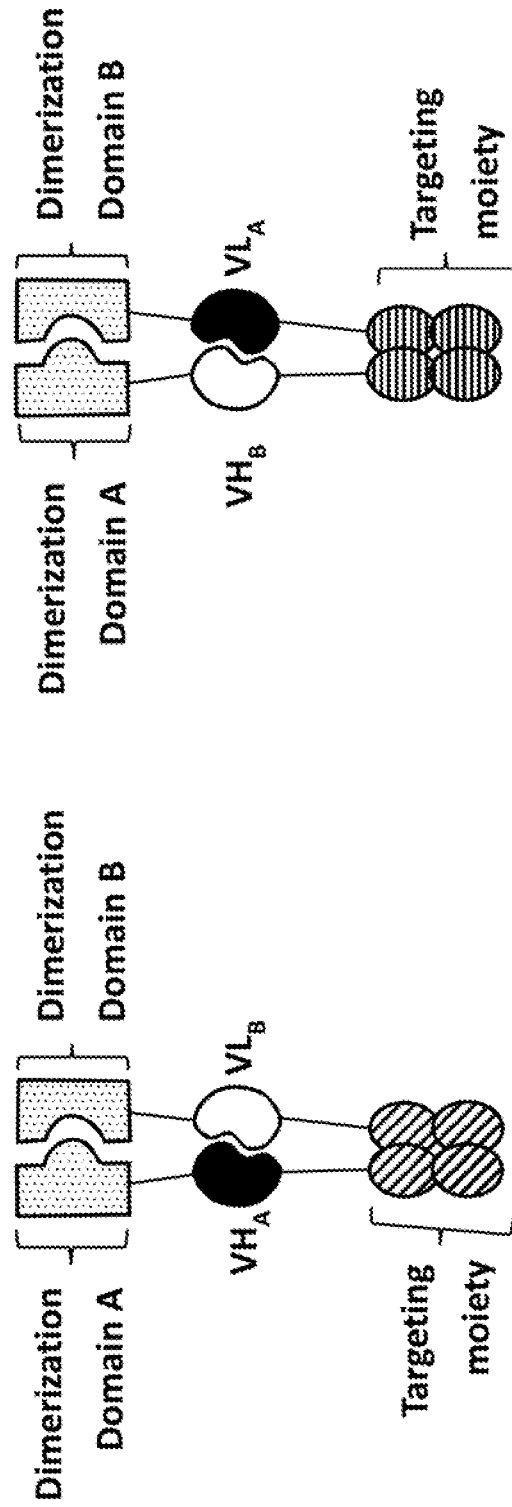


Fig. 6

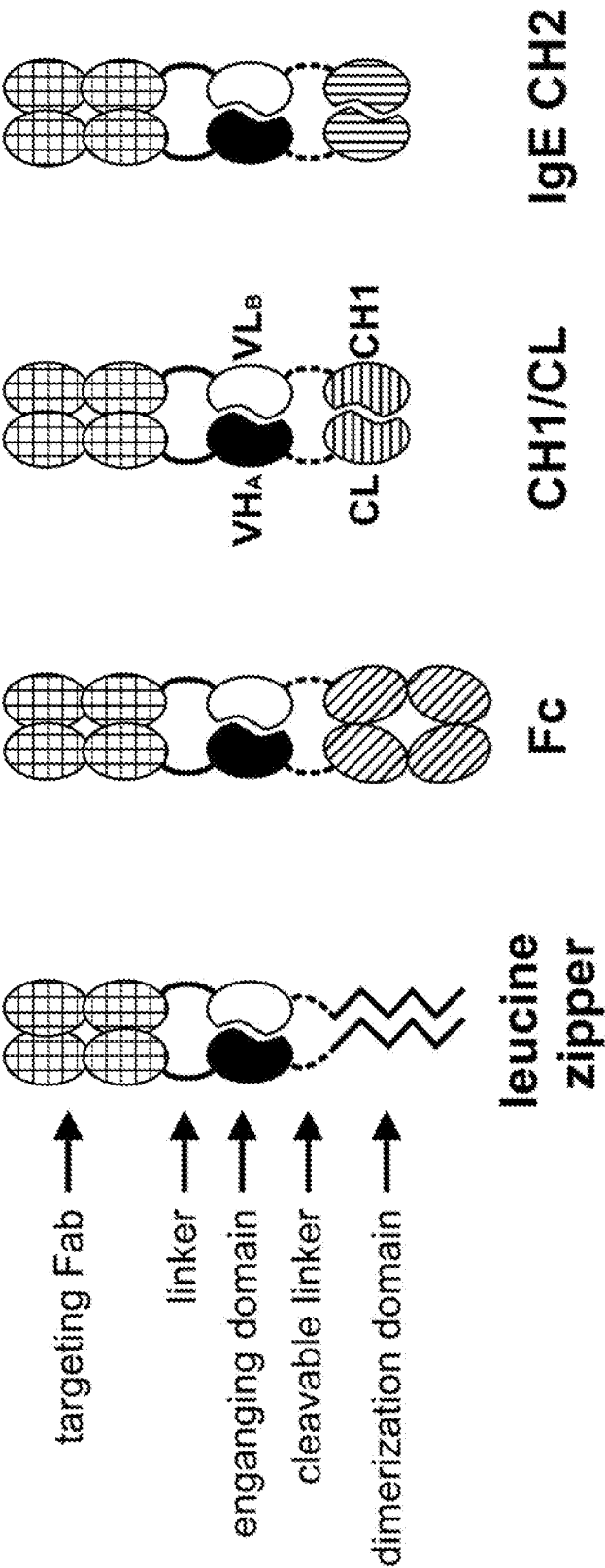


Fig. 7A

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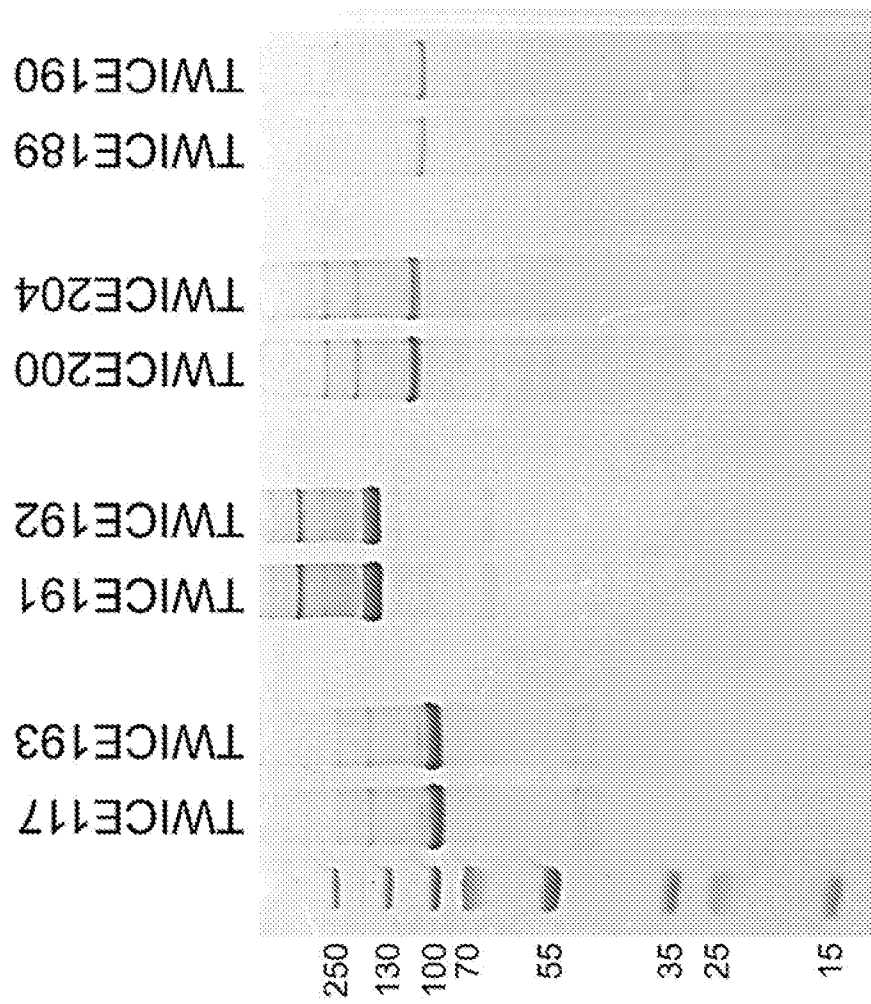


Fig. 7B

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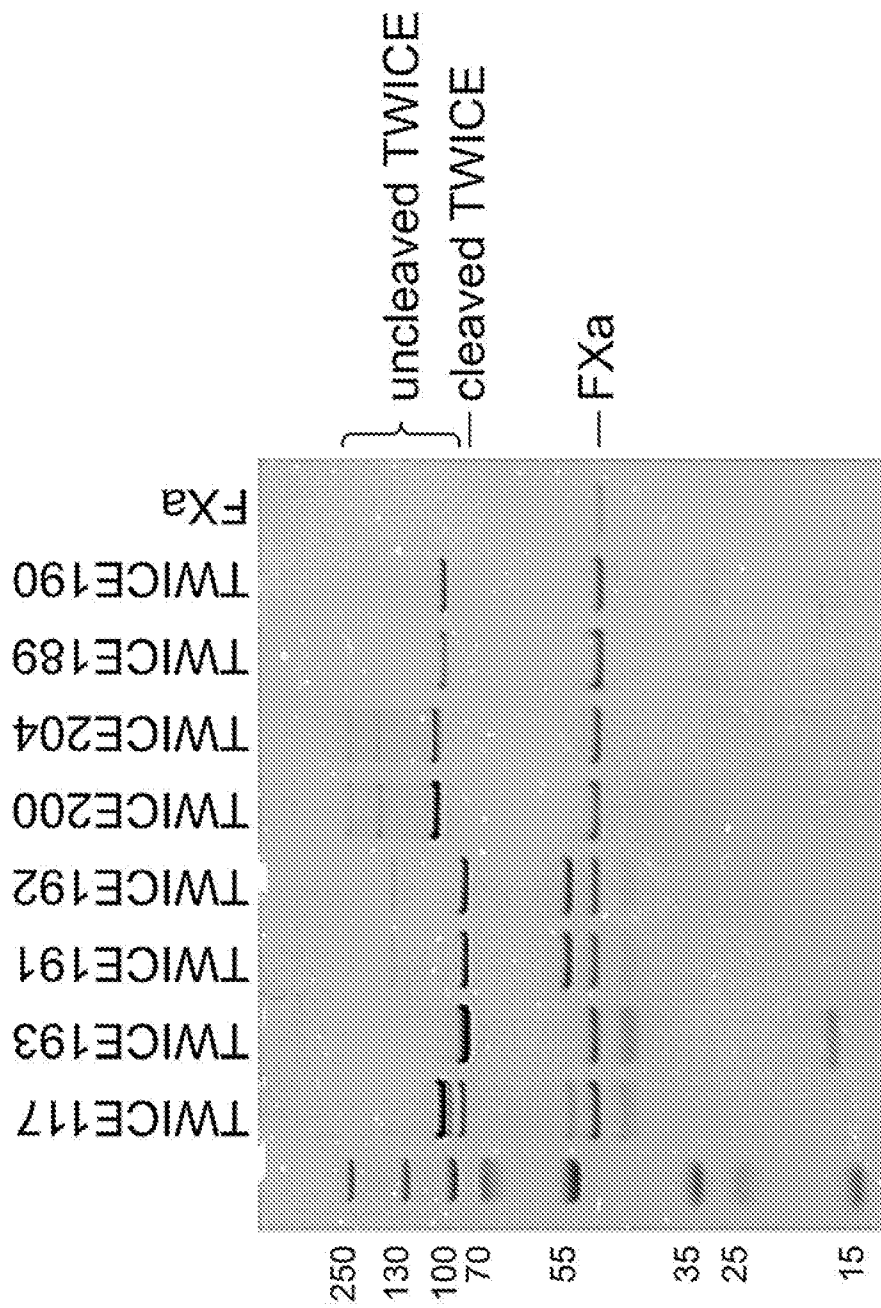


