SI Appendix

Supplemental Methods and Figure Legends

Title: Switch-Mediated Activation and Retargeting of CAR T cells for B cell Malignancies.

Running title: Switch-Mediated Activation of CAR-T cells.

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Supplemental Methods

Switch cloning and expression

Gene fragments encoding the FMC63 (anti-CD19) (1) or Ofatumumab (OFA, antiCD20) (2) heavy or light chains, with or without the PNE engraftment shown in Figure 1 (and described below), were synthesized (IDT) and sub-cloned into the pFUSE vector (InvivoGen, CA). Switches were expressed by transient transfection in the FreeStyle 293 Expression System (Thermo Fisher Scientific, CA). Heavy chain Fab constructs were expressed in vectors in which the Fc region was removed and a stop codon included after the first cysteine of the hinge region. Full length IgG heavy chain constructs were expressed in vectors harboring the IgG1 sequence with the following mutations: E233P/L234V/L235A/ΔG236 + A327G/A330S/P331S to reduce the ADCC and CDC effector capacity of the Fc region (3, 4). Expression was carried out as follows: Briefly, HEK293F cells were transfected at a density of 1 x 10^6 cells/ml with a 1:2 ratio of plasmid DNA to 293fectin (Thermo). Small scale expressions (30-50ml) used a 1:1 ratio of heavy chain to light chain, whereas larger scale expressions (≥100ml) required a 3:2 ratio of heavy chain to light chain. Expression medium containing the secreted proteins was harvested 72 hours post-transfection by centrifugation at 400 x *g*. Fab or IgG switches were purified by Protein G or Protein A respectively (GE Healthcare Life Sciences, CA). Switches were eluted with 0.1 M glycine pH 2.8 and neutralized by the addition of 10% v/v 1M Tris-HCl pH 8. Switches were then buffer exchanged into phosphate buffered saline with PD10 desalting columns (GE Healthcare Life Sciences, CA) and filter sterilized for subsequent use. Switch integrity was confirmed by SDS page and QTOF analysis (see below).

Switch sequences

PNE peptide sequence:

NYHLENEVARLKKL

Linker sequence (all constructs except HCNT):

GGGGS

Linker HCNT only:

LVGEAAAKEAAAKA

Anti-CD19 FMC63 Light chain WT:

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQE

DIATYFCQQGNTLPYTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ

ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Anti-CD19 FMC63 Light chain LCC1 (graft and linker underlined):

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQE

DIATYFCQQGNTLPYTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ

ESVTEQDSGGGGSNYHLENEVARLKKLGGGGSDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Anti-CD19 FMC63 Heavy chain (Fab) WT:

EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLK

MNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV

SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

Anti-CD19 FMC63 Heavy chain (Fab) HCC1 (graft and linker underlined):

EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLK

MNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV

SWNSGALTSGVHTFPAVLQSGGGGSNYHLENEVARLKKLGGGGSLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK

KVEPKSC

Anti-CD20 OFA Light chain WT:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPED
FAVYYCQQRSNWPITFGQGTRLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Anti-CD20 OFA Heavy chain (Fab) WT:

EVQLVESGGGLVQPGRSLRLSCAASGFTFNDYAMHWVRQAPGKGLEWVSTISWNSGSIGYADSVKGRFTISRDNAKK
SLYLQMNSLRAEDTALYYCAKDIQYGNYYYGMDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

IgG1 Fc region (E233P/L234V/L235A/ΔG236 + A327G/A330S/P331S):

DKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST
YRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Immunogenicity assessment of PNE

An assessment of the immunogenic potential of the PNE in humans was carried out by contract research organization EpiVax (Providence, RI). To understand PNE immunogenicity in the context of an approved therapeutic antibody, the PNE was engrafted onto the N-terminus of the light chain or the heavy chain of the humanized Herceptin antibody sequence (anti-Her2, 4D5, Trastuzumab). Herceptin was used for the basis of this analysis instead of the FMC63 antibody because FMC63 is a murine variable sequence that may already contain T cell epitopes that could skew the analysis. For *in silico* analysis of overall immunogenicity, the PNE-engrafted heavy and light chain sequences were compared with the wild type 4D5 heavy and light chains to understand the relative contribution of immunogenicity of the PNE. Briefly,

sequences were parsed into overlapping 9-mer frames (including the PNE sequence, linker, Fab variable and Fab constant domains) and each frame evaluated for binding to the most common human MHC Class II alleles (T cell epitopes) as described (5). The score was adjusted by the presence of regulatory T cell epitopes (Tregitopes) as described (6). The result of this assessment showed that given that of all the submitted fusions, when paired with Herceptin, were not significantly greater than Herceptin Fab alone and fall on the low end of the immunogenicity scale. Based on this analysis we expect a low probability of anti-drug-antibody response to a PNE-graft in a humanized Fab-based switch.

Switch sequences

Anti-Her2 4D5 Light chain LCNT (graft and linker underlined):

NYHLENEVARLKKLGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPS
RFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR
EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Anti-Her2 4D5 Heavy chain HCNT (graft and inker underlined):

NYHLENEVARLKKLLVGEAAAKEAAAKAEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVAR
IYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSSASTKGPS
VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSC

Lentivirus generation and transduction of human PBMC

To create the sCAR lentiviral construct, the 52SR4 scFv (7) specific for GCN4(7P14P) was sub-cloned into a lentiviral vector downstream of a EF1a promoter and CD8 leader sequence, and upstream of a CD8 hinge, CD8 transmembrane, CD137(4-1BB) costimulatory and CD3ζ domains. Conventional CART-19 was constructed in similar fashion using the scFv from the anti-CD19 antibody FMC63 as previously described

(8). For the IgG4 and IgG4m constructs of the sCAR, the 45 amino acid CD8 hinge region was replaced with peptide sequences from IgG4 or IgG4m (below) (9). For virus production, HEK293FT cells (ATCC) were sub-cultured in DMEM supplemented with 10% heat inactivated FCS, 0.1mM MEM non-essential amino acids, 6mM L-glutamine, 1mM MEM sodium pyruvate, 1% penicillin/streptomycin and 1mg/ml Geneticin (all from Life Technologies). $5x10^6$ HEK293FT cells were transferred to a biocoat (Corning) 90mm petri dish in antibiotic free medium and transfected with pMDL (6 µg), pRev (6 µg), pMDG (2 µg) and the CAR containing lentiviral vector (7.5 µg) using the Lipofectamin-2000 transfection reagent as per manufacturer's instruction (Life Technologies). Cells were incubated with the DNA-lipofectamine complexes for 6 hours before the medium was replaced and the cells cultured for 48 hours. After 48 hours the lentivirus containing supernatant was removed, clarified of cell debris by centrifugation (3000 RPM at 4 ^{o}C) and stored at -80 ^{o}C. Peripheral blood mononuclear cells (PBMCs) were purified from fresh healthy human donor blood (TSRI Normal Blood Donor Services) by conventional gradient centrifugation using Ficoll-Pacque PLUS (GE healthcare) and were then rested in a tissue-culture flask in AimV medium at a concentration of $1x10^6$/ml. The non-adherent fraction was removed after 1 hour, washed with warm AimV medium and then activated with anti-CD3/CD28 human T cell activation and expansion beads (Life Technologies; 3;1 bead to PBMC ratio) and IL-2 (300 IU/ml) for 24 hours. Activated PBMC were then transduced with lentivirus containing serum mixed with protamine sulphate (Sigma; 10 µg/ml) by spinfection (1000 xg for 90 minutes). Following spinfection, cells were incubated for 24 hours with the virus before the medium was replaced with AimV. Between 50-75% of the PBMC expressed the CAR by day 12, and cells were maintained at a concentration of 0.5-$2x10^6$/ml at all times during expansion. CAR expression on CART19 cells was detected using the AF647-conjugated goat-anti-mouse IgG (H&L; Life Technologies) and CAR expression on sCAR-T cells was detected using the same antibody or a soluble AF488-conjugated synthetic PNE peptide (Anaspec).

