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Supporting Information

Design of Switchable Chimeric Antigen Receptor T Cells Targeting Breast Cancer

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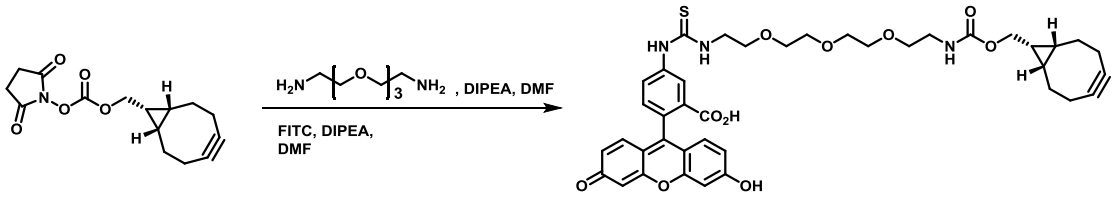
Supplementary Information

Cell lines and cell culture. Breast cancer cell lines SKBR3, HCC1954, MDA MB453, MDA MB361, BT-20, MDA MB231, and MDA MB468 were obtained from American Type Culture Collection (ATCC). The cell line MDA MB435 and its Her2-transfected cell line, MDA MB435/Her2 were kindly provided by Dr. Brunhide H. Felding (The Scripps Research Institute, La Jolla, CA). All cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, plus 1% antibiotics and 2mM L-glutamine. The virus-producing cell line, HEK293FT was purchased from Life Technologies, and maintained in DMEM containing 10% FBS, 1% antibiotics, 6mM L-glutamine, 0.1mM MEM-non-essential amino acids, 1mM sodium pyruvate and 500μg/mL Geneticin.

Synthesis of FITC-linker. 5-(3-(1-((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10,13-tetraoxa-4-azapentadecan-15-yl)thio-ureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (FITC linker) was synthesized as previously described[1].



Preparation of anti-Her2-FITC by site-specific conjugation. Anti-Her2 Fab (clone 4D5) was subcloned into the pBAD vector and site-directed mutagenesis was carried out in order to introduce the TAG amber nonsense codon using the Quickchange Site-directed Mutagenesis Kit (Agilent Technologies). Antibodies were co-expressed in *E.coli* with an orthogonal *Methanococcus jannaschii* tRNA/aminoacyl-tRNA synthetase specific for p-azido phenylalanine (pAzF) and purified as described previously [2;3]. The mutant antibodies containing pAzF were conjugated with 50-fold molar excess of FITC-linker in PBS (pH7.4) and incubated overnight at 37°C. Excess linker was removed by size exclusion chromatography on a Superdex 200 column (GE Life Sciences). The molecular weight and purity of final products was confirmed by mass spectrometry (QTOF) and SDS-PAGE.

Preparation of anti-Her2-PNE fusions. For GCN4 (PNE sequence: NYHLENEVARLKKL) fusions, gene fragments encoding 4D5 heavy or light chains, with or without GCN4 were subcloned into the pFuse vector. Fusions of the different combinations of heavy or light chains were expressed by transient transfection in the FreeStyle 293 Expression System. Briefly, HEK293F cells were transfected at a density of 1×10^6 cells/mL with a 1:2 ratio plasmid DNA to 293fectin (Life Technologies). Cells were

incubated at 37°C with 5% CO_2 for 96 hours. The recombinant protein-containing supernatant was harvested at 48 hours and 96 hours and further purified by Protein G chromatography.

Lentivirus vector generation and T cell transduction. The second generation lentivirus vector plasmid, pRRL-SIN-EF1α-WPRE and the three packaging plasmids (pMDL, pRev and pVSVG) were kindly provided by Dr. Inder M. Verma (Salk Institute for Biological Studies, La Jolla, CA). To create different CAR constructs, coding sequences for the anti-FITC scFv (clone FITC-E2)[4], anti-GCN4 scFv (clone 52SR4)[5] and anti-Her2 scFv (clone 4D5) were subcloned into the lentiviral plasmid and linked to the hinge, CD8 transmembrane region, and the cytoplasmic regions of human 4-1BB and CD3ζ. Both the 45 amino acid CD8 hinge (TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD) and the 12 amino acid mutated IgG4 (IgG4m) hinge (ESKYGPPCPPCP) were evaluated for each CAR construct in order to determine the optimal extracellular spacer.

Lentiviral particles were produced via transfection of the lentiviral constructs into HEK293FT human embryonic kidney cells [6]. Cells were transfected with pRRL-SIN-EF1α-WPRE, pMDL, pREV and pVSVG plasmids using FugeneHD (Promega). Medium was changed 6h after transfection and the viral supernatant was harvested at 24, 48 and 72h post transfection. Viral particles were concentrated 20-fold by ultracentrifugation for 2h at 25,000rpm with a Beckman Ti70 rotor (Beckman Coulter). Human T cells were purified from peripheral blood mononuclear cells (PBMC) using EasySep human T cell enrichment kit (StemCell Technologies Inc), and further activated for 24h with CD3/CD28-coated magnetic beads (Life Technologies) before infection. Concentrated lentivirus was applied to the activated human T cells in the presence of 5 μg/mL protamine sulfate and 50 IU/mL IL-2, and centrifuged at 1000 × g for 2h at 32°C. The sCAR expression level was determined by flow cytometry with Alexa Fluor 647 (Thermo Fisher)-labeled anti-human or anti-mouse IgG.

Binding assays

- **Target cells.** Flow cytometry was performed to evaluate Her2 binding of different tag (FITC and GCN4) labeled anti-Her2 Fab on breast cancer cells (SKBR3, MDA MB453, MDA MB231, MDA MB435 and MDA MB468). Cells were incubated with 25 nM antibody switches for 1h at 4°C. After washing, cells were incubated with Alexa Fluor 647-conjugated anti-human IgG (H+L) antibody and analyzed on a LSR II flow cytometer (Becton Dickinson). Mean fluorescence intensity (MFI) was further calculated by FlowJo 10. 1r5 software [7].
- **CAR-T cells.** Anti-FITC and anti-GCN4 CAR-T cells were incubated with different concentrations of the corresponding tag-labeled antibodies for 1h at 4°C, and washed with PBS (with 1% BSA). Antibody switches were then stained with Alexa Fluor 647-conjugated anti-human kappa chain antibody. The samples were analyzed on LSR II flow cytometer. The EC_{50} values were calculated by Graphpad Prism 5.0 based on the MFI values generated by FlowJo software.

Cytotoxicity assay. Co-cultures containing 1×10^4 target cells and 1×10^5 CAR-T cells with different concentrations of corresponding antibody switches were incubated at 37 °C for 24h. To test the effects of E: T ratio, 1×10^4 target cells were incubated with 25pM antibody switches with different numbers of the corresponding CAR-T cells ($0.01 - 10 \times 10^5$) at 37°C for 24h. Cytotoxicity was measured using the Cytotox-96 Nonradioactive cytotoxicity assay kit (Promega), which quantifies the amount of lactate dehydrogenase (LDH) released from lysed cells into the supernatant. The cytotoxicity was calculated with the following formula: (values used represent absorbance at 490nm)

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental LDH release} - \text{Spontaneous LDH release}}{\text{Maximum LDH release} - \text{Spontaneous LDH release}}$$

CAR-T cell activation analysis. CAR-T cells were incubated with switch bound target cells for 24h at 37°C. T cell activation was analyzed by flow cytometry with phycoerythrin-conjugated anti-human CD69 and APC-Cy7 conjugated anti-human CD25. In addition, the levels of cytokines (IL-2, IFN-γ and TNF-α) produced in the culture medium were measured by human IL-2, IFN-γ and TNF-α test kits (R&D System). All the tests were conducted in triplicate and results are shown as mean ± SD.

