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(71) Applicant(s)
Revitope Limited

(72) Inventor(s)
Cobbold, Mark

(74) Agent / Attorney
Pizzeys Patent and Trade Mark Attorneys Pty Ltd, GPO Box 1374, BRISBANE, QLD, 4001, AU

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(71) Applicant: **REVITOPE LIMITED** [GB/GB]; Angel Building, 407 St. John Street, London EC1V4AD (GB).

(72) Inventor; and
(71) Applicant : **COBBOLD, Mark** [GB/US]; 5 Everett Avenue, Winchester, Massachusetts 01890 (US).

(74) Agents: **MCNEILL, Rebecca** et al.; 125 Cambridge Park Drive, Suite 301, Cambridge, Massachusetts 02140 (US).

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(54) **Title**: FUNCTIONAL ANTIBODY FRAGMENT COMPLEMENTATION FOR A TWO-COMPONENTS SYSTEM FOR RE-DIRECTED KILLING OF UNWANTED CELLS

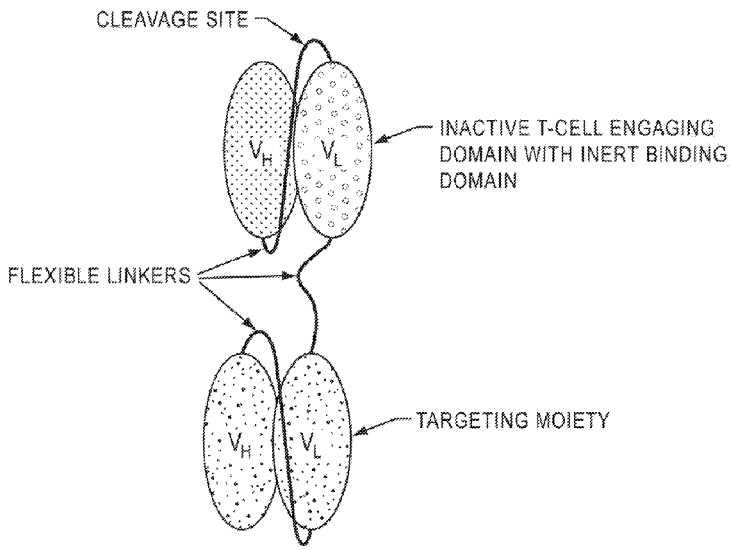


FIGURE 1

(57) **Abstract**: A targeted T-cell engaging agent for treating a condition characterized by the presence of unwanted cells includes (a) a targeting moiety that is capable of targeting the unwanted cells; (b) a first T-cell engaging domain capable of T-cell engaging activity when binding a second T-cell engaging domain, wherein the second T-cell engaging domain is not part of the agent; (c) at least one inert binding partner capable of binding to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed; and (d) at least one cleavage site separating the first T-cell engaging domain and the inert binding partner, wherein the cleavage site is: (i) cleaved by an enzyme expressed by the unwanted cells; (ii) cleaved through a pHsensitive cleavage reaction inside the unwanted cell; (iii) cleaved by a complementdependent cleavage reaction.

FUNCTIONAL ANTIBODY FRAGMENT COMPLEMENTATION FOR A TWO-COMPONENTS SYSTEM FOR REDIRECTED KILLING OF UNWANTED CELLS

DESCRIPTION

FIELD

[001] This application relates to targeted T-cell engaging agents for treating a condition characterized by the presence of unwanted cells. In particular, it relates to agents that can be used to treat a condition characterized by the presence of unwanted cells, such as cancer or other disease-causing cells.

BACKGROUND

[002] Cancer and other diseases caused by the presence of unwanted cells create significant loss of life, suffering, and economic impact. Immunotherapeutic strategies for targeting cancer have been an active area of translational clinical research.

[003] A variety of other approaches have been explored for immunotherapy, but many of these prior approaches lack sufficient specificity to particular unwanted cells. For example, demibodies have been designed each having an scFv portion binding to different antigens on a target cell, an Fc domain allowing pairing to a complementary demibody, and a binding partner capable of forming an association to another binding partner on a complementary demibody. WO 2007/062466. These demibodies, however, are not necessarily specific to cancer cells and could bind and have activity on other cells expressing the same antigens. See also WO 2013/104804, which provides a first polypeptide with a targeting moiety binding to a first antigen and a first fragment of a functional domain, along with a second polypeptide with a targeting moiety binding to a second antigen and a second fragment of a functional domain that is complementary to the first fragment of the functional domain. Likewise, this approach is not necessarily specific to cancer cells and could bind and have activity on other cells expressing the same antigens.

[004] While some positive test data has been shown with prior approaches, clinically-effective therapeutic strategies must be able to elicit a strong immune response in an individual suffering from a disease such as cancer. Additionally, effective therapies should be very specific and not cause unwanted side effects to other cell types in the body. Therefore, additional developments in this field of re-directed immunotherapy are required.

SUMMARY

[005] In accordance with the description, the inventors describe a targeted T-cell engaging agent for treating a condition characterized by the presence of unwanted cells. This agent includes (a) a targeting moiety that is capable of targeting the unwanted cells; (b) a first T-cell engaging domain capable of activity when binding a second T-cell engaging domain, wherein the second T-cell engaging domain is not part of the agent; (c) at least one inert binding partner capable of binding the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed; and (d) at least one cleavage site separating the first T-cell engaging domain and the inert binding partner.

[006] In one embodiment, a two-component system for treating a condition characterized by the presence of unwanted cells is encompassed comprising a first component comprising a targeted T-cell engaging agent comprising:

a. a first component comprising a targeted T-cell engaging agent comprising:
   i. a first targeting moiety that is capable of targeting the unwanted cells;
   ii. a first T-cell engaging domain capable of T-cell engaging activity when binding a second T-cell engaging domain, wherein the second T-cell engaging domain is not part of the first component;
   iii. a first inert binding partner for the first T-cell engaging domain binding to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed; and
   iv. a cleavage site separating the first T-cell engaging domain and the first inert binding partner, wherein the cleavage site is:
      (1) cleaved by an enzyme expressed by the unwanted cells;
      (2) cleaved through a pH-sensitive cleavage reaction inside the unwanted cell;
      (3) cleaved by a complement-dependent cleavage reaction; or
      (4) cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent,

b. a second component comprising a second T-cell engaging domain capable of T-cell engaging activity when binding the first T-cell engaging domain, wherein the

first and second T-cell engaging domains are capable of binding when neither is bound to an inert binding partner.

[007] In another embodiment, the second component of the two-component system further comprises a second targeting moiety that is capable of targeting the unwanted cells.

[008] In another embodiment, the second component of the two-component system further comprises a second inert binding partner for the second T-cell engaging domain binding to the second T-cell engaging domain such that the second T cell engaging domain does not bind to the first T-cell engaging domain unless the inert binding partner is removed and

a. a cleavage site separating the second T-cell engaging domain and the second inert binding partner, wherein the cleavage site is:

i. cleaved by an enzyme expressed by the unwanted cells;

ii. cleaved through a pH-sensitive cleavage reaction inside the unwanted cell;

iii. cleaved by a complement-dependent cleavage reaction; or

iv. cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent,

wherein cleavage of the cleavage site causes loss of the inert binding partner and complementation with the first T-cell engaging domain of the two-component system.

[009] In some embodiments, the first and second targeting moieties of the two-component system are the same.

[0010] In some embodiments, the first and second targeting moieties of the two-component system are different

[0011] In some embodiments, the first and second cleavage sites are the same.

[0012] In some embodiments, the first and second cleavage sites are different.

[0013] In some embodiments, at least one cleavage site is a protease cleavage site. In some embodiments, the at least one cleavage site is capable of being cleaved outside the unwanted cells.

[0014] In some embodiments of the two-component system, at least one enzyme expressed by the unwanted cells is a protease.

[0015] In some embodiments of the two-component system, at least one inert binding partner specifically binds the T-cell engaging domain.

[0016] In some embodiments of the two-component system, at least one inert binding partner is a VH or VL domain.

[0017] In some embodiments of the two-component system, the T-cell engaging domain is a VH domain, the inert binding partner is a VL domain and when the T-cell engaging domain is a VL domain, the inert binding partner is a VH domain.

[0018] In some embodiments of the two-component system, at least one targeting moiety is an antibody or functional fragment thereof. In some embodiments of the two-component system, the at least one inert binding partner is capable of dissociation once at least one cleavage site has been cleaved and after dissociation the two T-cell engaging domains are capable of binding to each other and exhibiting T-cell engaging activity.

[0019] In some embodiments of the two-component system, a set of nucleic acid molecules encodes the first and second component of the two-component system. In some embodiments of the two-component system, a nucleic acid molecule encodes the component for use in a two-component system.

[0020] In some embodiments of the two-component system, one T-cell engaging domain is a VH domain and the other T-cell engaging domain is a VL domain.

[0021] In another embodiment, a component for use in a two-component system for treating a condition characterized by the presence of unwanted cells comprising a first targeted T-cell engaging agent comprises:

a. a targeting moiety that is capable of targeting the unwanted cells;

b. a first T-cell engaging domain capable of T-cell engaging activity when binding a second T-cell engaging domain, wherein the second T-cell engaging domain is not part of the first targeted T-cell engaging agent;

c. an inert binding partner for the first T-cell engaging domain binding to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed; and

d. a cleavage site separating the first T-cell engaging domain and the inert binding partner, wherein the cleavage site is:

i. cleaved by an enzyme expressed by the unwanted cells;

ii. cleaved through a pH-sensitive cleavage reaction inside the unwanted cell;

iii. cleaved by a complement-dependent cleavage reaction; or

cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent, wherein cleavage of the cleavage site causes loss of the inert binding partner and allows for complementation with the second T-cell engaging domain that is not part of the agent.

[0022] In some embodiments, a method of treating a disease in a patient characterized by the presence of unwanted cells is encompassed that comprises administering the two-component system to the patient. In some embodiments, a method of targeting an immune response of a patient to unwanted cells is encompassed that comprises administering the two-component system. In some embodiments, these unwanted cells are cancer cells. In some embodiments, the cancer 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.

[0023] 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.

[0024] 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.

[0025] 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

[0026] Figure 1 shows one embodiment of a first component of a two-component system, where the first component is a targeted T-cell engaging agent in an inactive state with an inert binding partner.

[0027] Figure 2 shows the process by which the cleavable linker is cleaved and the inert binding partner is released to create an active entity.

[0028] Figure 3 illustrates the creation of an active targeted, T-cell engaging agent after the inert binding partner is released from a pair of complementary components in a two-component system.

[0029] Figures 4A-C illustrate the cleavage of the stepwise process of the pair of complementary components in a two-component system binding to the target cell (A), cleavage of linker attaching the inert binding partners (A and B), and binding to create an active moiety capable of T-cell

[0030] Figures 5A-B provide evaluation of constructs by SDS PAGE and Coomassie blue staining.

[0031] Figure 6 shows IFNγ expression as a proxy for T cell response when cancer cells were treated with various individual constructs and combinations, with 6245 serving as a positive control and the combination of 6248 and 6249 showing beneficial results.

[0032] Figure 7 shows IFNγ expression as a proxy for T cell response when cancer cells were treated with various individual constructs and combinations, with 6245 as a positive control and the combination of 6248 and 6249 showing beneficial results.

[0033] Figure 8 shows IFNγ expression as a proxy for T cell response when cancer cells were treated with different concentrations of constructs, with 6245 as a positive control and the combination of 6248 and 6249 showing beneficial results.

[0034] Figures 9A-B shows IFNγ expression as a proxy for T cell response when cancer cells were treated with controls or different concentrations of constructs, with 6245 as a positive control and the combination of 6248 and 6249 showing beneficial results. PHA also served as a positive control for nonspecific T-cell activation.

[0035] Figure 10 shows IFNγ expression as a proxy for T cell response when cancer cells were treated with controls or different concentrations of constructs, with very low levels with constructs having only a VH or VL for the anti-CDE3 scFv, but positive control bispecific constructs (both 9332 and 9333) showed higher levels of activity.

[0036] Figure 11 provides a stoichiometric assessment of complementary constructs of a two-component system.

[0037] Figure 12 shows IFNγ expression as a proxy for T cell response when MCF-7 cancer cells were treated with controls or different concentrations of constructs.

[0038] Figure 13 shows IFNγ expression as a proxy for T cell response when cancer cells were treated with controls or different concentrations of constructs targeting EpCAM.

[0039] Figure 14 shows IFNγ expression as a proxy for T cell response when cancer cells were treated with controls or different concentrations of constructs targeting either biparatopic EGFR epitopes or a combination of EpCAM and EGFR targeting.

[0040] Figure 15 shows the impact of protease inhibitors on constructs either containing protease cleavage sites or not containing protease cleavage sites.

[0041] Figure 16 shows that different types of targeting moieties may be used, by successfully pairing a construct having a VHH targeting moiety with a construct having an scFv moiety.

[0042] Figure 17 shows a sequence schematic for constructs 6248 and 6249 with the various linkers boxed and the protease cleavage site in bold and underline. The His tag is also in bold.

DESCRIPTION OF THE SEQUENCES

[0043] Tables 1A and 1B provide a listing of certain sequences referenced herein.

Table 1A: 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	DPMMFFRL	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	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)	GGGGS	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)	UGCCGCUAUAAUGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	95
Anti-EGFR aptamer	UGGCGCUAAAUAGCACGGAAAUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	96
Anti-EGFR aptamer	UGCUAGUAUAUCGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	97
Anti-EGFR aptamer	UGCCGCCAUAUCACACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	98

Anti-EGFR aptamer	UUCCGCUGUAUAACACGGACUUAAUCGC CGUAGUAAAGCAUGUCAAAGCCG	99
Anti-EGFR aptamer	UGUCGCUCUAUUGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	100
Anti-EGFR aptamer	UGCUGCUUUAUCCCACAUAUUUUUUCCC CUCAUAACAAUAUUUCUCCCCCC	101
Anti-EGFR aptamer	UGCNGCUAUAUCGCNCGUAUUUAAUCGC CGUAGAAAAGCAUGUCNANGCCG	102
Anti-EGFR aptamer	UGCAAAGAAAACGCACGUAUUUAAUCGC CGUAGUAAAGCAUGUCAAAGCCG	103
Anti-EGFR aptamer	UGCAUCACUAUCGAACCUAUUUAAUCCA CCAAAAUAAUUGCAAGUCCAUACUC	104
Anti-EGFR aptamer	UGCCNNAAUAACACACNUAUAUAAUCGC CGUACAAAAUCAUGUCAAANCCG	105
Anti-EGFR aptamer	UGCAGCUGUAUUGCACGUAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	106
Anti-EGFR aptamer	UUCCGAUAAUCCCGCGUACUAAAUCACC AUAGUCAACAAUUUCCAACCUC	107
Anti-EGFR aptamer	UCCACUAUAUCACACGUAUUUAAUCGCC GUAGAAAAGCAUGUCAAAGCCG	108
Anti-EGFR aptamer	UCCCUCAACCUCGCUACUAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCU	109
Anti-EGFR aptamer	UGCCGCUAUAUCACACGAAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	110
Anti-EGFR aptamer	AGCCCCUAGAACACACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	111
Anti-EGFR aptamer	UGCCAAUAUAUAACACGGAAUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	112
Anti-EGFR aptamer	UGCCGCUAUAGCGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	113
Anti-EGFR aptamer	UGCAGAUAUAUGUCACUCAUUAAUCCCC GUAUAAAAACAUAACUAAGCUC	114
Anti-EGFR aptamer	UGUAGCUGUAUUGCACACAUUAAAUCGC CGUAGUAAAGCAUGUCAAAGCCG	115
Anti-EGFR aptamer	UACCAAUAUAUCGCCACACAUAAUCGCC GUAGAAAAGCAUGUCAAAGCCG	116
Anti-EGFR aptamer	UGCCGCUAUGCCCACGGAAUUUAAUCGC CGUAGAAAAACAUGUCAAAGUCG	117
Anti-EGFR aptamer	UGCCGCUAUUUAGCACGGAUUAAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	118
Anti-EGFR aptamer	UGCCGCUAUUUAGCACGGAUUAAAUCGC CGUAGAAAAGCAUGUCNAAGCCG	119
Anti-EGFR aptamer	UGUAGUAAUAUGACACGGAUUUAAUCGC CGUAGAAAAGCANGUCAAAGCCU	120
Anti-EGFR aptamer	UGUCGCCAUUACGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	121
Anti-EGFR aptamer	UGCCCCCAAACUACACAAAUUUAAUCGC CGUAUAAAAGCAUGUCAAAGCCG	122
Anti-EGFR aptamer	UGCACUAUCUCACACGUACUAAUCGCCG UAUAAAAGCAUGUCAAAGCCG	123
Anti-EGFR aptamer	UGUCGCAAUAAUACACUAAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	124

Anti-EGFR aptamer	UGCAACAAUAUAGCACGUAUUUAAUCGC CGUAGUAAAGCAUGUCAAAGG	125
Anti-EGFR aptamer	CUACCACAAAUCCCACAUAUUUAAUCUC CCAAUCAAAUCUUGUCCAUUCCC	126
Anti-EGFR aptamer	UGCCCUAAACUCACACGGAUAUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	127
Anti-EGFR aptamer	UUGUCGUAUGUCACACGUAUUAAAUCGC CGUAUAAAAGCAUGUCAAAGCCG	128
Anti-EGFR aptamer	UUCCGCUAUAACACACGGAGAAAAUCGC CGUAGUAAAGCAUGUCAAAGCCG	129
Anti-EGFR aptamer	UGCCGAUAUAACGCACGGAUAUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	130
Anti-EGFR aptamer	UGCCAUUAUACAGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	131
Anti-EGFR aptamer	UCCAGAAAUAUGCACACAUUUAAUCGCC GUAGAAAAGCAUGUCAAAGCCG	132
Anti-EGFR aptamer	UCCGCUAAACAACACGGAUACAAUCGCC GUAGAAAAGCAUGUCCAAGCCG	133
Anti-EGFR aptamer	UGCACUAUCUCACACGUACUAAUCGCCG UAUAAAAGCAUGUCAAANNNG	134
Anti-EGFR aptamer	AUNGCNANNNUACACGUAUUNAAUCGCC GUAGAAAAGCAUGUCANAGCCG	135
Anti-EGFR aptamer	UGCUGCUAUAUUGCAAUUUUUUAAACUA AGUAGAAAACCAUGUACAAGUCG	136
Anti-EGFR aptamer	UGUCGCCAUAUUGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	137
Anti-EGFR aptamer	UGCCGUUAUAACCCACGGAAUUUAACCU CCGUAGAAAAGCAUGUCAAAGCCG	138
Anti-EGFR aptamer	UGUGAAUAUAUAUCACGGAUUUAAUCGC CGUAUAAAAGCNAUGUCAAAGCCG	139
Anti-EGFR aptamer	UGCCGAUAUNNANCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	140
Anti-EGFR aptamer	UGUCACUAAAUUGCACGUAUAUAAUCGC CGUAGUAAGCAUGUCAAAGCCG	141
Anti-EGFR aptamer	UGCAACCAUAAAGCACGUAAUAAAUCGC CGUAUAUAAGCAUGUCaAAGCCG	142
Anti-EGFR aptamer	UGCCGCUAUAUAGCACGUAUUAAUCGCC GUAGUAAAGCAUGUCaAAGCCG	143
Anti-EGFR aptamer	UGCCGCUAUAGCACACGGAAUUUAAUCG CCGUAGUAAAGCAUGUCAAAGCCG	144
Anti-EGFR aptamer	UGCAGGUAUAUAACNCGGAUUUAAUCGC CGUAGAAAAGCAUGUCNAAGCCG	145
Anti-EGFR aptamer	UGCUCCUAUAACACACGGAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	146
Anti-EGFR aptamer	UGCCCGUAAUUGCACGGAUUUAAUCGCC GUAGAAAAGCAUGUCCAAGCCGG	147
Anti-EGFR aptamer	ACUCCCUAUAUNGCAACUACAUAAUCGC CGUAAAUAAGCAUGUNCAAGCCG	148
Anti-EGFR aptamer	UGAAGCUAGAUCACACUAAAUUAAUCGC CGUAGAAAAGCAUGUCAAAAAAGCCG	149
Anti-EGFR aptamer	UGACUCUUUAUCCCCCGUACAUUAUUcA CCGAACCAAAGCAUUACCAUCCCC	150

Anti-EGFR aptamer	UGACGCCCUAACACACGUAUAUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	151
Anti-EGFR aptamer	UGUCGCAAAAUAGCACGUAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	152
Anti-EGFR aptamer	UGAGUGUAUAAUUCACGUAUUUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	153
Anti-EGFR aptamer	UGCUACUAUAUCGUAGGUAACUAAUCGC CCUACAAACUCACUCUAAAACCG	154
Anti-EGFR aptamer	UUACGCUAUAUCACACGGAAUUUUAAUC GCCGUAGAAAAGCAUGUCCAAGCCG	155
Anti-EGFR aptamer	CCCAUCUGUACUACAGGAAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	156
Anti-EGFR aptamer	UGCCCAUAAAUAGCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	157
Anti-EGFR aptamer	UGCCGCAAUAACAUACACAUAUAAUCGC CGUAGAAAAGCAUGUCAAAGCCG	158
Anti-EGFR aptamer	UGCAACUAUAUCGCACGUAUGUAAUCGC CGUAGAAAAAGCAUGUCAAAGCC	159
Anti-EGFR aptamer	UUCCGCUAUAUAGCACGGAAUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	160
Anti-EGFR aptamer	UUCCGCUAAGUCACACGAAAUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	161
Anti-EGFR aptamer	UGUAGCAAUAUCACACGUAAUUAAUCGC CGUAUAUAAGCAUGUCAAAGCCG	162
Anti-EGFR aptamer	UGCCGUUAUAUAUCACGGAUUUAAUCGC CGUAGAAAAGCAUGUCCAAGCCG	163
Anti-EGFR aptamer	UAACACAUAUAUCAAGUAACUUAUCUCC UUAGUAACCAUCUCCAAGCCG	164