Fig. 7C

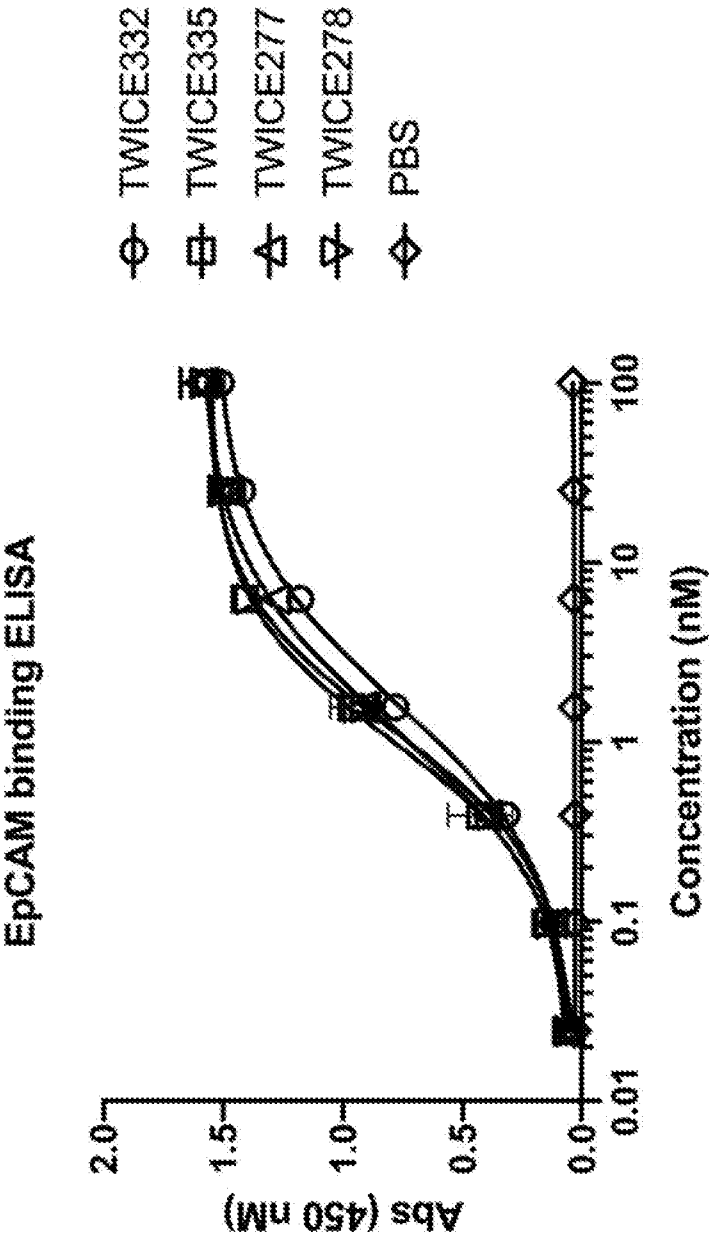


Fig. 8A

	TWICE332	TWICE335	TWICE277	TWICE278
EC50 (nM)	1.647	1.039	1.216	1.206

Fig. 8B

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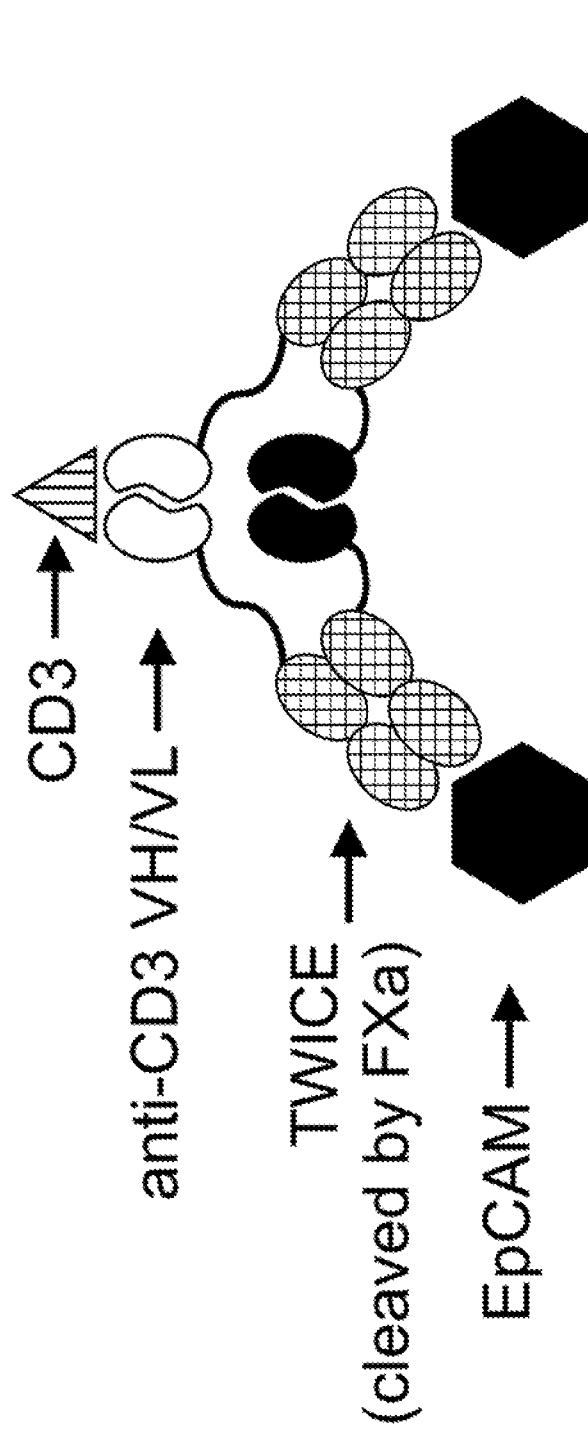


Fig. 9A

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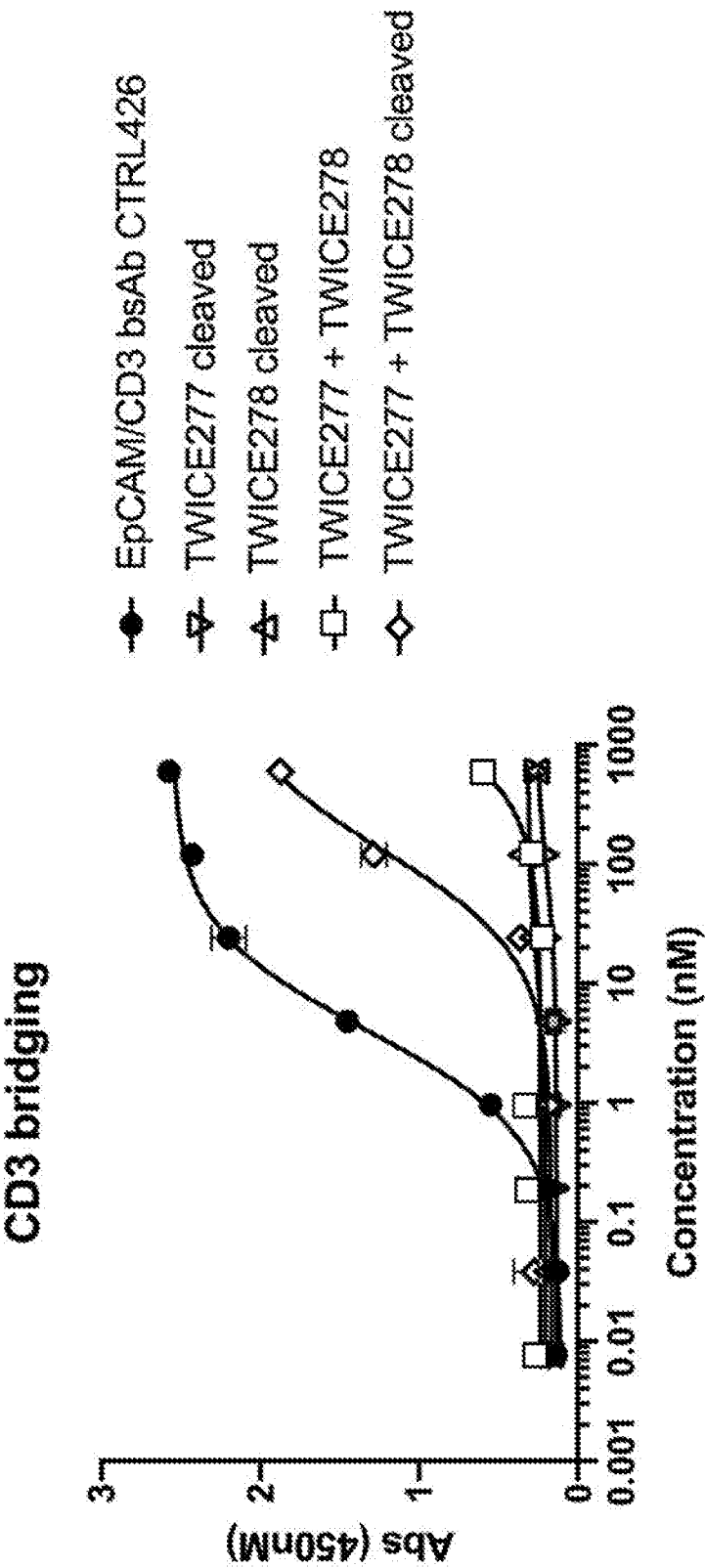