Tumor Distribution Study. Anti-Her2 Fab was labeled with IRDye800CW (LI-COR Biosciences) according to the manufacturer's protocol, and administered intravenously at 1 nmol per tumor-bearing mouse. Eight-week-old female NSG mice (Jackson Laboratory) were subcutaneously inoculated with 5×10^6 MDA MB435/Her2 cells and tumors were allowed to reach 500 mm^3 prior to injections. Bioluminescent imaging of mice bearing tumors was achieved using IVIS imaging by injecting mice intraperitoneally with 150mg/kg of D-luciferin. The distribution of intravenous IRDye800 labeled anti-Her2 Fab as a correlate of tumor size was assessed at 16min, 6h, 24h, 48h and 72h post injection.

***In vivo* efficacy study.** Efficacy studies were conducted in 8-week old female NSG mice. The representative tumor models included Her2 3+ (HCC1954), 2+ (MDA MB453) and 1+ (MDA MB231) cells. For Her2 3+ and 2+ xenografts, 5×10^6 HCC1954 or MDA MB453 cells in 50% Matrigel (BD Biosciences) were subcutaneously implanted into the right flank of the mice. For Her2 1+ models, 5×10^6 MDA MB231 cells in 50% Matrigel were orthotopically injected into the abdominal mammary fat pad. 10 days later, the mice were infused i.v. with 30×10^6 CAR-T cells, followed by i.v. administration of corresponding switch antibodies or wild type antibodies at 0.5mg/kg every other day for 7 times. In parallel, saline and conventional anti-Her2 CAR-T cells were injected as control groups. Mice were monitored and tumors were measured twice weekly using calipers.

Statistical analysis. Statistical analyses were conducted with Microsoft Excel 2013. Data are shown as mean ± SD, and significance was determined using a 2-sided Student's *t* test, unless otherwise noted. ***= $p<0.005$, **=$p<0.05$ and *=$p>0.05$, and a p value < 0.05 was considered significant.

Reference List

[1.] J. S. Ma, J. Y. Kim, S. A. Kazane, S. H. Choi, H. Y. Yun, M. S. Kim, D. T. Rodgers, H. M. Pugh, O. Singer, S. B. Sun, B. R. Fonslow, J. N. Kochenderfer, T. M. Wright, P. G. Schultz, T. S. Young, C. H. Kim, Y. Cao, *Proc.Natl.Acad.Sci.U.S.A* **2016**.

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Supplementary Table S1. QTOF-MS characterization of different anti-Her2 Fab and the FITC conjugates

Constructs	Non-reducing condition (-DTT) Expected/Observed Mass	Reducing condition (+DTT) Expected/Observed Mass
Wild type	47762/ 47755	23457/ 23454 (light chain) 24305/ 24303 (heavy chain)
LG68X	47893/ 47886	23588 (23562[a])/ 23559[a] (LG68X light chain) 24305/ 24303 (heavy chain)
LG68X-FITC	48651/ 48644	24345/ 24343 (LG68X-FITC light chain) 24305/ 24303 (heavy chain)
LS202X	47863/ 47856	23558 (23532[a])/ 23529[a] (LS202X light chain) 24305/ 24303 (heavy chain)
LS202X-FITC	48621/ 48614	24315/ 24313 (LS202X-FITC light chain) 24305/ 24303 (heavy chain)
HS75X	47863/ 47856	23457/ 23454 (light chain) 24406 (24380[a])/ 24378[a] (HS75X heavy chain)
HS75X-FITC	48621/ 48614	23457/ 23454 (light chain) 25163/ 25161 (HS75X-FITC heavy chain)
HK136X	47822/ 47815	23457/ 23454 (light chain) 24365 (24339[a])/ 24336[a] (HK136X heavy chain)
HK136X-FITC	48580/ 48572	23457/ 23454 (light chain) 25122/ 25120 (HK136X-FITC heavy chain)
LG68X/HS75X	47884/ 47987	23588 (23562[a])/ 23559[a] (LG68X light chain) 24406 (24380[a])/ 24378[a] (HS75X heavy chain)
LG68X/HS75X -$(FITC)_2$	49510/ 49503	24345/ 24343 (LG68X-FITC light chain) 25163/ 25161 (HS75X-FITC heavy chain)
LS202X/HK136X	47923/ 47915	23558 (23532[a])/ 23529[a] (LS202X light chain) 24365 (24339[a])/ 24336[a] (HK136X heavy chain)
LS202X/HK136X-$(FITC)_2$	49439/ 49432	24315/ 24313 (LS202X-FITC light chain) 25122/ 25120 (HK136X-FITC heavy chain)

[a] The reduction of azide group to amine caused 26 Dal decrease.

Supplementary Table S2. QTOF-MS characterization of different anti-Her2 Fab and the GCN4 fusions

Constructs	Non-reducing condition (-DTT) Expected/Observed Mass	Reducing condition (+DTT) Expected/Observed Mass
Wild type	47762/ 47755	23457/ 23454 (light chain) 24305/ 24302 (heavy chain)
LCNT	49786/ 49780	25481/ 25479 (LC-NT light chain) 24305/ 24302 (heavy chain)
LCCT	49786/ 49779	25481/ 25479 (LC-CT light chain) 24305/ 24302 (heavy chain)
HCNT	49787/ 49779	23457/ 23454 (light chain) 26330/ 26327 (HC-NT heavy chain)
HCCT	49787/ 49779	23457/ 23454 (light chain) 26330/ 26327 (HC-CT heavy chain)
NTBV	51811/ 51806	25481/ 25479 (LC-NT light chain) 26330/ 26327 (HC-NT heavy chain)
CTBV	51811/ 51804	25481/ 25478 (LC-NT light chain) 26330/ 26326 (HC-NT heavy chain)

Supplementary Table S3. Comparison of binding activity of different FITC labelled anti-Her2 conjugates to breast cancer cells

Cell line	Her2 Level	MFI[a] / Relative Binding Index[b]							
		anti-CD19 Fab	anti-Her2 Fab	LG68	LS202	HS75	HK136	LG68/HS75	LS202/HK136
SKBR3	Her2 3+	144	13606/96	14299/99	18414/128	15513/108	17204/119	13575/94	20323/141
MDA MB453	Her2 2+	151	8645/57	8493/56	8539/57	8195/54	7133/47	7306/48	8332/55
MDA MB231	Her2 1+	89	586/7	411/5	439/5	456/5	444/5	427/5	416/5
MDA MB435	Her2 1+	94	228/2	203/2	193/2	213/2	216/2	216/2	222/2
MDA MB468	Her2 0	86	82/1	85/1	85/1	90/1	83/1	84/1	85/1

[a]MFI: mean fluorescence intensity calculated by software FlowJo X10.0.6

[b]Relative Binding index represent MFI of indicated antibody / MFI of anti-CD19 Fab

Supplementary Table S4. Comparison of binding activity of different GCN4 tagged anti-Her2 fusions to breast cancer cells

Cell line	Her2 Level	MFI[a] / Relative Binding Index[b]							
		anti-CD19 Fab	anti-Her2 Fab	LCNT	LCCT	HCNT	HCCT	NTBV	CTBV
SKBR3	Her2 3+	151	14306/95	18068/120	14977/99	17535/116	17381/115	19253/128	22931/152
MDA MB453	Her2 2+	147	8081/55	7342/50	7041/48	7309/50	7947/54	8195/56	6910/47
MDA MB231	Her2 1+	87	424/5	475/5	334/4	414/5	418/5	458/5	406/5
MDA MB435	Her2 1+	94	245/3	213/2	188/2	191/2	206/2	210/2	216/2
MDA MB468	Her2 0	80	86/1	84/1	89/1	84/1	90/1	92/1	86/1

[a]MFI: mean fluorescence intensity calculated by software FlowJo X10.0.6

[b]Relative Binding index represent MFI of indicated antibody / MFI of anti-CD19 Fab

Supplementary Table S5. Cytotoxic activity of anti-FITC CAR-T induced by different FITC conjugates against various human breast cancer cells.