CAR sequences

sCAR (CD8) sequence:

MALPVTALLLPLALLLHAARPDAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWVQEKPDHLFTGLIGGTNNRAPG
VPARFSGSLIGDKAALTITGAQTEDEAIYFCVLWYSDHWVFGGGTKLTVLGGGGGSGGGGSGGGGSGGGGSDVQLQE
SGPGLVAPSQSLSITCTVSGFLLTDYGVNWVRQSPGKGLEWLGVIWGDGITDYNSALKSRLSVTKDNSKSQVFLKMNSL
QSGDSARYYCVTGLFDYWGQGTTLTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPL
AGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLY
NELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT
KDTYDALHMQALPPR

CART-19 sequence:

MALPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPS
RFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSL
SVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKH
YYYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTC
GVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNL
GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD
ALHMQALPPR

sCAR (CD8) hinge:

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

sCAR (IgG4) hinge:

ESKYGPPCPSCP

sCAR (IgG4m) hinge:

ESKYGPPCPPCP

Western blot

The Western blot assay presented in **Fig S6B** was performed by incubating 10^6 CAR-T cells with 10^6 RS4;11 or K562 cells along with 10 nM of the LCNT switch in 1 ml of RPMI 5% FCM media for 30 minutes before being washed and lysed in ice cold RIPA buffer (supplemented with protease and phosphatase inhibitors; Sigma) for 15 minutes on ice, this was followed by a 15 minute centrifuge (12,000 xg at 4 ^{o}C). Reduced or non-reduced whole cell lysates (15 µg) were boiled in SDS for 10 minutes and then resolved on a 4-12% Bis-Tris pre-cast gel (Life Technologies). Proteins were transferred onto a nitrocellulose membrane using the iBlot system (Life Technologies) and then incubated in Odyssey blocking buffer (LiCor) for 1 hour at room temperature followed by mouse-anti-CD3ζ (BD; 1mg/ml) or rabbit-anti-GAPDH over night at 4 ^{o}C. The membrane was then washed using PBS (0.01% Tween-20; Sigma) and incubated with the IRDye-688-conjugated donkey anti-mouse (green bands in **Fig S6B**) or IRDye-800-conjugated donkey-anti-rabbit (red bands in **Fig S6B**) antibodies (LiCor) for 1 hour at room temperature before being washed several times and imaged using the LiCor scanner.

Live cell imaging

The live cell imaging data presented in **Fig S8D** was performed using the IncuCyte ZOOM® microscope (Essen BioScience). Cytotoxicity assays were set up using CAR-T cells with RS4;11 or Nalm-6 cells as previously described but with the exception of using a flat bottomed 96-well plate. Plates were imaged every 30 minutes at 10 x magnification for 16-24 hours. Nalm-6 cells were used for clustering analysis because they expressed GFP. RS4;11 cells were used to detect cytotoxicity using the Cell Tox Green® (Promega), which was added at a 1:4000 dilution. T cells were labelled with the CellVue Claret® Far Red fluorescence stain (Sigma; Red). Images were analyzed using software provided by the manufacturer and

using the following settings for Cell Tox Green® staining: Top-hat (400 µm and 2.0 GCU) with edge sensitivity of -38 and filters of 2000 μm^2 area, 70.0 mean intensity and 2E5 integrated intensity.

Mass spectrometry

Mass spectrometry analysis of protein samples was acquired on an Agilent 6520 Accurate-Mass Quadrupole-Time-of-Flight (Q-TOF) mass spectrometer equipped with an electrospray (ESI) ionization source and liquid chromatography stack (Agilent). Ionization settings were: capillary voltage 3500 kV; positive mode; fragmentor voltage 200 V; drying nebulizer gas, 350 °C. Instrument was configured to standard 2 GHz, extended dynamic range and deconvolution was performed by Agilent MassHunter Qualitative Analysis software using the maximum entropy setting. To separate antibodies a 2.1x150 mm, C8 reverse phase, wide pore (5 µm, 300 Å, Phenomenex) column was used. The gradient was: water (A)/acetonitrile (B) (0.1% formic acid) gradient (2% B for 3 minutes, followed by a 2-95% B gradient over 15 minutes, and 95% B for 7 minutes). Results from mass spectrometry can be found tabulated in **Fig S2B**.

Supplemental Figure Legends

Supplementary Figure 1: Schematic of direct versus switchable CAR-T cells. **(A)** Conventional CAR-T cells (left) utilize an scFv that directly targets tumor associated cell surface antigens. Upon binding, signaling through the CAR drives an effector response that leads to the killing of target cells and the propagation of an anti-tumor immune response. The same response is achieved with switchable CAR-T cells (sCAR-T cells; right), however, antigen recognition is uncoupled from T cell signaling by the introduction of an antibody based "switch" that targets tumor associated antigens. The switch is then recognized by the sCAR-T cells through a high affinity scFv that binds to a peptide neo-epitope (PNE) that is engrafted onto the switch. Thus sCAR-T cells only target antigens in the presence of a PNE engrafted switch and offer controlled activation, deactivation and re-targeting of CAR-T cell therapy. The LCCT switch is shown as an example.

(B) The relative immunogenicity of PNE engrafted Herceptin heavy or light chains (HCNT and LCNT respectively) was measured *in silico* by EpiVax and showed minimal increases compared with the wild type heavy and light chains (HCWT and LCWT respectively). Using this analysis, scores of >20 are considered to be potentially immunogenic.

Supplementary Figure 2: Characterization of switches. (A) Denaturing SDS-page gel depicting the anti-CD19 Fab, anti-CD19 IgG switches derived from the FMC63 clone under non-reducing (top) and reducing (bottom) conditions. The switches with monovalent engrafting positions on the heavy and light chains resolve into two bands on the reduced gel (e.g. LCNT), whereas the bivalent (e.g. NTBV) and wildtype antibodies overlap and appear as one band when reduced. **(B)** Table of high resolution anti-CD19 Fab switch masses obtained on an Agilent Quadruple Time-of-Flight (QTOF) mass spectrometer. Masses were deconvoluted from charge envelopes using Agilent Qualitative Analysis software with the Bioconfirm plugin. In general accuracy was ± 2 Da. **(C)** SDS-page gel showing the anti-CD20 Fab switches derived from the ofatumumab clone under non-reducing (top) and reducing (bottom) conditions with accompanying table of high resolution QTOF analysis.

Supplementary Figure 3: thermal stability and antigen binding of switches: (A) Thermal stability analysis for the anti-CD19 Fab and IgG switches with a table showing the melt temperatures of the WT, LCNT, HCNT, NTBV, LCC1, HCC1, C1BV, LCCT, HCCT and CTBV switches, in both IgG and Fab formats, using the Protein Thermal Shift™ kit (Thermo). The first melt temperature indicates the Fab melt, where all PNE engrafts resided. The second melt temperature indicates the Fc region and is only present for full length IgG constructs. Representative analysis is shown on the right. **(B)** Switch binding to $CD19^+$ RS4;11 cells was measured by incubating the cells with 100 nM to 0.1 pM of each switch for 30 minutes at 4 ^oC in FACs buffer (PBS, 1% FCS, 1 mM EDTA). This was followed by two wash steps and then a 30 minute incubations

with a PE-conjugated goat-anti-human-kappa chain antibody (50 µg/ml; Southern Biotech) at 4 ^{o}C; cells were analyzed by flow cytometry and data are presented as total MFI of switch binding. The EC_{50} of binding for each switch against RS4;11 cells is indicated in the legend. The binding specificity of the LCNT IgG and Fab switches was tested against $CD19^{-}$ K562 cells, this data demonstrates that the N-terminally engrafted switches retained their specificity for CD19.