Fig. 9B

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CTLA-4 bridging

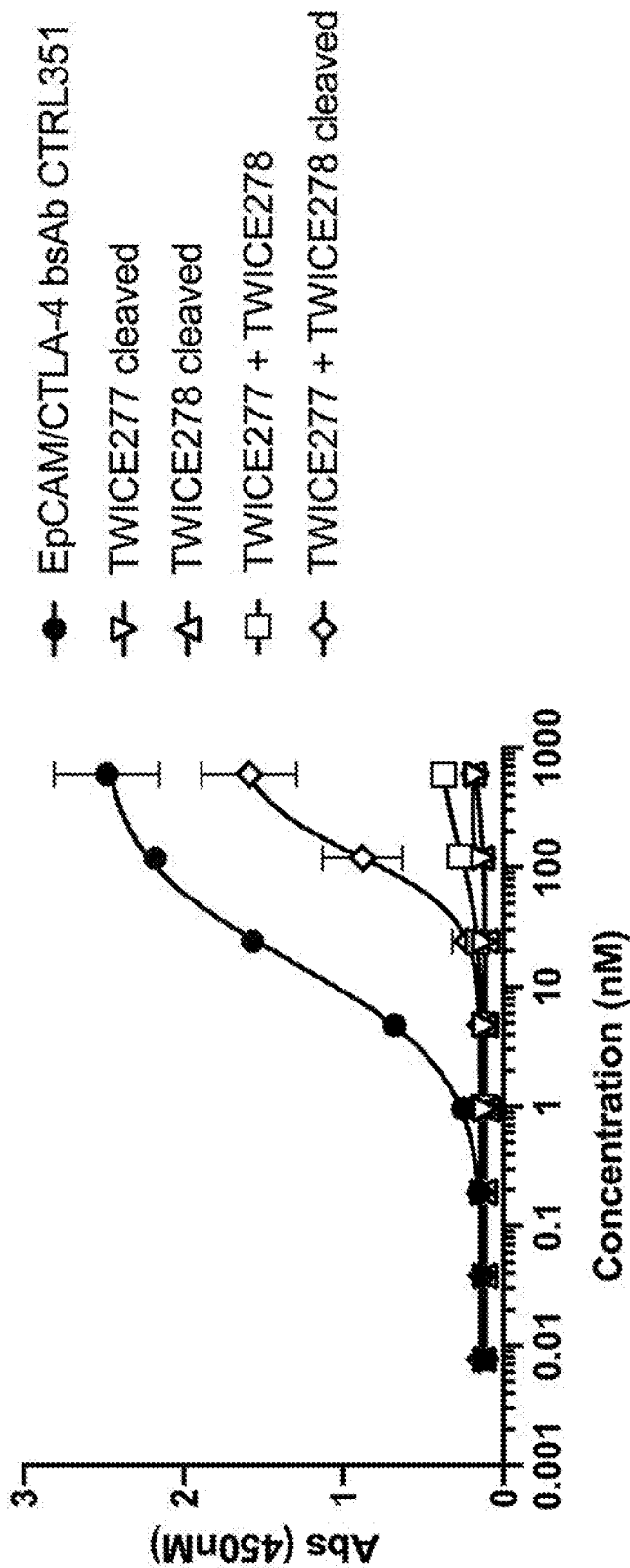


Fig. 9C

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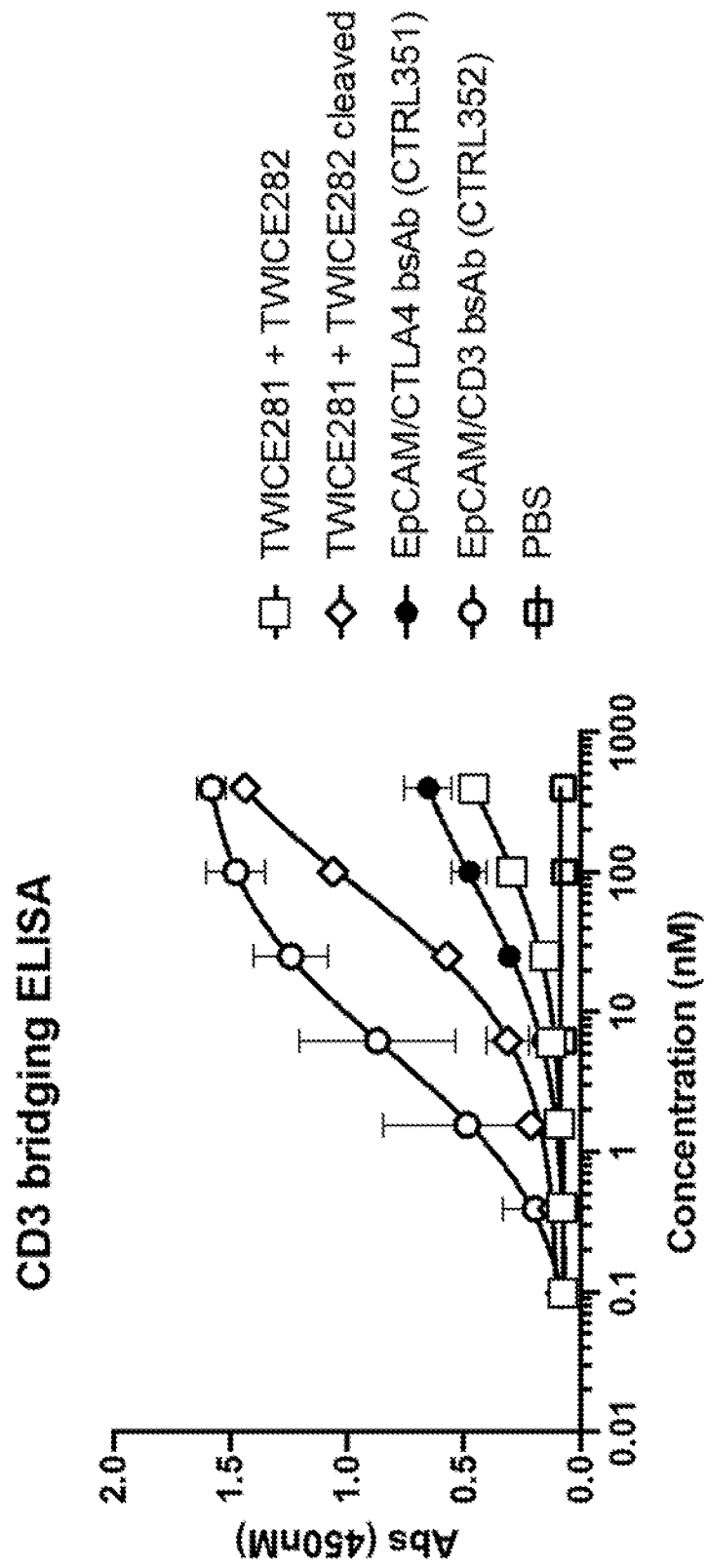