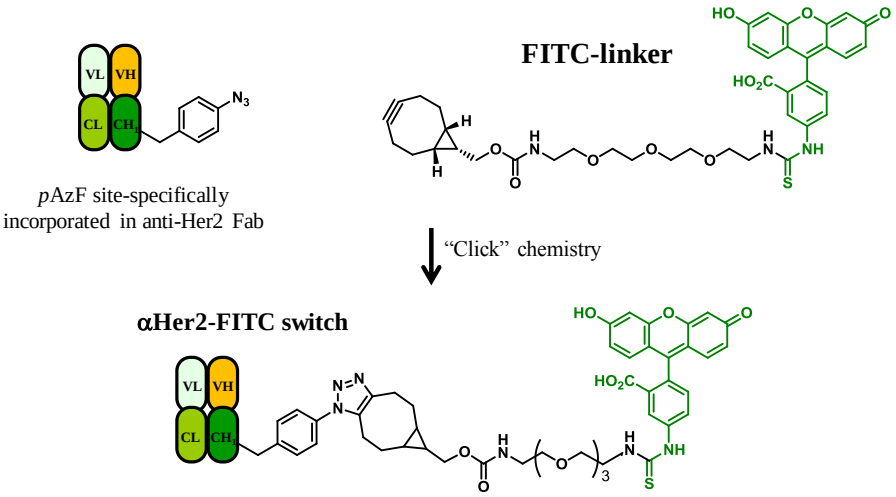
Cell line	Her2 level	IC_{50} (pM)					
		LG68	LS202	HS75	HK138	LG68/HS75	LS202/HK138
BT20	1+	33.8±3.8	27.7±4.0	77.1±8.2	30.9±4.2	54.0±5.2	2.9±0.2
MDA MB231	1+	32.1±5.2	33.5±5.1	106.1±9.3	68.5±9.2	70.1±8.1	9.9±0.6
MDA MB435	1+	72.1±6.2	113.5±9.3	215.5±12.2	201.9±15.2	102.0±13.2	18.3±2.4

Abbreviations: IC_{50}, half-maximal inhibitory concentration; N.D., not determined.

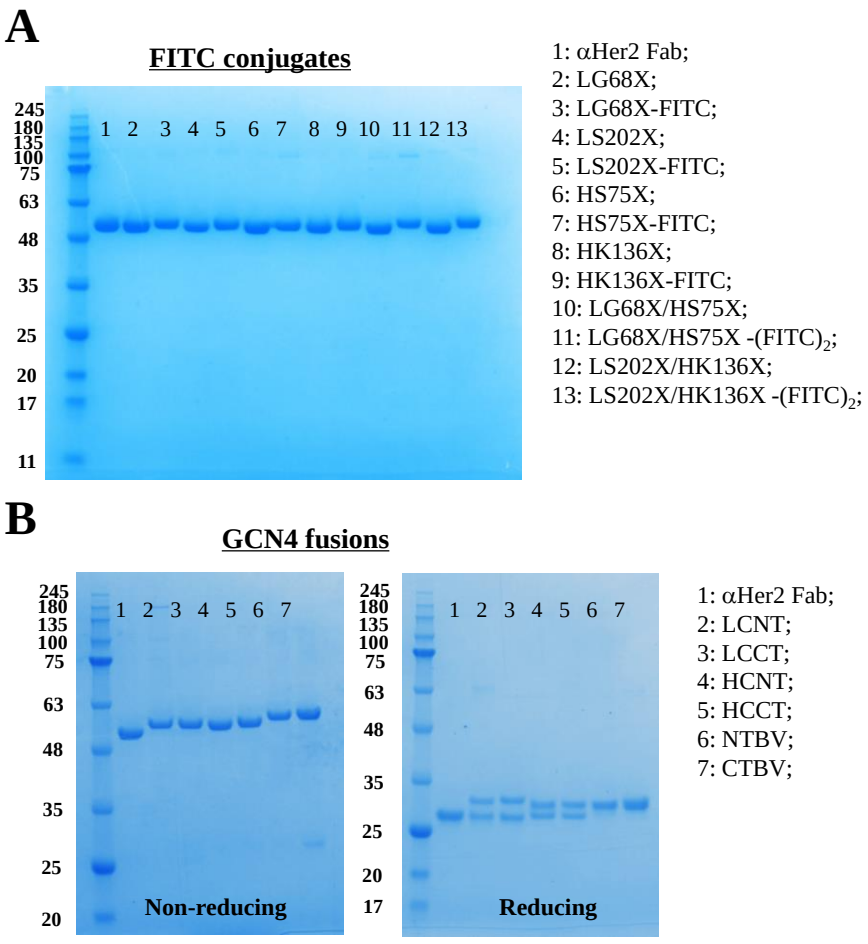
Supplementary Table S6. Cytotoxic activity of anti-GCN4 CAR-T induced by different GCN4 fusions against various human breast cancer cells.

Cell line	Her2 level	IC_{50} (pM)					
		LC-NT	LC-CT	HC-NT	HC-CT	LC-NT/HC-CT	LC-CT/HC-CT
BT20	1+	41.4±7.2	53.1±4.1	N.D.	65.9±5.3	N.D.	2.8±0.4
MDA MB231	1+	N.D.	69.8±7.2	N.D.	47.2±5.2	N.D.	2.0±0.2
MDA MB435	1+	N.D.	207.4±15.5	N.D.	135.7±9.2	N.D.	4.0±0.7

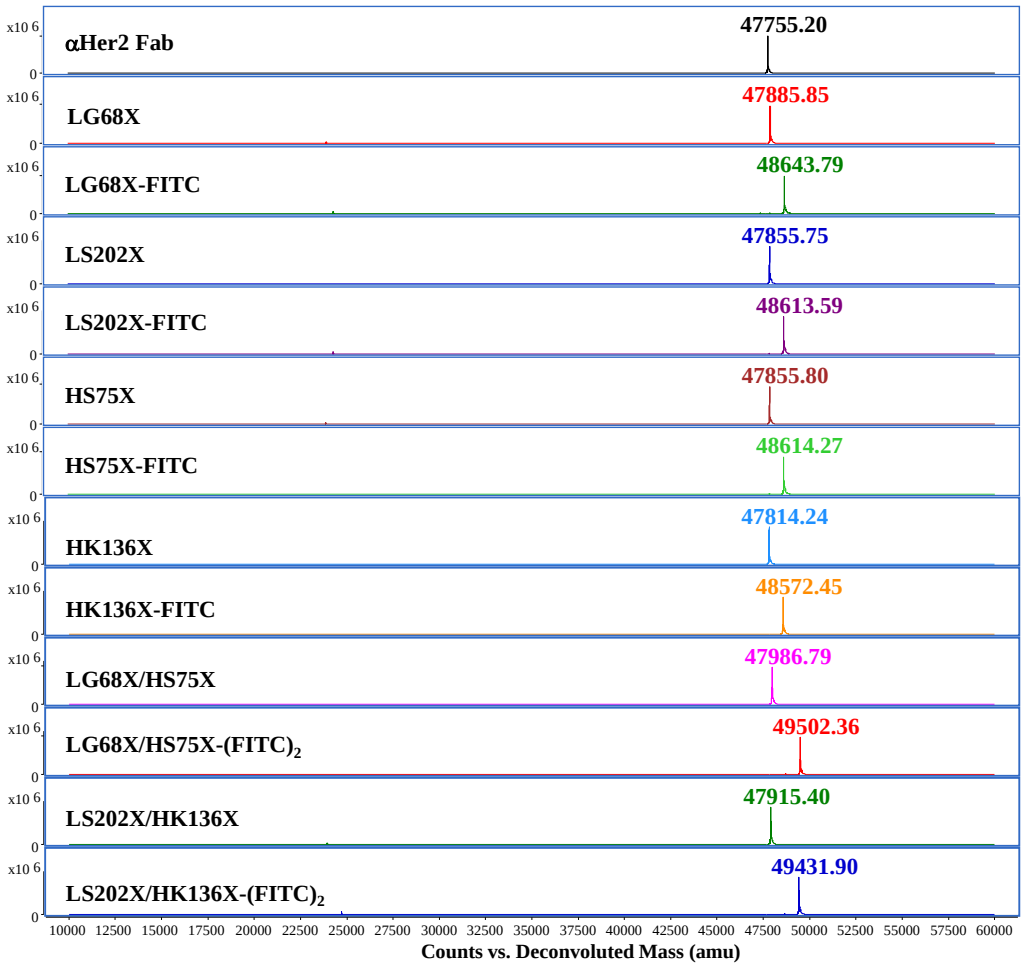
Abbreviations: IC_{50}, half-maximal inhibitory concentration; N.D., not determined.
Data shown are a mean of duplicate samples ± SD.