Table 1B: Description of Construct Sequences and SEQ ID NOs		
Description	**Sequence**	**#**
Construct 6245 single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (254-372) -18-mer linker (373-390) -V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (493) [5.3.9] (391-496)] -hexahistidine (497-502)	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDVQLVQSGAEVKKPGASVKVSCKASGY TFTRYTMHWVRQAPGQGLEWIGYINPSR GYTNYADSVKGRFTITTDKSTSTAYMEL SSLRSEDTATYYCARYYDDHYCLDYWGQ GTTVTVSSGEGTSTGSGGSGGSGGADDI VLTQSPATLSLSPGERATLSCRASQSVS YMNWYQQKPGKAPKRWIYDTSKVASGVP ARFSGSGSGTDYSLTINSLEAEDAATYY CQQWSSNPLTFGGGTKVEIKHHHHHH	165

CAS Registry Number 1005198-65-1 ChemID: 1005198-65-1		
Construct 6246 single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (254-372) -18-mer linker (373-390) -hexahistidine (391-396)	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDVQLVQSGAEVKKPGASVKVSCKASGY TFTRYTMHWVRQAPGQGLEWIGYINPSR GYTNYADSVKGRFTITTDKSTSTAYMEL SSLRSEDTATYYCARYYDDHYCLDYWGQ GTTVTVSSGEGTSTGSGGSGGSGGADHH HHHH	166
Construct 6247 single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -anti-CD3E-V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (356) [5.3.9] (254-359)] -hexahistidine (360-365)	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDIVLTQSPATLSLSPGERATLSCRASQ SVSYMNWYQQKPGKAPKRWIYDTSKVAS GVPARFSGSGSGTDYSLTINSLEAEDAA TYYCQQWSSNPLTFGGGTKVEIKHHHHH H	167
Construct 6248 single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (254-372) -25-mer linker (373-397 containing MMP2 cleavage site AIPVSLR (SEQ ID NO: 46)) -V-KAPPA	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDVQLVQSGAEVKKPGASVKVSCKASGY TFTRYTMHWVRQAPGQGLEWIGYINPSR GYTNYADSVKGRFTITTDKSTSTAYMEL SSLRSEDTATYYCARYYDDHYCLDYWGQ GTTVTVSSGEGTSTGSGAIPVSLRGSGG SGGADDIVLTQSPATLSLSPGERATLSC RASQSVSSSYLAWYQQKPGQAPRLLIYG ASSRATGVPARFSGSGSGTDFTLTISSL	168

(Homo sapiens V-KAPPA from gantenerumab, CAS: 1043556-46-2; 398-505); 87-hexahistidine (506-511)	EPEDFATYYCLQIYNMPITFGQGTKVEI KHHHHHH	
Construct 6249 single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -scFv anti-CD3E V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (493)[5.3.9] (254-359)] -25-mer linker (360-384 containing MMP2 cleavage site AIPVSLR (SEQ ID NO: 46)) - Ig heavy chain V region (clone alpha-MUC1-1, GenBank Accession S36265; 385-502)-hexahistidine (503-508)	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDIVLTQSPATLSLSPGERATLSCRASQ SVSYMNWYQQKPGKAPKRWIYDTSKVAS GVPARFSGSGSGTDYSLTINSLEAEDAA TYYCQQWSSNPLTFGGGTKVEIKGEGTS TGSGAIPVSLRGSGGSGGADDVQLVQSG AEVKKPGASVKVSCKASGYTFTGYYMHW VRQAPGQGLEWMGWINPNSGGTNYAQKF QGRVTITRDTSASTAYMELSSLRSEDTA VYYCARDFLSGYLDYWGQGTLVTVSSHH HHHH	169
Construct 9327 EpCAM V_LV_H-V_HDV_L single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (254-372) -25-mer linker (373-397) -V-KAPPA (Homo sapiens V-KAPPA from gantenerumab, CAS: 1043556-46-2; 398-505); -hexahistidine (506-511) Anti-EpCAM sequence from Brischwein K et al, Mol. Immunol. (2006) 43:1129-43	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDVQLVQSGAEVKKPGASVKVSCKASGY TFTRYTMHWVRQAPGQGLEWIGYINPSR GYTNYADSVKGRFTITTDKSTSTAYMEL SSLRSEDTATYYCARYYDDHYCLDYWGQ GTTVTVSSGEGTSTGSGGGGSGGGGSGG SGGADDIVLTQSPATLSLSPGERATLSC RASQSVSSSYLAWYQQKPGQAPRLLIYG ASSRATGVPARFSGSGSGTDFTLTISSL EPEDFATYYCLQIYNMPITFGQGTKVEI KHHHHHH	170

Construct 9328 single chain; scFv anti-EPCAM [Mus musculus V-KAPPA (IGKV8-19*01 (98.00%)-IGKJ5*01 L126>I (112)) [12.3.9] (1-113) -15-mer tris(tetraglycyl-seryl) linker (114-128) -Mus musculus VH (IGHV1-54*01 (85.90%)-(IGHD)-IGHJ4*01, S123>T (243)) [8.8.14] (129-248)] -5-mer tetraglycyl-seryl linker (249-253) -scFv anti-CD3E V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (493)[5.3.9] (254-359)] -25-mer linker (360-384) - Ig heavy chain V region (clone alpha-MUC1-1, GenBank Accession S36265; 385-502)-hexahistidine (503-508)	ELVMTQSPSSLTVTAGEKVTMSCKSSQS LLNSGNQKNYLTWYQQKPGQPPKLLIYW ASTRESGVPDRFTGSGSGTDFTLTISSV QAEDLAVYYCQNDYSYPLTFGAGTKLEI KGGGGSGGGGSGGGGSEVQLLEQSGAEL VRPGTSVKISCKASGYAFTNYWLGWVKQ RPGHGLEWIGDIFPGSGNIHYNEKFKGK ATLTADKSSSTAYMQLSSLTFEDSAVYF CARLRNWDEPMDYWGQGTTVTVSSGGGG SDIVLTQSPATLSLSPGERATLSCRASQ SVSYMNWYQQKPGKAPKRWIYDTSKVAS GVPARFSGSGSGTDYSLTINSLEAEDAA TYYCQQWSSNPLTFGGGTKVEIKGEGTS TGSGGGGSGGGGSGGSGGADDVQLVQSG AEVKKPGASVKVSCKASGYTFTGYYMHW VRQAPGQGLEWMGWINPNSGGTNYAQKF QGRVTITRDTSASTAYMELSSLRSEDTA VYYCARDFLSGYLDYWGQGTLVTVSSHH HHHH	171
Construct 9329 Glypican3 V_{HH}-CD3ε(V_H-MMP2-V_L) Anti-human Glypican-3 VHH sequence from US Patent 2012145469; residues 1-116) -5-mer tris(tetraglycyl-seryl) linker (117-122)- scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (123-241) -25-mer linker (242-266 containing MMP2 cleavage site AIPVSLR (SEQ ID NO: 46)) -V-KAPPA (Homo sapiens V-KAPPA from gantenerumab, CAS: 1043556-46-2; 267-374); -hexahistidine (375-380)	QVQLVQSGGGLVQPGGSLRLSCAASYFD FDSYEMSWVRQAPGKGLEWIGSIYHSGS TYYNPSLKSRVTISRDNSKNTLYLQMNT LRAEDTATYYCARVNMDRFDYWGQGTLV TVSSSGGGGSDVQLVQSGAEVKKPGASV KVSCKASGYTFTRYTMHWVRQAPGQGLE WIGYINPSRGYTNYADSVKGRFTITTDK STSTAYMELSSLRSEDTATYYCARYYDD HYCLDYWGQGTTVTVSSGEGTSTGSGAI PVSLRGSGGSGGADDIVLTQSPATLSLS PGERATLSCRASQSVSSSYLAWYQQKPG QAPRLLIYGASSRATGVPARFSGSGSGT DFTLTISSLEPEDFATYYCLQIYNMPIT FGQGTKVEIKHHHHHH	172
Construct 9330 anti-[Homo sapiens SDC1 (syndecan-1, CD138), scFv, from indatuximab CAS: 1238517-16-2, US Patent US20140010828], [Mus musculus V-KAPPA (IGKV10-94*01 -IGKJ1*01) [6.3.9] (1-108) -15-mer tris(tetraglycyl-seryl) linker (109-123) [Mus musculus VH (IGHV1-9*01 - (IGHD)-IGHJ4*01) [8.8.15] (124-245) -5-mer tris(tetraglycyl-seryl) linker (246-250)-scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-	DIQMTQSTSSLSASLGDRVTISCSASQG INNYLNWYQQKPDGTVELLIYYTSTLQS GVPSRFSGSGSGTDYSLTISNLEPEDIG TYYCQQYSKLPRTFGGGTKLEIKRGGGG SGGGGSGGGGSQVQLQQSGSELMMPGAS VKISCKATGYTFSNYWIEWVKQRPGHGL EWIGEILPGTGRTIYNEKFKGKATFTAD ISSNTVQMQLSSLTSEDSAVYYCARRDY YGNFYYAMDYWGQGTSVTVSSGGGGSDV QLVQSGAEVKKPGASVKVSCKASGYTFT RYTMHWVRQAPGQGLEWIGYINPSRGYT NYADSVKGRFTITTDKSTSTAYMELSSL RSEDTATYYCARYYDDHYCLDYWGQGTT VTVSSGEGTSTGSGAIPVSLRGSGGSGG ADDIVLTQSPATLSLSPGERATLSCRAS QSVSSSYLAWYQQKPGQAPRLLIYGASS	173

IGHJ6*01) [8.8.12] (251-369) -25-mer linker (370-394 containing MMP2 cleavage site AIPVSLR (SEQ ID NO: 46)) -V-KAPPA (Homo sapiens V-KAPPA from gantenerumab, CAS: 1043556-46-2; 395-502); -hexahistidine (503-508)	RATGVPARFSGSGSGTDFTLTISSLEPE DFATYYCLQIYNMPITFGQGTKVEIKHH HHHH	
Construct 9332 EGFR V_{HH}-CD3ε(V_H-V_L) Anti-human EGFR V_{HH} sequence from 7D12 sequence from Schmitz KR et al, Structure. 2013 Jul 2;21(7):1214-24; residues 1-124) -30-mer tris(tetraglycyl-seryl) linker (125-154)-scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (155-273) -18-mer linker (274-291) -V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (394) [5.3.9] (292-397)] -hexahistidine (398-403) Anti-human CD3ε sequence from Brischwein K et al, Mol. Immunol. (2006) 43:1129-43 US Patent US7919089	QVKLEESGGGSVQTGGSLRLTCAASGRT SRSYGMGWFRQAPGKEREFVSGISWRGD STGYADSVKGRFTISRDNAKNTVDLQMN SLKPEDTAIYYCAAAAGSAWYGTLYEYD YWGQGTQVTVSSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSDVQLVQSGAEVKKP GASVKVSCKASGYTFTRYTMHWVRQAPG QGLEWIGYINPSRGYTNYADSVKGRFTI TTDKSTSTAYMELSSLRSEDTATYYCAR YYDDHYCLDYWGQGTTVTVSSGEGTSTG SGGSGGSGGADDIVLTQSPATLSLSPGE RATLSCRASQSVSYMNWYQQKPGKAPKR WIYDTSKVASGVPARFSGSGSGTDYSLT INSLEAEDAATYYCQQWSSNPLTFGGGT KVEIKHHHHHH	174
Construct 9333 EGFR V_{HH}-CD3ε(V_H-V_L) Anti-human EGFR V_{HH} sequence from 9G8 sequence from Schmitz KR et al, Structure. 2013 Jul 2;21(7):1214-24; residues 1-127) -30-mer tris(tetraglycyl-seryl) linker (128-157)-scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (158-276) -18-mer linker (277-294) -V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (394) [5.3.9] (292-397)] -hexahistidine (401-406)	EVQLVESGGGLVQAGGSLRLSCAASGRT FSSYAMGWFRQAPGKEREFVVAINWSSG STYYADSVKGRFTISRDNAKNTMYLQMN SLKPEDTAVYYCAAGYQINSGNYNFKDY EYDYWGQGTQVTVSSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSDVQLVQSGAEV KKPGASVKVSCKASGYTFTRYTMHWVRQ APGQGLEWIGYINPSRGYTNYADSVKGR FTITTDKSTSTAYMELSSLRSEDTATYY CARYYDDHYCLDYWGQGTTVTVSSGEGT STGSGGSGGSGGADDIVLTQSPATLSLS PGERATLSCRASQSVSYMNWYQQKPGKA PKRWIYDTSKVASGVPARFSGSGSGTDY SLTINSLEAEDAATYYCQQWSSNPLTFG GGTKVEIKHHHHHH	175
Construct 9334 EGFR V_{HH}-CD3ε(V_H-MMP2-V_L)	QVKLEESGGGSVQTGGSLRLTCAASGRT SRSYGMGWFRQAPGKEREFVSGISWRGD STGYADSVKGRFTISRDNAKNTVDLQMN SLKPEDTAIYYCAAAAGSAWYGTLYEYD	176

Anti-human EGFR V_{HH} sequence from 7D12 sequence from Schmitz KR et al, Structure. 2013 Jul 2;21(7):1214-24; residues 1-124) -30-mer tris(tetraglycyl-seryl) linker (125-154)- scFv anti-CD3E [humanized VH (Homo sapiens IGHV1-46*01 (82.50%)-(IGHD)-IGHJ6*01) [8.8.12] (155-273) -25-mer linker (274-298 containing MMP2 cleavage site AIPVSLR (SEQ ID NO: 46)) -V-KAPPA (Homo sapiens V-KAPPA from gantenerumab, CAS: 1043556-46-2; 299-406); -hexahistidine (407-412)	YWGQGTQVTVSSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSDVQLVQSGAEVKKP GASVKVSCKASGYTFTRYTMHWVRQAPG QGLEWIGYINPSRGYTNYADSVKGRFTI TTDKSTSTAYMELSSLRSEDTATYYCAR YYDDHYCLDYWGQGTTVTVSSGEGTSTG SGAIPVSLRGSGGSGGADDIVLTQSPAT LSLSPGERATLSCRASQSVSSSYLAWYQ QKPGQAPRLLIYGASSRATGVPARFSGS GSGTDFTLTISSLEPEDFATYYCLQIYN MPITFGQGTKVEIKHHHHHH	
Construct 9335 EGFR V_{HH}-CD3ε(V_L-MMP2-V_H) Anti-human EGFR V_{HH} sequence from 9G8 sequence from Schmitz KR et al, Structure. 2013 Jul 2;21(7):1214-24; residues 1-127) -30-mer tris(tetraglycyl-seryl) linker (128-157)-scFv anti-CD3E V-KAPPA (Mus musculus IGKV4-59*01 (81.70%)-IGKJ1*01 L124>V (493)[5.3.9] (158-263)]-25-mer linker (264-288 containing MMP2 cleavage site AIPVSLR (SEQ ID NO: 46)) - Ig heavy chain V region (clone alpha-MUC1-1, GenBank Accession S36265; 289-406)-hexahistidine (307-412)	EVQLVESGGGLVQAGGSLRLSCAASGRT FSSYAMGWFRQAPGKEREFVVAINWSSG STYYADSVKGRFTISRDNAKNTMYLQMN SLKPEDTAVYYCAAGYQINSGNYNFKDY EYDYWGQGTQVTVSSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSDIVLTQSPATL SLSPGERATLSCRASQSVSYMNWYQQKP GKAPKRWIYDTSKVASGVPARFSGSGSG TDYSLTINSLEAEDAATYYCQQWSSNPL TFGGGTKVEIKGEGTSTGSGAIPVSLRG SGGSGGADDVQLVQSGAEVKKPGASVKV SCKASGYTFTGYYMHWVRQAPGQGLEWM GWINPNSGGTNYAQKFQGRVTITRDTSA STAYMELSSLRSEDTAVYYCARDFLSGY LDYWGQGTLVTVSSHHHHHH	177

DESCRIPTION OF THE EMBODIMENTS

I. A Two-Component System Comprising At Least One Targeted T-cell Engaging Agent

[0044] A variety of targeted T-cell engaging agents are described in different embodiments, and in some embodiment as part of a two-component system comprising a first component and a second component. In each of the embodiments, however, a targeting moiety may be used to deliver the targeted T-cell engaging agent to an area of unwanted cells, allowing for a therapeutic effect to be delivered locally. The targeted T-cell engaging agent also contains a first T-cell engaging domain capable of activity when binding

a second T-cell engaging domain, but the second T-cell engaging domain is not part of the targeted T-cell engaging agent. In other words, without the second T-cell engaging domain that is not part of the targeted T-cell engaging agent, the first T-cell engaging domain is not capable of T-cell engaging activity. The targeted T-cell engaging agent also comprises an inert binding partner capable of binding the first T-cell engaging domain and preventing it from binding to a second T-cell engaging domain. In other words, the inert binding partner binds to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed. By does not bind, the application does not exclude nonspecific binding or low levels of binding (for example, $\leq$ 1%, $\leq$5%, $\leq$10%). The concept is one of functional insufficiency with the de novo VH/VL complementation insufficient for T-cell target binding. Proteolytic cleavage liberates the inert VH or VL groups allowing the opportunity for re-pairing of active VH and VL pairs at the cell surface. Furthermore, the targeted T-cell engaging agent includes a cleavage site separating the first T-cell engaging domain and the inert binding partner. The cleavage site is cleaved when the targeted T-cell engaging agent is in the microenvironment of the unwanted cells.

[0045] In some embodiments, the second T-cell engaging domain is part of a second targeted T-cell engaging agent. Thus, in some embodiments, a kit or composition may comprise two targeted T-cell engaging agents, one with a first T-cell engaging domain and another with a second T-cell engaging domain. In such a kit or composition, the inert binding partners may be capable of dissociation once the cleavage site in each agent has been cleaved; after dissociation, the two T-cell engaging domains may be capable of binding to each other and exhibiting activity.

[0046] In some embodiments with two targeted T-cell engaging agents, the two-component system comprises one T-cell engaging domain that may be a VH domain and another T-cell engaging domain that may be a VL domain. In embodiments with two targeted T-cell engaging agents, the targeting moieties in the first component and the second component may be the same or they may be different.

[0047] In embodiments with two targeted T-cell engaging agents, the cleavage sites in the first component and the second component may be the same or they may be different.

[0048] Figure 1 shows one embodiment of a targeted T-cell engaging agent construct comprising (a) an scFv targeting domain comprising a VH domain and a VL domain that bind the target, wherein the VH and VL domain are connected by a flexible linker; (b) an

inactive T-cell engaging domain comprising a VL domain that binds to an inert VH domain, wherein the VH and VL domains are connected by a flexible linker having a cleavage site, and (c) a flexible linker joining the targeting domain and the inactive T-cell engaging domain.

[0049] In some embodiments, Figure 2 shows the process by which the cleavable linker is cleaved and the inert binding partner is released to create an entity without an inert binding partner. This entity is still inactive because the VL domain in the T-cell engaging domain is not active on its own.

[0050] In some embodiments, Figure 3 illustrates the creation of an active targeted T-cell engaging agent after the inert binding partner is released from a pair of complementary targeted T-cell engaging agents.

[0051] In some embodiments, Figures 4A-C illustrate the cleavage of the stepwise process of the targeted T-cell engaging agents binding to the target cell (4A), cleavage of the inert binding partners (4A and 4B), and binding to create an active targeted T-cell engaging agent (4C).

[0052] In some alternative embodiments, the second T-cell engaging domain may not be bound to a targeting moiety and/or may not comprise a cleavage site and inert binding partner. In some instances, the second T-cell engaging domain may be conjugated or linked to a targeting moiety (either the same targeting moiety or a different targeting moiety), but in such an embodiment it would not be conjugated or linked to an inert binding partner. In such an embodiment, only the first T-cell engaging domain is bound to an inert binding partner. In another embodiment, the second T-cell engaging domain may also comprise a targeting moiety, a cleavage site, and an inert binding partner, each as described herein.

[0053] In some embodiments, the structural arrangement from N-terminus to C-terminus of the first component comprises IBVL-L1-TCEVH-L2-TVL-L3-TVH. In some embodiments, the structural arrangement from N-terminus to C-terminus of the second component comprises TCEVL-L2-TVH-L3-TVL. In some embodiments, the structural arrangement from N-terminus to C-terminus of the second component comprises IBVH-L1-TCEVL-L2-TBVH-L3-TBVL. In each of these embodiments IB stands for inert binding partner and IBVL is a VL inert binding partner, whereas IBVH is a VH insert binding domain. TCE stands for T-cell engaging and an TCEVL is a VL portion of a T-cell engaging domain and a TCEVH is a VH portion of a T-cell engaging domain. TB stand for target

binding domain and a TBVH is a VH portion of a target binding domain and a TBVL is a VL portion of a target binding domain. L1 is a linker with a protease cleavage site, while L2 and L3 are optionally linkers that optionally are not cleavable by the same protease as L1.

A. Targeting Moiety

[0054] The targeting moiety functions in the targeted T-cell engaging agent by delivering the agent to the local environment of the unwanted cells, enabling a localized treatment strategy. In certain embodiments, the targeting moiety targets the unwanted cells by specifically binding to the unwanted cells. In some instances, the targeting moiety specifically binds the unwanted cells even while the inert binding partner is binding the first T-cell engaging domain.

[0055] In some embodiments, a first targeting moiety is bound, optionally by a linker, to a first T-cell engaging domain and, as part of a separate construct, a second targeting moiety is bound, optionally by a linker, to a second T-cell engaging domain. In this way, each complementary part of the T-cell engaging domain is delivered to the unwanted cells by a separate targeting moiety. In some embodiments, the targeting moieties are of the same type and, in some embodiments, the targeting moieties are different. When the targeting moieties are of different types, they can either target different epitopes (either overlapping or nonoverlapping) on the same target protein of the unwanted cell or they can target different target proteins. In situations when the targeting moieties target different proteins, the unwanted cell will express an antigen corresponding to each of the two types of targeting moieties, providing additional specificity for this approach.