Fig. 10A

CTLA4 bridging ELISA

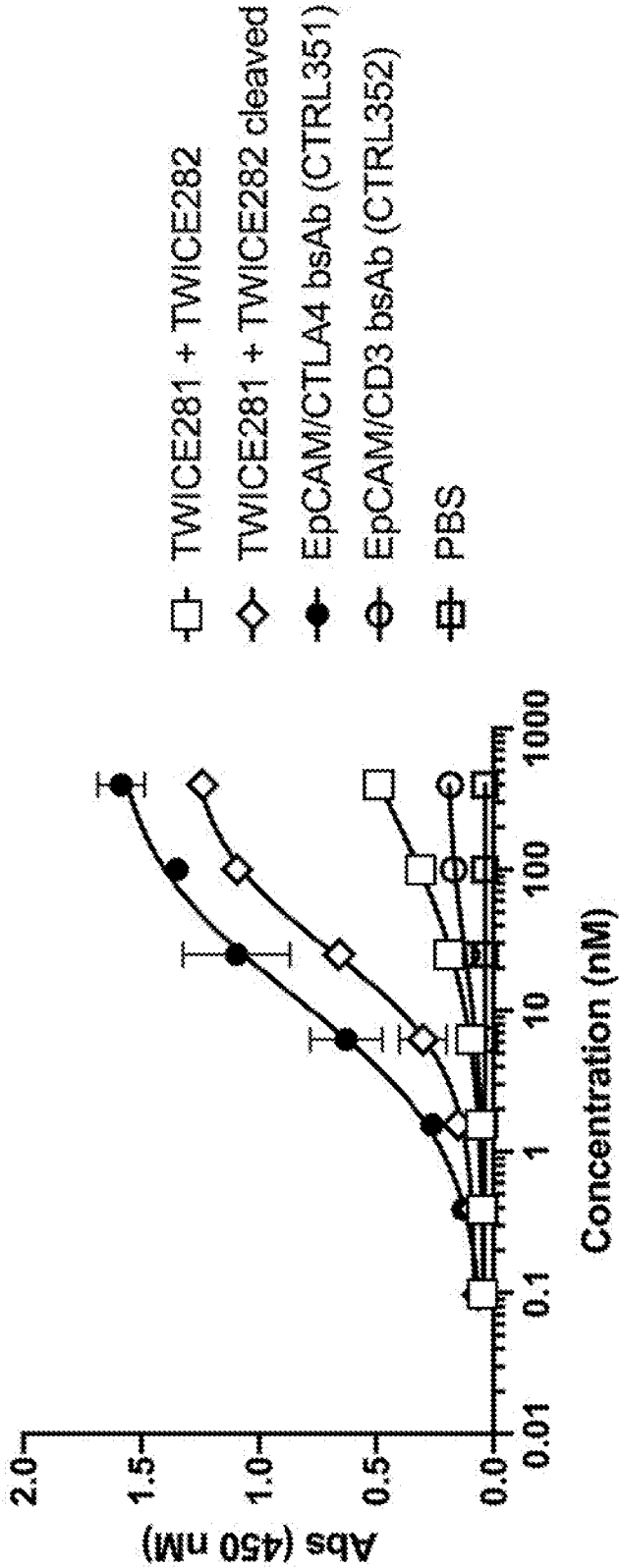


Fig. 10B

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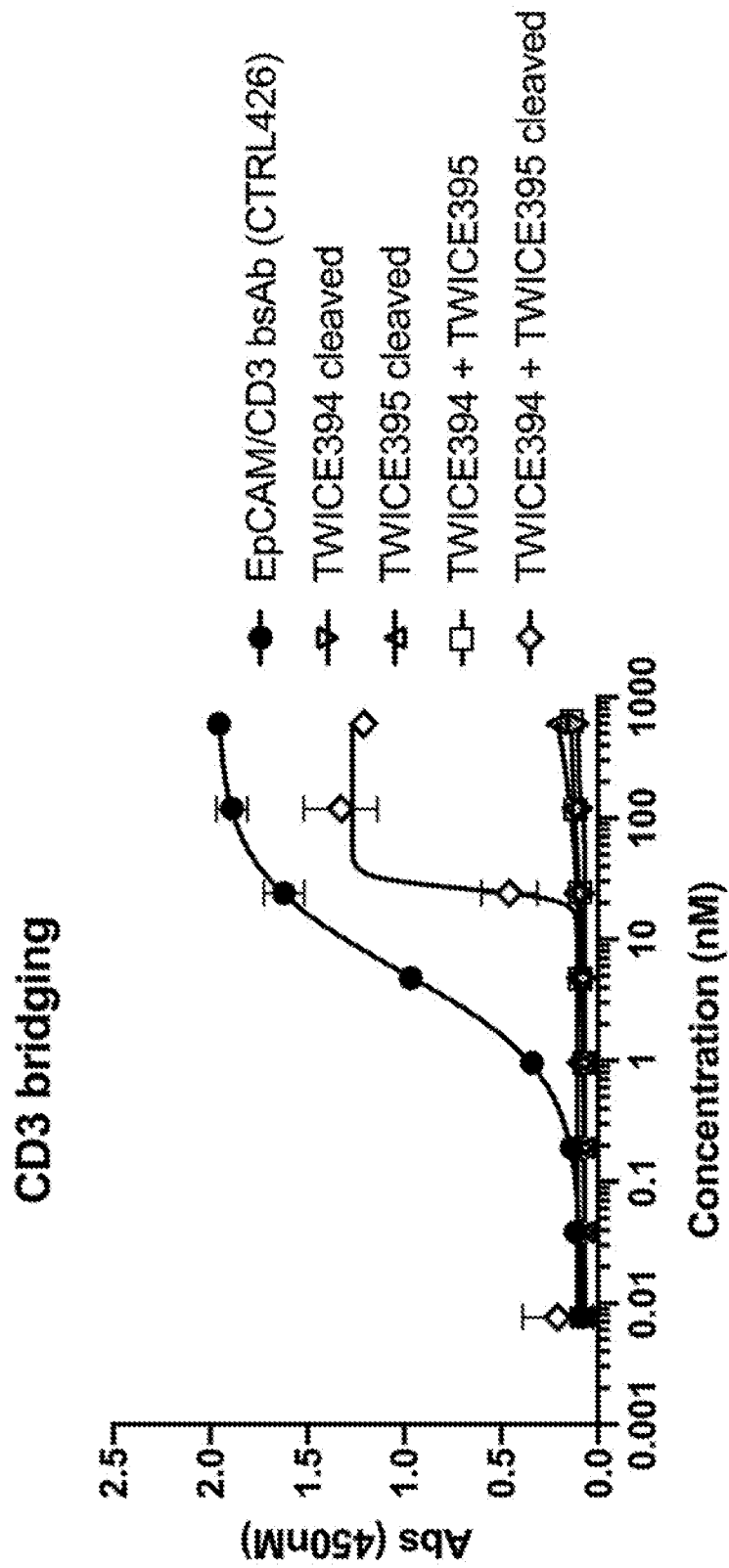