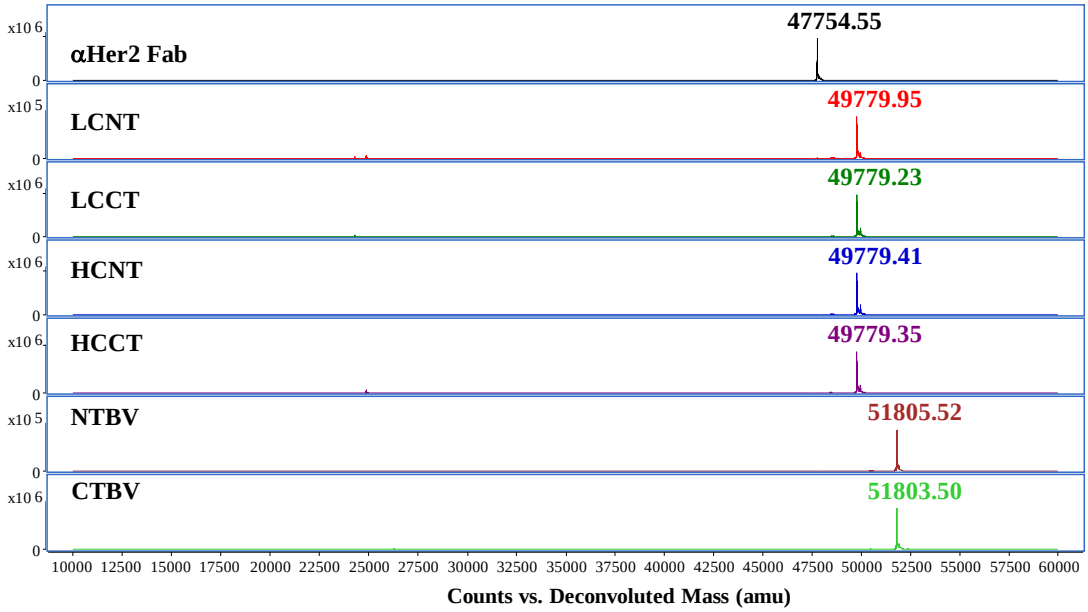
Supplementary Figure S1. General scheme for the generation of site-specific FITC antibody conjugates. Mutant antibodies containing pAzF were conjugated with BCN-PEG4-FITC by "Click" reaction.



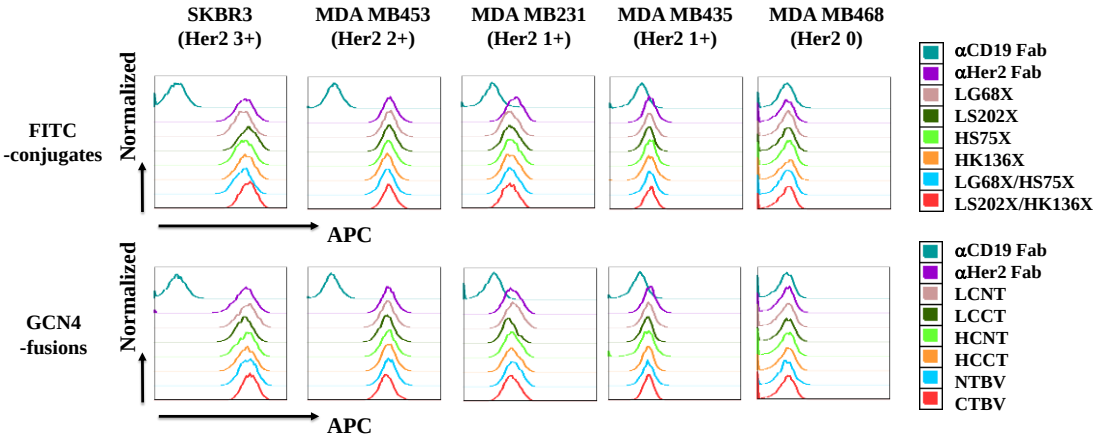
Supplementary Figure S2. SDS-PAGE analysis of switches before and after FITC conjugation (A), and anti-Her2 Fab fused with GCN4 (B) under non-reducing and reducing conditions.



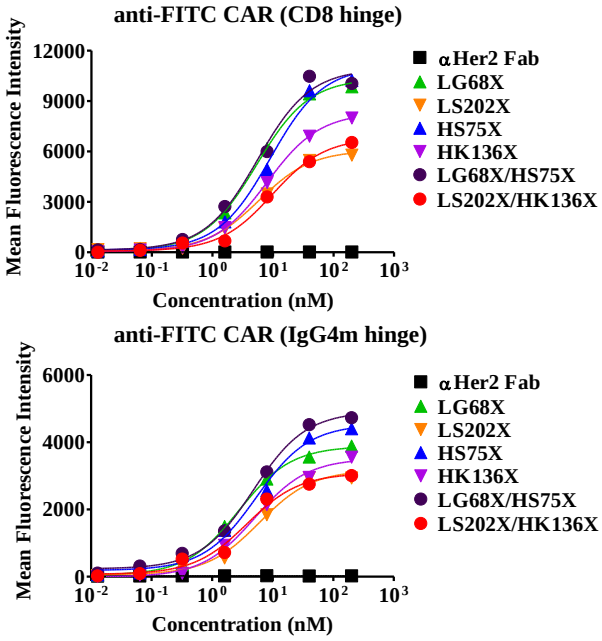
Supplementary Figure S3. Mass spectrometric analysis of site-specific anti-Her2-FITC conjugates obtained on an Agilent Quadruple Time-of-Flight (QTOF) mass spectrometer. Deconvoluted masses were obtained using Agilent Qualitative Analysis software.



Supplementary Figure S4. Mass spectrometric analysis of anti-Her2-GCN4 fusions obtained on an Agilent Quadruple Time-of-Flight (QTOF) mass spectrometer. Deconvoluted masses were obtained using Agilent Qualitative Analysis software.