Supplementary Figure 4: expression of CAR and binding to switch. **(A)** An AF647-conjugated goat-anti-mouse IgG (H&L) antibody (100 µg/ml; Thermo) was used to show surface expression of CART19 and the CD8 sCAR by flow cytometry (Accuri C6; BD); alternatively an AF488-conjugated PNE soluble peptide (10 µg/ml; Anaspec) was used to detect surface expression of the sCAR by flow cytometry. **(B)** Switch binding to CD8 sCAR-T cells but not mock transduced PBMC (sCAR negative) was measured for each switch using the method described in **Sup Fig. 3B**. The EC_{50} of binding for each switch is indicated in the graph key.

Supplementary Figure 5: *in vitro* sCART cell activity. (A) Cytotoxicity of CD8 sCAR-T cells RS4;11 cells using IgG based switches as described in the methods, after 20-24 hours the supernatants were assayed to detect LDH release as a proxy for target cell lysis. The EC_{50} values for each switch are denoted within parentheses and unless otherwise stated all *in vitro* cytotoxicity assays were conducted at an effector: target cell ratio of 10: 1. **(B)** This assay was repeated using $CD19^{-}$ K562 cells with the IgG switches. **(C)** CD8 sCAR-T cells from a cytotoxicity assay using Fab switches and either RS4;11 or K562 target cells were stained with anti-CD3 (APC), anti-CD25 (PE) and anti-CD69 (PerCP) for 30 minutes at 4^{o}C and the washed before being analyzed by flow cytometry to determine the proportion of T cells with an activated phenotype ($CD25^{+}CD69^{+}$) within the $CD3^{+}$ population.

Supplementary Figure 6: characterization of the sCAR hinge modifications. **(A)** T cells expressing the IgG4 or IgG4m sCAR designs were incubated with an APC-conjugated goat-anti mouse IgG (H&L) (100 µg/ml; top) or the AF488-conjugated PNE (10 µg/ml; bottom) for 30 minutes at 4^{o}C and then analyzed by flow cytometry: the reduced binding of the goat-anti mouse IgG (H&L) polyclonal antibody to the IgG4 and IgG4m sCAR-T cells compared with the CD8 sCAR and CART19 **(Fig S4A)** is likely due to there being less accessible binding epitopes with the shortened sCAR; reflecting this, binding of the AF488-PNE peptide is higher. **(B)** Western blot showing the dimerization of the chimeric antigen receptors of sCAR-T cells and CART19 cultured with RS4;11 (R) or K562 (K) cells. Anti-human CD3ζ (green) was used to detect endogenous CD3ζ (~20 KDa) and the CD3ζ present in the CAR signaling domain (monomers = 50-60 KDa; dimers (white arrow) = 100-125KDa): non-reduced samples are shown on the left and reduced samples on the right. GAPDH (Red) was used as an endogenous control. The differences in GAPDH levels of the RS4;11 and K562 cell lines reflects the relative size of these cells. CD3 zeta staining here is comparable to that found in (10-12).

Supplementary Figure 7: on-target cytotoxicity assays using the IgG4 and IgG4m sCAR-T cells. (A) IgG4 and IgG4m sCAR-T cells were cultured with RS4;11 cells and a panel of anti-CD19 Fab switches (LCNT switch data is presented in **Fig 2A**) at concentrations ranging from 1000 pM to 0.1 pM. Cytotoxicity was determined using the LDH release assay, EC_{50} values are denoted within parentheses.

Supplementary Figure 8: sCAR hinge modifications enhance activity. **(A)** IgG4 and **(B)** IgG4m sCAR T cells were cultured with K562 cells and the WT, LCNT, HCNT, NTBV, LCC1 or HCCT switches for 20-24 hours and cytotoxicity was analyzed by LDH release assay as described in methods. **(C)** IL-2 production was measured by CBA (BD). **(D)** IgG4m sCAR-T cells were cultured with RS4;11 cells, 1 nM to 0.1 pM of the LCNT switch and increasing concentrations of free soluble PNE (as indicated). Cytotoxicity was determined using the

LDH release assay using the supernatant from these assays. Supernatant samples from the wells where the IgG4m sCAR-T cells received 1 nM of the LCNT switch were used to determine cytokine release (CBA; BD) and the activation status of these cells ($CD25^{+}CD69^{+}$) were measured by flow cytometry. Finally, live cell imaging (Incucyte, Essen) was used to quantify the killing of RS4;11 cells by the IgG4m sCAR-T cells in the presences of the Cell Tox Green® cell viability dye. In some of these assays a non-binding mutated version of the PNE was used as a control (PNE-X; grey line/bar; peptide sequence = AAAAENEVARLKKL). This data demonstrated that the activity of IgG4m sCAR-T cells could be controlled *in vitro* by the addition of free PNE.

Supplementary Figure 9: sCAR-T cell expansion and localization during tumor clearance. NSG mice were inoculated with $CD19^{+}$ Nalm-6 as described in the methods section. 6 days later, mice were randomly sorted into groups with an even distribution of disease burden and then into 5 cohorts per group. One cohort from each group was culled every day for analysis. Each group received $40x10^{6}$ CART-19, sCAR-T cells with (+) LCNT Fab switch, or no T cells (PBS group). A fourth group consisted of two cohorts (n=3) of mice injected *I.V.* with sCAR-T cells without (-) LCNT Fab switch that were culled on the first day (6 hours post T cell infusion) and on the final day. LCNT Fab dosing (0.5 mg/kg) in the indicated group was carried out at alongside the initial T cell infusion and then daily for 5 days. Luminescence was measured at 8 hours and subsequently every 24 hours as indicated. Switch dosing, imaging and culling at the indicated itme points were done at consistent times of day throughout the experiment to minimize variance. **(A)** The spleen, lungs, liver and blood of NGS mice engrafted with Nalm-6 cells were harvested and then passed through a 40 µm cell trainer (BD) using the plunger of a 5 mL syringe to produce a single cell suspension. Lung samples were digested using 1 mg/ml Liberase LT (Roche) and 50 µg/ml DNaseI (Sigma) for 45 minutes shaking at 37 ^{o}C prior to homogenization. Red blood cells were then lysed using red cell lysis buffer (eBiosciences). Cells were then seeded into a round-bottom 96 well plate (10^{6} cells per well) and

stained with anti-CD3 (APC), anti-CD4 (PerCP) and anti-CD8-PE in 100µL of FACS buffer for 30 minutes at 4 ^{o}C. The cells were then washed twice and analyzed by flow cytometry (LSR-II; BD). Nalm-6 cells were detected in the lung and liver by day 5, but not in the spleen or blood; data are presented as % of lymphocytes ± SEM. **(B)** Prior to infusion, the T cells were labelled with the eFluor® 450 proliferation tracker dye as per the manufacturer's instructions (eBiosciences; 0.5 µM per 10^6 cells) which enabled cell proliferation to be analyzed by flow cytometry *ex vivo.* A representative histogram from the liver where IgG4m sCAR-T cells (+) or (-) the LCNT switch is shown with a blue or green trace respectively, data from day 5 or day 1 of this model are represented by solid and dashed traces respectively. **(C)** eFluor® 450 dilution in CART19 and IgG4m sCAR-T cells (+) or (-) the LCNT switch was measured by flow cytometry over 5 days in the spleen, lung and liver; data are presented as mean fluorescence intensity (MFI) of the proliferation dye ± SEM.

Supplementary Figure 10: T cell analysis in the peripheral blood. **(A)** Representative IVIS images from **Fig. 5A**. **(B)** $CD3^+CD4/8^+$ T cells were enumerated in the blood of the NSG mice (represented in **A** and **Fig. 5A**) every 10 days from days 10-70. For this, 10µL of CountBright beads (BD) were added to 30µL of heparinized blood along with 5µL of the anti-CD3, CD4 and CD8 antibodies used in Fig. S10. Samples were incubated at 4^{o}C for 30 minutes before the cells were incubated with 1mL of BD FACS lysing solution for 30 minutes at room temperature in the dark. Cells were then analyzed by flow cytometry. **(C)** Representative IVIS images from **Fig. 5D**. **(D)** Flow cytometry gating strategy to identify $CD3^+$ T cells that expressed CD4, CD8, CD45RA or CD62-L as presented in **Fig 5E**.