Fig. 11A

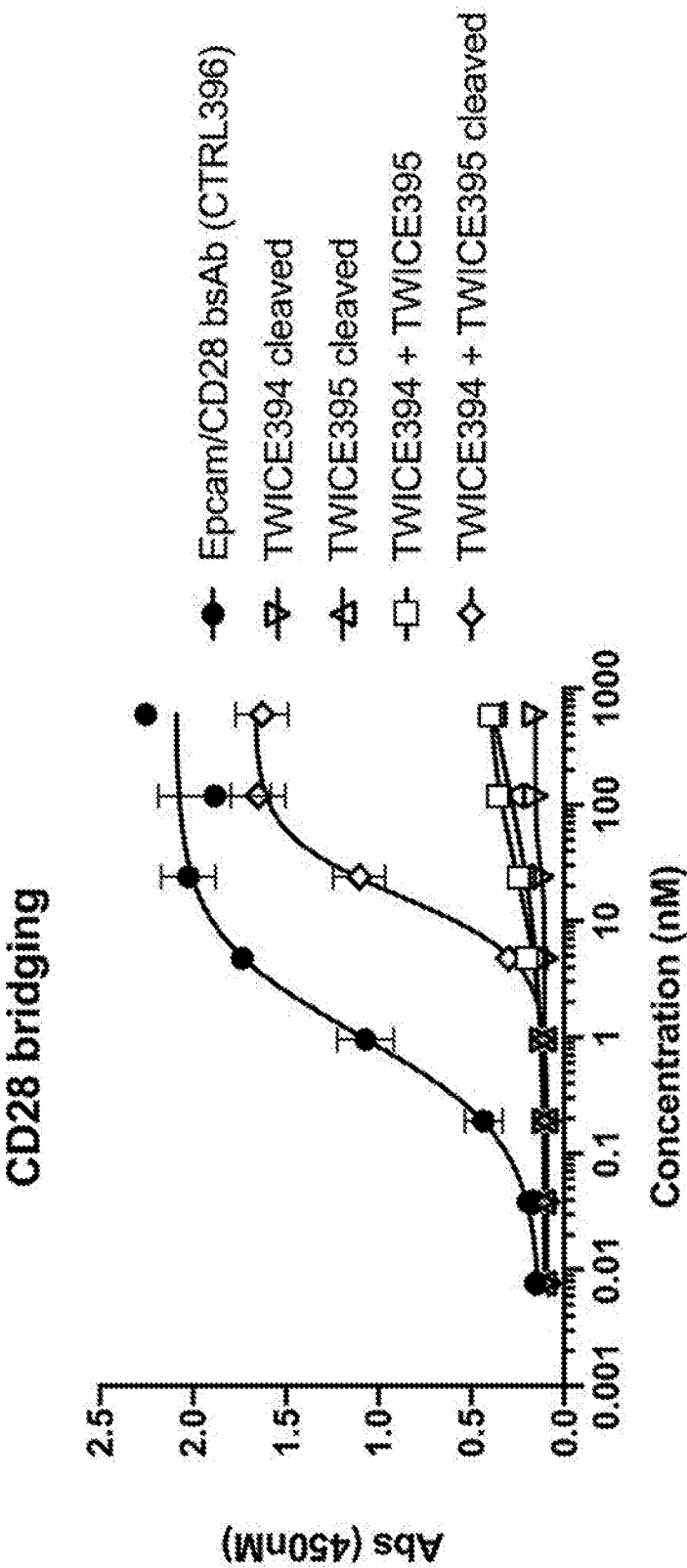


Fig. 11B

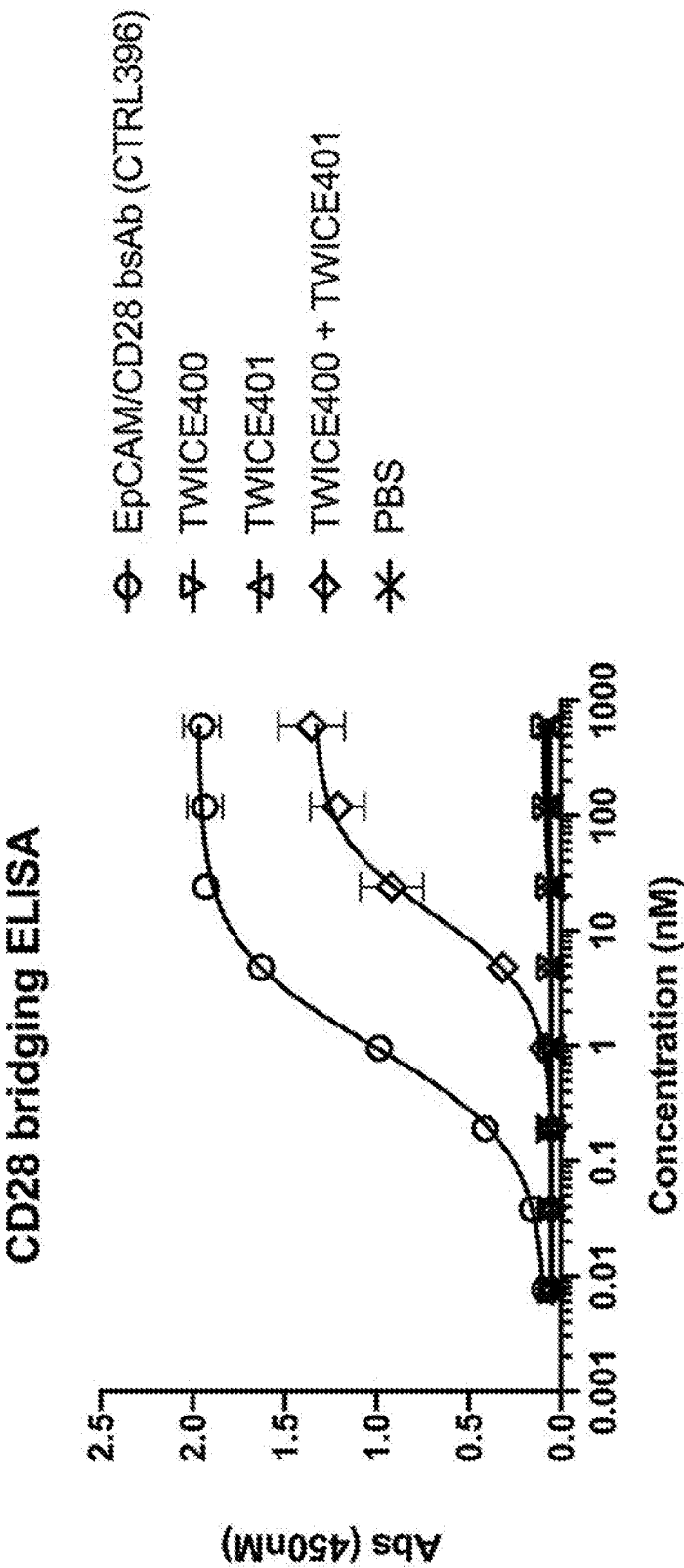


Fig. 12A

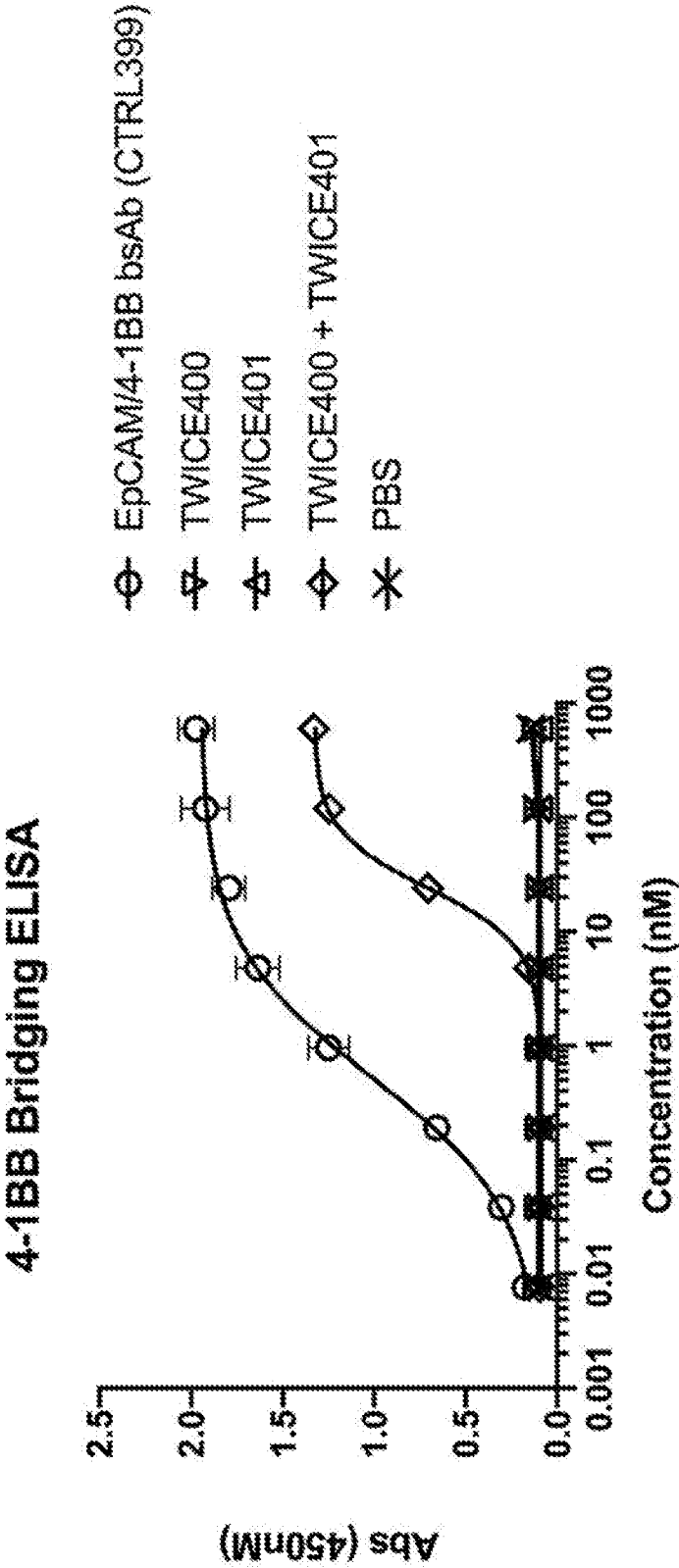


Fig. 12B

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PD-L1 bridging ELISA

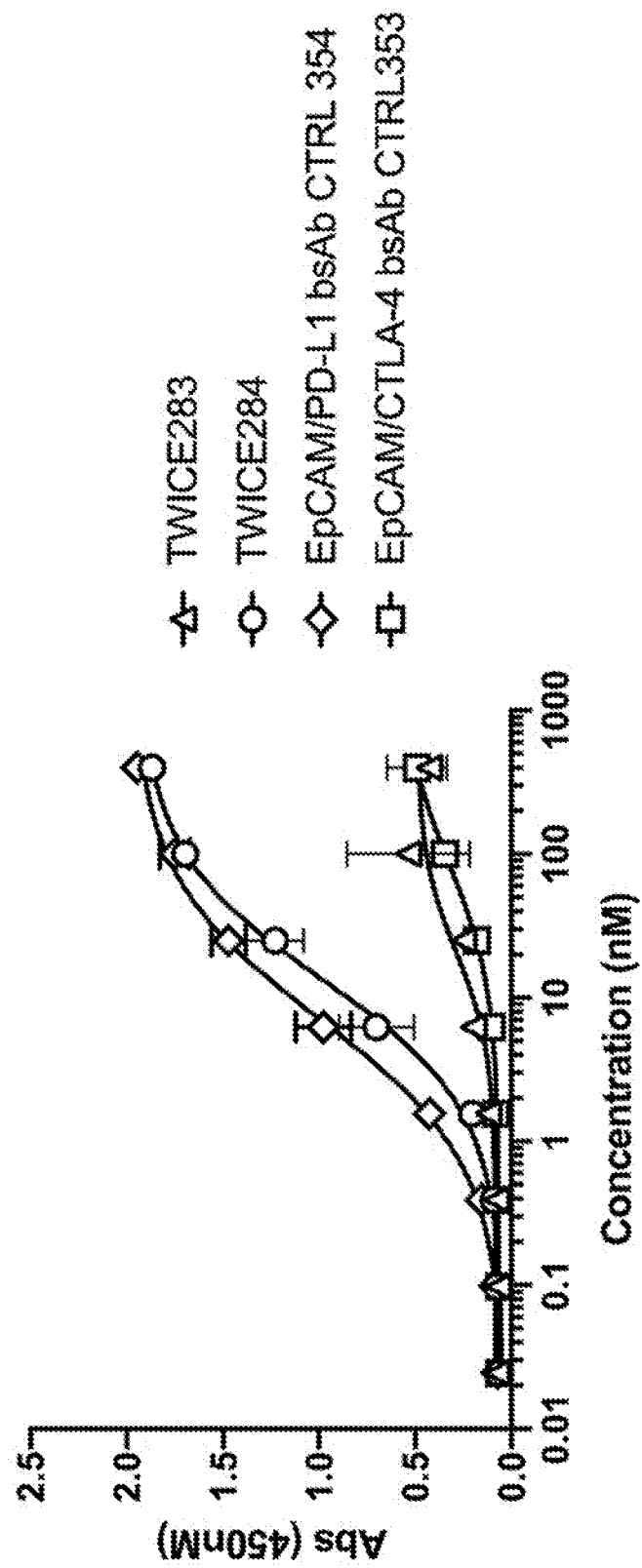


Fig. 13

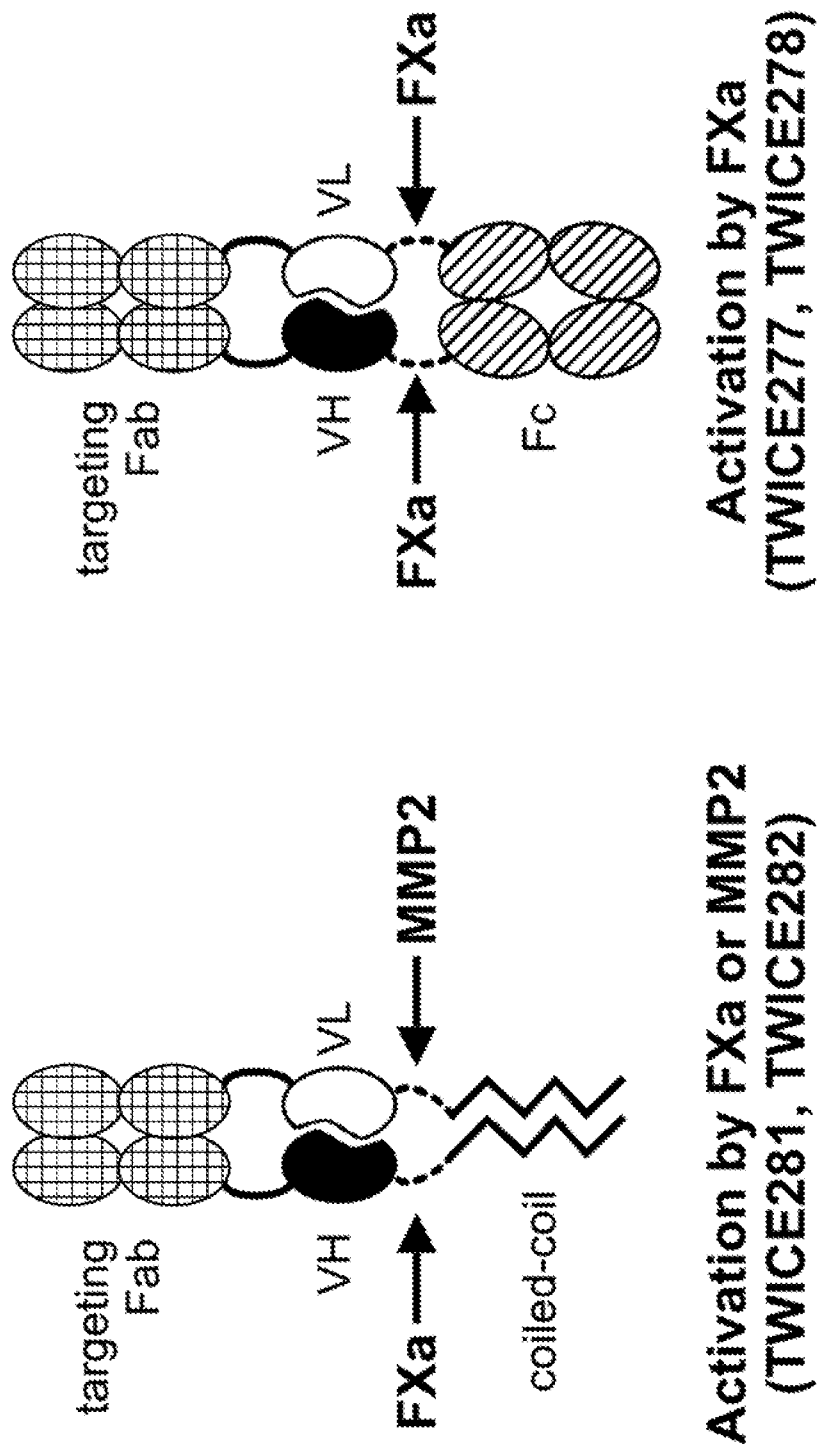


Fig. 14A