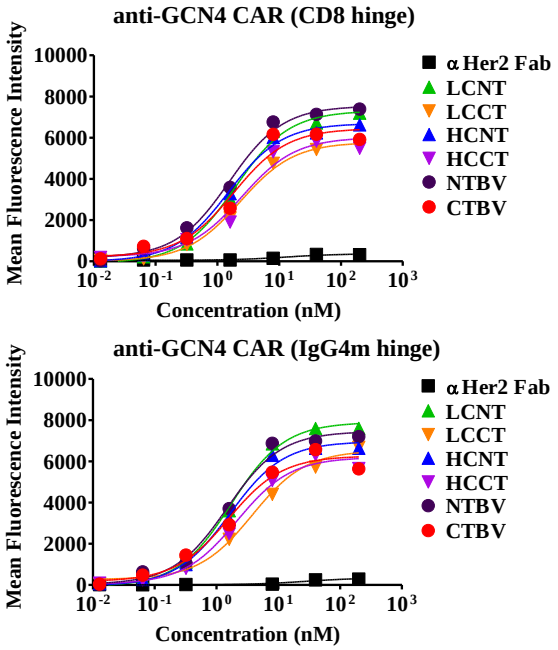
Supplementary Figure S5. Flow cytometry analysis of FITC and GCN4-based switches and wild type 4D5 Fab bound to breast cancer cells with a range of Her2 expression levels. Bound switches were detected with an Alexa Fluor647 conjugated anti-human IgG (H+L) secondary antibody.



	EC50 (nM)
αHer2 Fab	N.D.
LG68X	5.4±0.2
LS202X	5.8±0.3
HS75X	9.0±0.5
HK136X	8.0±0.4
LG68X/HS75X	5.6±0.4
LS202X/HK136X	9.7±0.5

	EC50 (nM)
αHer2 Fab	N.D.
LG68X	2.7±0.1
LS202X	6.1±0.4
HS75X	5.3±0.5
HK136X	5.1±0.4
LG68X/HS75X	4.8±0.4
LS202X/HK136X	3.8±0.3

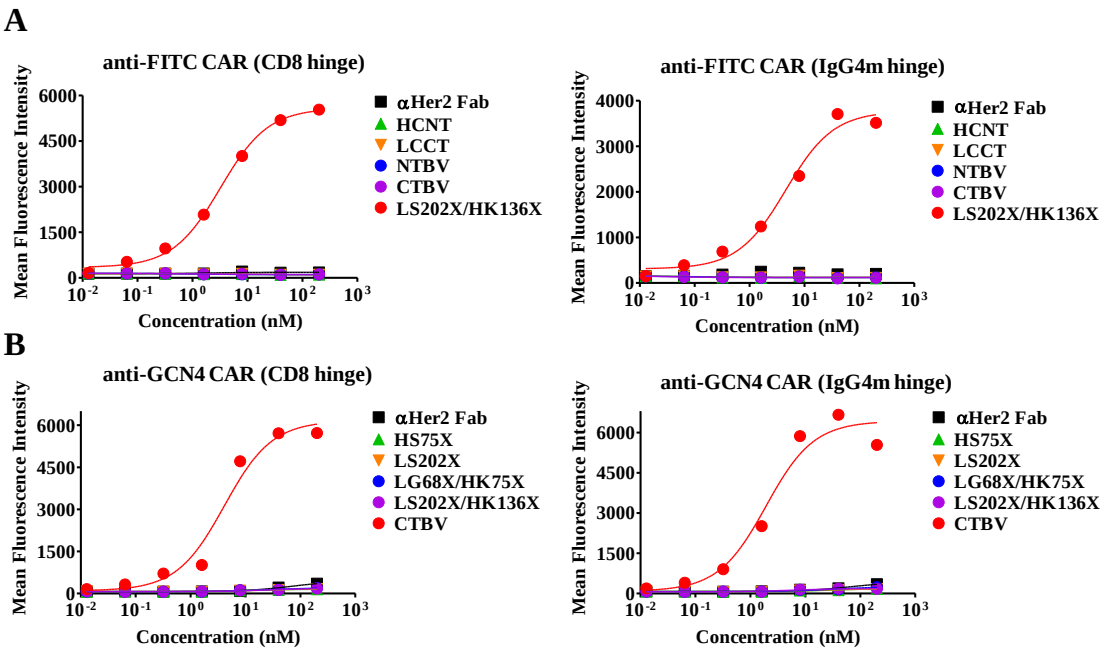
Supplementary Figure S6. Binding of FITC switches to anti-FITC CAR-T cells (both CD8 and IgG4m). Cells were incubated with indicated switches and detected with Alexa Fluor647 conjugated anti-human kappa chain antibody. The EC_{50} value was calculated with Graphpad Prism software.



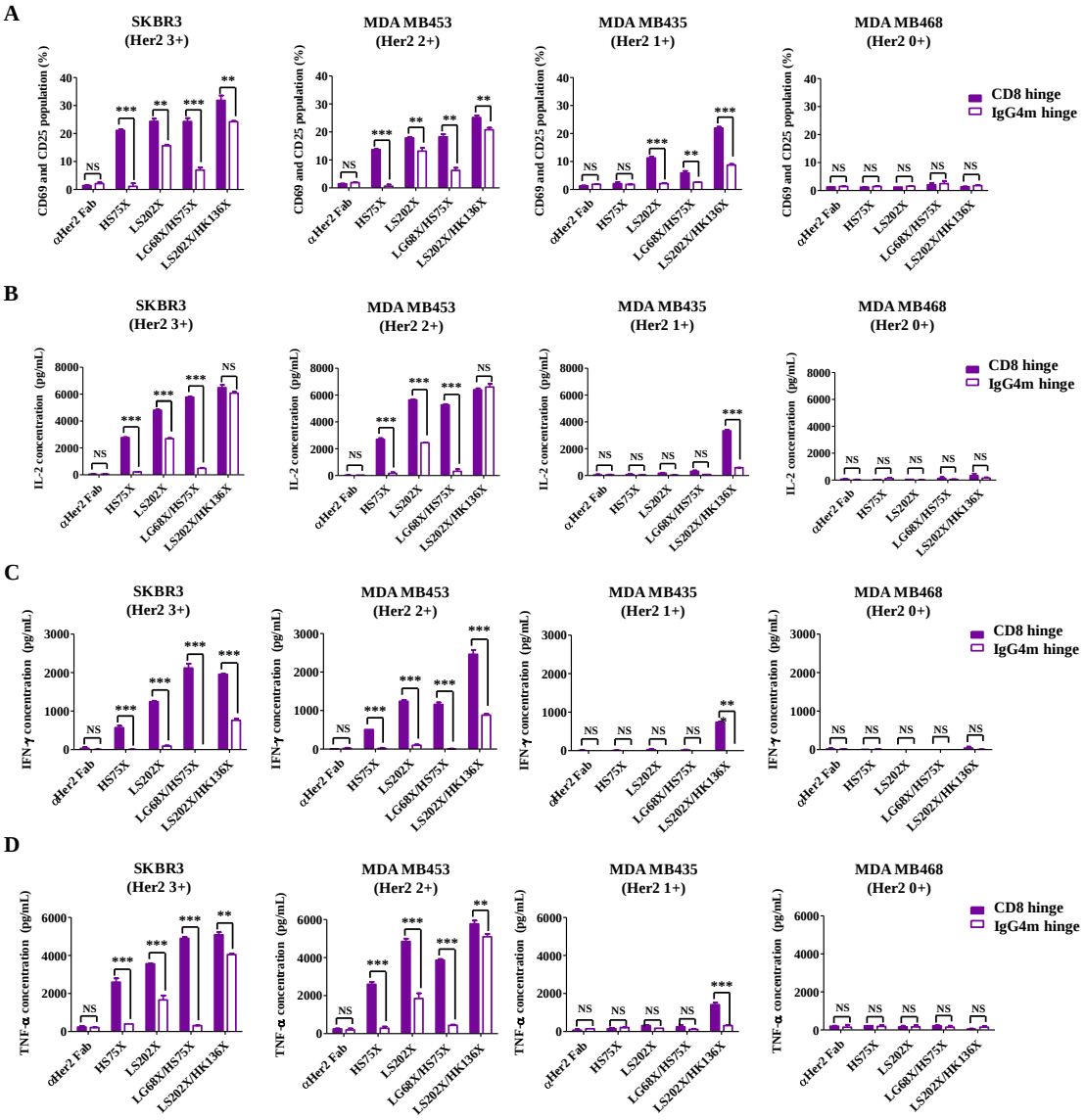
	EC50 (nM)
αHer2 Fab	N.D.
LCNT	2.0±0.2
LCCT	2.3±0.4
HCNT	1.5±0.2
HCCT	2.5±0.2
NTBV	1.6±0.2
CTBV	1.8±0.2