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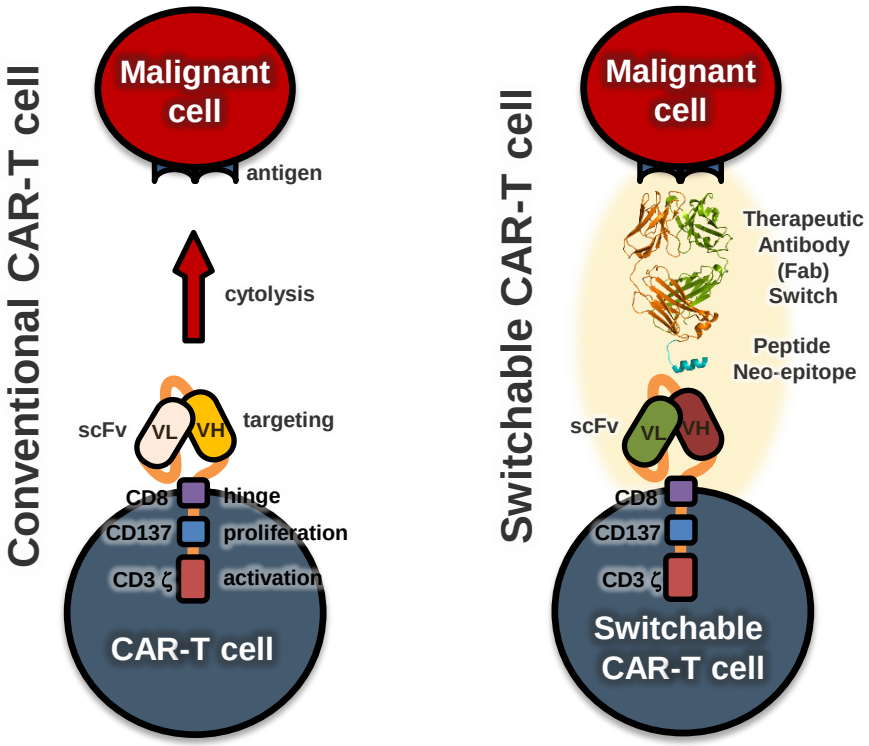
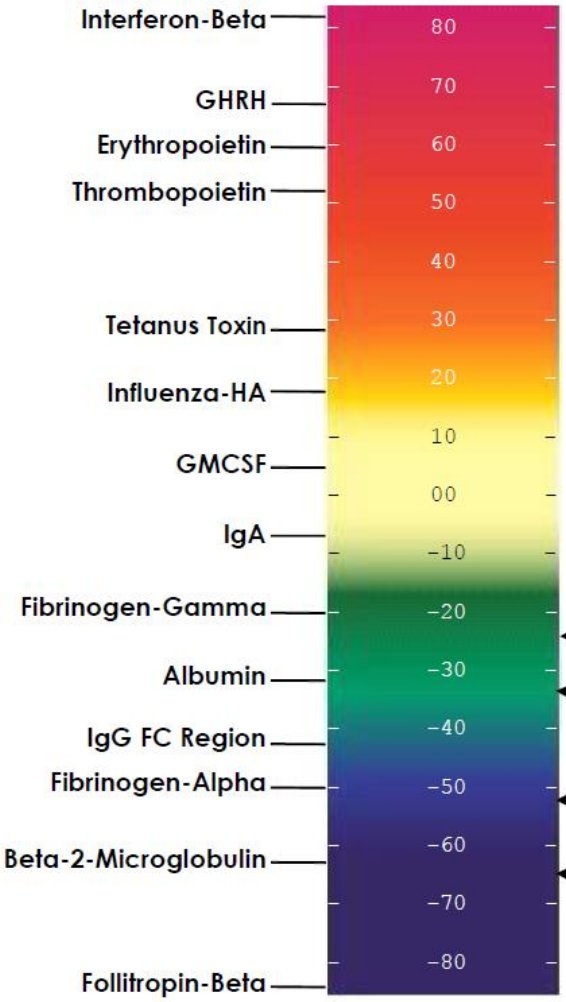
Figure S1

A



B

EpiMatrix Protein Immunogenicity Scale



LCNT (-25.17)

LCWT (-34.97)

HCNT (-53.73)

HCWT (-64.47)

PNE peptide:
NYHLENEVARLKKL

Peptide sequences analyzed:

LCNT: N-terminal graft of the PNE to the 4D5 light chain

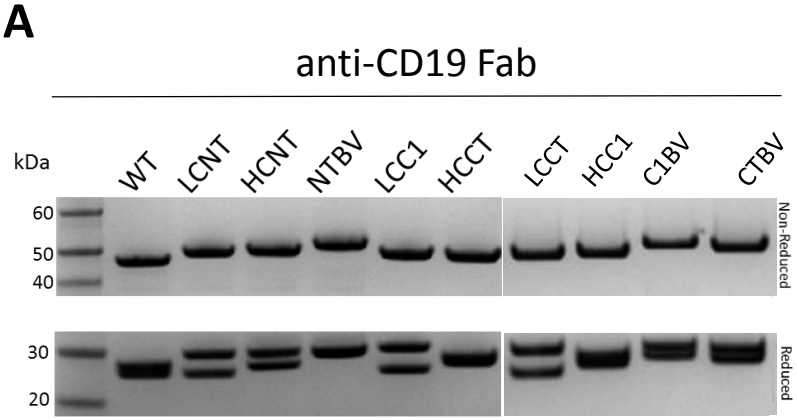
LCWT: wild type 4D5 light chain

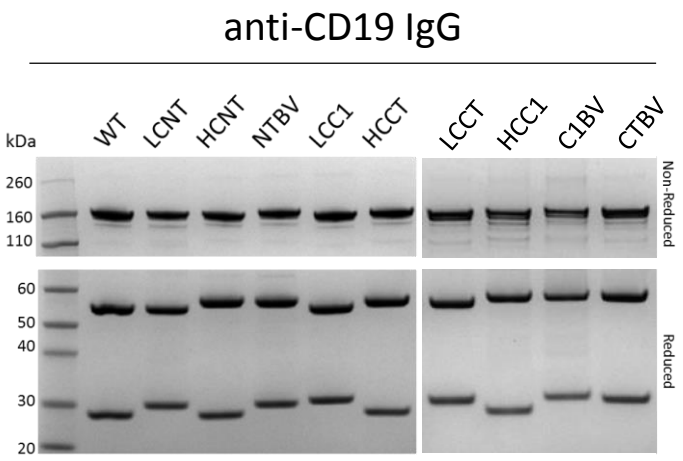
HCNT: N-terminal graft of the PNE to the 4D5 heavy chain

HCWT: wild type 4D5 heavy chain

Figure S2

A



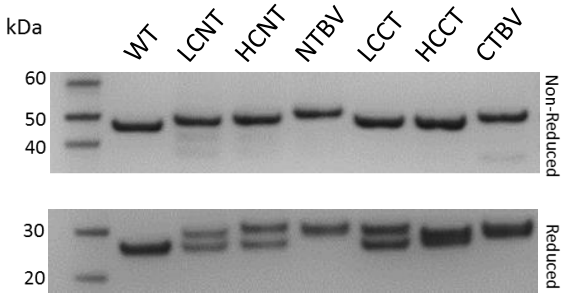


B

High resolution mass (QTOF) analysis for anti-CD19 Fab switches

FMC63	Protein mass (Da)		Error	
Protein	**Expected**	**Found**	**ppm**	**Da**
WT	47321.99	47323.03	-21.98	1.04
LCNT	49346.25	49346.93	-13.78	0.68
HCNT	50312.44	50314.54	-41.74	2.10
NTBV	52336.70	52339.41	-51.78	2.71
LCC1	49533.36	49534.46	-22.21	1.10
HCC1	49517.41	49518.86	-29.28	1.45
C1BV	51728.78	51730.26	-28.61	1.48
LCCT	49160.00	49161.32	-26.85	1.32
HCCT	49346.25	49347.13	-17.83	0.88
CTBV	51184.26	51185.83	-30.67	1.57