[0056] In certain embodiments, the targeting moiety is an antibody or functional part thereof. By functional part, we mean any antibody fragment that retains its binding activity to the target on the unwanted cell, such as an scFv or VHH or other functional fragment including an immunoglobulin devoid of light chains, 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. Unless specifically noted as "full length antibody," when the application refers to antibody it inherently includes a reference to a functional part thereof.

[0057] Certain antibody targets (with examples of unwanted cell types in parentheses) may include: Her2/Neu (Epithelial malignancies); CD22 (B cells, autoimmune or malignant); EpCAM (CD326) (Epithelial malignancies); EGFR (epithelial malignancies);

PMSA (Prostate Carcinoma); CD30 (B cell malignancies); CD20 (B cells, autoimmune, allergic or malignant); CD33 (Myeloid malignancies); membrane lgE (Allergic B cells); lgE Receptor (CD23) (Mast cells or B cells in allergic disease), CD80 (B cells, autoimmune, allergic or malignant); CD86 (B cells, autoimmune, allergic or malignant); CD2 (T cell or NK cell lymphomas); CA125 (multiple cancers including Ovarian carcinoma); Carbonic Anhydrase IX (multiple cancers including Renal Cell Carcinoma); CD70 (B cells, autoimmune, allergic or malignant); CD74 (B cells, autoimmune, allergic or malignant); CD56 (T cell or NK cell lymphomas); CD40 (B cells, autoimmune, allergic or malignant); CD19 (B cells, autoimmune, allergic or malignant); c-met/HGFR (Gastrointestinal tract and hepatic malignancies; TRAIL-R1 (multiple malignancies including ovarian and colorectal carcinoma); DRS (multiple malignancies including ovarian and colorectal carcinoma); PD-1 (B cells, autoimmune, allergic or malignant); PD1L (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 cells or T cells, autoimmune, allergic or malignant); CXCR4 (B cells or T cells, autoimmune, allergic or malignant); Neuropilin 1 (Epithelial malignancies, including lung cancer); Glypicans (multiple cancers including liver, brain and breast cancers); HER3/EGFR (Epithelial malignancies); PDGFRa (Epithelial malignancies); EphA2 (multiple cancers including neuroblastoma, melanoma, breast cancer, and small cell lung carcinoma); CD38 (Myeloma); CD138 (Myeloma); α4-integrin (AML, myeloma, CLL, and most lymphomas).

[0058] In certain modes, antibodies include an anti-epidermal growth factor receptor antibody such as Cetuximab, an anti-Her2 antibody, an anti-CD20 antibody such as Rituximab, an anti-CD22 antibody such as Inotuzumab, G544 or BU59, an anti-CD70

antibody, an anti-CD33 antibody such as hp67.6 or Gemtuzumab, an anti-MUC1 antibody such as GP1.4 and SM3, an anti-CD40 antibody, an anti-CD74 antibody, an anti-P-cadherin antibody, an anti-EpCAM antibody, an anti-CD138 antibody, an anti-E-cadherin antibody, an (anti-CEA antibody, an anti-FGFR3 antibody, and an anti α4-integrin antibody such as natalizumab.

[0059] Table 2A provides nonlimiting examples of cancer types, possible targeting moieties, and proteases that are expressed by those cancer types. In order to prepare a two-component system, the cancer may be identified from column 1, one or two targets chosen for the targeting moiety (as desired), and one or two proteases chosen for the cancer type, as well (as desired). Other sections of this application discuss when to use one versus two targeting moieties and one versus two protease cleavage sites.

Table 2A: Coordination of Cancer Type, Targets for Targeting Moiety, and Proteases that Can Cleave Cleavage Sites

Cancer	**Targets for Targeting Moiety**	**Proteases that can Cleave Cleavage Site**
Prostate Cancer	ADAM17, CD59, EpCAM, HER2, Integrin αV, Integrin αVβ3, MCP-1, PCLA, PSCA, PSMA, RANKL, RG1, SLC44A4 STEAP-1, VEGF-C	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 α6β4, 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, 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-L, 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 α5β1, Lewis y/b antigen, EGFL7, TPBG, DKK-1, NaPi2b, flt4, cMet, CD71	Cathepsin B, MMP2, MMP9, ST14, ADAM17
Pancreatic Carcinoma	SLC44A4, uPAR, MUC1, MUCH16, TACSTD-2, CEA, EphhA4, 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 α5β1, TYRP1, GD3, Integrin αV	Cathepsin B, MMP9
Ovarian Cancer	HER2/neu, EpCAM, CA125, DLL4, Integrin αVβ3, 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

[0060] For example, when targeting moieties in the first and second components are different, Table 2B provides a nonlimiting list of potential targeting moieties to use in combination with particular cancer types. In a two-component system, a targeting moiety for the first component would be present and a second targeting moiety for the second component may optionally be present. If only the first component has a targeting moiety or if the first and second components have the same targeting moiety, either the targeting moiety listed in column 1 or column 2 of the table may be used when the cancer type is listed in column 3.

Table 2B: Targeting Moieties for Use in Two-Component System

Targeting Moiety for First Component	**Optional Targeting Moiety for Second Component**	**Cancer Type**
Antibody against CD20 (such as Rituximab)	Antibody against CD80	Lymphoma
Antibody against CD20 (such as Rituximab)	Antibody against CD22 (such as Inotuzumab)	Lymphoma

Antibody against CD20 (such as Rituximab)	Antibody against CD70	Lymphoma
Antibody against HER2	Antibody against EpCAM	Epithelial malignancies
Antibody against EGFR (such as Cetuximab)	Antibody against mucin protein core	Breast cancer
Antibody against EGFR (such as Cetuximab)	Antibody against HER2	Epithelial malignancies
Antibody against EGFR (such as Cetuximab)	Antibody against transferrin receptor	Gliomas
Antibody against gp95/gp97	Antibody against p-glycoprotein	Drug-resistant melanomas
Antibody against TRAIL-R1	Antibody against DR5	Multiple malignancies, including ovarian and colorectal carcinoma
Antibody against IL-4	Antibody against IL-6	Lymphomas and leukemias
Antibody against CD19	Antibody against CD22	Lymphoma
Antibody against PMSA	Antibody against PSCA	Prostate carcinoma
Antibody against P-cadherin	Antibody against Cripto (TDGF1)	Epithelial malignancies
Antibody against CD74	Antibody against CD40	Lymphomas
Antibody against PD1L	Antibody against IGF-1R	Epithelial adenocarcinoma
Antibody against CD38	Antibody against CD138	Myeloma
Antibody against BCMA	Antibody against CD138 or CD38	Myeloma
Antibody against CD33	Antibody against CD133	Myeloid Malignancies, e.g. AML
Antibody against CD33	Antibody against CD123	Myeloid Malignancies such as AML
Antibody against CD49d	Antibody against CD33	Myeloid Malignancies
Antibody against PSMA	Antibody against PSCA	Prostate Cancer
Antibody against Glypican 3	Antibody against cMet or EpCAM	Hepatocellular carcinoma
Antibody against EpCAM	Antibody against EGFR	Lung Cancer
Antibody against EpCAM	Antibody against MUC1	Pancreatic Cancer
Antibody against EpCAM	Antibody against EGFR	Colorectal Carcinoma
Antibody against MUC1	Antibody against EGFR	Ovarian Carcinoma
Antibody against GD2	Antibody against HER2	Sarcoma
Antibody against HER2	Antibody against HER3	Breast Cancer
Antibody against IL-13R	Antibody against EGFR	Brain Cancer

[0061] In some embodiments, the targeting moiety is not an antibody, but is another type of targeting moiety. For example, a targeting moiety may be a binding partner for a protein known to be expressed on the unwanted cell. Such expression levels may include overexpression. For example, the following binding partners may bind to the following targets on an unwanted cell:

Table 3: Non-Antibody Binding Partners and Corresponding Targets	
Binding Partner	**Target on Unwanted 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 TGFα	EGFR (EGF receptor)
PD1	PD-L1 and/or PD-L2
IL13	IL-13R (Glioblastoma)
Stem cell factor	CXCR4
Insulin-like growth factor (IGF)	IGFR
CD40	CD40L

[0062] The binding partner need not comprise the full length or wildtype sequence for the binding partners listed in Table 3. All that is required is that the binding partner bind to the target on the unwanted cell and can thus include truncated forms, analogs, variants, and derivatives that are well known in the art.

[0063] 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 unwanted cell. Aptamers that bind unwanted cells, such as cancer cells, are well known and methods for designing them are known.

[0064] Cell-based SELEX systems may be used to select a panel of target cell-specific aptamers from a random candidate library. A ssDNA pool may be dissolved in binding buffer and denatured and then incubated with target cells. After washing the bound DNAs 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 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 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.

[0065] 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 unwanted cells. Other aptamers against other targets on unwanted cells are equally part of the description herein and incorporated by reference as described in Zhu et al., Progress in Aptamer Mediated Drug Delivery Vehicles for Cancer Targeting, Theranostics 4(9):931-944 (2014).

[0066] In some embodiments, aptamers for use herein bind to the target on the unwanted cell with a K_d in the nanomolar to picomolar range (such as 1 picomolar to 500 nanomolar or 1 picomolar to 100 nanomolar).

B. T-Cell Engaging Domain

[0067] The targeted T-cell engaging agent comprises a first T-cell engaging domain that is unable of engaging a T-cell alone. Instead, the first T-cell engaging domain is capable of activity when binding a second T-cell engaging domain, which is not part of the targeted T-cell engaging agent. Thus, the first and second T-cell engaging domains may be any two moieties that do not possess T-cell engaging activity alone, but do possess it when paired with each other. In other words, the first and second T-cell engaging domains are complementary halves of a functional active protein.

[0068] When the two T-cell engaging domains are associated together in the two-component system, they may bind to the CD3 antigen and/or T-cell receptor on the surface of the T-cell as these activate T cells. CD3 is present on all T cells and consists of subunits designated γ, δ, ε, ζ, and η. 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. In a normal situation, 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 two-component system binds 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.

[0069] In some embodiments, the first T-cell engaging domain is a VH domain and the second T-cell engaging domain is a VL domain. In other embodiments, the first T-cell engaging domain is a VL domain and the second T-cell engaging domain is a VH domain. In such embodiments, when paired together the first and second T-cell engaging domains may comprise an scFv.

[0070] If the first and second T-cell engaging 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. If the antigen is CD3, one potential T-cell engaging domain may be derived from muromonab.

C. Inert Binding Partner

[0071] The targeted T-cell engaging agent also comprises at least one inert binding partner capable of binding the first T-cell engaging domain and preventing it from binding to a second T-cell engaging domain unless certain conditions occur. When the first T-cell engaging domain is bound to the at least one inert binding partner, it does not possess a T-cell engaging activity. In other words, the at least one inert binding partner cripples the function of the first T-cell engaging domain by blocking it from binding its complementary pair (the second T-cell engaging domain) and preventing the two domains from joining together to have a T-cell engaging activity. In other words, the inert binding partner binds to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed. By does not bind, the application does not exclude nonspecific binding or low levels of binding (for example, $\leq$ 1%, $\leq$5%, $\leq$10%).

[0072] In some embodiments, the inert binding partner binds specifically to the T-cell engaging domain.

[0073] In some embodiments, the at least one inert binding partner is a VH or VL domain. In some embodiments, when the T-cell engaging domain in the targeted T-cell

engaging agent is a VH domain, the inert binding partner may be a VL domain and when the first T-cell engaging domain is a VL domain, the inert binding partner may be a VH domain.

[0074] If a first component comprises a targeting moiety and a VL T-cell engaging domain and a VH inert binding partner, in some embodiments, the VH inert binding partner has an equilibrium dissociation constant for binding to the VL T-cell engaging domain, which is greater than the equilibrium dissociation constant of the VL T-cell engaging domain for its partner VH T-cell engaging domain in the second component. In some embodiments, the prior sentence is equally true when VH is switched for VL and vice versa.

[0075] Based on empirical evidence in the examples, it is believed that using the inert binding partner as a mispairing partner with the T-cell engaging domain in the construct results in constructs that are more stable and easier to manufacture.

D. Cleavage Site

[0076] By way of overview, the cleavage site may be (i) cleaved by an enzyme expressed by the unwanted cells; (ii) cleaved through a pH-sensitive cleavage reaction inside the unwanted cell; (iii) cleaved by a complement-dependent cleavage reaction; or (iv) cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent. In some embodiments, the cleavage site is a protease cleavage site.

[0077] The cleavage sites function to release the inert binding partner from the first T-cell engaging domain. The cleavage sites can function in different ways to release the inert binding partner from the first T-cell engaging domain T-cell epitopes in the microenvironment of the unwanted cells. The cleavage may occur inside the unwanted cell or outside the unwanted cell, depending on the strategy employed. If cleavage occurs outside the unwanted cell, the T-cell engaging domain can be presented without first being internalized into a cell and being engaged in the classical antigen-processing pathways.

[0078] In certain embodiments, at least one cleavage site may be cleaved by an enzyme expressed by the unwanted cells. Cancer cells, for instance, are known to express certain enzymes, such as proteases, and these may be employed in this strategy to cleave the targeted T-cell engaging agent's cleavage site. 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 1A or 2A 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 invention 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 PMAP (www.proteolysis.org), ExPASy Peptide Cutter (ca.expasy.org/tools/peptidecutter) and PMAP.Cut DB (cutdb.burnham.org).

[0079] In some embodiments, at least one cleavage site may be cleaved through a pH-sensitive cleavage reaction inside the unwanted cell. If the targeted T-cell engaging agent is internalized into the cell, the cleavage reaction may occur inside the cell and may be triggered by a change in pH between the microenvironment outside the unwanted cell and the interior of the cell. Specifically, some cancer types are known to have acidic environments in the interior of the cancer cells. Such an approach may be employed when the interior unwanted cell type has a characteristically different pH from the extracellular microenvironment, such as particularly the glycocalyx. Because pH cleavage can occur in all cells in the lysozymes, selection of a targeting agent when using a pH-sensitive cleavage site may require, when desired, more specificity. For example, when a pH-sensitive cleavage site is used, a targeting agent that binds only or highly preferably to cancer cells may be desired (such as, for example, an antibody binding to mesothelin for treatment of lung cancer).

[0080] In certain embodiments, at least one cleavage site may be cleaved by a complement-dependent cleavage reaction. Once targeted T-cell engaging agents bind to the unwanted cell, the patient's complement cascade may be triggered. In such a case, the complement cascade may also be used to cleave the inert binding partner from the first T-cell engaging domain by using a cleavage site sensitive to a complement protease. For example, C1r and C1s and the C3 convertases (C4B,2a and C3b,Bb) are serine proteases. C3/C5 and C5 are also complement proteases. Mannose-associated binding proteins (MASP), serine proteases also involved in the complement cascade and responsible for cleaving C4 and C2 into C4b2b (a C3 convertase) may also be used. For example, and

without limitation, C1s cleaves YLGRSYKV and MQLGRX. MASP2 is believed to cleave SLGRKIQI. Complement component C2a and complement factor Bb are believed to cleave GLARSNLDE.

[0081] In some embodiments, at least one cleavage site may be cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the targeted T-cell engaging agent. For example, any protease may be simultaneously directed to the microenvironment of the unwanted cells by conjugating the protease to a targeting agent that delivers the protease to that location. The targeting agent may be any targeting agent described herein. The protease may be affixed to the targeting agent through a peptide or chemical linker and may maintain sufficient enzymatic activity when bound to the targeting agent.

[0082] In some embodiments, both the first component and second component are mispaired with an inert binding partner. In some embodiments, the protease cleavage site in the first component and the second component are the same. In other embodiments, the protease cleavage sites in the first component and the second component are different cleavage sites for the same protease. In other embodiments, the protease cleavage sites in the first component and the second component are cleavage sites for different proteases. In some embodiments employing two different proteases, the unwanted cell expresses both proteases.

[0083] In some embodiments, in a first component, the inert binding partner in an uncleaved state interferes with the specific binding of a VL or VH T-cell engaging domain to its partner VH or VL, respectively, T-cell engaging domain in a second component. In some embodiments, the inert binding partner in an uncleaved state inhibits the binding of the VL or VH T-cell engaging domain to its partner VH or VL, respectively, T-cell engaging domain in a second component such that the dissociation constant (Kd) of the VL or VH T-cell engaging domain to its partner VH or VL, respectively, T-cell engaging domain in a second component in an uncleaved state is at least 100 times greater than the Kd of the VL or VH T-cell engaging domain to its partner VH or VL, respectively, T-cell engaging domain in a second component in a cleaved state.

E. Linkers

[0084] In addition to the cleavage site, linkers may optionally be used to attach the separate parts of the targeted T-cell engaging agents together. By linker, we include any chemical moiety that attaches these parts together. In some embodiments, the linkers 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).

[0085] In some embodiments, the linker is a maleimide (MPA) or SMCC linker.

F. Methods of Making

[0086] The targeted T-cell engaging agents as described herein can be made using genetic engineering techniques. Specifically, a nucleic acid may be expressed in a suitable host to produce a targeted T-cell engaging agent. For example, a vector may be prepared comprising a nucleic acid sequence that encodes the targeted T-cell engaging agent including all of its component parts and linkers and that vector may be used to transform an appropriate host cell.

[0087] 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.

[0088] Chemical linkage techniques, such as using maleimide or SMCC linkers, may also be employed.

[0089] In instances where the binding partner is an aptamer, a person of ordinary skill in the art would appreciate how to conjugate an aptamer to a protein, namely the T-cell engaging 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 T-cell engaging domain.

II. Pharmaceutical Compositions

[0090] The targeted T-cell engaging agents 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 targeted T-cell engaging agents may be provided in sterile, pyrogen-free water for injection or sterile, pyrogen-free

saline. Alternatively, the targeted T-cell engaging agents may be provided in lyophilized form for resuspension with the addition of a sterile liquid carrier.

III. Methods of Treatment

A. Reduction of Unwanted Cells, Targeting of Immune Response, and Treatment of Cancer

[0091] The targeted T-cell engaging agents described herein may be used in a method of treating a disease in a patient characterized by the presence of unwanted cells comprising administering a two-component system comprising at least one targeted T-cell engaging agent and a second component to the patient, as each of the components have been described in detail in various embodiments above. Additionally, the agents described herein may also be used in a method of targeting a patient's own immune response to unwanted cells comprising administering a two-component system to the patient.

[0092] 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 two-component system may be administered in the same formulation or two different formulations within a sufficiently close period of time to be active in the patient.

[0093] 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).

[0094] The condition characterized by unwanted cells may include cancer. The cancer may be a solid or non-solid malignancy. 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.

[0095] The two-component system may be administered alone or in conjunction with other forms of therapy, including surgery, radiation, or traditional chemotherapy.

EXAMPLES

Example 1. Preparation of Constructs

[0096] Various constructs, both control and experimental, were prepared and used in the examples.

A. Single Chain scFv Bispecific Constructs

[0097] A single chain scFv construct was used in this application in order to serve as a positive control. Construct 6245 (SEQ ID NO: 165) was prepared as a bispecific antibody comprising an anti-EPCAM scFv and anti-CD3E scFv. This construct does not comprise any mispairing with an inert binding partner and has both active targeting and T-cell engaging moieties.

B. Precleaved Two-Component System Constructs Using a Targeting scFv

[0098] Construct 6246 (SEQ ID NO: 166) and 6247 (SEQ ID NO: 167) are complementary precleaved constructs in a two-component system. By precleaved, the description refers to a construct with a functional targeting moiety and an unpaired T-cell engaging moiety (i.e., one that is not mispaired to an inert binding partner and one that is also not yet paired with its correct partner to form a functional T-cell engaging complex). Both constructs comprise an anti-EPCAM scFv. Construct 6246 comprises an anti-CD3E VH domain, whereas construct 6247 comprises an anti-CD3E VL domain. Neither construct contains an inert binding partner as a mispairing partner.

C. Two-Component System Constructs Using a Targeting scFv and a T-cell Engaging Domain Mispaired to an Inert binding partner, as Well as a Protease Cleavage Site for Releasing the Inert binding partner

[0099] Construct 6248 (SEQ ID NO: 168) and 6249 (SEQ ID NO: 169) are complementary constructs in a two-component system. Both constructs comprise an anti-EPCAM scFv. Construct 6248 comprises an anti-CD3E VH 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. Construct 6249 comprises an anti-CD3E VL 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.

D. Two-Component System Constructs with a Targeting Moiety, and a T-Cell Engaging Domain Mispaired with an Inert binding partner Without a Protease Cleavage Site for Releasing the Inert binding partner

[00100] Constructs 9327 (SEQ ID NO: 170) and 9328 (SEQ ID NO: 171) are two-component system constructs using an scFv targeting domain; however, they do not

have a protease cleavage site for releasing the inert binding partner that serves as a mispairing moiety. Both constructs comprise an anti-EpCAM scFv for targeting the constructs to the unwanted cells expressing EpCAM. Construct 9327 comprises an anti-CD3E VH domain linked by a 25-mer linker that does not have a protease cleavage site corresponding to a protease used in the examples to an inert binding partner VL domain from gantenerumab. Construct 9328 comprises an anti-CD3E VL domain linked by a 25-mer linker that does not have a protease cleavage site corresponding to a protease used in the examples to an inert binding partner VH domain from clone alpha-MUC1-1 antibody.

Because these constructs do not have a protease cleavage site corresponding to a protease used in the examples, the inert binding partner will remain attached to the construct, preventing the two would-be complementary components of the two-component system from coming together to create an active anti-CD3E scFv.

E. Constructs Providing Different Targeting Moieties

[00101] Constructs 9329 (SEQ ID NO: 172) and 9330 (SEQ ID NO: 173) provide different targeting moieties. These constructs were intended to be used in two-component systems where one construct targets a first antigen on a cancer cell and the second construct targets a second antigen on the same cancer cell. Because the relative size of scFv and VHH targeting moieties are similar, these constructs were intended to "mix-and-match" with the pairable constructs having an anti-CD3E VL domain.