	EC50 (nM)
αHer2 Fab	N.D.
LCNT	1.7±0.1
LCCT	3.8±0.3
HCNT	1.7±0.4
HCCT	2.2±0.3
NTBV	1.4±0.2
CTBV	1.6±0.2

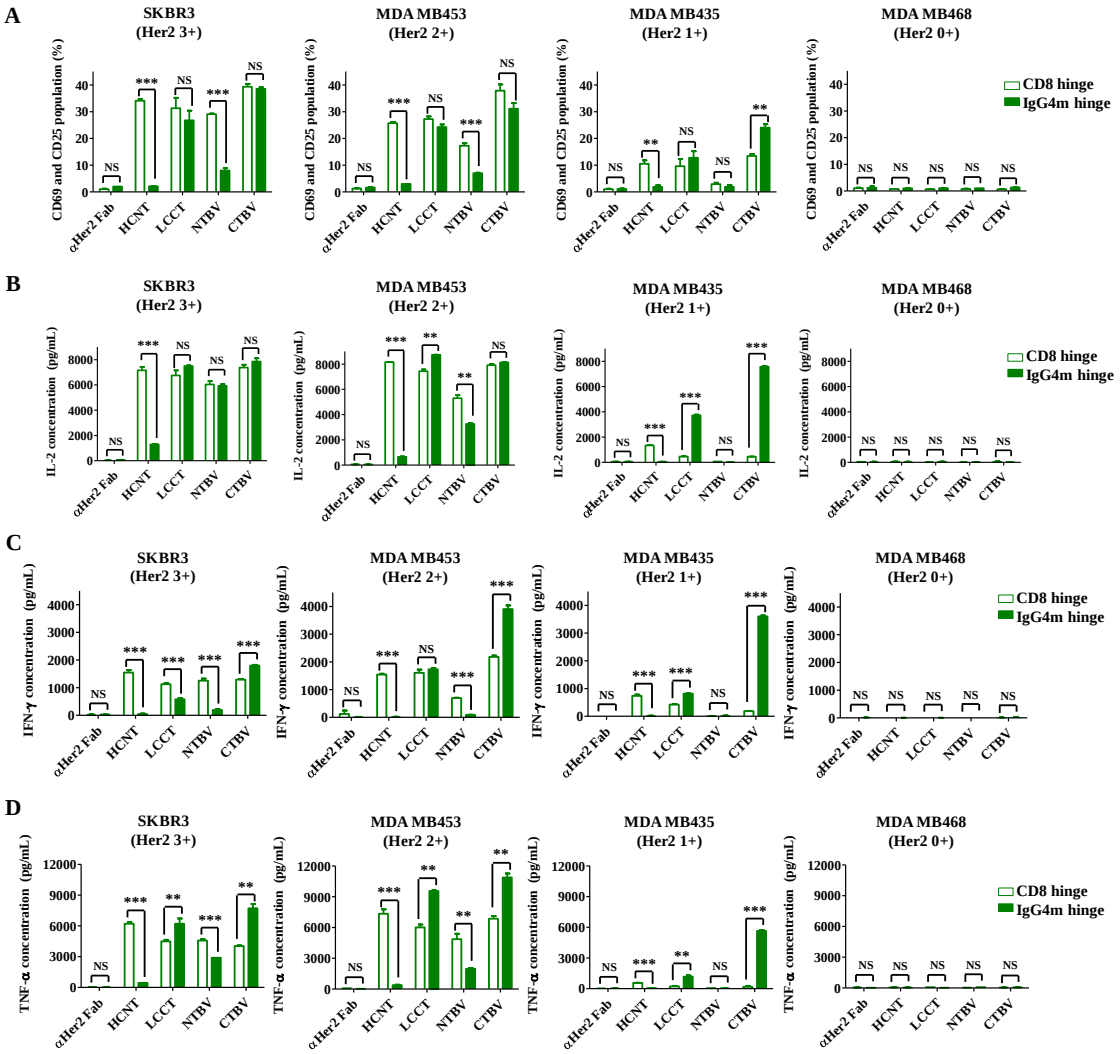
Supplementary Figure S7. Binding of different GCN4 switches to GCN4 specific CAR-T cells (both CD8 and IgG4m). Cells were incubated with indicated switches and detected with Alexa Fluor647 conjugated anti-human kappa chain antibody. The EC_{50} value was calculated with Graphpad Prism software.



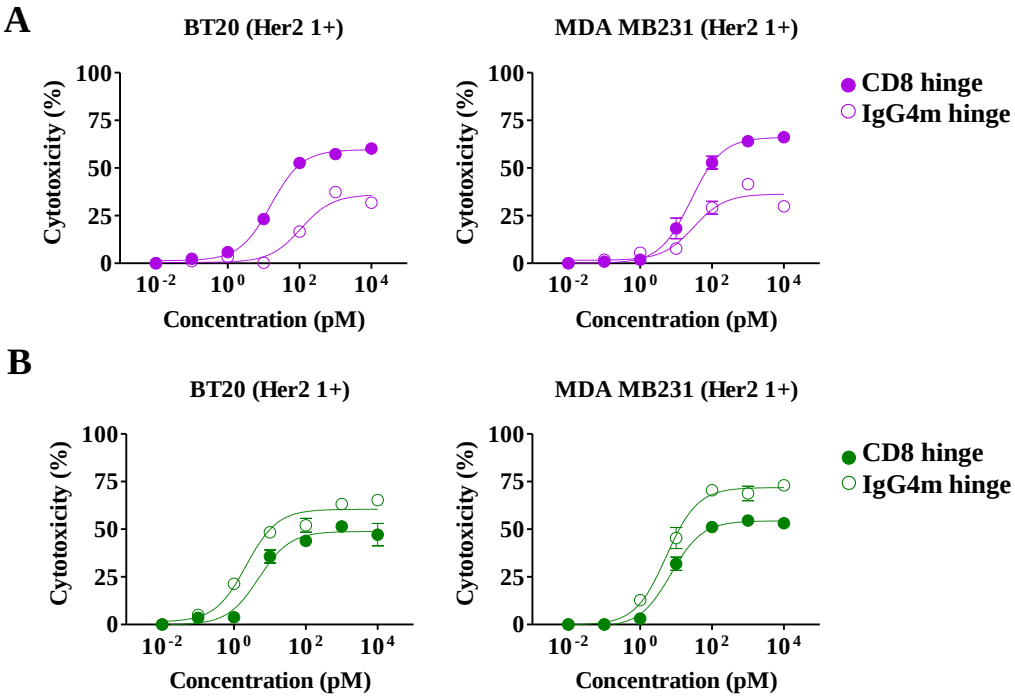
Supplementary Figure S8. Validation of binding specificity of FITC (A) or GCN4 (B) specific CAR-T cells with irrelevant tag-based antibody switches. Cells were incubated with indicated switches and detected with Alexa Fluor647 conjugated anti-human kappa chain antibody. No cross binding was observed to occur between GCN4-based sCAR-T cells and FITC-based switches or between FITC-based sCAR-T cells and GCN4-based switches.