C



High resolution mass (QTOF) analysis for anti-CD20 Fab switches

OFA	Protein mass (Da)		Error	
Protein	**Expected**	**Found**	**ppm**	**Da**
WT	47447.42	47448.52	-29.51	1.4
LCNT	49471.38	49473.84	-49.73	2.46
HCNT	50437.57	50439.74	-43.02	2.17
NTBV	52461.83	52465.00	-60.42	3.17
LCCT	49471.38	49472.77	-28.10	1.39
HCCT	49471.38	49473.05	-33.76	1.67
CTBV	51495.64	51497.13	-28.93	1.49

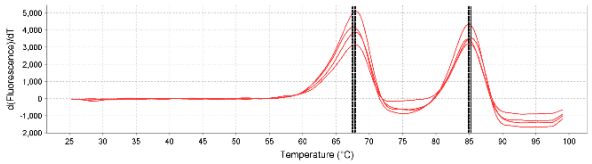
Figure S3

A Thermal melt curves for anti-CD19 Fab and IgG switches

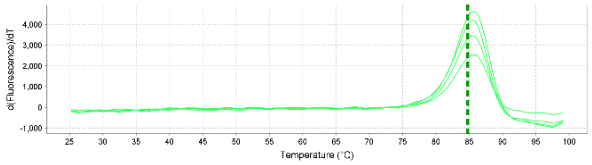
Switch	Sample	T_m 1 (Fab)	St. Error	T_m 2 (Fc)	St. Error
Fab	WT	84.77	0.19	--	--
	LCNT	83.93	0.16	--	--
	HCNT	83.86	0.22	--	--
	NTBV	82.68	0.16	--	--
	LCC1	76.75	0.25	--	--
	HCCT	84.33	0.19	--	--
	LCCT	73.96	0.08	--	--
	CTBV	73.29	0.1	--	--
	HCC1	82.98	0.19	--	--
	C1BV	75.25	0.12	--	--
IgG	WT	85	0.09	67.78	0.09
	LCNT	84.26	0.09	66.85	0.18
	HCNT	84.45	0.09	67.87	0
	NTBV	83.34	0.23	67.87	0
	LCC1	77.13	0	67.97	0.09
	HCCT	85.1	0.19	67.5	0
	LCCT	73.42	0	70.92	2.89
	CTBV	73.51	0.19	67.68	0.21
	HCC1	83.6	0.21	67.68	0.21
	C1BV	72.21	0.06	68.23	0

Example of melt curves depicting Fab and Fc melts

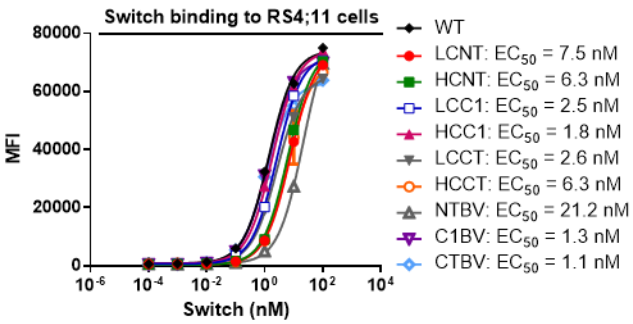
Representative anti-CD19 IgG melt curve



Representative anti-CD19 Fab melt curve



B Biding of switches to CD19⁺ RS4;11 cells but not CD19⁻ K562 cells



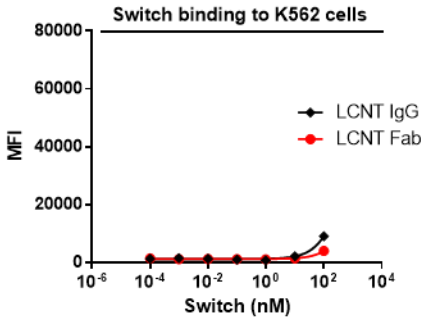
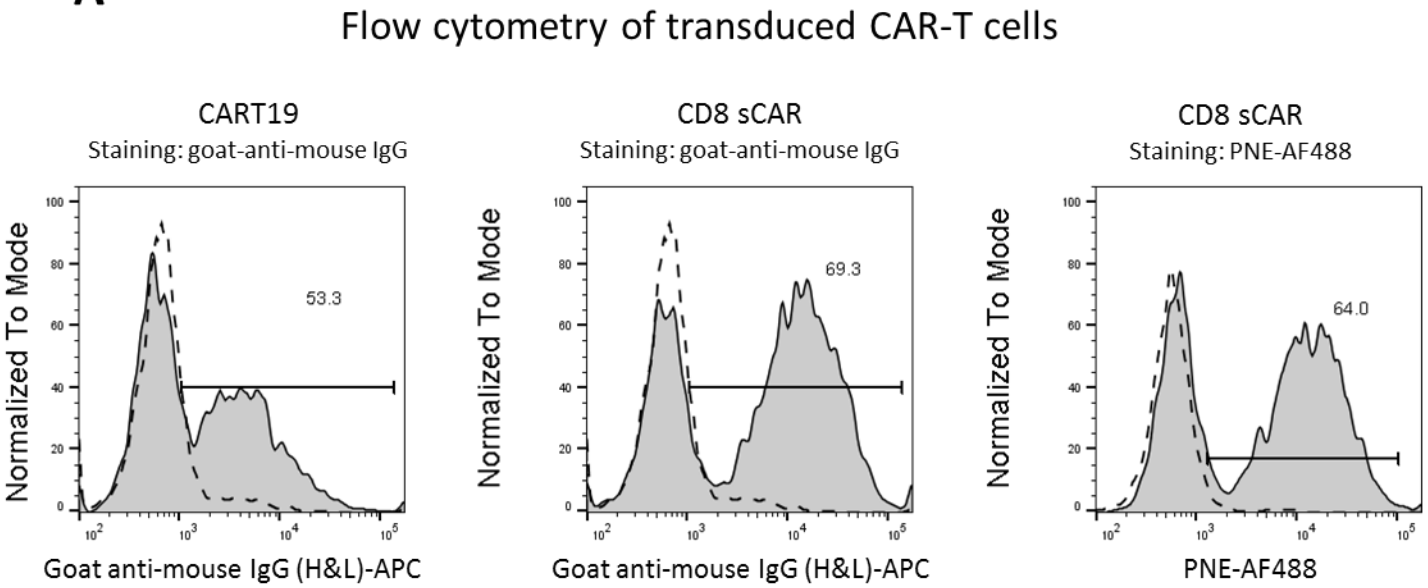


Figure S4

A


Flow cytometry of transduced CAR-T cells
CART19
Staining: goat-anti-mouse IgG
CD8 sCAR
Staining: goat-anti-mouse IgG
CD8 sCAR
Staining: PNE-AF488
Normalized To Mode
53.3
69.3
64.0
Goat anti-mouse IgG (H&L)-APC
Goat anti-mouse IgG (H&L)-APC
PNE-AF488