[00102] Construct 9329 comprises an anti-glypican-3 VHH sequence. It also comprises an anti-CD3E VH domain attached by a 25-mer linker comprising an MMP2 cleavage site (AIPVSLR (SEQ ID NO: 46)) to an inert binding partner VL domain from gantenerumab.

[00103] Construct 9330 comprises an anti-SDC1 scFv from indatuximab as the targeting moiety. It also comprises an anti-CD3E VH domain attached by a 25-mer linker comprising an MMP2 cleavage site (AIPVSLR (SEQ ID NO: 46)) to an inert binding partner VL domain from gantenerumab.

F. VHH/scFv Bispecific Constructs

[00104] Construct 9332 (SEQ ID NO: 174) and 9333 (SEQ ID NO: 175) are both VHH/scFv bispecific constructs comprising an anti-EGFR VHH portion and an anti-CD3E scFv portion. These two constructs do not comprise any insert binding domain.

G. Two-Component System Constructs Using a Targeting VHH Domain, a T-Cell Engaging Domain Mispaired to an Inert binding partner and Comprising a Protease Cleavage Site for Releasing the Inert binding partner

[00105] Constructs 9334 (SEQ ID NO: 176) and 9335 (SEQ ID NO: 177) are complementary two-component system constructs using a targeting VHH domain. Both constructs comprise an anti-EGFR VHH domain for targeting to the unwanted cells expressing EGFR. Construct 9334 comprises an anti-CD3E VH domain linked by a 25-mer linker having an MMP2 cleavage site (AIPVSLR (SEQ ID NO: 46)) to an inert binding partner VL domain from gantenerumab. Construct 9335 was prepared comprising an anti-CD3E VH domain linked by 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.

[00106] Thus, as a summary, the constructs are as provided in Table 4, with more detail and sequences provided above in Table 1B, with IBD standing for Inert binding partner.

Table 4: Construct Summary

No.	Targeting Moiety	T-Cell Engaging Moiety?	IBD? Cleavage?	Pair with?
6245	anti-EpCAM scFv	anti-CD3E scFv	no IBD, no cleavage	no pairing necessary for TCE activity (positive control)
6246	anti-EPCAM scFv	anti-CD3E VH	no IBD, no cleavage	pairs with at least 6247
6247	anti-EPCAM scFv	anti-CD3E VL	no IBD, no cleavage	pairs with at least 6246
6248	anti-EPCAM scFv	anti-CD3E VH	MMP2 cleavage site and inert VL	pairs with at least 6249
6249	anti-EPCAM scFv	anti-CD3E VL	MMP2 cleavage site and inert VH	pairs with at least 6248
9327	anti-EpCAM scFv	anti-CD3E VH	no cleavage site and inert VL	cannot easily pair with 9328 because no cleavage site (negative control)
9328	anti-EpCAM scFv	anti-CD3E VL	no cleavage site and inert VH	cannot easily pair with 9327 because no cleavage site (negative control)
9329	anti-Glypican-3 VHH	anti-CD3E VH	MMP2 cleavage site and inert VL	"mix-and-match" with pairable constructs having an anti-CD3E VL domain

9330	anti-SDC1 scFv from indatuximab	anti-CD3E VH	MMP2 cleavage site and inert VL	"mix-and-match" with pairable constructs having an anti-CD3E VL domain
9332	anti-EGFR VHH (7D12)	anti-CD3E scFv	no IBD, no cleavage	no pairing necessary for TCE activity (positive control)
9333	anti-EGFR VHH (9D8)	anti-CD3E scFv	no IBD, no cleavage	no pairing necessary for TCE activity (positive control)
9334	anti-EGFR VHH	anti-CD3E VH	MMP2 cleavage site and inert VL	pairs with at least 9335
9335	anti-EGFR VHH	anti-CD3E VL	MMP2 cleavage site and inert VH	pairs with at least 9334

H. Preparation and Storage of All Constructs

[00107] Constructs were generated by DNA2.0 (Newark, California) and expressed in HEK293T cells. Single-stranded oligonucleotides were designed to cover a specified sequence with a C-terminal hexahistidine tag to aid with down-stream purification. The oligonucleotides were chemically synthesized, then assembled using a variety of proprietary protocols depending on the sequence characteristics. In some instances, template independent PCR was used. In some instances, smaller sequences were assembled to create larger sequences by use of standard restriction enzyme digestion and ligase-mediated assembly. The assembled oligonucleotides were then cloned into standard E. coli plasmids and the complete double strand sequence verified by automated Sanger sequencing on ABI hardware. Constructs were expressed by transient transfection in HEK293T cells at the 150ml scale and antibody fragments purified using affinity chromatography.

[00108] Before experiments began, constructs were thawed on ice and aliquoted under sterile conditions into low protein-binding tubes. Aliquots were stored at -80°C until required. Aliquots were thawed on ice immediately prior to use. Aliquots were used for a maximum of five freeze-thaw cycles.

Example 2. Evaluation Construct Manufacturing

A. Figure 5A: Evaluation of Constructs by SDS PAGE and Coomassie Blue Staining

[00109] Aliquots of antibody were thawed on ice, and diluted in 25 mM Tris pH7.4 to a final concentration of 2.0 mg/ml. If the constructs were already more dilute than this, the dilution step was omitted. An appropriate volume of 6X gel sample buffer (0.5 M

Tris pH 6.8, 12% (w/v) SDS, 25% (v/v) glycerol, 5 mM EDTA, 200 mM N-ethylmaleimide) was added to each sample, which was then heated to 90°C for 10 minutes.

[00110] 10 μg of each construct was run on a 4-20% pre-cast gradient gel. Once run, gels were fixed for 30 minutes in stain buffer (10% (v/v) acetic acid, 50% (v/v) methanol and 40% (v/v) dH_2O) and then stained for 2 hours in Coomassie blue R-250 (0.25% in stain buffer), followed by de-staining for 2 to 3 hours in stain buffer with several changes of buffer as required. Gels were stored in 7% (v/v) acetic acid before documentation.

[00111] Results are shown in Figure 5A. This shows that the proteins have been made and have very high purity, along with the correct molecular mass predicted by their sequence.

B. Figure 5B

[00112] Additional constructs were evaluated in Figure 5B. The method used for Figure 5A was used for 5B.

[00113] Constructs 6245 (the bispecific construct not requiring pairing), 6248 and 6248 (pairable constructs with an inert binding partner and an MMP2 cleavage site) were produced adequately. Constructs 6246 and 6247 (not containing an inert binding partner) were produced at low yields and are believed to be unstable. It is likely that the VH/VL pairing is important for fragment stability.

[00114] Thus, we believe that the constructs mispaired with an inert binding partner are more stable and easier to manufacture.

C. Yield

[00115] The yield of the constructs assessed in Figure 5B was as follows:

Table 5: Yields

Construct	Yield (mg)
6245	13.29
6246	0.57
6247	3.24
6248	17.54
6249	43.52

Example 3. Evaluation of IFNγ Expression in T-Cells Cells Mixed with Tumor Cell Lines and Treated with Various Constructs

[00116] Preparations of single constructs and mixed constructs were tested for their IFNγ expression in order to test the ability of the complementary constructs in a two-component system to elicit a T-cell response.

[00117] **Background of IFNγ Assays Generally:** Expression of cytokine markers *in vitro*, such as IFNγ expression, is known to have a predictive value for T cell responses and, thus, predicts *in vivo* results. As described in Ghanekar et al., Clin Diag Lab Immunol j8(3):628-31 (2001), IFNγ expression in CD8+ T cells measured by cytokine flow cytometry (CFC) is a surrogate marker for the response of cytotoxic T lymphocytes. Ghanekar at 628. Prior work showed that there is a strong correlation between the expression of IFNγ by CD8+ T cells and the activity of CTL effector cells. Ghanekar at 630. Prior work shows that the use of data on IFNγ expression allows greater accuracy in assessing CD8+ T-cell responses in a clinical setting. *Id.* at 631. This demonstrates that the cytokine expression assays herein were known to have predictive value for *in vivo* and clinical responses. While the methods herein do not follow the exact method steps of Ghanekar because there are multiple ways to assess IFNγ expression, Ghanekar demonstrates that IFNγ expression is a proxy for T-cell activity.

[00118] **T Cell Line Culture:** Cytotoxic T cells were used in the IFNγ assays and cultured in RPMI-1640 medium containing 4.0 mM L-glutamine, 1% penicillin and streptomycin, 10% heat-inactivated FBS, 1% heat-inactivated human serum (pooled AB serum, TCS Bioscience) and 1,000 U/ml IL-2. Cells were kept at a density of 1-2 x 10^6 cells/ml, and were fed by replacement of three quarters of the medium every 48 hours. They were originally generated by adding 10ug HLA-A*0201-restricted viral peptide NLVPMVATV to 10 million peripheral blood mononuclear cells (PBMCs) from an HLA-A*0201+ donor. Cells were cultured in RPMI-1640 medium containing 4.0 mM L-glutamine, 1% penicillin and streptomycin, 10% heat-inactivated FBS and 1% heat-inactivated human serum (pooled AB serum, TCS Bioscience) for four days before the media was changed to include 1000 U/ml IL-2. The T cells were predominantly CD8+ T cells with a small amount of CD4+ T cells as well.

[00119] **Tumor Cell Line Culture:** The following cell lines were used: SW620, MCF-7, SNU398, and U266. Cells were cultured in DMEM containing 10% FBS,

and 1% penicillin/streptomycin solution except SNU398 and U266 cells which were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mM glutamine and 1% penicillin/streptomycin solution. SW620 cells are derived from a human colon cancer metastasis. MCF-7 cells are derived from a human breast cancer metastatic site (pleural effusion). SNU398 cells are derived from a human anaplastic hepatocellular carcinoma patient in 1990. U266 cells are derived from a human male multiple myeloma patient secreting IgE.

[00120] **Impact of Constructs on IFNγ Production**: Adherent cell lines were plated in a 96 well plate (100,000 cells per well) for at least 16 hours. Non-adherent cells (100,000 cells per well) were plated on the day of the experiment by centrifuging the culture at 400 x g for 5 minutes and resuspending the cells in T cell medium. 20,000 T cells per well in T cell medium were added. Constructs were made up in T cell medium and added to the cultures. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures. The final volume in the culture was 200 µl per well. Cultures were incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The cultures were centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00121] **IFNγ ELISA**: IFNγ levels in tissue culture supernatants were assayed using either an eBioscience Ready-Set-Go ELISA kit (cat. no. 88-7316-88) or a BioLegend Human IFNγ ELISA Max kit (cat. no. 430106) as per the manufacturer's instructions.

A. Figure 6

[00122] IFNγ was evaluated for various single constructs and mixed constructs. The IFNγ production and ELISA assay protocols provided above were used, except as noted. SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 µg/ml) were made up in T cell medium and added to the culture. Controls were PHA-M (final concentration 10 µg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 µl per well. The culture was incubated for 24 hours at 37°C, 5% CO2 and 100% relative humidity. The culture was centrifuged at 400

x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00123] IFNγ was evaluated for various single constructs and mixed constructs. Single constructs were assessed, with construct 6425 (a bispecific scFv for EpCAM and CD3E) was serving as a positive control. Baseline IFNγ was assessed in T-cells with SW620 cancer cells, SW620 cancer cells alone, and T-cells stimulated nonspecifically with phytohemagglutinin (PHA) to show the capacity of T-cells for IFNγ expression.

[00124] In SW620 tumor cells, constructs were used at a final concentration of 1 μg/ml. Cultures were incubated for 4 hours and the supernatants were assayed for IFNγ. Mean ± standard deviation of triplicates are provided. was evaluated for various single constructs and mixed constructs. Cultures were incubated for 4 hours and the supernatants were assayed for IFNγ. Mean ± standard deviation of triplicates are provided. Constructs were used at a final concentration of 1 μg/ml. Cultures were incubated for 24 hours and the supernatants were assayed for IFNγ. Mean ± standard deviation of triplicates are provided.

[00125] Construct 6245 serves as a positive control because this construct has both a targeting anti-EpCAM scFv and an anti-CD3E scFv; thus, it is a bispecific construct not requiring pairing for T-cell engaging (TCE) activity.

[00126] Constructs 6248 and 6249 (pairs of a two-component system each having an inert binding partner separated from the anti-CD3E T-cell engaging VL or VH, respectively, by a linker with an MMP2 cleavage site) showed more IFNγ expression when paired together than when administered alone. The combination of 6246 and 6247 (pairs of a two-component system without any mispairing to an inert binding partner or protease cleavage site required for them to associate) yield a much lower response than the combination of 6248 and 6249 likely because the 6246 and 6247 are not protected during manufacturing by the inert binding partner, which is believed to stabilize the unpaired anti-CD3E VH and VL domains in each construct, respectively. Thus, we believe that the mispaired constructs having an inert binding partner are more stable and easier to manufacture than precleaved constructs having an unpaired anti-CD3E VH or VL domain.

B. Figure 7

[00127] IFNγ was evaluated for various single constructs and mixed constructs. SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well)

on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 μg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures (final concentration of constructs was 1 μg/ml per construct). Controls were PHA-M (final concentration 10 μg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ. Mean ± standard deviation of triplicates are provided.

[00128] Construct 6245 serves as a positive control because this construct has both a targeting anti-EpCAM scFv and an anti-CD3E scFv; thus, it is a bispecific construct not requiring pairing for T-cell engaging (TCE) activity.

[00129] Constructs 6248 and 6249 (pairs of a two-component system each having an inert binding partner separated from the anti-CD3E T-cell engaging VL or VH, respectively, by a linker with an MMP2 cleavage site) showed more IFNγ expression when paired together than when administered alone. The combination of 6246 and 6247 (pairs of a two-component system without any binding domain or protease cleavage site required for them to associate) yield a much lower response than the combination of 6248 and 6249 likely because the 6246 and 6247 are not protected during manufacturing by the inert binding partner, which is believed to stabilize the unpaired anti-CD3E VH and VL domains in each construct, respectively. Thus, we believe that the mispaired constructs having an inert binding partner are more stable and easier to manufacture than constructs with an unpaired anti-CD3E VH or VL domain.

C. Figure 8

[00130] SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration ranging from 1 ng/ml to 1 μg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before

addition to the cultures (final concentration of constructs ranged from 1 ng/ml to 1 μg/ml per construct). Controls were SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ. Mean ± standard deviation of triplicates were shown in Figure 8.

[00131] Construct 6245 served as a positive control and paired constructs 6248 and 6249 were assessed. Both the control construct and the paired two-component system showed IFNγ expression. This demonstrates that the inert VL and VH domains are being cleaved from constructs 6248 and 6249, respectively, and that these two constructs are pairing to create a complete anti-CD3E scFv, which is capable of engaging T cells.

[00132] The two-component system (6248 and 6249) has a lower potency than the bispecific 6245 construct, yet is still in an acceptable range and may actually offer dosing advantages in avoiding side effects.

D. Figures 9A-B

[00133] SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration ranging from 1 ng/ml to 1 μg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures (final concentration of constructs ranged from 10 ng/ml to 10 μg/ml per construct). Controls were PHA-M (final concentration 10 μg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -200°C until analyzed for IFNγ. Mean ± standard deviation of triplicates were shown in Figures 9A-B.

[00134] Both the control construct and the paired two-component system showed IFNγ expression. This demonstrates that the inert VL and VH domains are being

cleaved from constructs 6248 and 6249, respectively, and that these two constructs are pairing to create a complete anti-CD3E scFv, which is capable of engaging T cells.

[00135] The two-component system (6248 and 6249) has a lower potency than the bispecific 6245 construct, yet is still in an acceptable range and may actually offer dosing advantages in avoiding side effects.

E. Figure 10

[00136] SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 μg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures (final concentration of constructs was 1 μg/ml per construct). Controls were PHA-M (final concentration 10 μg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ. Figure 10 provides mean ± standard deviation of triplicates.

[00137] Figure 10 shows a very low level of IFNγ expression for constructs with only a VH or VL for the anti-CD3E scFv; however, positive bispecific constructs with a full scFv (9332 and 9333) showed higher IFNγ expression levels.

F. Stoichiometric Assessment of Complementary Constructs of a Two-Component System (Figure 11)

[00138] Complementary constructs of a two-component system (6248 and 6249) were added together in varying ratios, as shown in Figure 11.

[00139] SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs were pre-mixed in the specified ratios in T cell medium and added to the culture (final concentration of constructs was 1 μg/ml in total). The two components 6248 and 6249, one containing

the VH domain of the CD3 activating moiety (6249) and the other containing the VL domain of the CD3 activating moiety (6248), were pre-mixed at ratios 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100. The mixtures of the two components were added to 100,000 unwanted tumor cells and 20,000 T cells. Controls were PHA-M (final concentration 10 μg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00140] The results in Figure 11 demonstrate an increasing activation of T cells as the ratio of the two components reaches equilibrium. When there is an excess of either the component containing the VH domain of the CD3 activating moiety (6249) or the other component containing the VL domain of the CD3 activating moiety (6248), the activation of T cells is decreased as the activation is reliant on both the VH and VL of the CD3 activating moiety coming together. Therefore, IFNγ expression levels were much lower when all or nearly all of the constructs provided were of one type or the other. The highest IFNγ expression level corresponds to the scenario where an equal amount of each of the two complementary constructs were provided. This provides further evidence demonstrating that the IFNγ expression is caused by the two halves of the anti-CD3E scFv coming together from the two constructs in the two-component system.

G. Use of MCF-7 Cells (Figure 12)

[00141] Figure 12 shows experiments conducted in the MCF-7 tumor cell line. MCF-7 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 μg/ml) were made up in T cell medium and added to the culture. Controls were PHA-M (final concentration 10 μg/ml), MCF-7 cells plus T cells with no additions, and MCF-7 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the

supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00142] Similar results were achieved to the other cell lines used. In Figure 12, positive control constructs 6245, 9332, and 9333 (each having a full anti-CD3E scFv) showed much higher IFNγ expression levels than any of the single components comprising only a VH or VL domain from the anti-CD3E antibody. Figure 12 also provides baseline IFNγ expression levels for MCF-7 cells alone or T-cells stimulated nonspecifically with PHA.

H. Figures 13-14

[00143] SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 μg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures (final concentration of constructs was 1 μg/ml per construct). Controls were PHA-M (final concentration 10 μg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 μl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00144] In Figure 13, the data show that the two-component system of 6248 and 6249 functions as expected because these constructs have an inert binding partner that can be cleaved by an MMP2 cleavage site and pairable anti-CD3E variable domains (one VH in 6248 and one VL in 6249). Constructs 9327 and 9328 do not generate a strong IFNγ signal because neither of these constructs has a cleavage site between the inert binding partner and the anti-CD3E variable domain.

[00145] Constructs 9327 and 6248 do not show any activity because they both have VH domains for the anti-CD3E antibody and cannot make a functional anti-CD3E scFv; additionally, 9327 does not have a cleavage site. 9327 and 6249 show a very low level

of activity because 9327 has no cleavage site and 6249 has a cleavage site, but the two together can make an anti-CD3E scFv if some low level of spontaneous cleavage occurs.

[00146] In Figure 14, the pairing of constructs 9334 and 9335 (providing biparatopic approach to targeting EFGR, with targeting antibody scFvs to different epitopes on EGFR) did not create an IFNγ signal. It is believed that either the epitopes on EGFR were too far apart for the two anti-CD3E variable domains to reach each other or the epitopes were too close and creating steric hindrance for binding on the antibody side. It is, however, very reasonable to test biparatopic combinations. Another antibody for EGFR can be identified and tested for combinations in this approach.

[00147] Figure 14 also shows targeting two different proteins expressed on the same cancer cell. Construct 6248 binds EpCAM and is successfully paired with 9335, which binds EGFR. Construct 6249 also binds EpCAM and is successfully paired with 9334. This establishes that different molecules on a cancer cell may be targeted, providing yet a further layer of specificity for some embodiments of the two-component system described herein. This also provides further evidence that components 9335 and 9334 work in other contexts and further suggests these components were either too close or too far from each other in their combination with each other described above.

[00148] The combination of 9334 and 6249 provides useful information in this figure, demonstrating that dual targeting can be achieved because construct 9334 targets EGFR and 9249 targets EpCAM.

[00149] The combination of 9334 and 6248 was not expected to have activity because both constructs comprise a VH from the anti-CD3E antibody and neither construct comprises a VL from that antibody.

I. Figure 15

[00150] SNU398 cells were cultured in RPMI1640 containing 10% FBS, 2 mM glutamine and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 μg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures (final concentration of constructs was 1 μg/ml per construct). Controls were PHA-M (final concentration 10 μg/ml), SNU398 cells plus T cells with no

additions, and SNU398 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 µl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00151] Figure 15 shows that adding a protease inhibitor reduces the IFNγ expression of the two-component system having a protease cleavage site (constructs 6248 and 6249). The protease inhibitor does not impact the 6245 bispecific construct as cleavage and pairing are not required for activity.

J. Figure 16

[00152] SW620 cells were cultured in DMEM containing 10% FBS, and 1% penicillin/streptomycin solution. Cells were plated in a 96 well plate (100,000 cells per well) on the day prior to the experiment. On the day of the experiment the medium was aspirated and discarded. 20,000 T cells per well in T cell medium were added. Constructs (final concentration of 1 µg/ml) were made up in T cell medium and added to the culture. Where mixtures of constructs were used, these were pre-mixed before addition to the cultures (final concentration of constructs was 1 µg/ml per construct). Controls were PHA-M (final concentration 10 µg/ml), SW620 cells plus T cells with no additions, and SW620 cells without T cells or other additions. Each condition was run in triplicate. The final volume in the culture was 200 µl per well. The culture was incubated for 24 hours at 37°C, 5% CO_2 and 100% relative humidity. The culture was centrifuged at 400 x g for 5 minutes and the supernatants aspirated and placed in a separate plate. Supernatants were stored at -20°C until analyzed for IFNγ.

[00153] The functional combination of 9335 and 6248 shows that different kinds of antibody fragments may be combined in a first component and second component, respectively. 9335 employs an anti-EGFR VHH as the targeting moiety and 6248 employs an anti-EPCAM scFv as the targeting moiety.