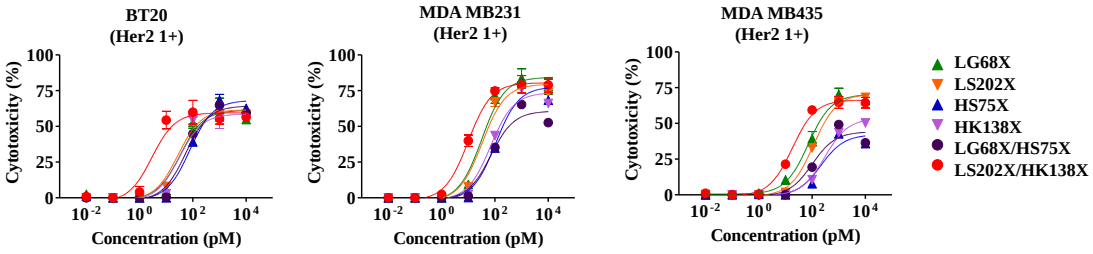
Supplementary Figure S9. sCAR-T activation by combinations of various switches and hinge length anti-FITC sCAR-T cells against different Her2 expressing cancer cells. FITC-based sCAR-T cells were cocultured with SKBR3, MDA MB453, MDA MB435 or MDA MB468 cells at E: T = 10: 1 with 100 pM of the corresponding switch for 24h. T cell activation was evaluated by flow cytometry with staining for CD69 and CD25 (A) redirected sCAR-T cells. IL-2 (B), IFN-γ (C) and TNF-α (D) levels from the incubation medium were measured by ELISA kit. ***= p<0.005, **=p<0.05 and NS=p>0.05 were calculated using the Student's t-test, and a p value < 0.05 was considered significant.



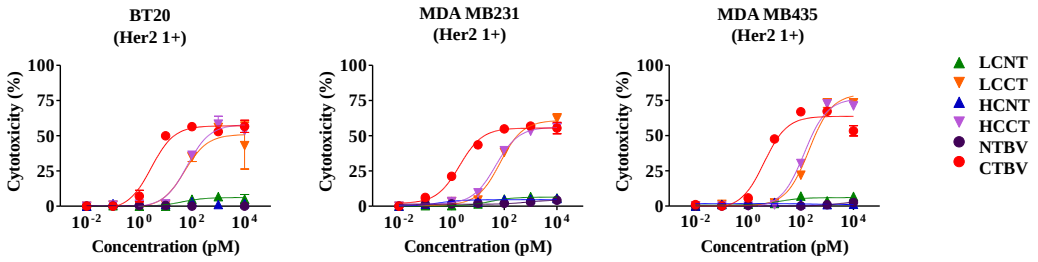
Supplementary Figure S10. sCAR-T activation by combinations of various switches and hinge length anti-GCN4 sCAR-T cells against different Her2 expressing cancer cells. GCN4-based sCAR-T cells were cocultured with SKBR3, MDA MB453, MDA MB435 or MDA MB468 cells at E: T = 10: 1 with 100 pM of the corresponding switches for 24h. T cell activation was evaluated by flow cytometry with staining for CD69 and CD25 (A) redirected sCAR-T cells. IL-2 (B), IFN-γ (C) and TNF-α (D) levels from the incubation medium were measured by ELISA kit. ***= p<0.005, **=p<0.05 and NS=p>0.05 were calculated using the Student's t-test, and a p value < 0.05 was considered significant.



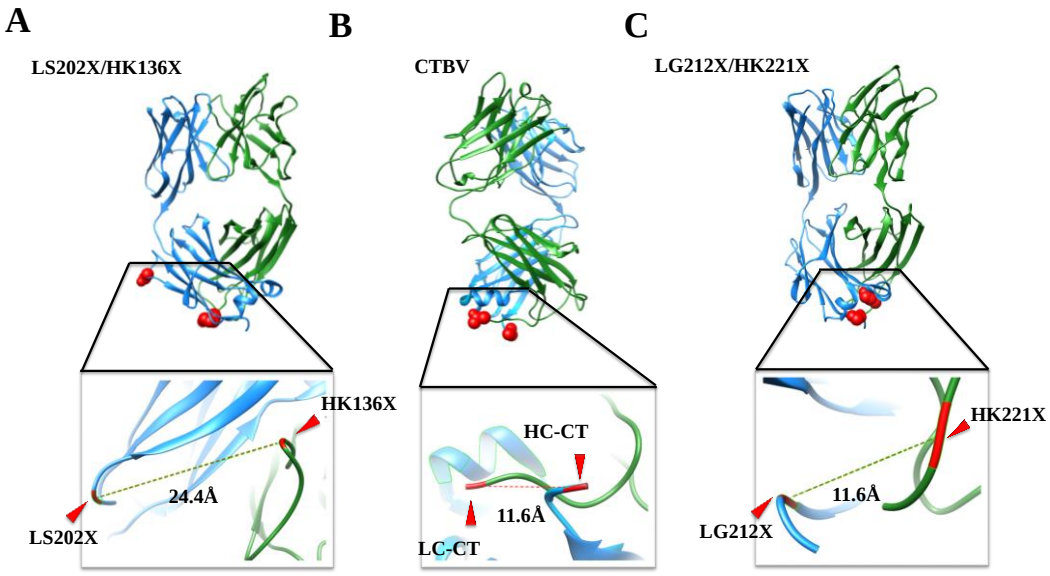
Supplementary Figure S11. *In vitro* cytotoxicity of sCAR-T cells with different hinge lengths on Her2 1+ BT20 and MDA MB231 cancer cells. Different concentrations of FITC LS202X/HK136X conjugate (A) and GCN4 CTBV fusion (B) were used to induce the corresponding CAR-T activity on target cells. Cytotoxicity was determined by measuring the amount of LDH released into cultured medium.



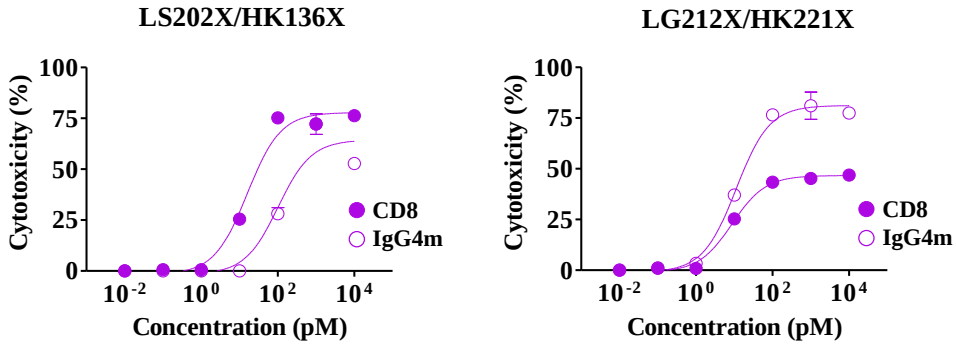
Supplementary Figure S12. *In vitro* cytotoxicity comparison of different FITC-based switches with anti-FITC CAR-T (CD8 hinge) cells against Her2 1+ cancer cells. The cytolytic activity was determined by measuring the amount of LDH released into cultured medium.



Supplementary Figure S13. The *in vitro* cytotoxicity comparison of different GCN4 switches redirected anti-GCN4 CAR-T (IgG4m hinge) cells against different Her2 1+ cancer cells. The cytolytic activity was determined by measuring the amount of LDH released into cultured medium.