B

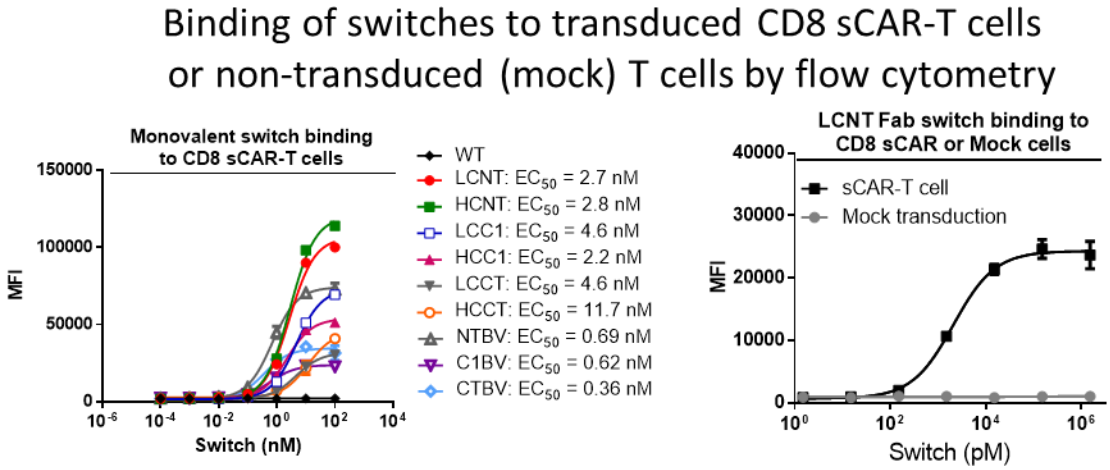
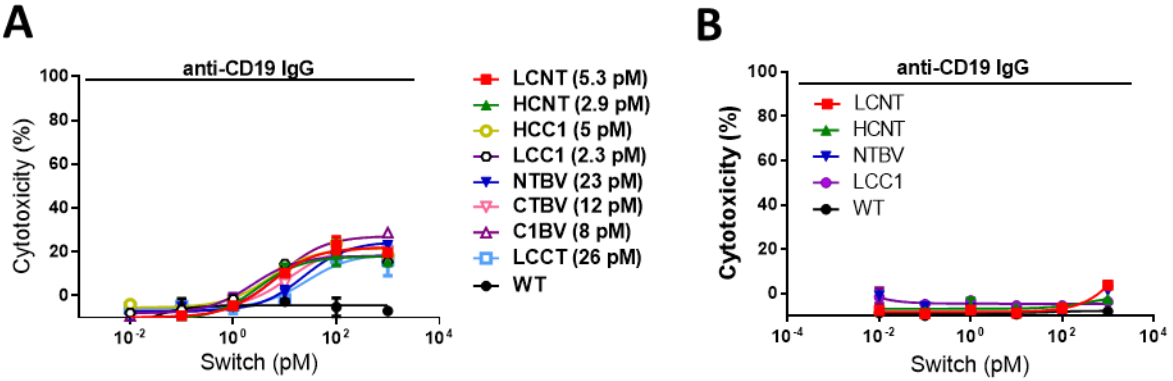

Binding of switches to transduced CD8 sCAR-T cells
or non-transduced (mock) T cells by flow cytometry
Monovalent switch binding
to CD8 sCAR-T cells
MFI
Switch (nM)
WT
LCNT: EC50 = 2.7 nM
HCNT: EC50 = 2.8 nM
LCC1: EC50 = 4.6 nM
HCC1: EC50 = 2.2 nM
LCCT: EC50 = 4.6 nM
HCCT: EC50 = 11.7 nM
NTBV: EC50 = 0.69 nM
C1BV: EC50 = 0.62 nM
CTBV: EC50 = 0.36 nM
LCNT Fab switch binding to
CD8 sCAR or Mock cells
sCAR-T cell
Mock transduction
MFI
Switch (pM)

Figure S5

Cytotoxicity of anti-CD19 IgG switch designs against RS4;11 and K562



Quantification of activation markers for anti-CD19 Fab switches against RS4;11 or K562

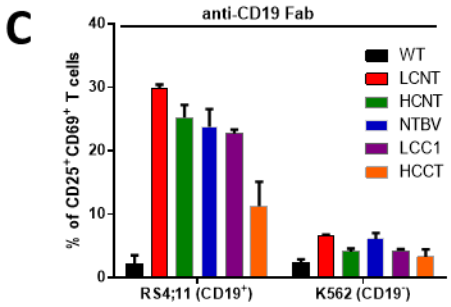
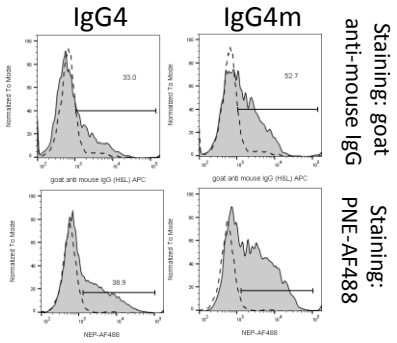