Example 4. *In Vivo* Targeting of B Cell Lymphoma Using a Two-Component System

[00154] A two-component system comprising a first component and a second component are administered to a patient having lymphoma. The first component comprises Rituximab or an anti-CD22 antibody as a targeting moiety, a VH domain from an antibody

binding CD3 as a T-cell engaging domain, a VL domain as an inert binding partner, and the ADAM28 cleavage site KPAKFFRL. The second component also comprises Rituximab or an anti-CD22 antibody as a targeting moiety, the complementary VL domain from an antibody binding CD3 as a T-cell engaging domain, VH as an inert binding partner, and the ADAM28 cleavage site KPAKFFRL. The VH domain from an antibody binding CD3 as a T-cell engaging domain of the first component and the VL domain from an antibody binding CD3 as a T-cell engaging domain of the second component are capable of binding to each other when not bound to an inert binding partner and possessing the activity to engage a T-cell.

[00155] The patient is infused with the agent, which targets all B cells, healthy and malignant. Upon binding malignant cells, the agent comes into contact with proteases whereby cleavage of the protease recognition domain releases the inert binding partners from both the first and the second T-cell engaging domains.

[00156] The malignant B cells that are bound by the now-activated two-component system complex attracts the host immune system for cytolysis by T-cells due to the presence and activity of the complex of the first and second T-cell engaging domains.

Example 5. Specific Embodiments of Two-Component Systems

[00157] A two-component system chosen from System A-E is prepared according to Table 3 and administered to a patient having cancer. If an item is described as optional, the row of the table describes both two-component systems having or not having that item.

Table 6: Certain Embodiments of the Two-Component System					
A	First Component				
	Targeting Moiety	T-Cell Engaging Moiety	Cleavage Site	Inert binding partner	Optional Linker(s) & Location(s)
	Antibody targeting HER2	V_H of antibody targeting CD3	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
	Second Component				
	Optional Targeting Moiety	T-Cell Engaging Moiety	Optional Cleavage Site	Optional Inert binding partner	Optional Linker(s) & Location(s)
	Antibody targeting HER2	V_L of antibody targeting CD3	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
B	First Component				
	Targeting Moiety	T-Cell Engaging Moiety	Cleavage Site	Inert binding partner	Optional Linker(s) & Location(s)

	antibody targeting EGFR, such as Cetuximab	V_H of antibody targeting CD4	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
	Second Component				
	Optional Targeting Moiety	T-Cell Engaging Moiety	Optional Cleavage Site	Optional Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting EGFR, such as Cetuximab	V_L of antibody targeting CD4	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
C	First Component				
	Targeting Moiety	T-Cell Engaging Moiety	Cleavage Site	Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting CD20, such as Rituximab	V_H of antibody targeting CD8	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).

	Second Component				
	Optional Targeting Moiety	T-Cell Engaging Moiety	Optional Cleavage Site	Optional Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting CD20, such as Rituximab	V_L of antibody targeting CD8	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
D	First Component				
	Targeting Moiety	T-Cell Engaging Moiety	Cleavage Site	Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting CD22, such as Inotuzumab	V_H of antibody targeting CD28	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
	Second Component				
	Optional Targeting Moiety	T-Cell Engaging Moiety	Optional Cleavage Site	Optional Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting CD22, such as Inotuzumab	V_L of antibody targeting CD28	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting

				domain without creating any binding specificity	moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
E	First Component				
	Targeting Moiety	T-Cell Engaging Moiety	Cleavage Site	Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting CD33, such as Gemtuzumab	V_H of antibody targeting T cell receptor (TCR)	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).
	Second Component				
	Optional Targeting Moiety	T-Cell Engaging Moiety	Optional Cleavage Site	Optional Inert binding partner	Optional Linker(s) & Location(s)
	antibody targeting CD33, such as Gemtuzumab	V_L of antibody targeting T cell receptor (TCR)	Any ADAM28 cleavage site	Any VH domain that binds to the VL domain of the T-cell engaging domain without creating any binding specificity	For example, GGGGS (SEQ ID No:45). Located between the V_H and V_L of the targeting moiety, between the targeting moiety and the inactive T-cell engaging domain, and/or between the V_H and V_L of the inactive T-cell engaging domain (See Figure 1).

Example 6. Embodiments

[00158] The following numbered items provide embodiments as described herein, though the embodiments recited here are not limiting.

[00159] Item 1. A two-component system for treating a condition characterized by the presence of unwanted cells comprising:

a. a first component comprising a targeted T-cell engaging agent comprising:

i. a first targeting moiety that is capable of targeting the unwanted cells;

ii. a first T-cell engaging domain capable of T-cell engaging activity when binding a second T-cell engaging domain, wherein the second T-cell engaging domain is not part of the first component;

iii. a first inert binding partner for the first T-cell engaging domain binding to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed; and

iv. a cleavage site separating the first T-cell engaging domain and the first inert binding partner, wherein the cleavage site is:

(1) cleaved by an enzyme expressed by the unwanted cells;

(2) cleaved through a pH-sensitive cleavage reaction inside the unwanted cell;

(3) cleaved by a complement-dependent cleavage reaction; or

(4) cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent,

b. a second component comprising a second T-cell engaging domain capable of T-cell engaging activity when binding the first T-cell engaging domain,

wherein the first and second T-cell engaging domains are capable of binding when neither is bound to an inert binding partner.

[00160] Item 2. The two-component system of item 1, wherein the second component further comprises a second targeting moiety that is capable of targeting the unwanted cells.

[00161] Item 3. The two-component system of any one of items 1-2, wherein the second component further comprises a second inert binding partner for the second T-cell engaging domain binding to the second T-cell engaging domain such that the second T cell engaging domain does not bind to the first T-cell engaging domain unless the inert binding partner is removed and

a. a cleavage site separating the second T-cell engaging domain and the second inert binding partner, wherein the cleavage site is:

i. cleaved by an enzyme expressed by the unwanted cells;

ii. cleaved through a pH-sensitive cleavage reaction inside the unwanted cell;

iii. cleaved by a complement-dependent cleavage reaction; or

iv. cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent,

wherein cleavage of the cleavage site causes loss of the inert binding partner and complementation with the first T-cell engaging domain of the two-component system.

[00162] Item 4. The two-component system of any one of items 1-3, wherein the first and the second targeting moieties are the same.

[00163] Item 5. The two-component system of any one of items 1-3, wherein the first and the second targeting moieties are different.

[00164] Item 6. The two-component system of any one of items 1-5, wherein the first and second cleavage site are the same.

[00165] Item 7. The two-component system of any one of items 1-5, wherein the first and second cleavage site are different.

[00166] Item 8. The two-component system of any one of items 1-7, wherein at least one cleavage site is a protease cleavage site.

[00167] Item 9. The two-component system of any one of items 1-8, wherein at least one cleavage site is capable of being cleaved outside the unwanted cells.

[00168] Item 10. The two-component system of any one of items 1-9, wherein at least one enzyme expressed by the unwanted cells is a protease.

[00169] Item 11. The two-component system of any one of items 1-10, wherein at least one inert binding partner specifically binds the T-cell engaging domain.

[00170] Item 12. The two-component system of any one of items 1-11, wherein at least one inert binding partner is a VH or VL domain.

[00171] Item 13. The two-component system of any one of items 1-12, wherein

a. when the T-cell engaging domain is a VH domain, the inert binding partner is a VL domain and

b. when the T-cell engaging domain is VL domain, the inert binding partner is a VH domain.

[00172] Item 14. The two-component system of any one of items 1-13, wherein at least one targeting moiety is an antibody or functional fragment thereof.

[00173] Item 15. The two-component system of any one of items 1-14, wherein the at least one inert binding partner is capable of dissociation once at least one cleavage site has been cleaved and after dissociation the two T-cell engaging domains are capable of binding to each other and exhibiting T-cell engaging activity.

[00174] Item 16. The two-component system of item 1-15, wherein one T-cell engaging domain is a VH domain and the other T-cell engaging domain is a VL domain.

[00175] Item 17. A component for use in a two-component system for treating a condition characterized by the presence of unwanted cells comprising a first targeted T-cell engaging agent comprising:

a. a targeting moiety that is capable of targeting the unwanted cells;

b. a first T-cell engaging domain capable of T-cell engaging activity when binding a second T-cell engaging domain, wherein the second T-cell engaging domain is not part of the first targeted T-cell engaging agent;

c. an inert binding partner for the first T-cell engaging domain binding to the first T-cell engaging domain such that the first T-cell engaging domain does not bind to the second T-cell engaging domain unless the inert binding partner is removed; and

d. a cleavage site separating the first T-cell engaging domain and the inert binding partner, wherein the cleavage site is:

i. cleaved by an enzyme expressed by the unwanted cells;

ii. cleaved through a pH-sensitive cleavage reaction inside the unwanted cell;

iii. cleaved by a complement-dependent cleavage reaction; or

iv. cleaved by a protease that is colocalized to the unwanted cell by a targeting moiety that is the same or different from the targeting moiety in the agent,

wherein cleavage of the cleavage site causes loss of the inert binding partner and allows for complementation with the second T-cell engaging domain that is not part of the agent.

[00176] Item 18. The component for use in a two-component system of item 17, wherein the cleavage site is a protease cleavage site.

[00177] Item 19. The component for use in a two-component system of any one of items 17-18, wherein the cleavage site is capable of being cleaved outside the unwanted cells.

[00178] Item 20. The component for use in a two-component system of any one of items 17-19, wherein the enzyme expressed by the unwanted cells is a protease.

[00179] Item 21. The component for use in a two-component system of any one of items 17-20, wherein at least one inert binding partner specifically binds the T-cell engaging domain.

[00180] Item 22. The component for use in a two-component system of any one of items 17-21, wherein the inert binding partner is a VH or VL domain.

[00181] Item 23. The component for use in a two-component system of any one of items 17-22, wherein

a. when the T-cell engaging domain is a VH domain, the inert binding partner is a VL domain and

b. when the T-cell engaging domain is VL domain, the inert binding partner is a VH domain.

[00182] Item 24. The component for use in a two-component system of any one of items 17-23, wherein the targeting moiety is an antibody or functional fragment thereof.

[00183] Item 25. A set of nucleic acid molecules encoding the first and second component of the two component system of any one of items 1-16.

[00184] Item 26. A nucleic acid molecule encoding the component for use in a two-component system of any one of items 17-24.

[00185] Item 27. A method of treating a disease in a patient characterized by the presence of unwanted cells comprising administering the two-component system of any one of items 1-16 to the patient.

[00186] Item 28. A method of targeting an immune response of a patient to unwanted cells comprising administering the two-component system of any one of items 1-16 to the patient.

[00187] Item 29. The method of any one of items 27-28, wherein the unwanted cells are cancer cells.

[00188] Item 30. The method of item 29, wherein the cancer 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.

EQUIVALENTS

[00189] 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.

[00190] 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.

[00191] In the specification and the claims the term "comprising" shall be understood to have a broad meaning similar to the term "including" and will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. This definition also applies to variations on the term "comprising" such as "comprise" and "comprises".

[00192] The reference to any prior art in this specification is not, and should not be taken as an acknowledgement or any form of suggestion that the referenced prior art forms part of the common general knowledge in Australia.

What is Claimed is:

1. A kit or composition when used for treating cancer in a patient comprising:

a. a first component comprising a targeted T-cell binding agent comprising:

i. a first targeting moiety that is an antibody or antigen binding fragment thereof that binds a tumor antigen expressed by the cancer;

ii. a first T-cell binding domain capable of T-cell binding activity when binding a second T-cell binding domain, wherein the second T-cell binding domain is not part of the first component, and wherein the first T-cell binding domain is either a VH domain or VL domain;

iii. a first inert binding partner for the first T-cell binding domain binding to the first T-cell binding domain such that the first T-cell binding domain does not bind to the second T-cell binding domain unless the inert binding partner is removed, wherein if the first T-cell binding domain is a VH domain, the inert binding partner is a VL domain and if the first T-cell binding domain is a VL domain, the inert binding partner is a VH domain; and

iv. a protease cleavage site separating the first T-cell binding domain and the first inert binding partner, wherein the protease cleavage site is capable of releasing the inert binding domain from the T-cell binding domain in the presence of a protease:

(1) expressed by the cancer;

(2) 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 the agent,

a second component comprising a second T-cell binding domain capable of T-cell binding activity when binding the first T-cell binding domain, wherein the first and second T-cell binding domains are capable of binding CD3 or the T cell receptor (TCR) when neither is bound to an inert binding partner, and further wherein if the first T-cell binding domain is a VH domain, the second T-cell binding domain is a VL domain and if the first T-cell binding domain is a VL domain, the second T-cell binding domain is a VH domain.

2. The kit or composition when used according to claim 1, wherein the second component further comprises a second targeting moiety that is an antibody or antigen binding fragment thereof that binds a tumor antigen expressed by the cancer.

3. The kit or composition when used according to claim 2, wherein the second component further comprises a second inert binding partner for the second T-cell binding domain binding to the second T-cell binding domain such that the second T cell binding domain does not bind to the first T-cell binding domain unless the inert binding partner is removed, wherein if the second T-cell binding domain is a VH domain, the second inert binding partner is a VL domain and if the second T-cell binding domain is a VL domain, the second inert binding partner is a VH domain and

a. a protease cleavage site separating the second T-cell binding domain and the second inert binding partner, wherein the protease cleavage site is

(i) cleaved by a protease expressed by the cancer

(ii) 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 the agent,

wherein cleavage of the protease cleavage site causes loss of the inert binding partner and complementation with the first T-cell binding domain of the kit or composition.

4. The kit or composition when used according to claim 3, wherein the first and the second targeting moieties are different.

5. The kit or composition when used according to claim 3, wherein the protease cleavage sites of the first component and second component are different.

6. The kit or composition when used according to claim 3, wherein the protease cleavage sites of the first component and second component are cleaved by a protease expressed by the cancer.

7. The kit or composition when used according to claim 3, wherein the protease cleavage sites of the first component and/or second component 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 the agent.

8. The kit or composition when used according to claim 3, wherein each inert binding partner is capable of dissociation once at least one protease cleavage site for each inert binding partner has been cleaved and after dissociation the two T-cell binding domains are capable of binding to each other and exhibiting T-cell binding activity.

9. A component for use in a kit or composition when used for treating cancer in a patient comprising a first targeted T-cell binding agent comprising:

a. a targeting moiety that is an antibody or antigen binding fragment thereof that binds a tumor antigen expressed by the cancer;

b. a first T-cell binding domain capable of T-cell binding activity when binding a second T-cell binding domain, wherein the second T-cell binding domain is another component of the kit or composition that is not part of the first targeted T-cell binding agent, and wherein the first T-cell binding domain is either a VH domain or VL domain and wherein the first T-cell binding domain and the second T-cell binding domain are capable of binding CD3 or TCR;

c. an inert binding partner for the first T-cell binding domain binding to the first T-cell binding domain such that the first T-cell binding domain does not bind to the second T-cell binding domain unless the inert binding partner is removed, wherein if the first T-cell binding domain is a VH domain, the inert binding partner is a VL domain and if the first T-cell binding domain is a VL domain, the inert binding partner is a VH domain; and

a protease cleavage site separating the first T-cell binding domain and the inert binding partner, wherein the cleavage site is 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 the agent,

wherein cleavage of the protease cleavage site causes loss of the inert binding partner and allows for complementation with the second T-cell binding domain that is not part of the agent, further wherein if the first T-cell binding domain is a VH domain, the second T-

cell binding domain is a VL domain and if the first T-cell binding domain is a VL domain, the second T-cell binding domain is a VH domain.

10. A set of nucleic acid molecules encoding the first and second components of the kit or composition of claim 1.
11. A nucleic acid molecule encoding the first targeted T-cell binding agent of claim 9.
12. A method of treating cancer expressing a tumor antigen that binds the first targeting moiety in a patient comprising administering the composition of claim 1 to the patient.
13. A method of treating cancer expressing a tumor antigen that binds the first targeting moiety in a patient comprising administering the composition of claim 3 to the patient.
14. The method of claim 13, wherein the cancer expressing a tumor antigen that binds the first 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.
15. A method of targeting T cells expressing CD3 or TCR to cancer expressing a tumor antigen that binds the first targeting moiety in a patient comprising administering the composition of claim 3 to the patient.
16. The kit or composition when used according to claim 1, wherein the first and second T-cell binding domains are capable of forming an scFv when not bound to an inert binding domain.

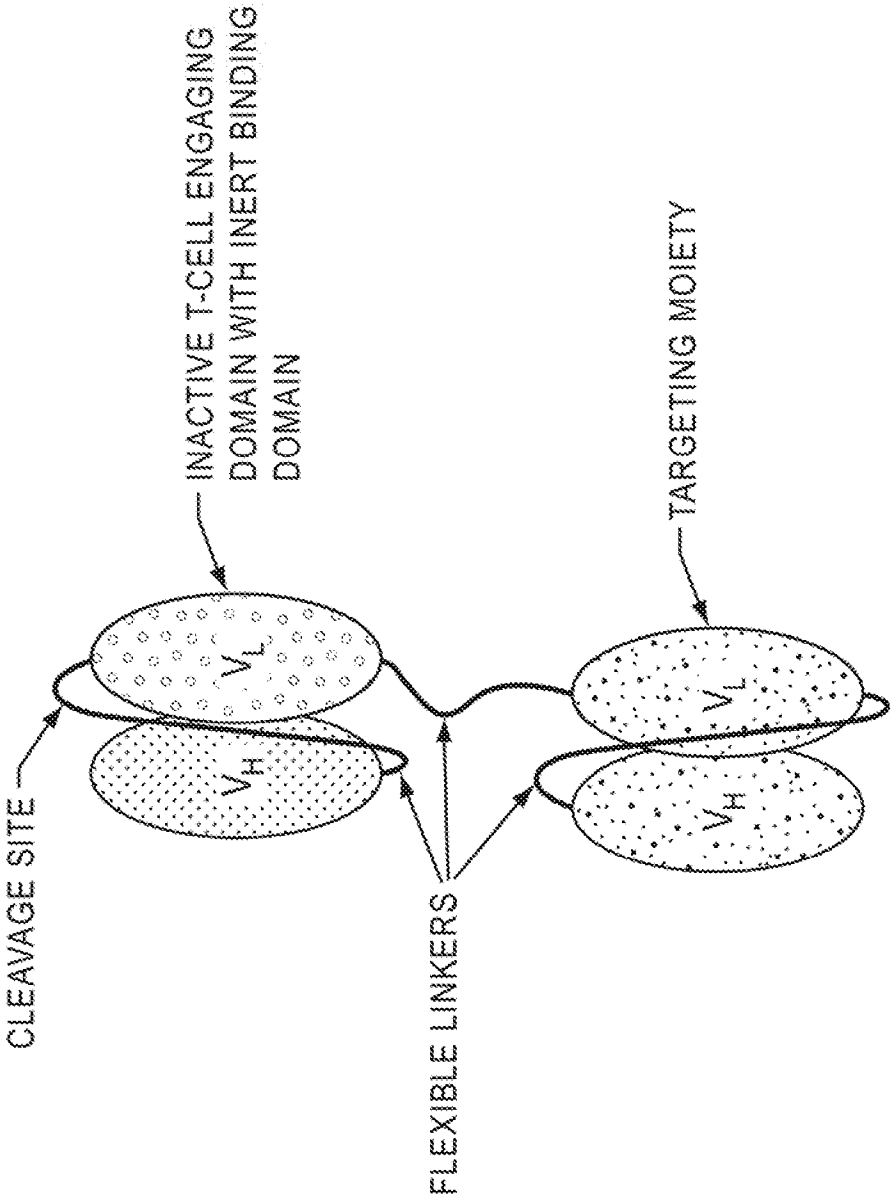

INACTIVE T-CELL ENGAGING DOMAIN WITH INERT BINDING DOMAIN
TARGETING MOIETY
V_L
V_H
V_L
V_H
CLEAVAGE SITE
FLEXIBLE LINKERS