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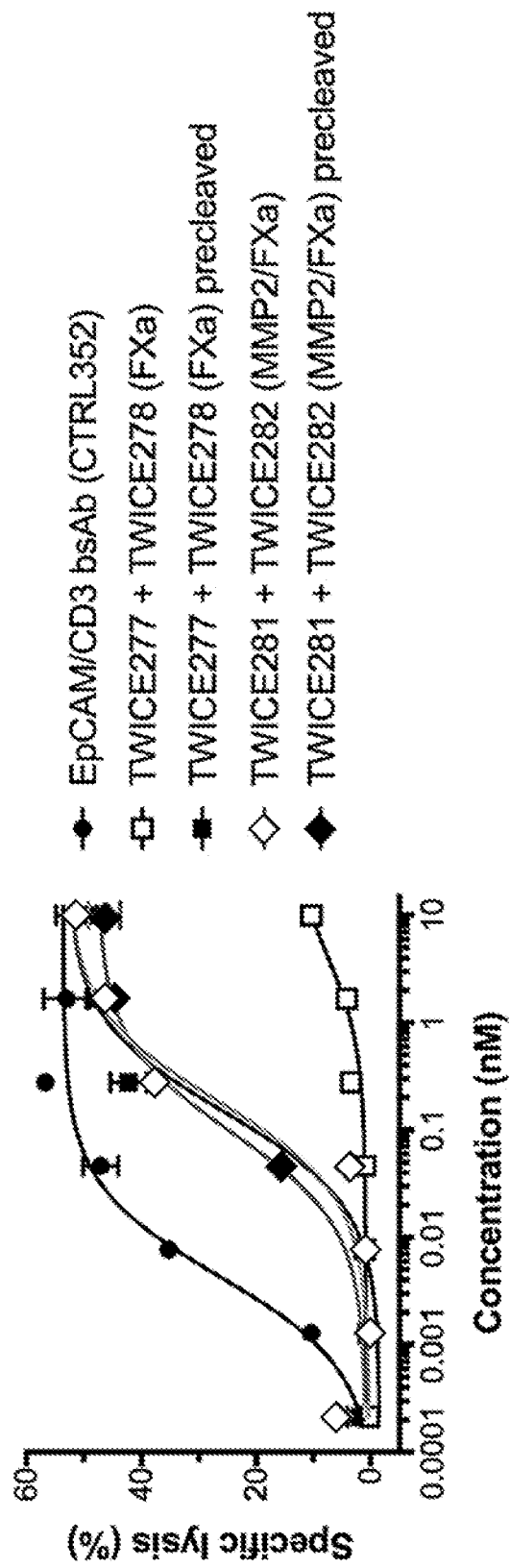


Fig. 14B

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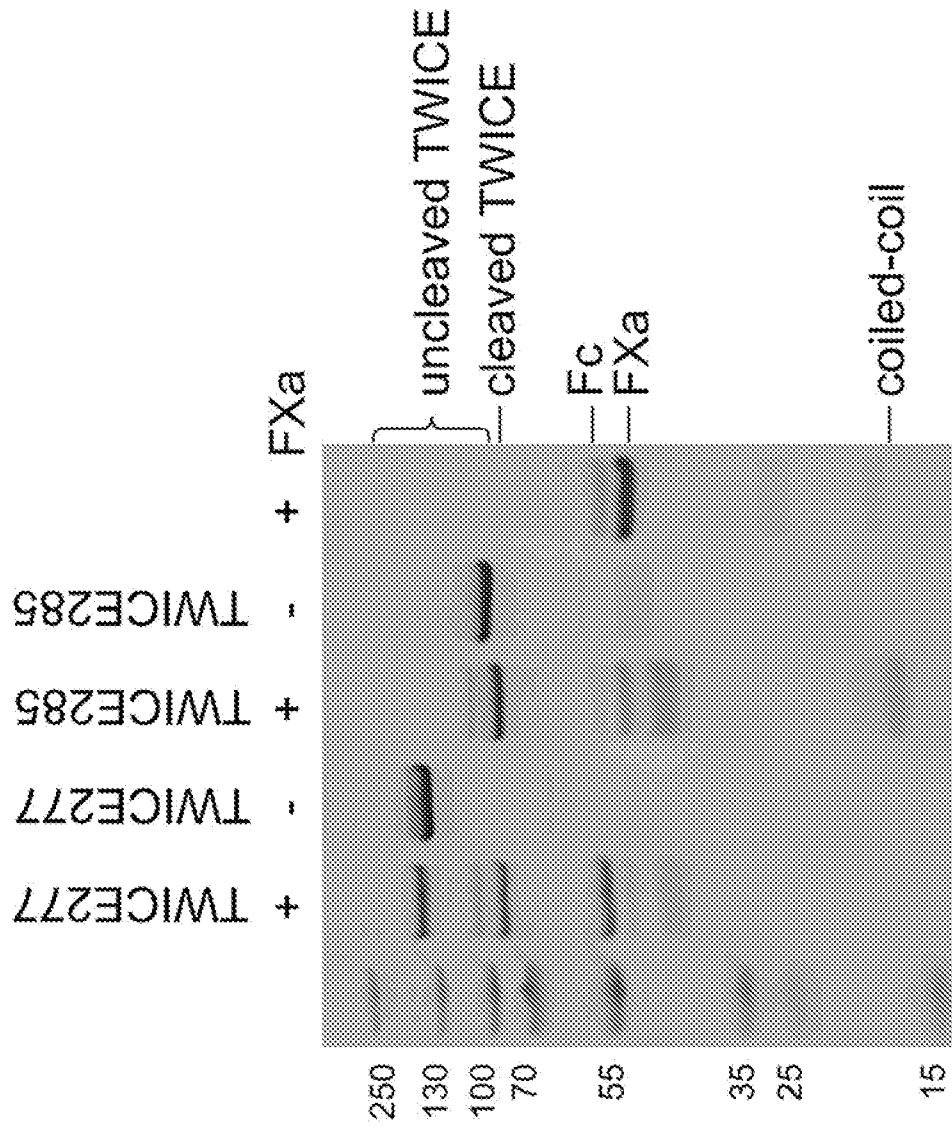


Fig. 15A

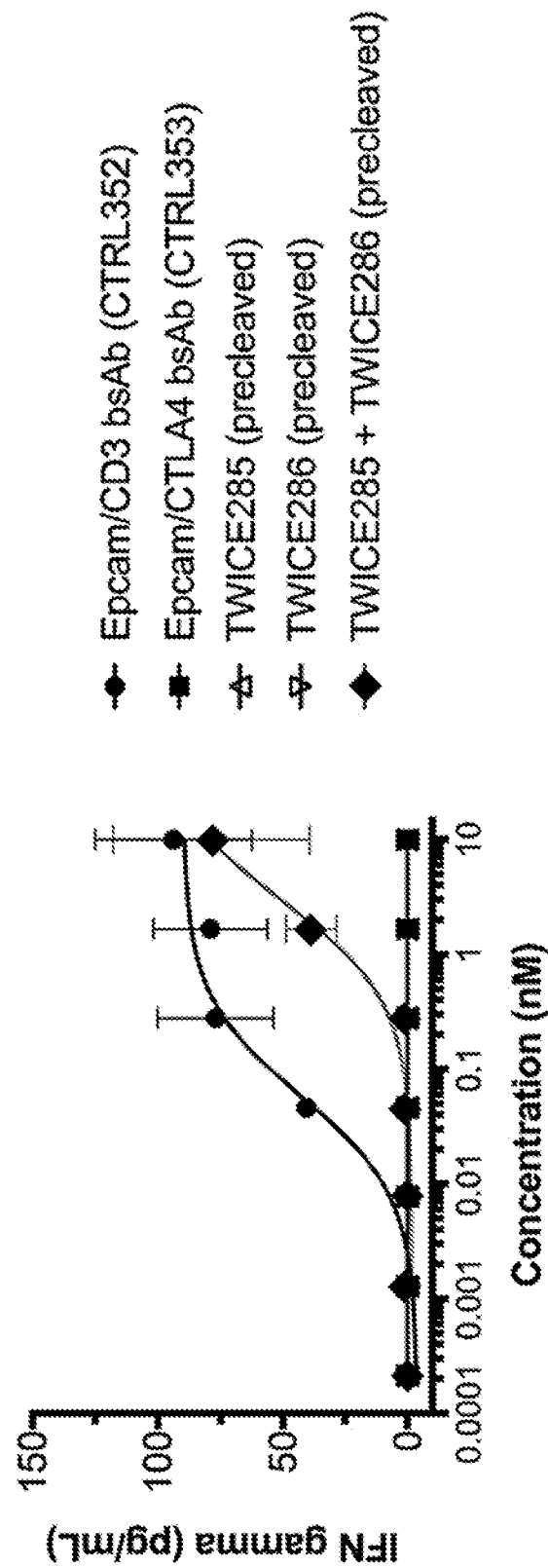


Fig. 15B

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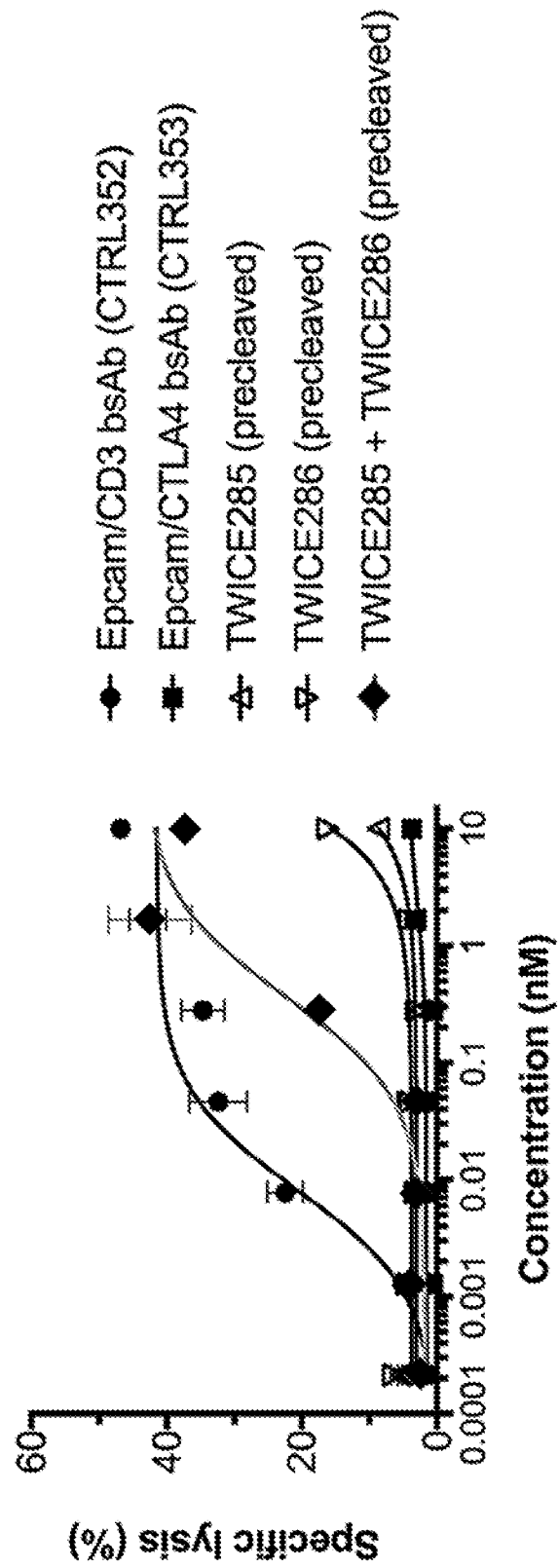