Figure S6

A

Flow cytometry of transduced CAR-T cells



B

Western blot analysis of hinge designs

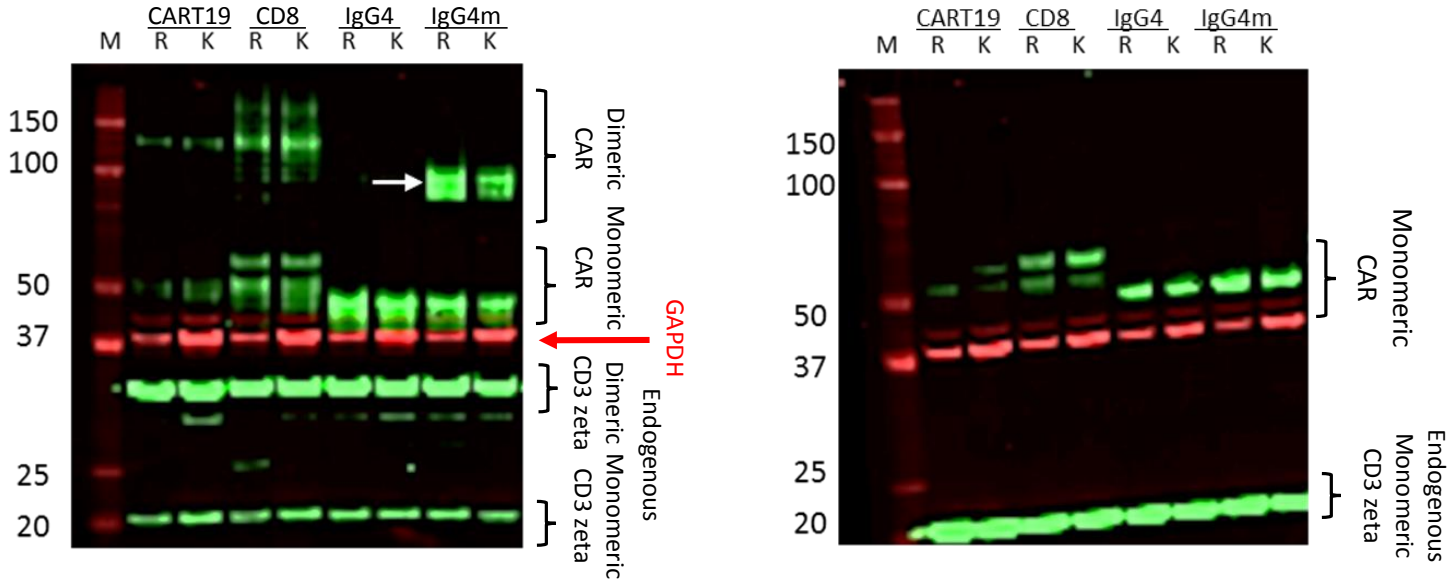


Figure S7

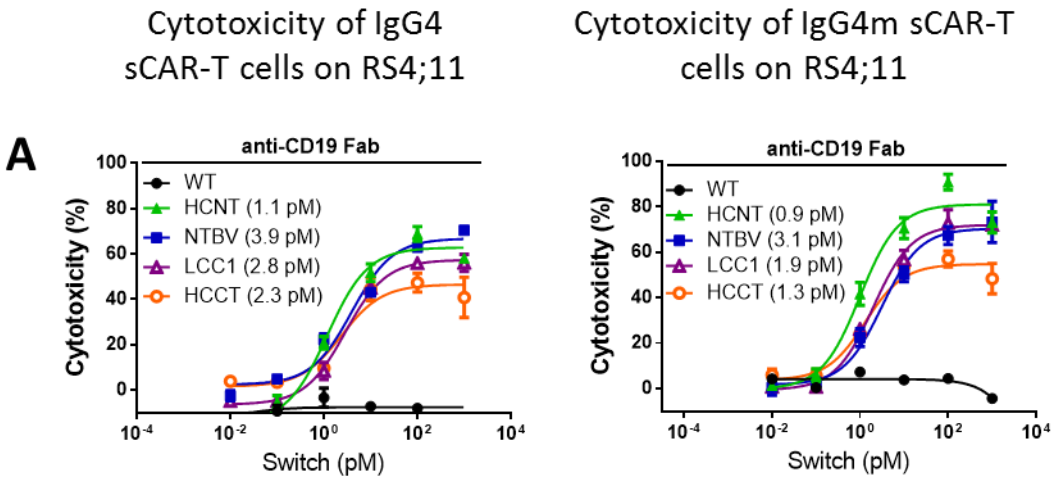
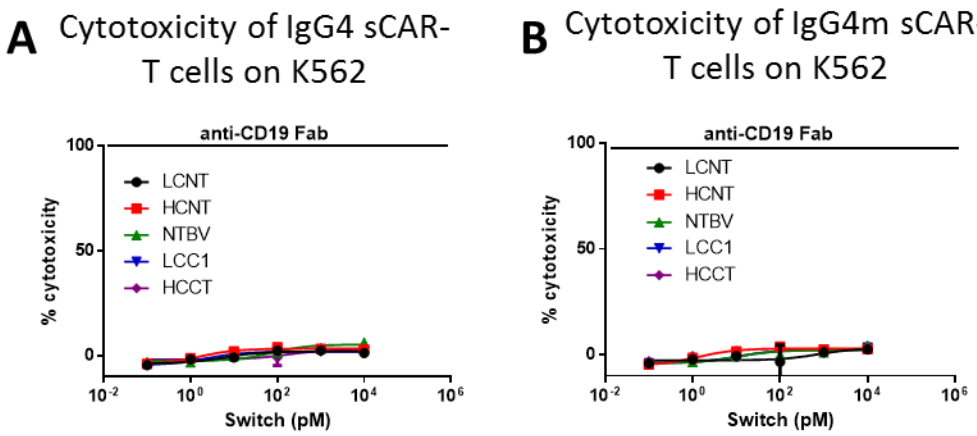
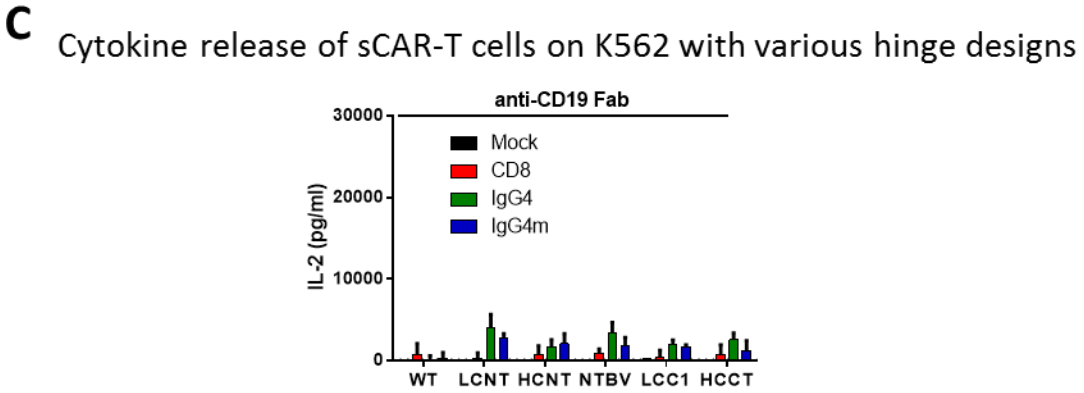
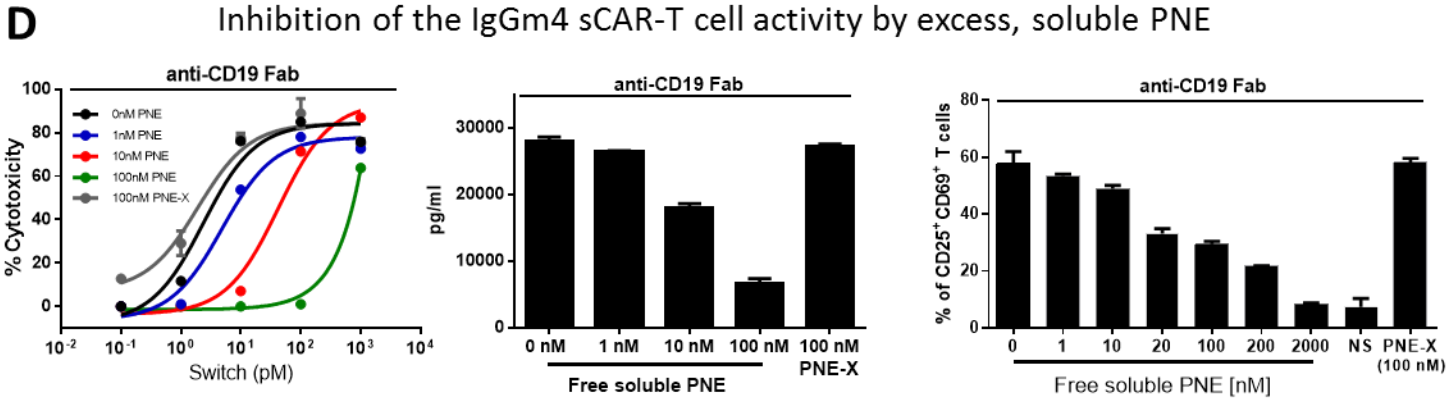

A
Cytotoxicity of IgG4 sCAR-T cells on RS4;11
anti-CD19 Fab
WT
HCNT (1.1 pM)
NTBV (3.9 pM)
LCC1 (2.8 pM)
HCCT (2.3 pM)
Cytotoxicity (%)
Switch (pM)
Cytotoxicity of IgG4m sCAR-T cells on RS4;11
anti-CD19 Fab
WT
HCNT (0.9 pM)
NTBV (3.1 pM)
LCC1 (1.9 pM)
HCCT (1.3 pM)
Cytotoxicity (%)
Switch (pM)

Figure S8


A
Cytotoxicity of IgG4 sCAR-T cells on K562
B
Cytotoxicity of IgG4m sCAR-T cells on K562
anti-CD19 Fab
LCNT
HCNT
NTBV
LCC1
HCCT
% cytotoxicity
Switch (pM)


C
Cytokine release of sCAR-T cells on K562 with various hinge designs
anti-CD19 Fab
Mock
CD8
IgG4
IgG4m
IL-2 (pg/ml)
WT
LCNT
HCNT
NTBV
LCC1
HCCT


D
Inhibition of the IgGm4 sCAR-T cell activity by excess, soluble PNE
anti-CD19 Fab
0nM PNE
1nM PNE
10nM PNE
100nM PNE
100nM PNE-X
% Cytotoxicity
Switch (pM)
pg/ml
0 nM
1 nM
10 nM
100 nM
100 nM PNE-X
Free soluble PNE
% of CD25+CD69+ T cells
0
1
10
20
100
200
2000
NS
PNE-X (100 nM)
Free soluble PNE [nM]