FIGURE 1

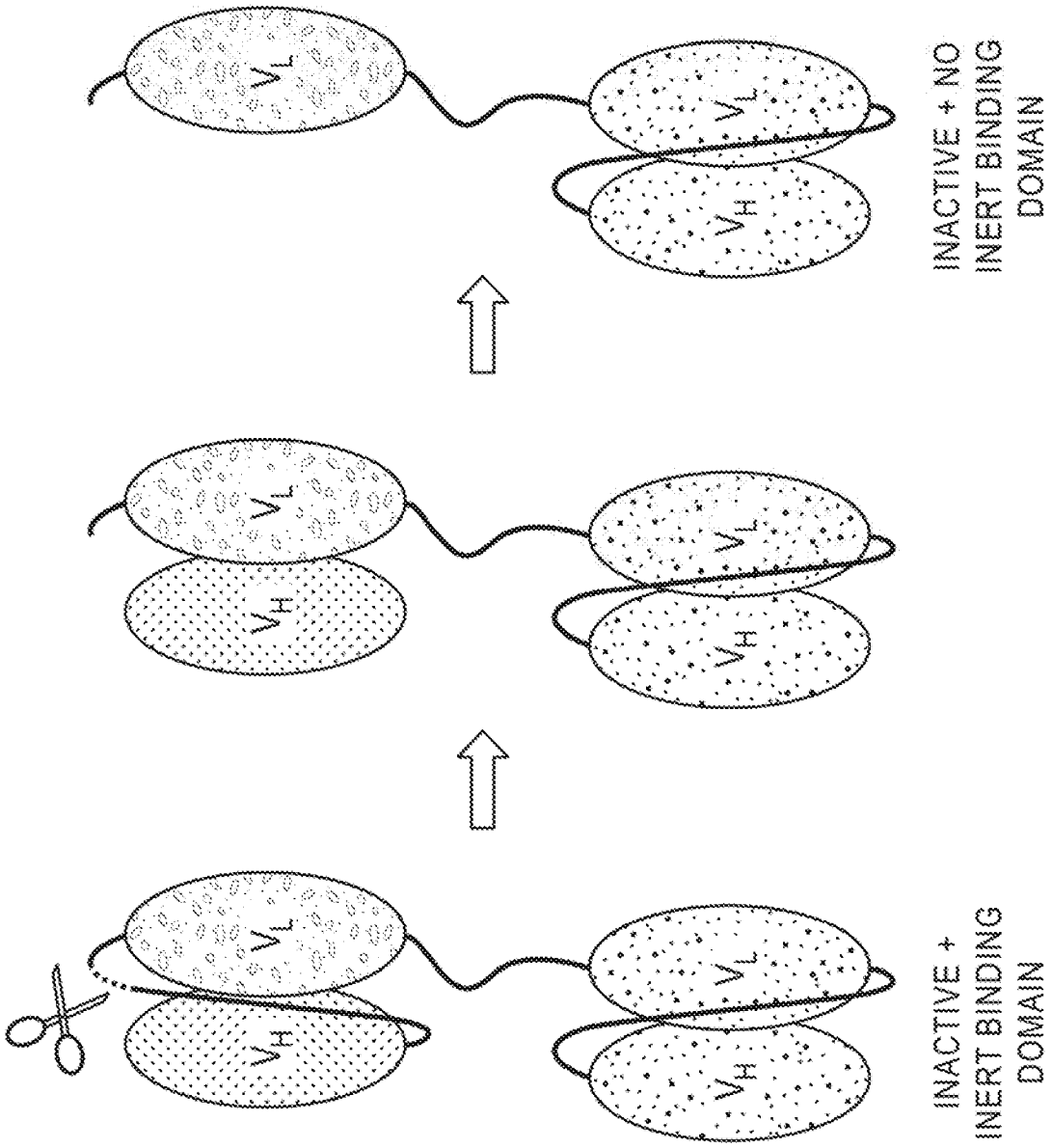

V_L
V_H
INACTIVE + NO INERT BINDING DOMAIN
INACTIVE + INERT BINDING DOMAIN

FIGURE 2

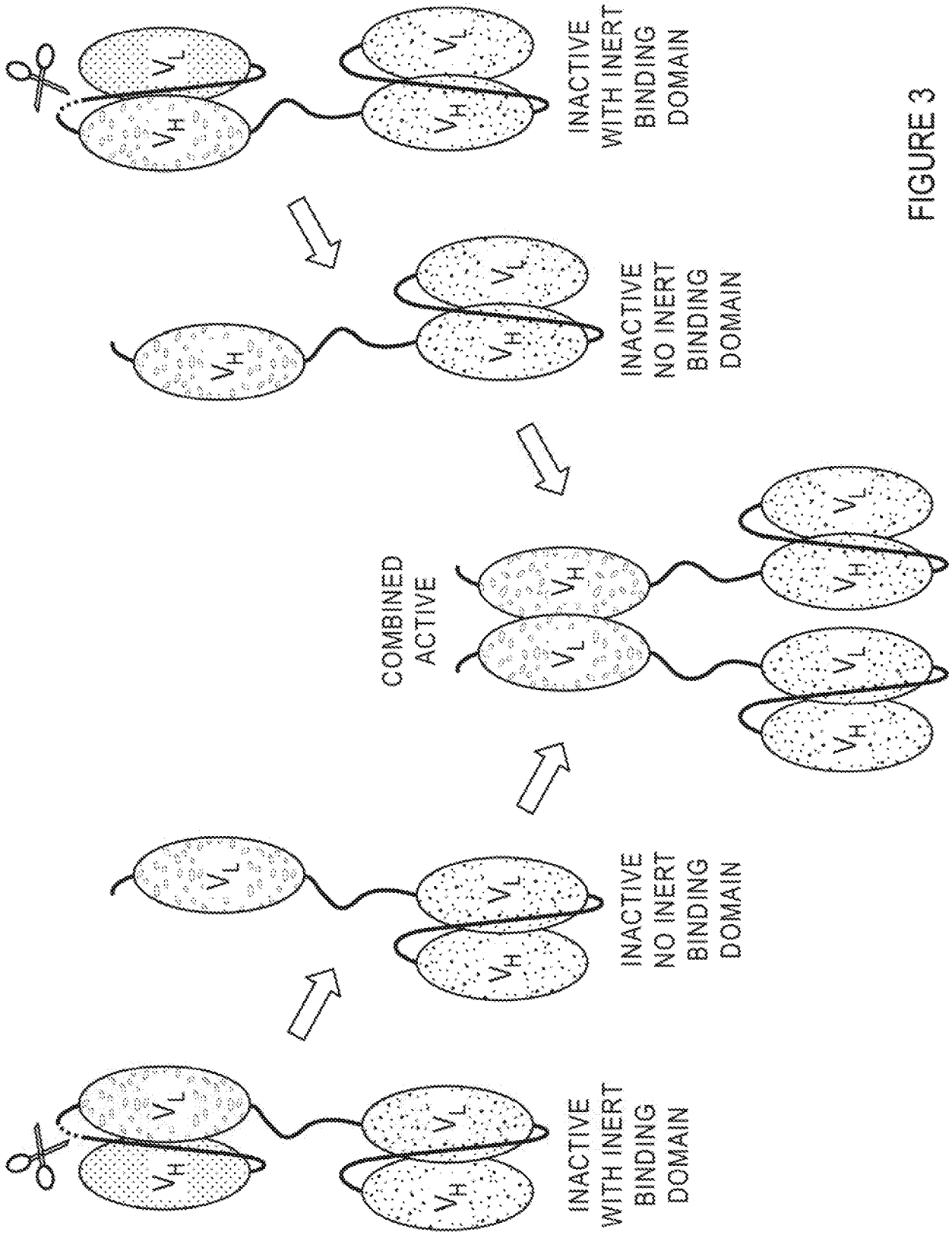

INACTIVE WITH INERT BINDING DOMAIN
INACTIVE NO INERT BINDING DOMAIN
COMBINED ACTIVE
INACTIVE NO INERT BINDING DOMAIN
INACTIVE WITH INERT BINDING DOMAIN