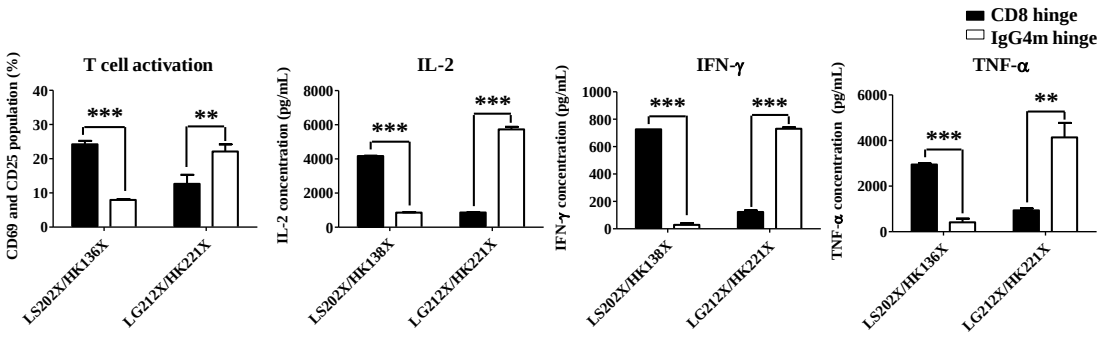
Supplementary Figure S14. The location of LS202X/HK136X (A), CTBV (B) and LG212X/HK221X (C) sites and relative distances between bivalent sites are shown on the 4D5 Fab structure (Protein Data Bank ID 1N8Z). Distance were calculated with UCSF Chimera 1.10.2.



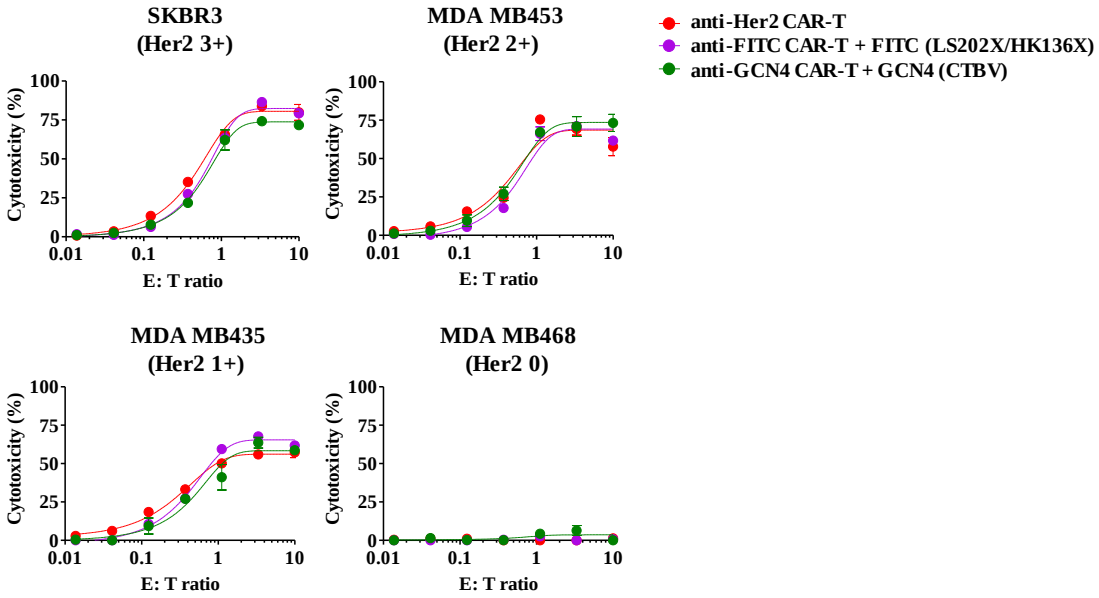
	Maximal killing (%)	EC_{50} (pM)
CD8	77.9±3.5	16.1±0.2
IgG4m	64.4±2.9	102.0±1.5

	Maximal killing (%)	EC_{50} (pM)
CD8	46.7±2.2	18.8±0.2
IgG4m	81.2±3.3	10.2±0.3

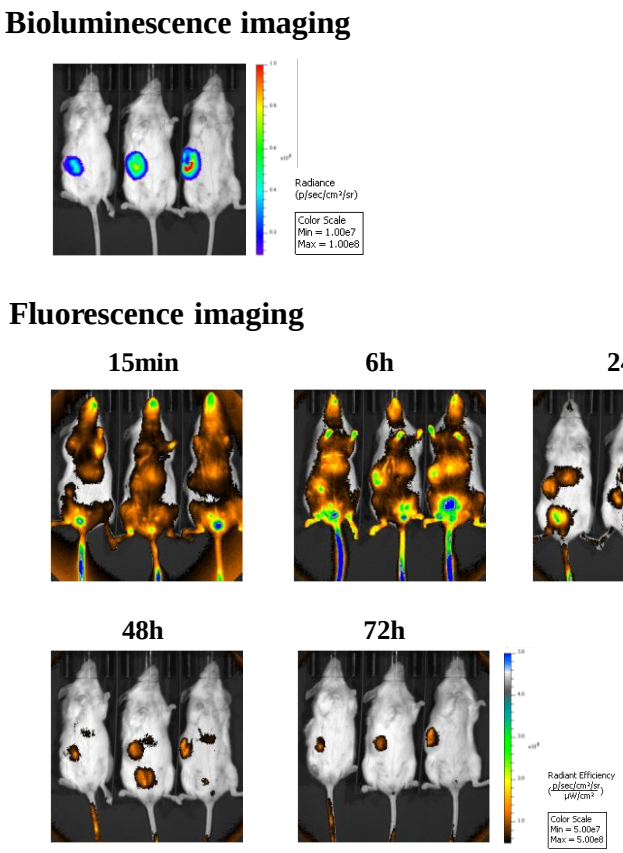
Supplementary Figure S15. Cytotoxicity of different bivalent FITC conjugates (LS202X/HK136X and LG212X/HK221X) with different anti-FITC CAR-T cells (CD8 or IgG4m hinge) using Her2 1+ MDA MB435 cancer cells. The cytolytic activity was determined by measuring the amount of LDH released into culture medium.



Supplementary Figure S16. sCAR-T activation by combinations of various switches and anti-FITC sCAR-T cells with different hinge lengths on Her2 1+ MDA MB435 cancer cells. T cell activation and cytokine release with 100 pM switch against MDA MB435 cells at an E: T ratio of 10: 1. ***= p<0.005, **=p<0.05 and NS=p>0.05 were calculated using the Student's t-test.



Supplementary Figure S17. *In vitro* comparison of anti-Her2 CAR-T cells with optimized anti-FITC and anti-GCN4 sCAR-T cells. The anti-Her2 CAR-T or sCAR-T cells were co-cultured with Her2 expressing cancer cells at the indicated E: T ratios with 50 pM of the corresponding bivalent switches. Target cell lysis was determined by LDH release.



Supplementary Figure S18. Optical imaging with IRDye800 labeled anti-Her2 Fab in MDA MB435/Her2 tumor-bearing mice. (A) 5×10^6 tumor cells were subcutaneous implanted in the right flank of female NSG mice. When tumor reached 500mm^3, bioluminescence imaging of tumors was taken after intraperitoneal injection of D-luciferin. (B) Whole-body fluorescence imaging of mice after intravenous administration of IRDye800 labeled Fab at 1nmol. Images were acquired by IVIS imaging at the indicated time point after injection of Fab. Arrows indicate the location of implanted tumor.