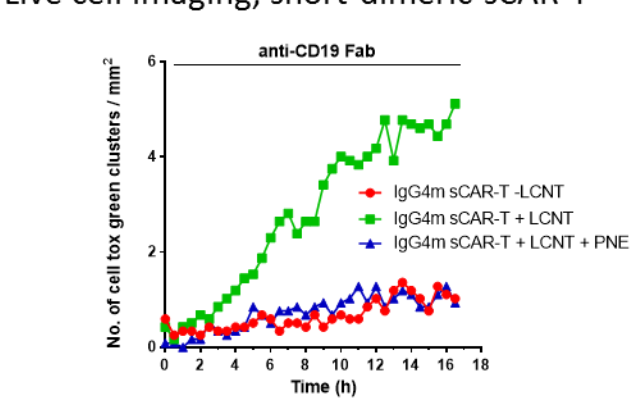
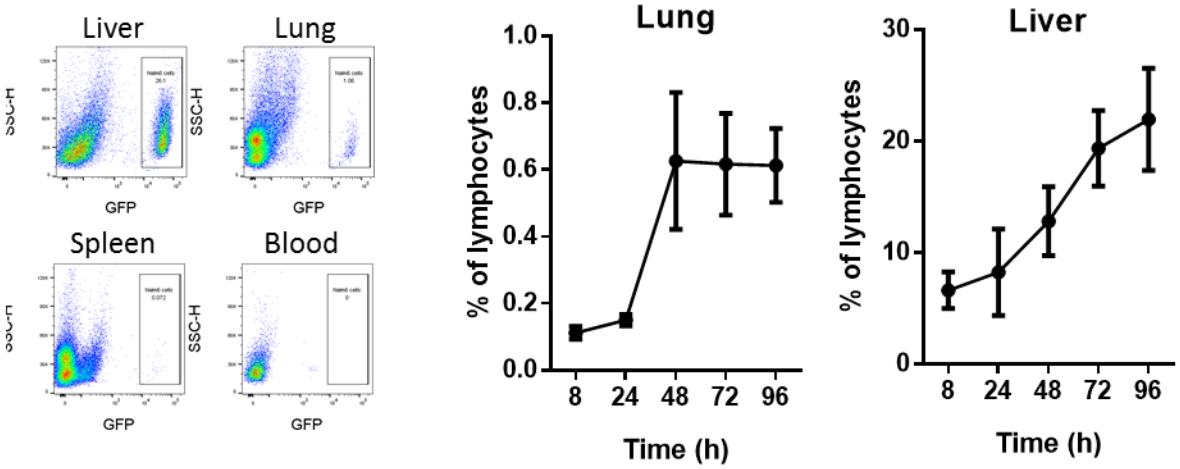

Live cell imaging, short-dimeric sCAR-T
anti-CD19 Fab
IgG4m sCAR-T -LCNT
IgG4m sCAR-T + LCNT
IgG4m sCAR-T + LCNT + PNE
No. of cell tox green clusters / mm2
Time (h)

Figure S9

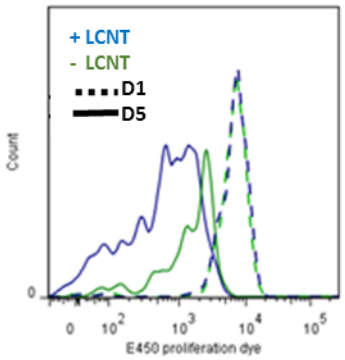
A

Location of Nalm-6 cells by flow cytometry
in untreated mice (PBS) from main text Figure 4A



B

Dye-dilution by flow cytometry for IgG4m sCAR-T
cells treated with and without switch *in vivo*



C

Dye-dilution by flow cytometry for IgG4m sCAR-T
cells compared with CART-19 in spleen, lung, liver

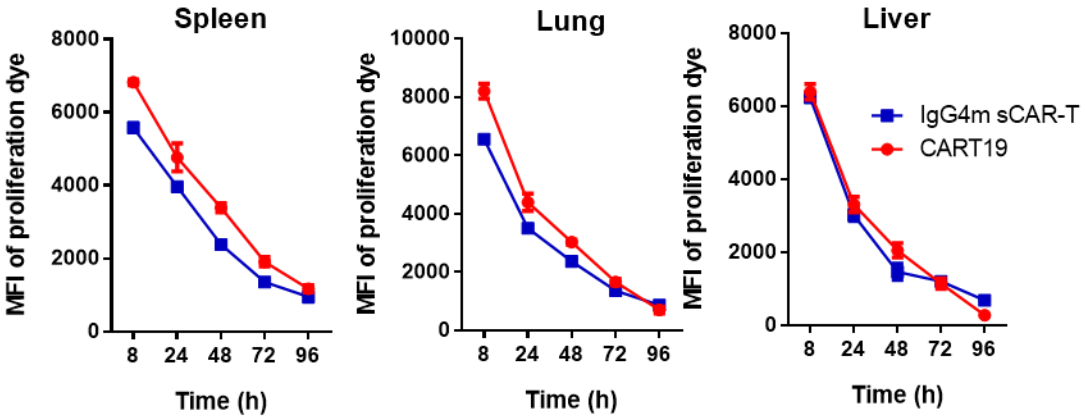
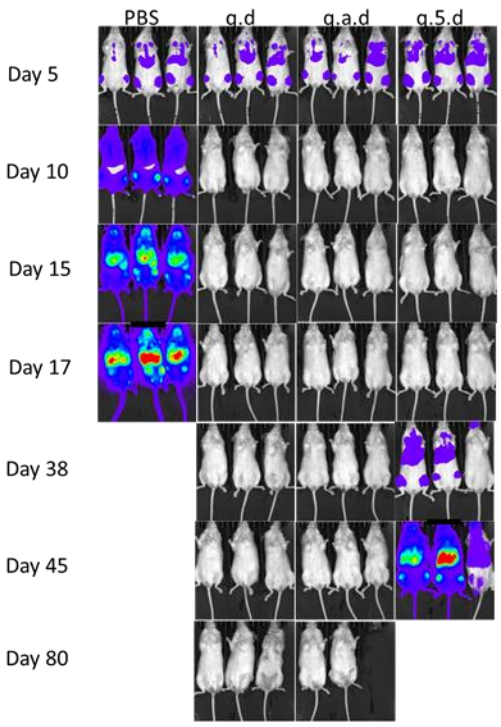
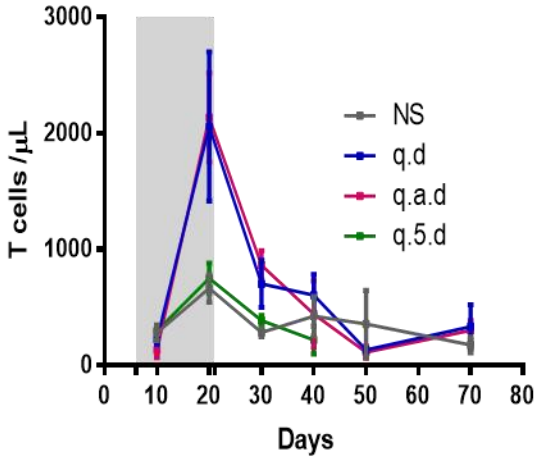


Figure S10

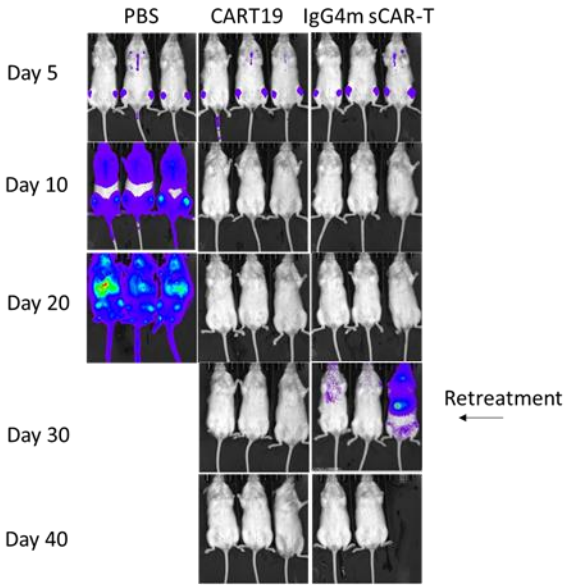
A Representative IVIS images from main text **Fig 5A**



B IgG4m sCAR-T cell expansion in the model shown in main text **Fig 5A**



C Representative IVIS images from main text **Fig 5D**



D Representative flow plots demonstrating the gating strategy for phenotype determination in main text **Fig 5D**