Fig. 15C

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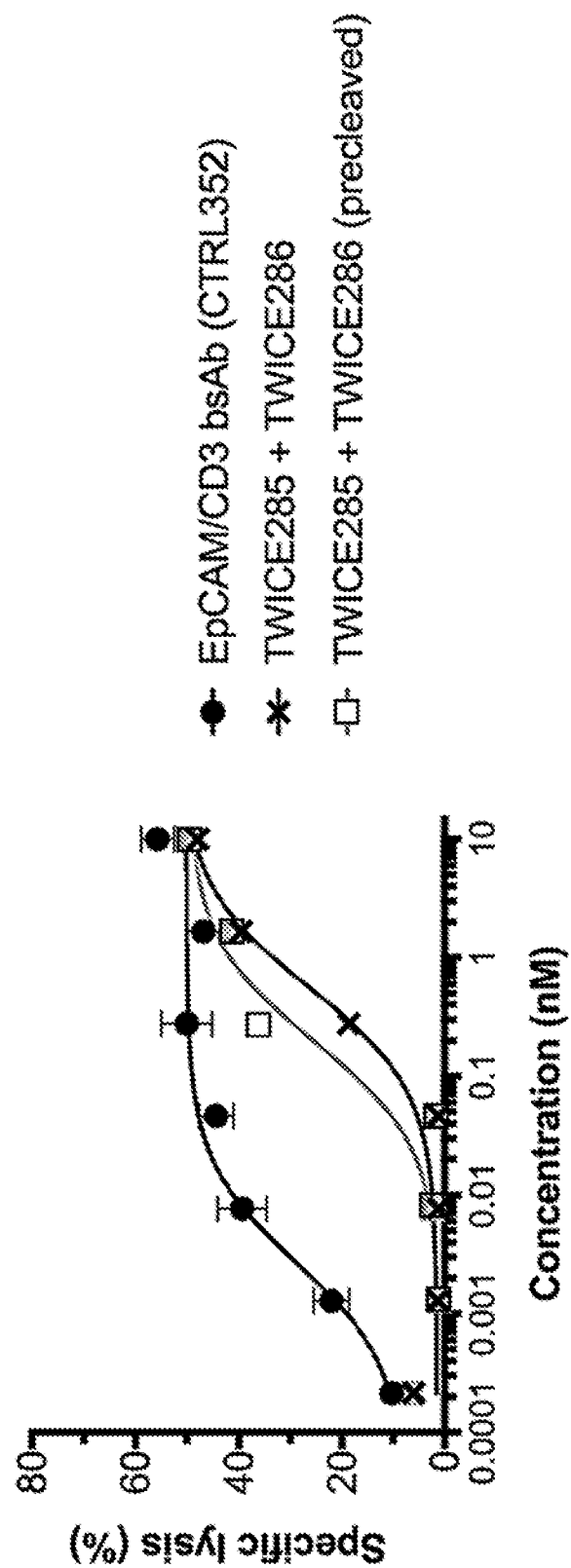