FIGURE 3

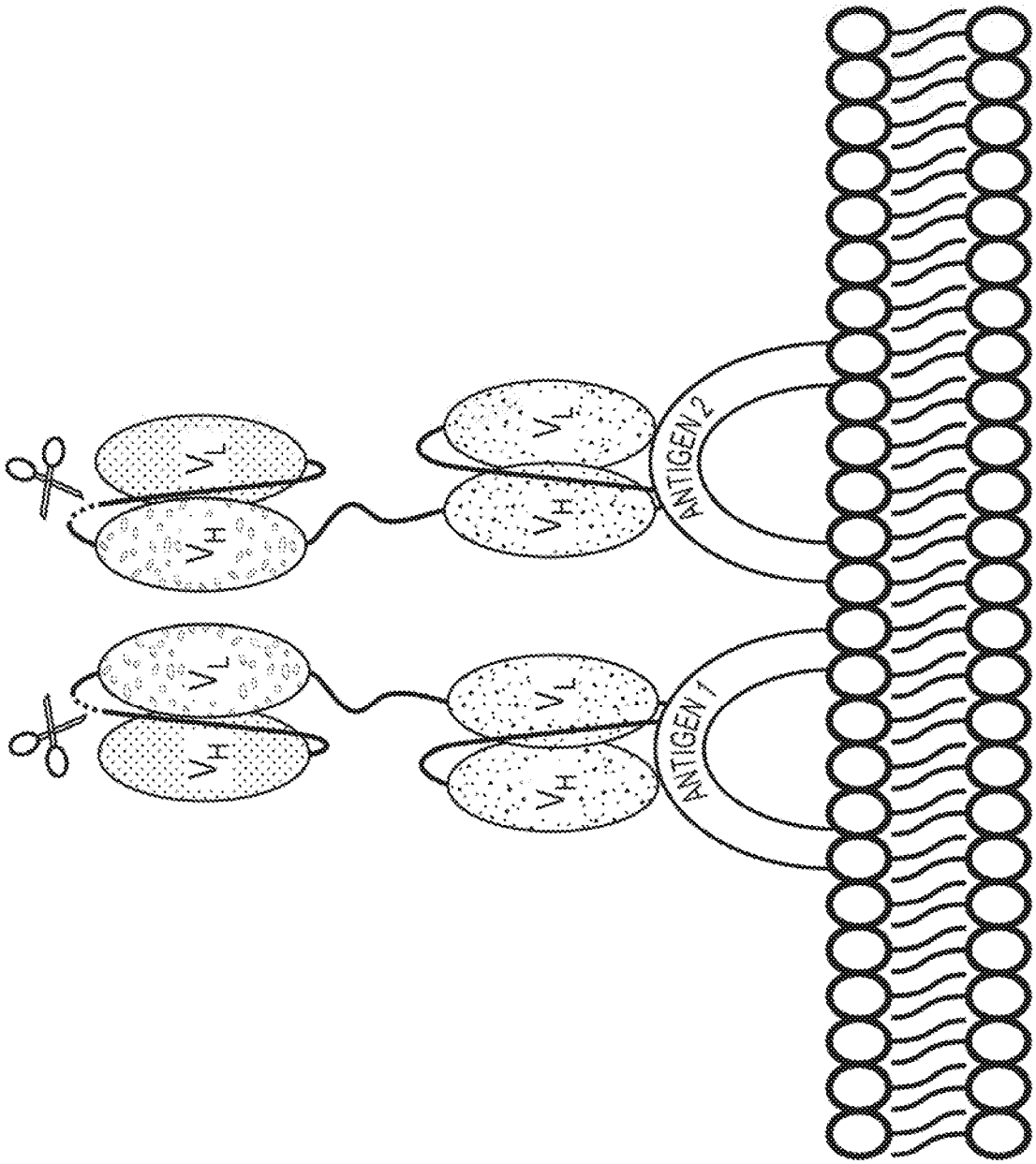


FIGURE 4A

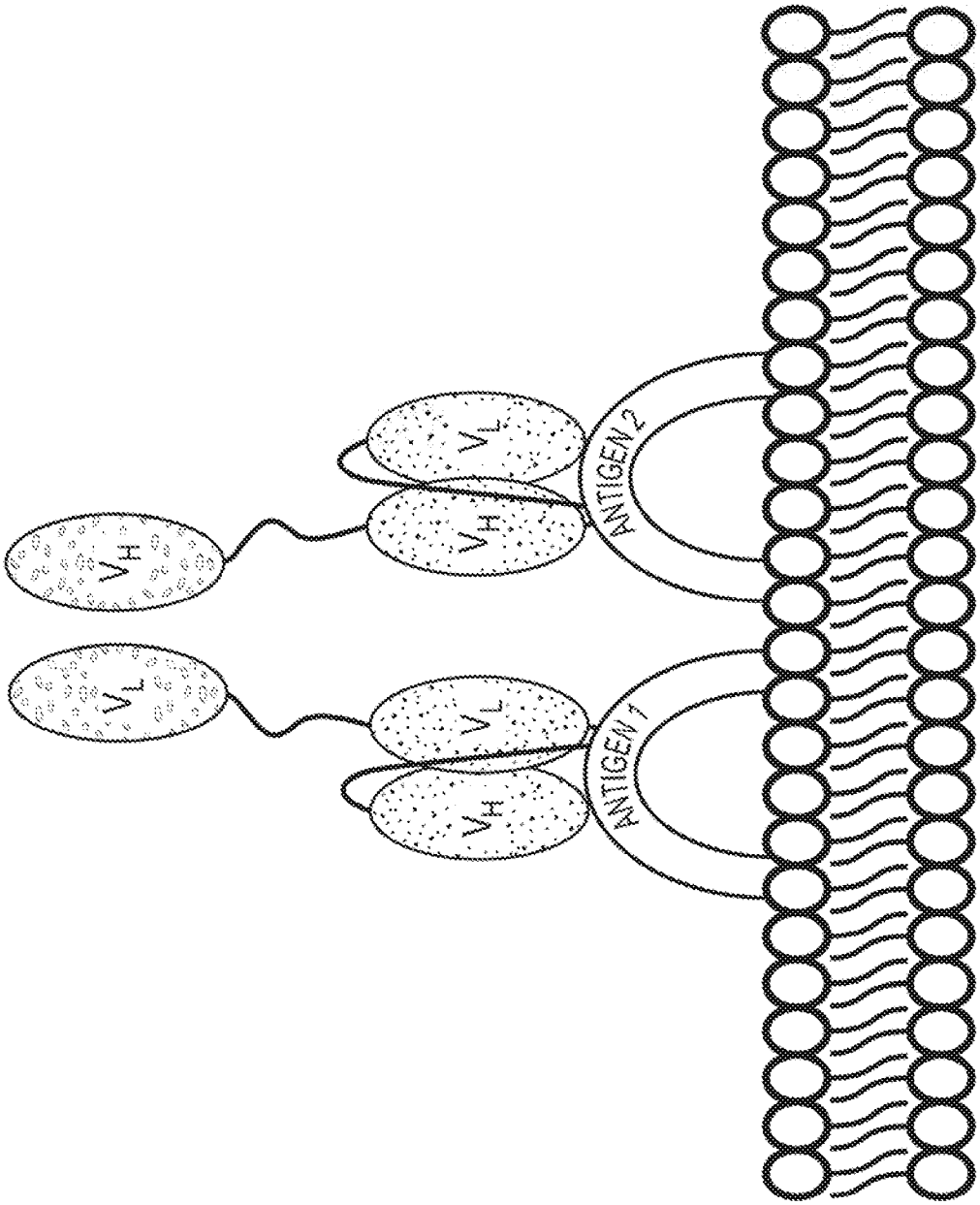


FIGURE 4B

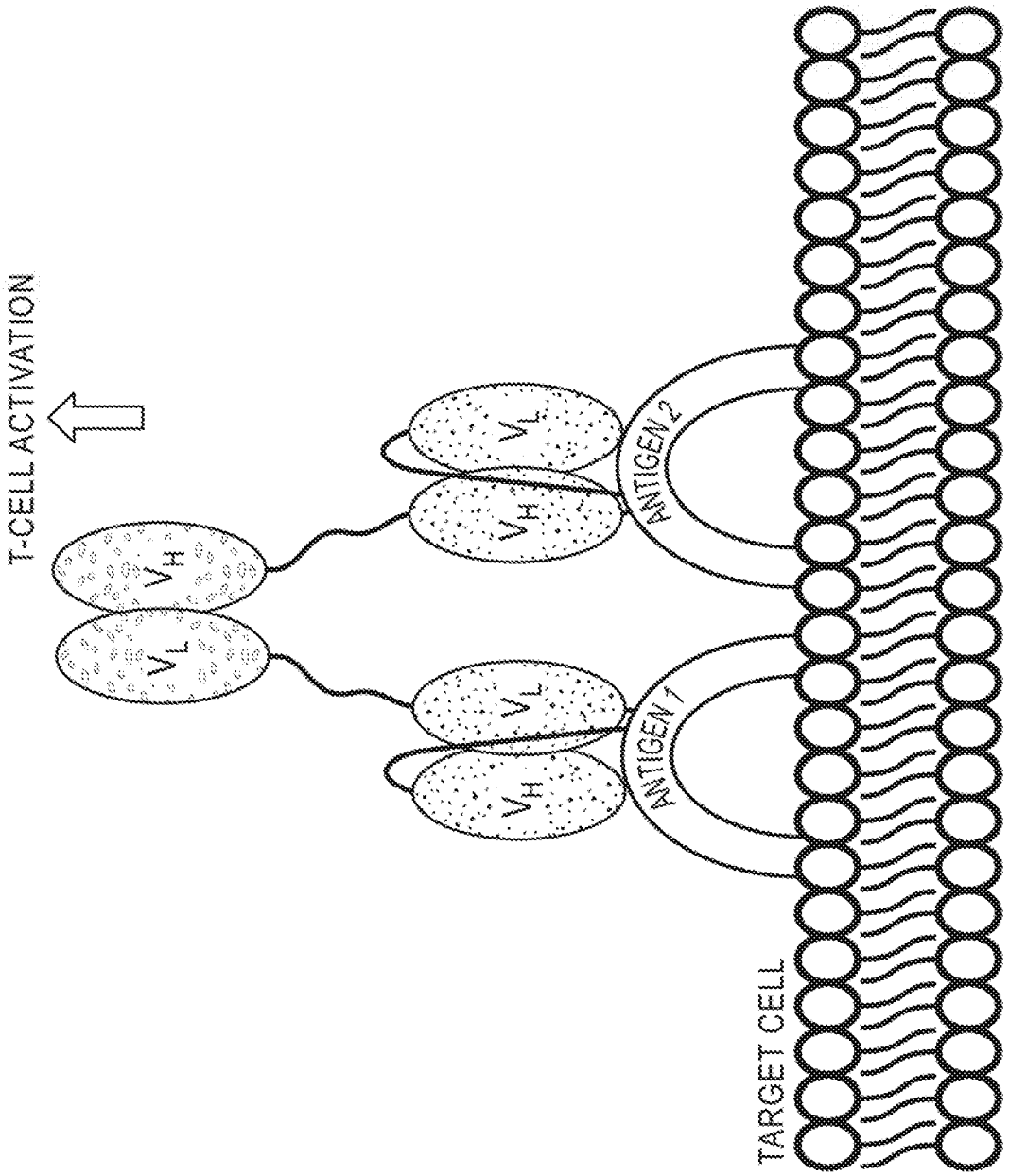

T-CELL ACTIVATION
V_H
V_L
V_L
V_H
ANTIGEN 2
V_L
V_H
ANTIGEN 1
TARGET CELL

FIGURE 4C

FIGURE 5A

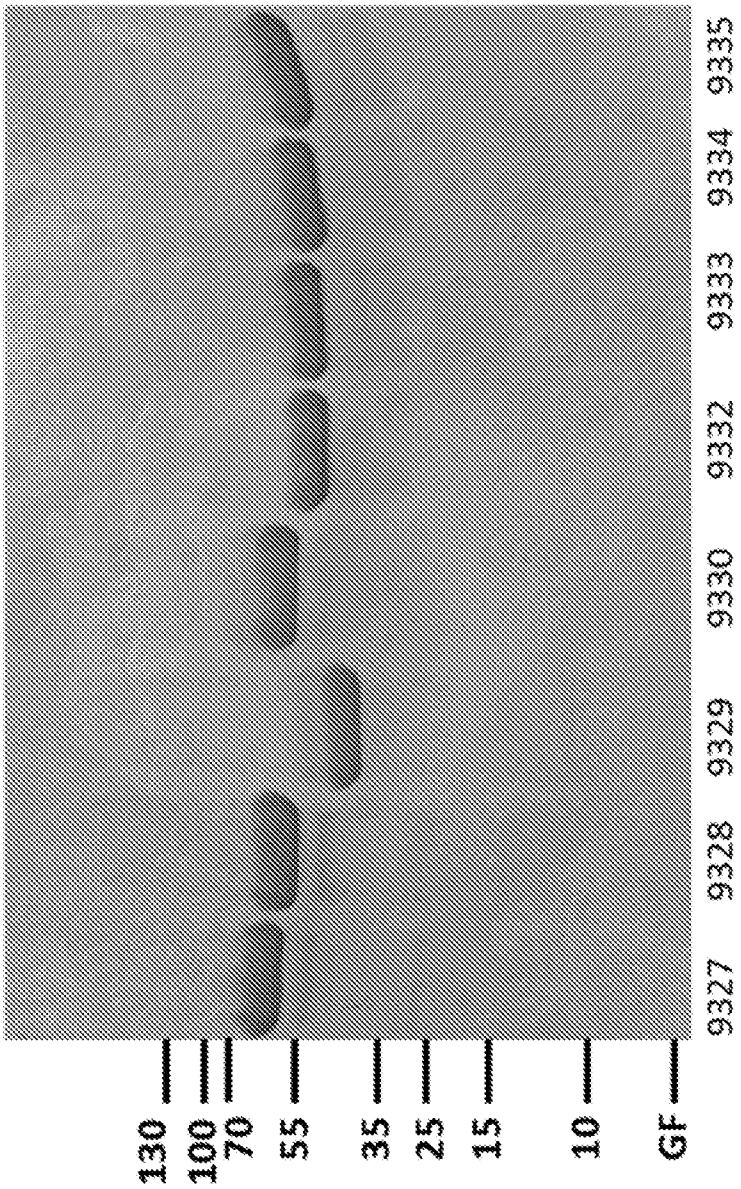

9327
9328
9329
9330
9332
9333
9334
9335
130
100
70
55
35
25
15
10
GF

FIGURE 5B

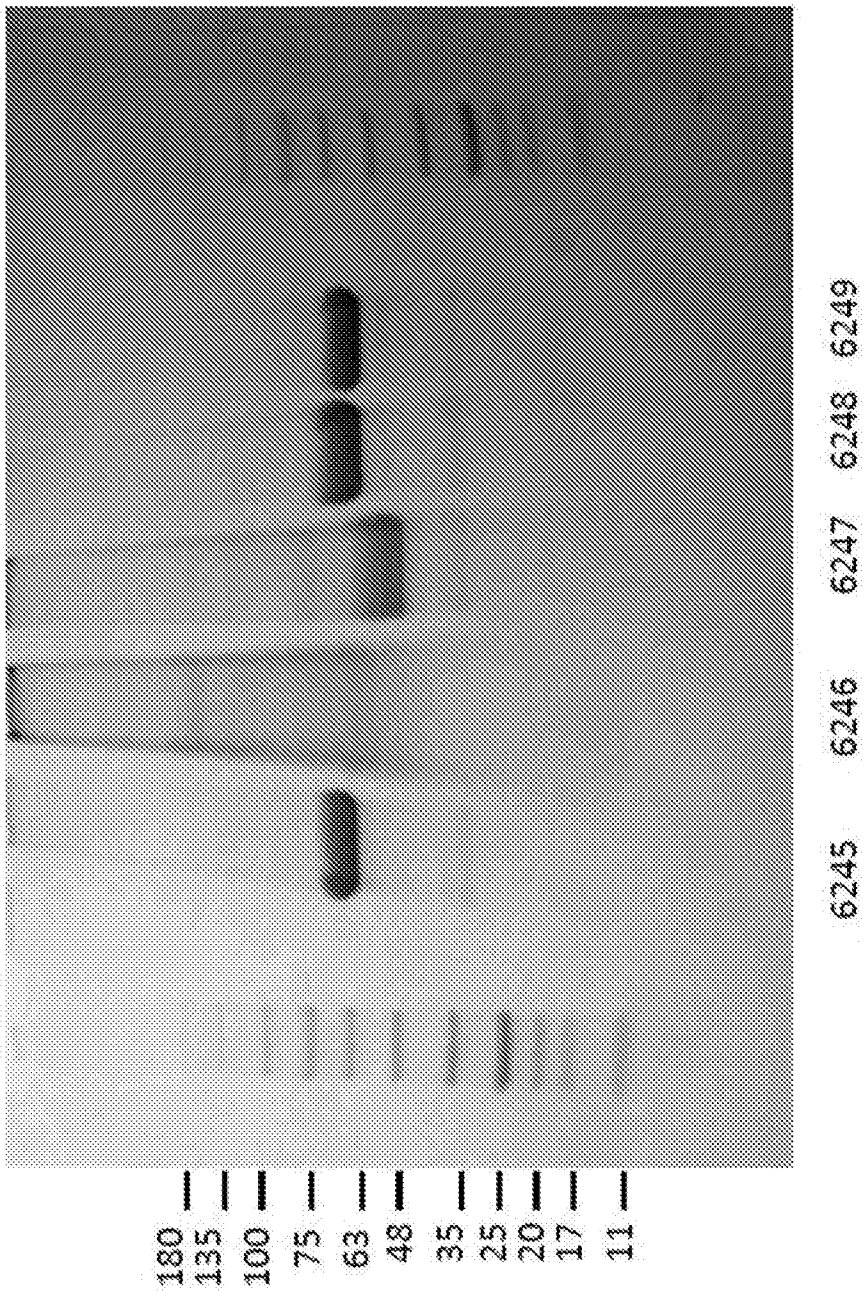


FIGURE 6

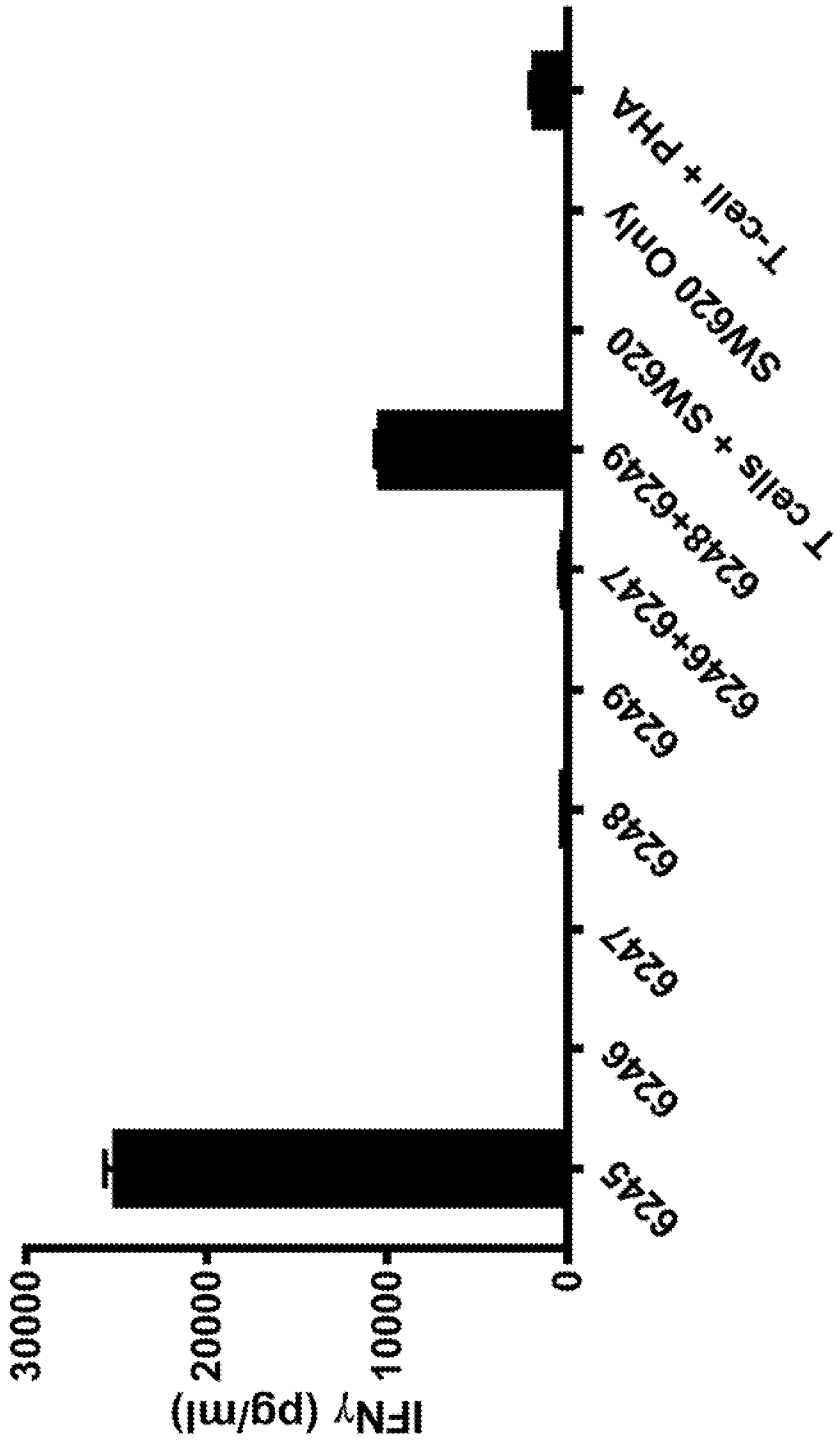

IFNγ (pg/ml)
30000
20000
10000
0
6245
6246
6247
6248
6249
6246+6247
6248+6249
T cells + SW620
SW620 Only
T-cell + PHA

FIGURE 7

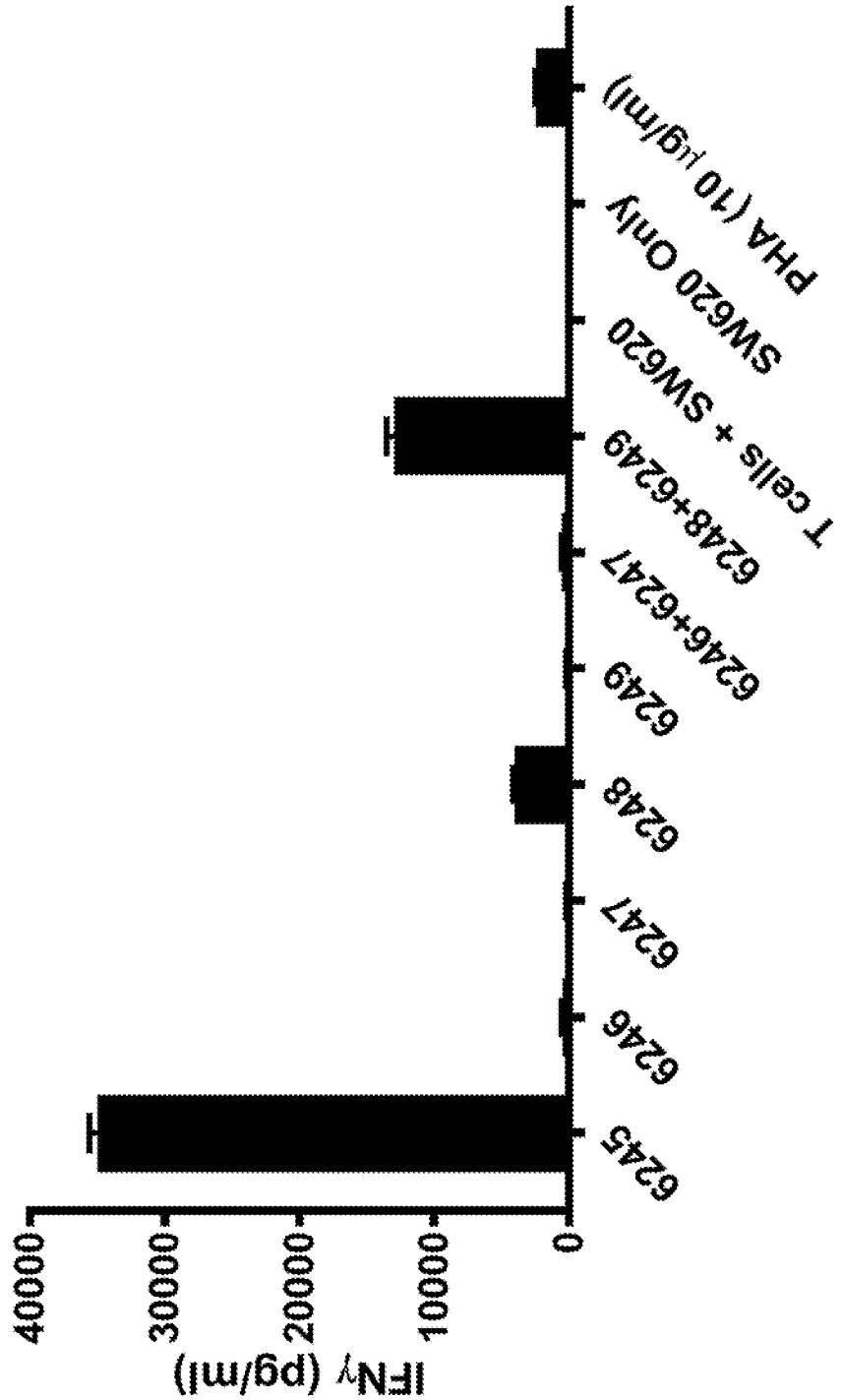
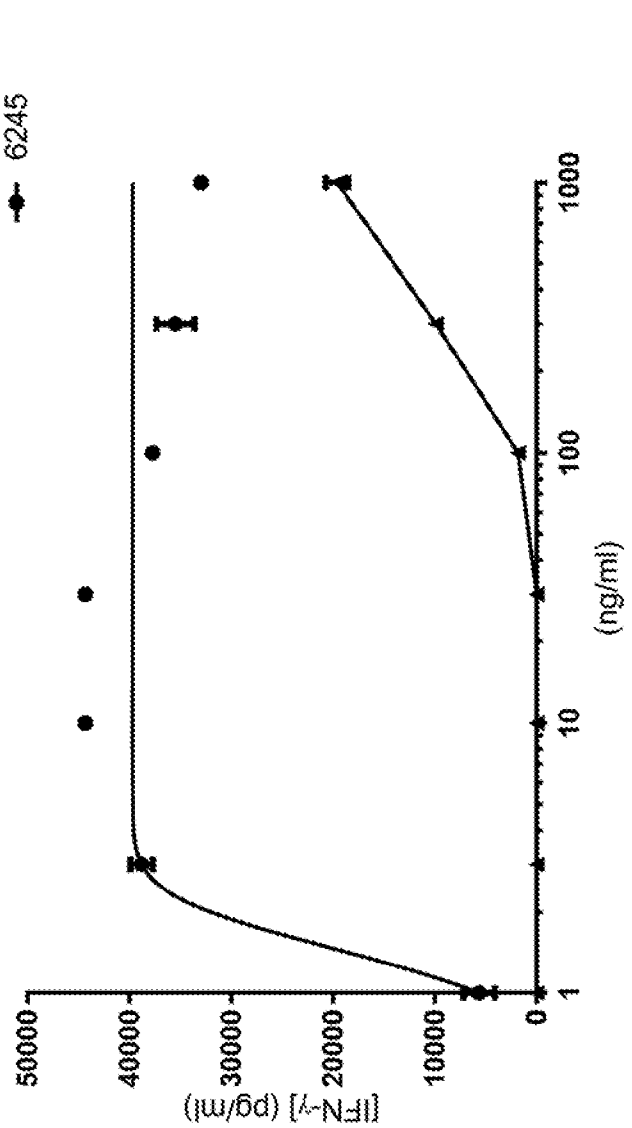

IFNγ (pg/ml)
40000
30000
20000
10000
0
6245
6246
6247
6248
6249
6246+6247
6248+6249
T cells + SW620
SW620 Only
PHA (10 μg/ml)

FIGURE 8


6248+6249
6245
50000
40000
30000
20000
10000
0
[IFN-γ] (pg/ml)
1
10
100
1000
(ng/ml)

FIGURES 9A-B

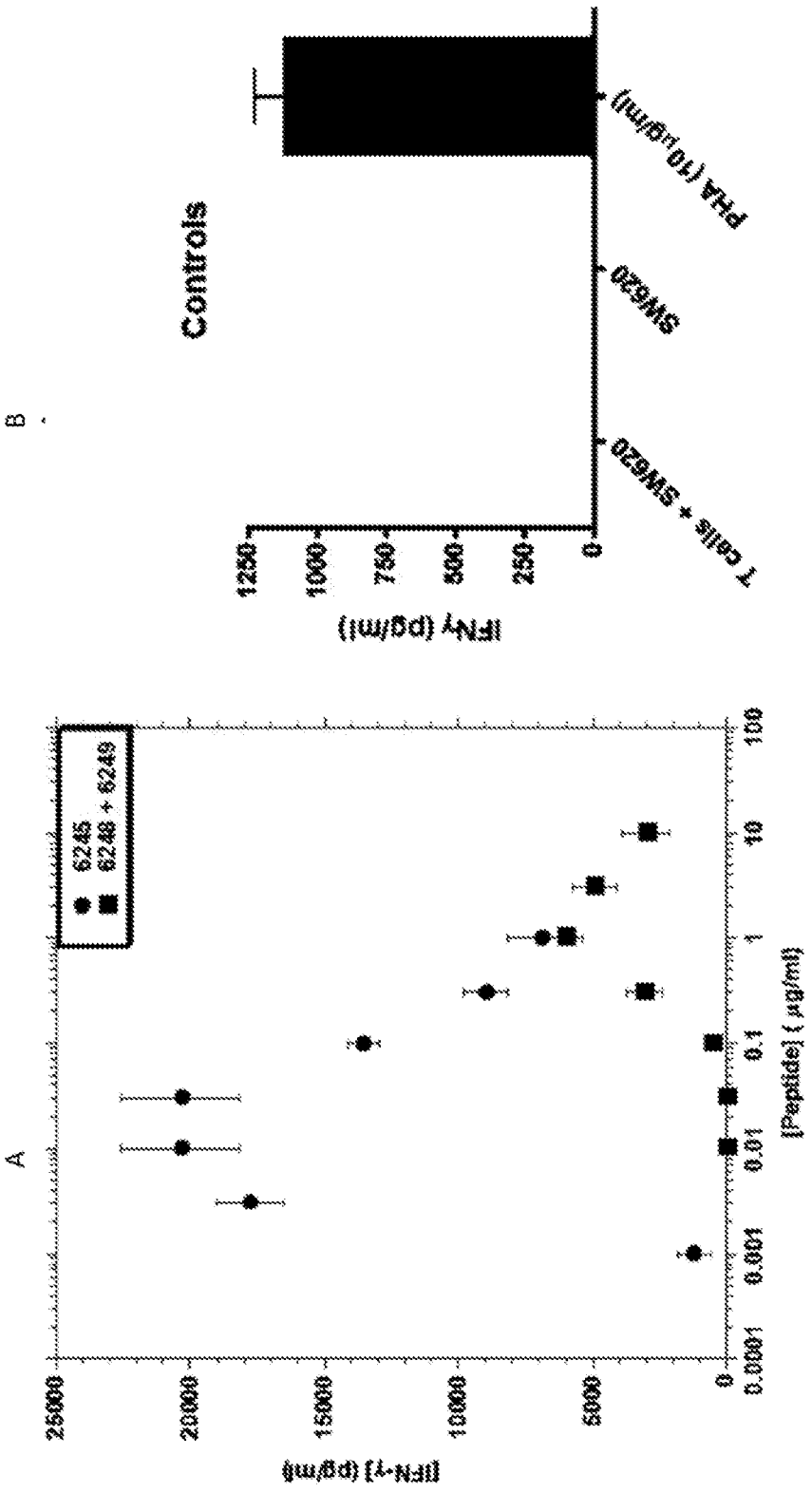

A
6245
6248 + 6249
[IFN-γ] (pg/ml)
25000
20000
15000
10000
5000
0
0.0001
0.001
0.01
0.1
1
10
100
[Peptide] (μg/ml)
B
Controls
T Cells + SW620
SW620
PHA (10 μg/ml)
IFNγ (pg/ml)
1250
1000
750
500
250
0

FIGURE 10

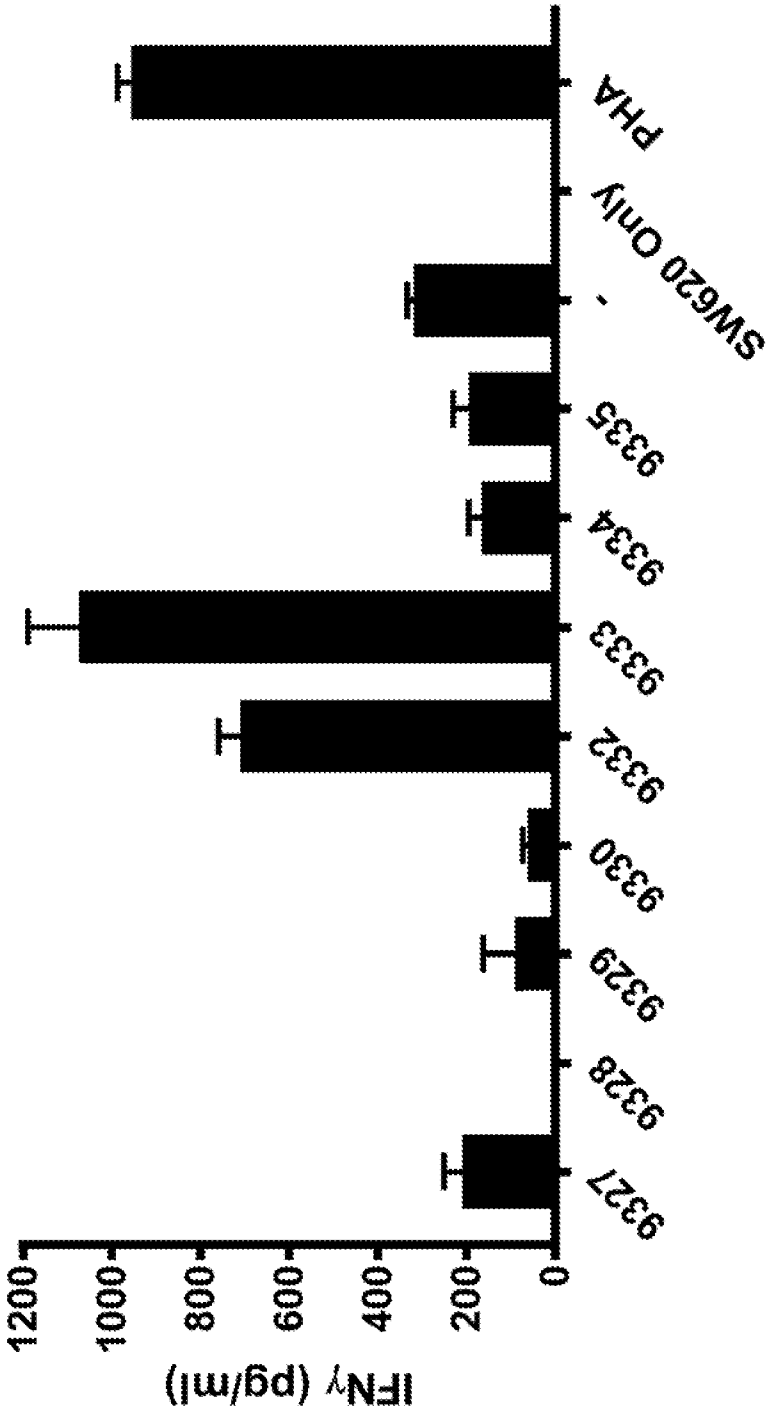

IFNγ (pg/ml)
0
200
400
600
800
1000
1200
9327
9328
9329
9330
9332
9333
9334
9335
-
SW620 Only
PHA