Fig. 15D

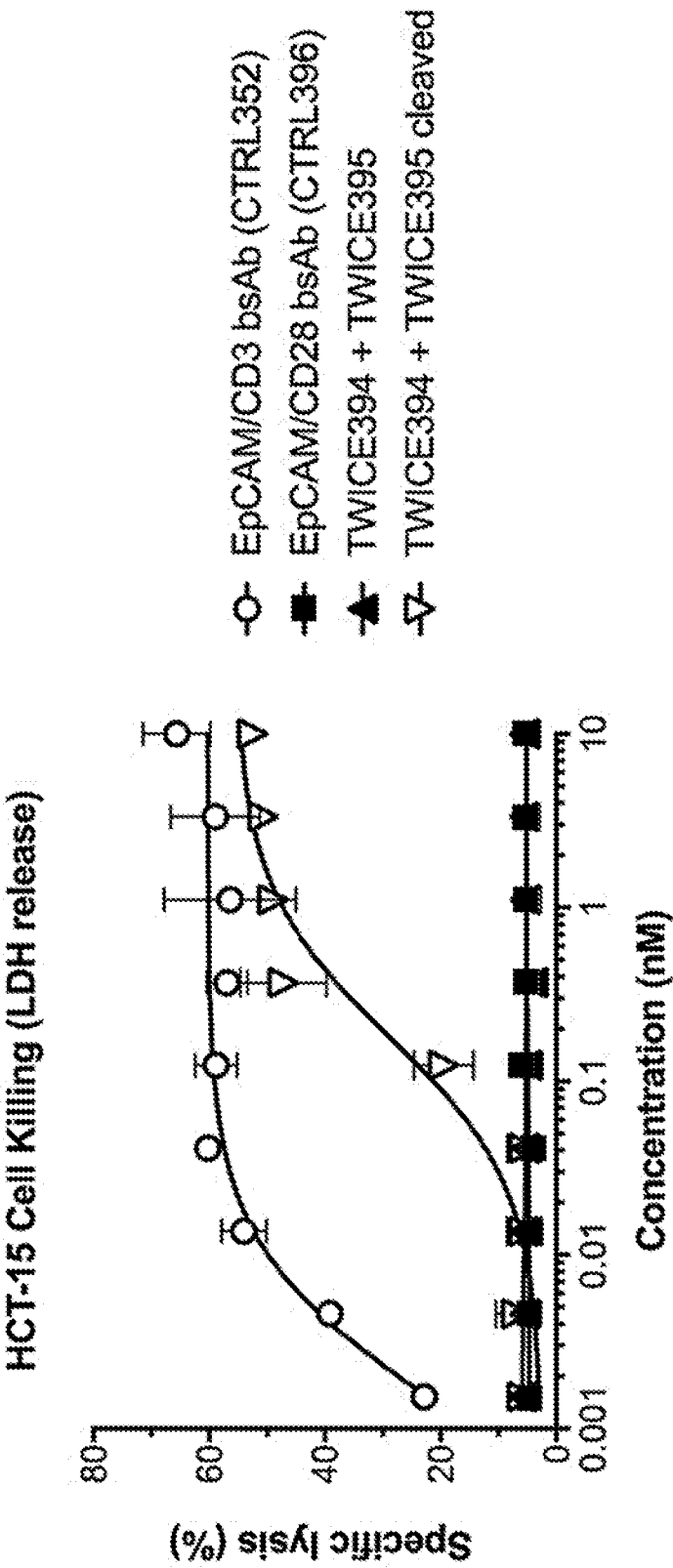


Fig. 16

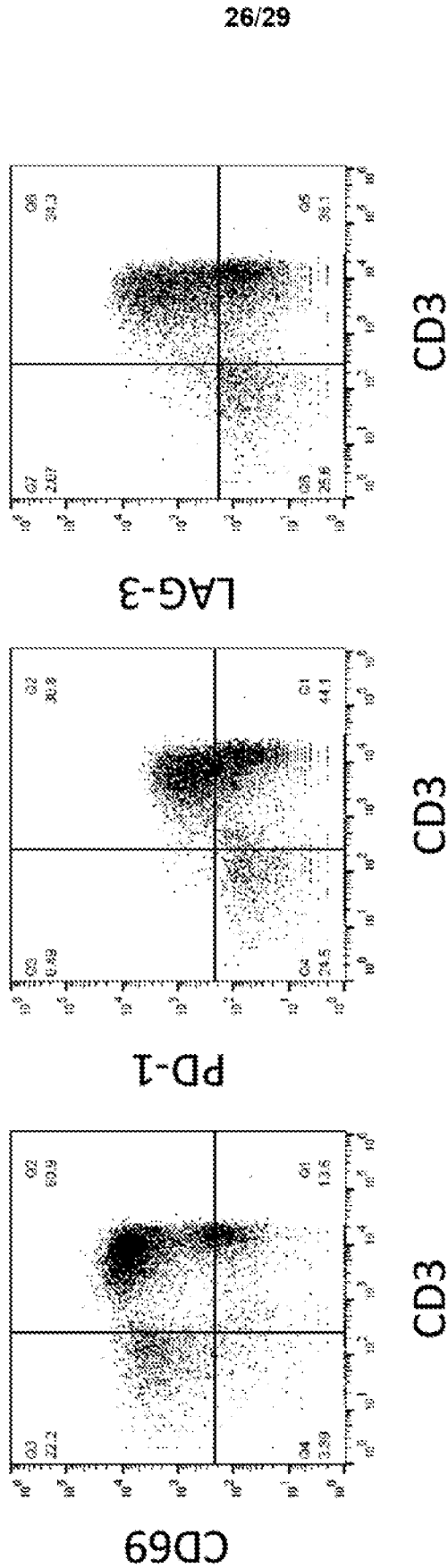


Fig. 17A

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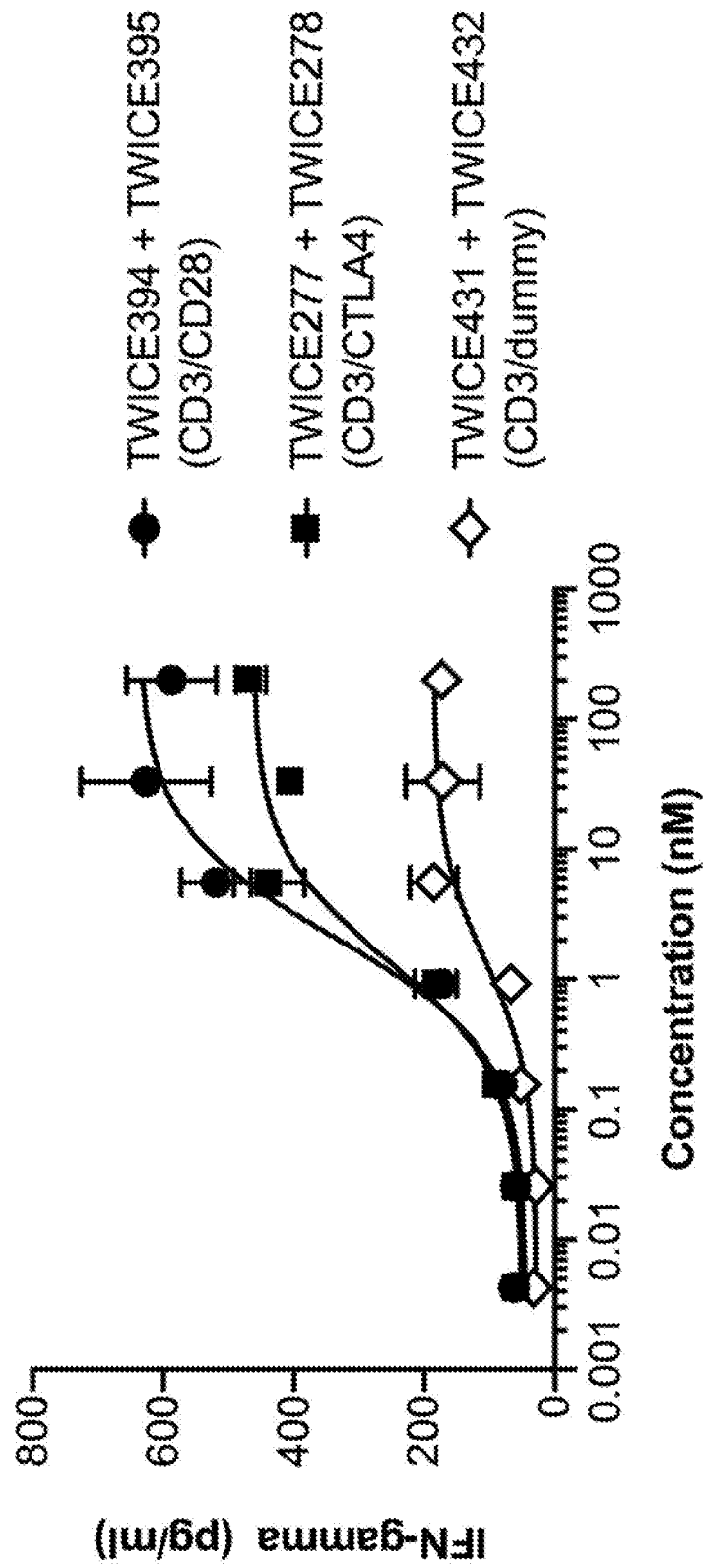


Fig. 17B

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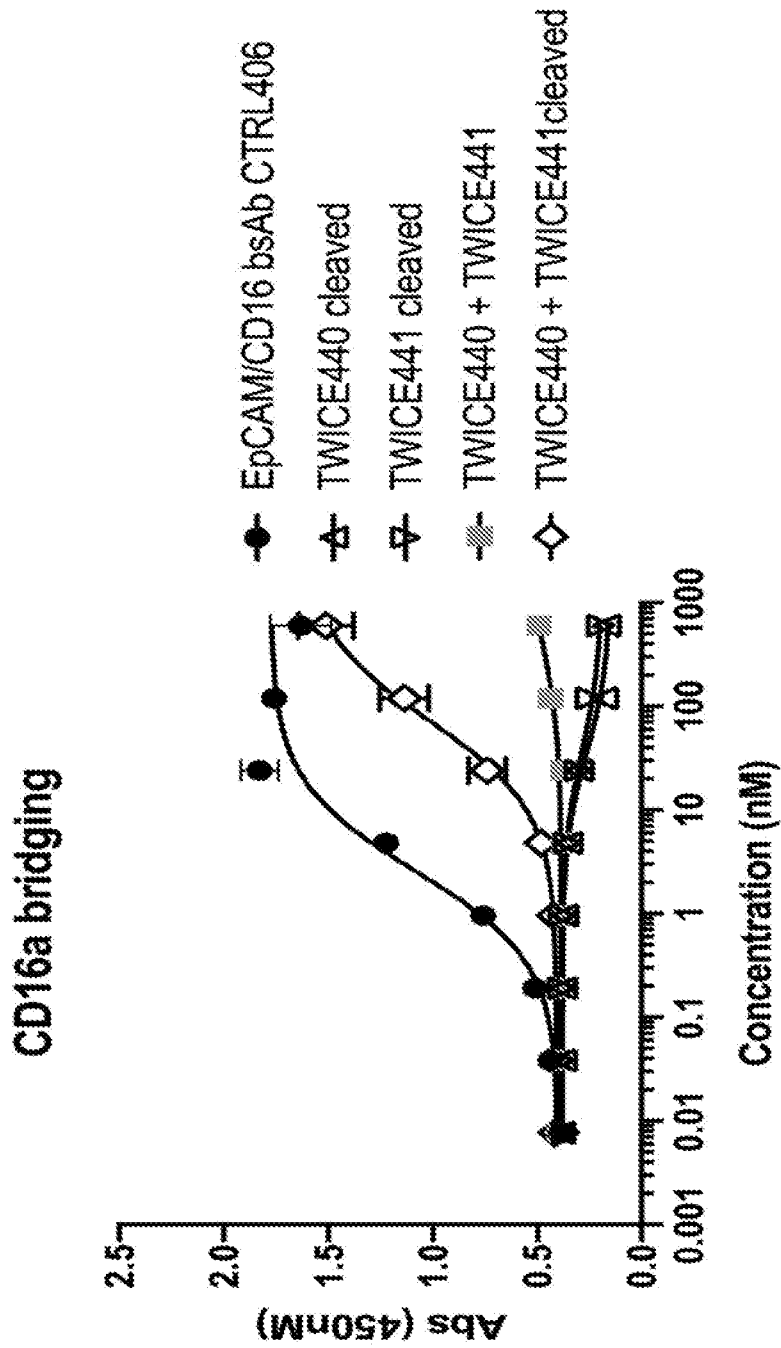


Fig. 18A

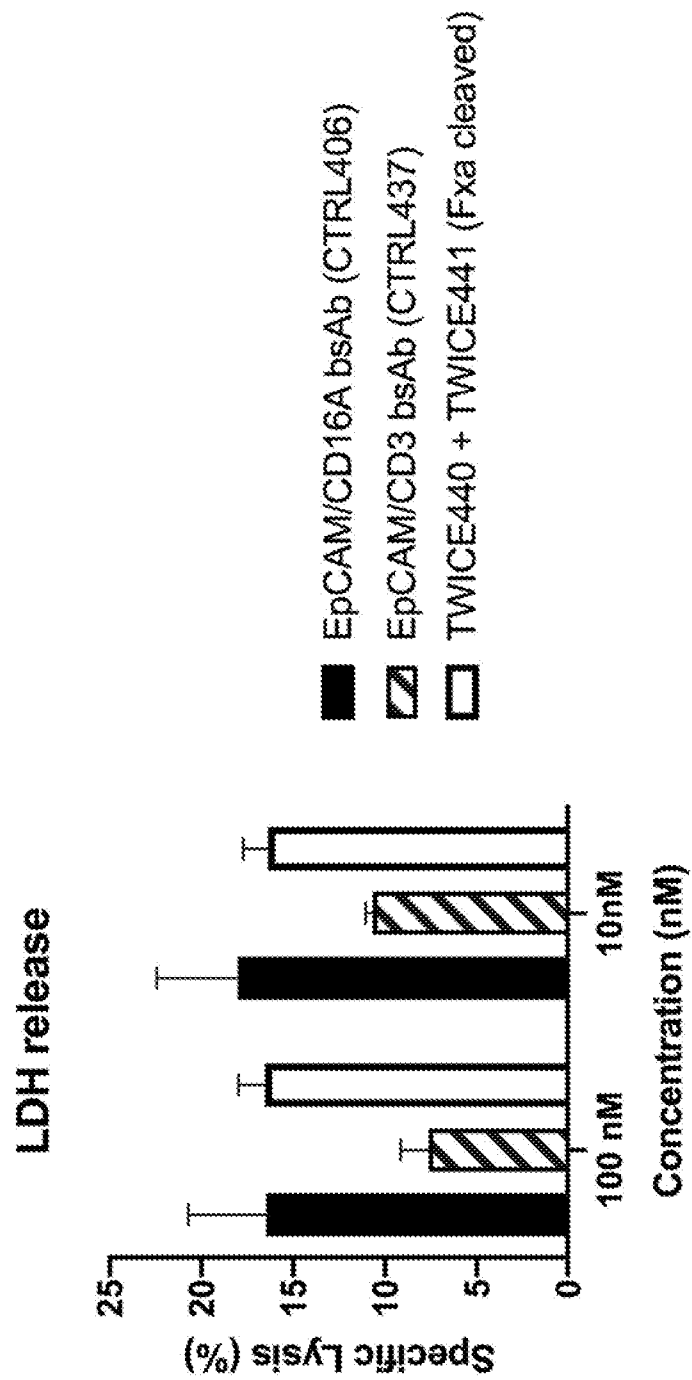


Fig. 18B

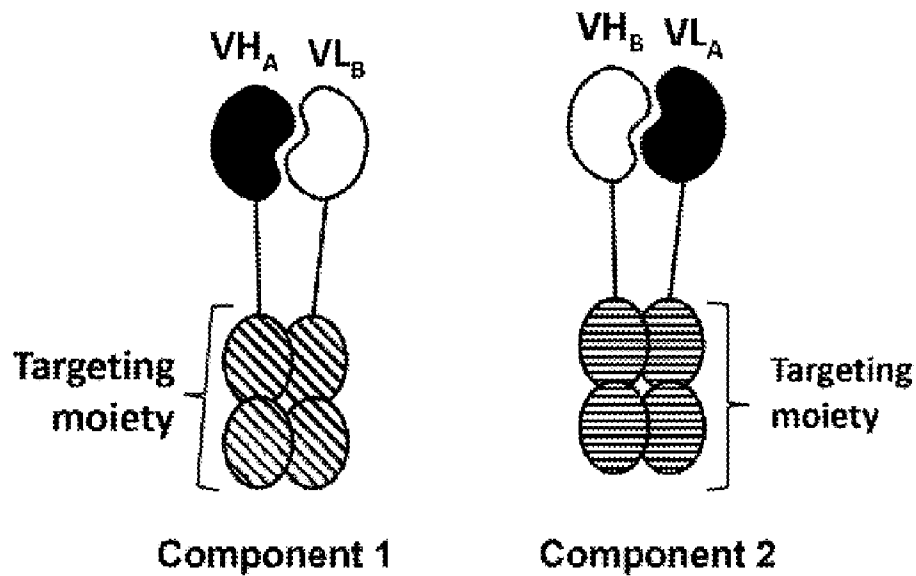


Fig. 1A