FIGURE 11

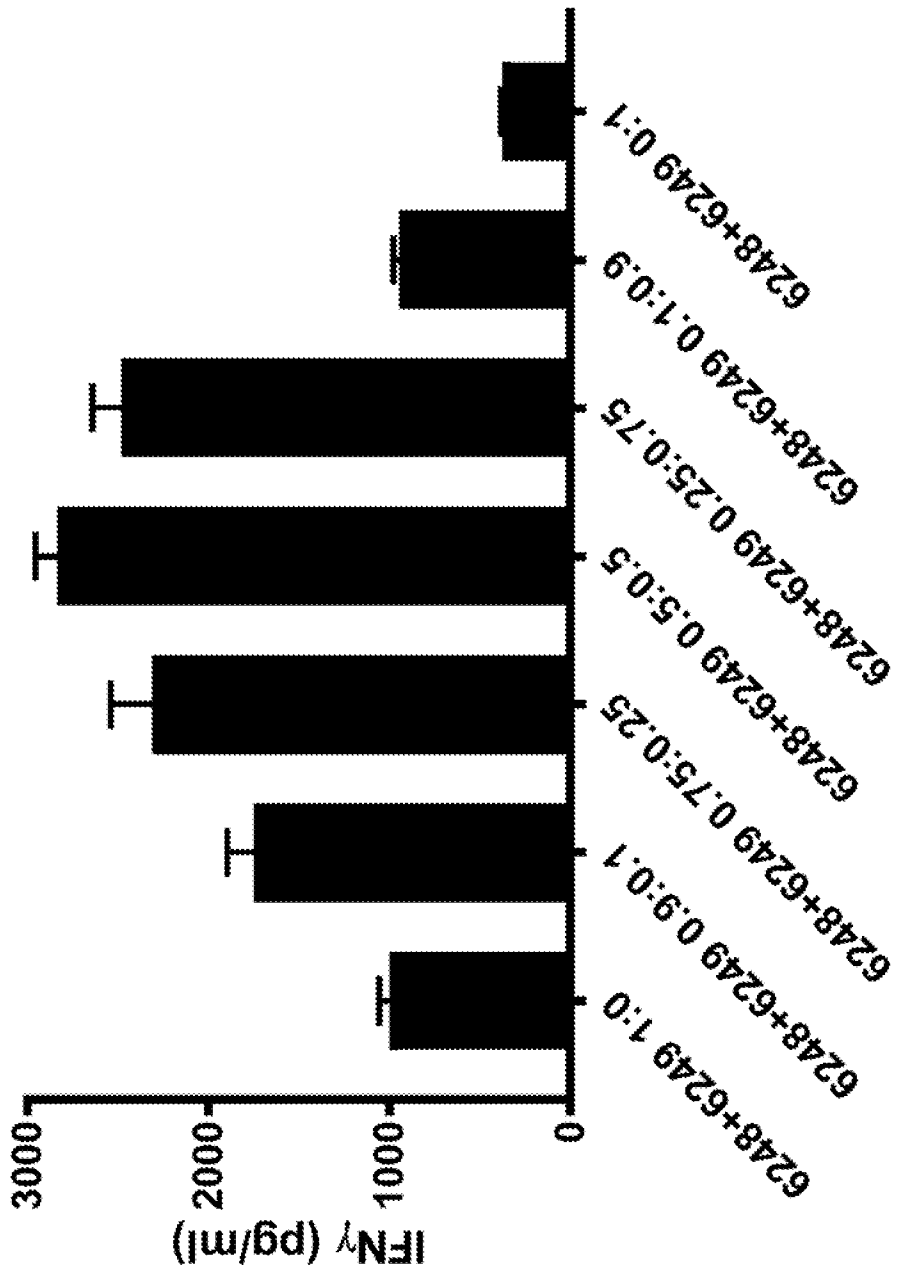

IFNγ (pg/ml)
3000
2000
1000
0
6248+6249 1:0
6248+6249 0.9:0.1
6248+6249 0.75:0.25
6248+6249 0.5:0.5
6248+6249 0.25:0.75
6248+6249 0.1:0.9
6248+6249 0:1

FIGURE 12

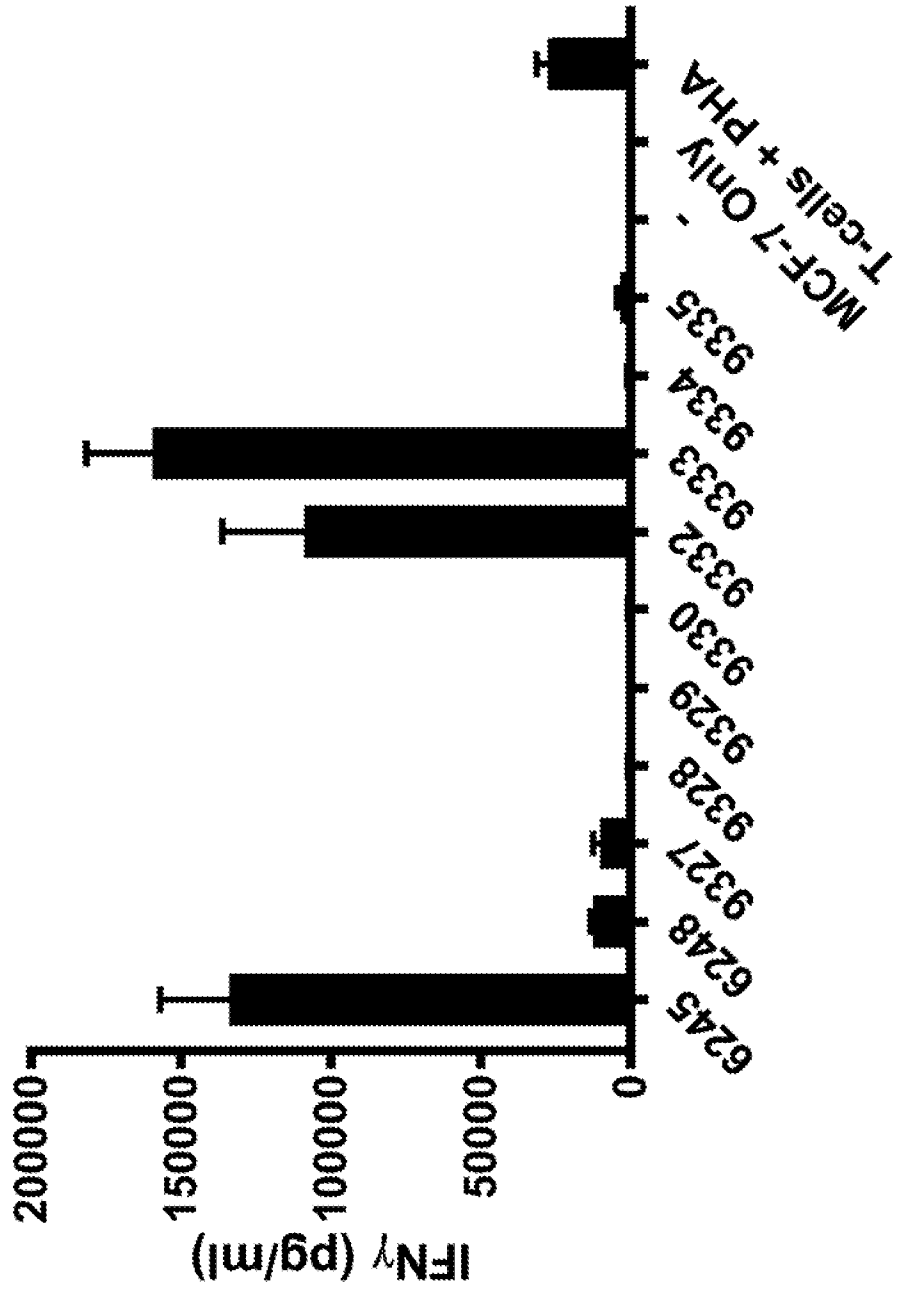

IFNγ (pg/ml)
200000
150000
100000
50000
0
6245
6248
9327
9328
9329
9330
9332
9333
9334
9335
MCF-7 Only
T-cells + PHA

FIGURE 13

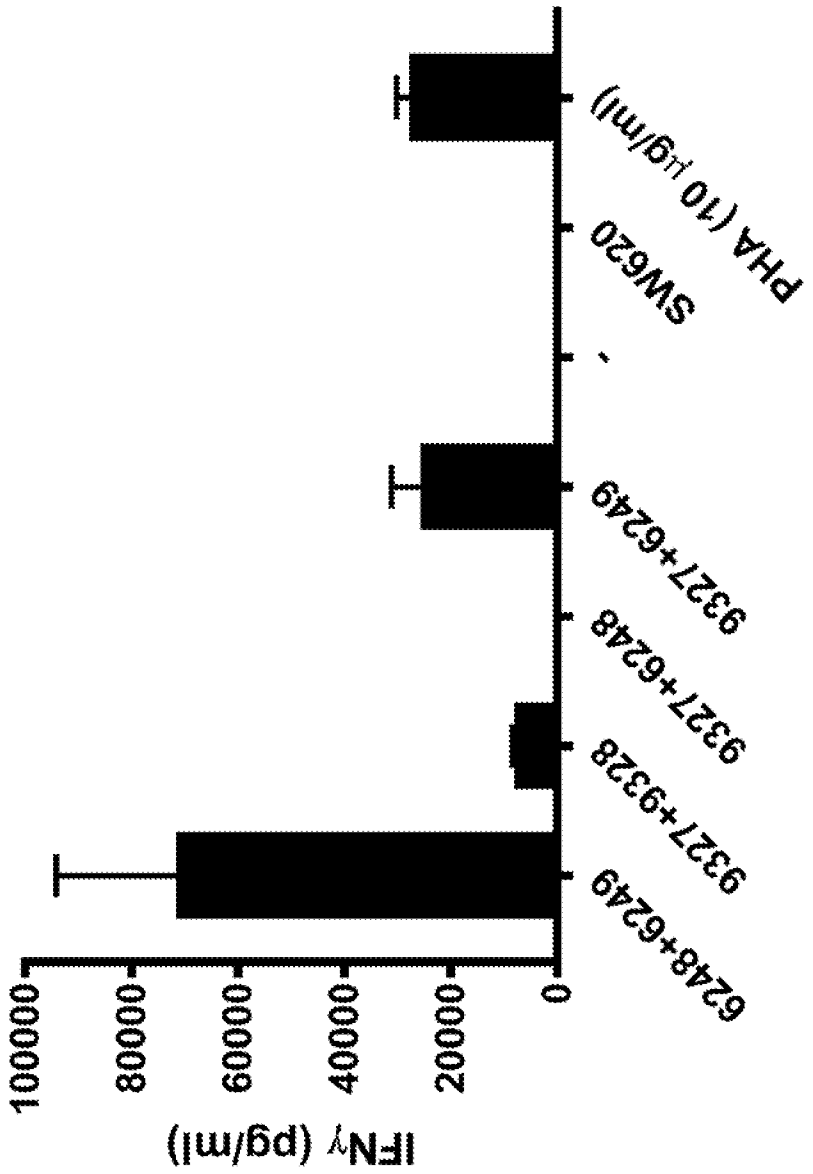

IFNγ (pg/ml)
100000
80000
60000
40000
20000
0
6248+6249
9327+9328
9327+6248
9327+6249
-
SW620
PHA (10 μg/ml)

FIGURE 14

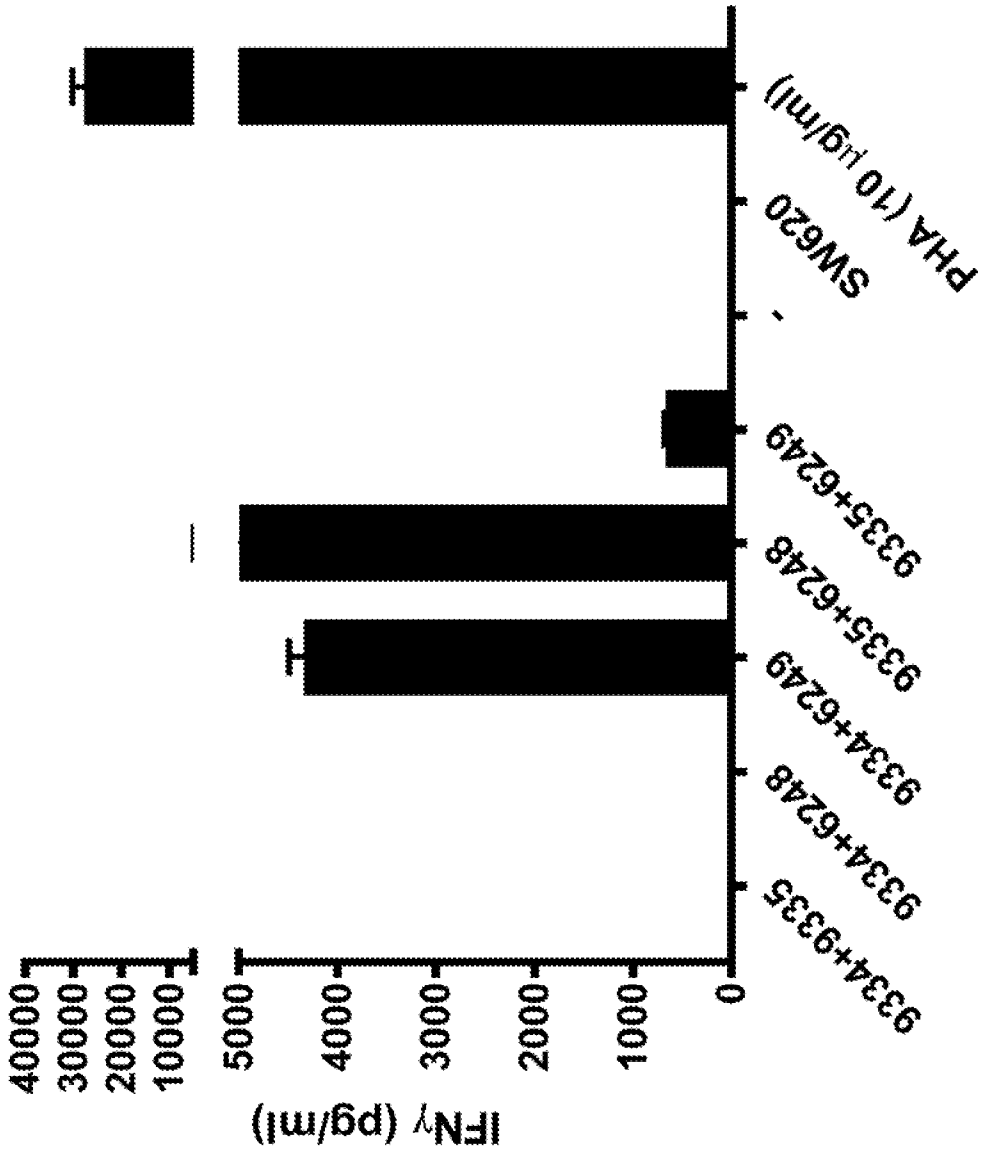

IFNγ (pg/ml)
40000
30000
20000
10000
5000
4000
3000
2000
1000
0
PHA (10 µg/ml)
SW620
-
9335+6249
9335+6248
9334+6249
9334+6248
9334+9335

FIGURE 15

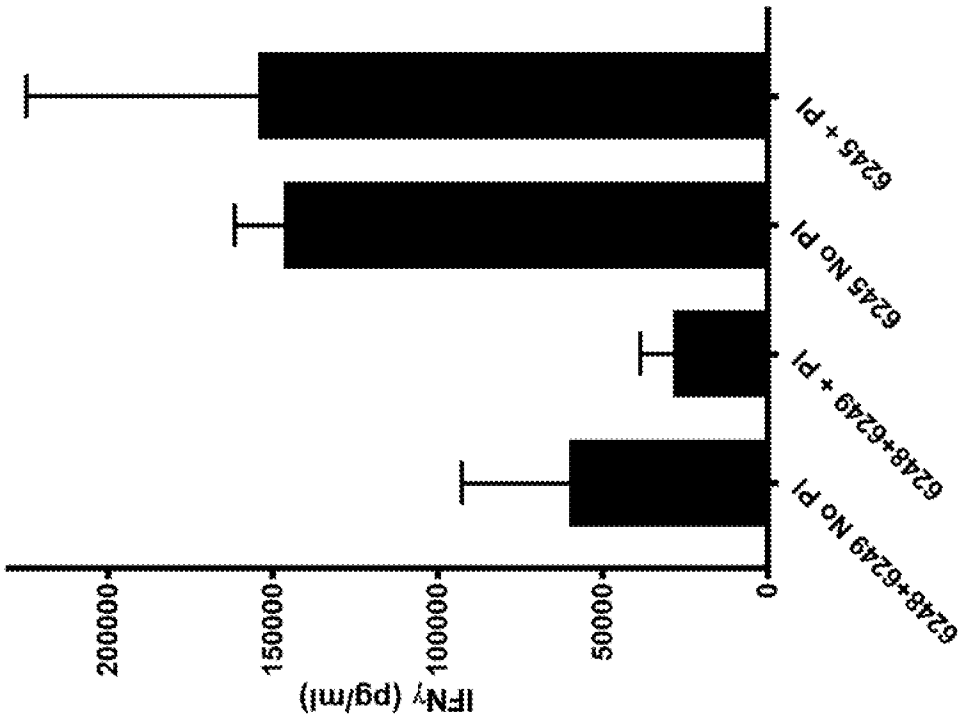

IFNγ (pg/ml)
200000
150000
100000
50000
0
6248+6249 No PI
6248+6249 + PI
6245 No PI
6245 + PI

FIGURE 16

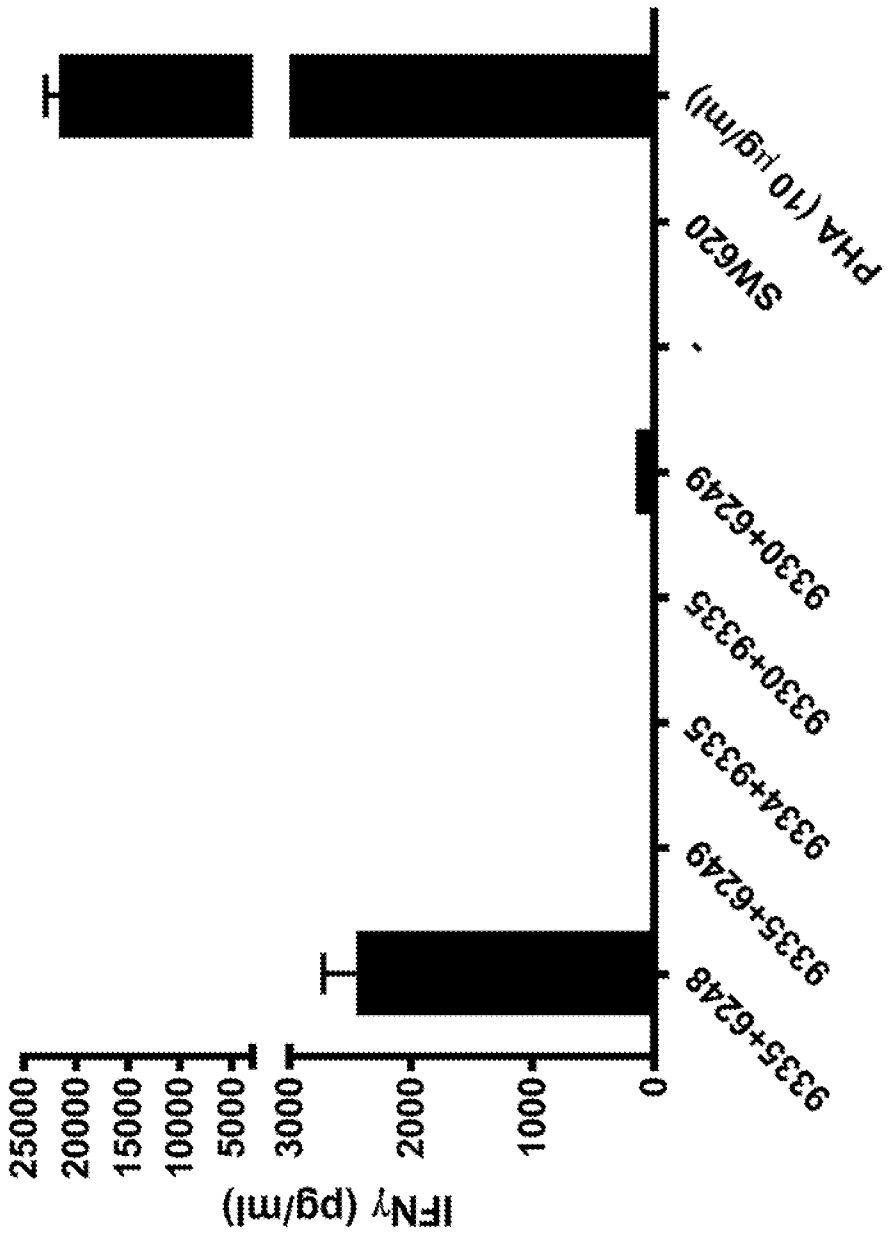

IFNγ (pg/ml)
25000
20000
15000
10000
5000
3000
2000
1000
0
9335+6248
9335+6249
9334+9335
9330+9335
9330+6249
-
SW620
PHA (10 µg/ml)

FIGURE 17

6248 EpCAM scFv (V_L LINKER V_H) LINKER scFv(Anti-CD3ε V_H CLEAVABLE LINKER inert V_L) **hexahistidine tag** (MMP2 cleavage site bold underlined)

ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGVPDR
FTGSGSGTDFTLTISSVQAEDLAVYYCQNDYSYPLTFGAGTKLEIKGGGGSGGGGSGGGGSEVQLLE
QSGAELVRPGTSVKISCKASGYAFTNYWLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKFKGKATLTA
DKSSSTAYMQLSSLTFEDSAVYFCARLRNWDEPMDYWGQGTTVTVSSGGGGSDVQLVQSGAEVKKPG
ASVKVSCKASGYTFTRYTMHWVRQAPGQGLEWIGYINPSRGYTNYADSVKGRFTITTDKSTSTAYME
LSSLRSEDTATYYCARYYDDHYCLDYWGQGTTVTVSS**GEGTSTGSGAIPVSLRG**SGGGGGADDIVLT
QSPATLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGVPARFSGSGSGTDF
TLTISSLEPEDFATYYCLQIYNMPITFGQGTKVEIK**HHHHHH** (SEQ ID NO: 168)

6249 EpCAM scFv (V_L LINKER V_H) LINKER scFv(Anti-CD3ε V_L CLEAVABLE LINKER inert V_H) **hexahistidine tag** (MMP2 cleavage site underlined)

ELVMTQSPSSLTVTAGEKVTMSCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGVPDR
FTGSGSGTDFTLTISSVQAEDLAVYYCQNDYSYPLTFGAGTKLEIKGGGGSGGGGSGGGGSEVQLLE
QSGAELVRPGTSVKISCKASGYAFTNYWLGWVKQRPGHGLEWIGDIFPGSGNIHYNEKFKGKATLTA
DKSSSTAYMQLSSLTFEDSAVYFCARLRNWDEPMDYWGQGTTVTVSSGGGGSDIVLTQSPATLSLSP
GERATLSCRASQSVSYMNWYQQKPGKAPKRWIYDTSKVASGVPARFSGSGSGTDYSLTINSLEAEDA
ATYYCQQWSSNPLTFGGGTKVEIK**GEGTSTGSGAIPVSLR**GSGGSGGGADDVQLVQSGAEVKKPGASV
KVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPNSGGTNYAQKFQGRVTITRDTSASTAYMELSS
LRSEDTAVYYCARDFLSGYLDYWGQGTLVTVSS**HHHHHH** (SEQ ID NO: 169)