Supplementary Materials for

Remote control of therapeutic T cells through a small molecule-gated chimeric receptor

Chia-Yung Wu, Kole T. Roybal, Elias M. Puchner, James Onuffer, Wendell A. Lim

correspondence to: jamesonuffer@gmail.com, wendell.lim@ucsf.edu

This PDF file includes:

Materials and Methods
Figs. S1 to S11
Captions for Movies S1 to S4

Other Supplementary Materials for this manuscript includes the following:

Movies S1 to S4

MATERIALS AND METHODS

Construction of ON-switch CARs

The nucleotide sequence encoding a signal sequence, the anti-human CD19 antigen ligand binding scFv, and the human CD8α hinge and transmembrane domain was generously provided by Dr. Michael Milone and Dr. Carl June at the University of Pennsylvania. Insertion of a Myc epitope tag (EQKLISEEDL) immediately downstream of the signal sequence was performed by PCR. The human 4-1BB co-stimulation and CD3ζ ITAM signaling chains were cloned from cDNAs supplied by Open Biosystems (clones 2924109 and LIFESEQ3227409). Sequences encoding the FKBP and the T2089L mutant of FRB domains were obtained from Addgene (plasmids #20160 and #20148). Sequences encoding the gibberellin-binding GID1 and GAI were also obtained from Addgene (plasmids #37306 and #37315). The sequence encoding the anti-mesothelin scFv HN1 (PMID 20635390) was synthesized by assembly PCR (PMID 15980526) with oligonucleotides purchased from Integrated DNA Technologies. Standard molecular cloning techniques (PCR, restriction digestion, ligation, etc.) were applied to construct CAR expression plasmids using a second-generation self-inactivating lentiviral vector.

Culturing conditions for T cells and target cells

A Jurkat T cell line engineered with a NFAT-dependent EGFP reporter gene was a gift from Dr. Arthur Weiss at UCSF. Raji and Daudi B cells were acquired from ATCC. Jurkat T cells, Raji B cells and Daudi B cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin and streptomycin. K562 target cell lines were provided by Dr. Carl June's laboratory at the University of Pennsylvania and were cultured in IMDM medium supplemented with 10% FBS. Cell density and average cell size (to help with assessing activated or resting states of primary T cells) in cultures were quantified using a Countess cell counter (Life Technologies).

Primary human $CD4^+$ or $CD8^+$ T cells were isolated from anonymous healthy donor's blood after apheresis (Trima residuals from Blood Centers of the Pacific, San Francisco, CA) as approved by University Institutional Review Board. Cells were enriched by negative selection using RosetteSep Human $CD4^+$ or $CD8^+$ T Cell Enrichment Cocktail (STEMCELL Technologies #15062 & 15063). Isolated T cells were cryopreserved in RPMI-1640 medium supplemented with 20% human AB serum and 10% DMSO until use. Two days prior to lentiviral transduction, cells were thawed and cultured in human T cell medium, consisting of X-VIVO15 (Lonza #04-418Q), 5% human AB serum (Valley Biomedical Inc., #HP1022), and 10mM N-acetyl L-Cysteine (Sigma-Aldrich #A9165). Recombinant human IL-2 (NCI BRB Preclinical Repository) was added to a final concentration of 30 IU/mL for $CD4^+$ cells and to 100 IU/mL for $CD8^+$ T cells.

Lentiviral engineering of T cells and K562 target cells

Pantropic VSV-G pseudotyped lentivirus was produced from Lenti-X 293T cells (Clontech Laboratories #632180) co-transfected with a pHR'SIN:CSW transgene expression vector and the viral packaging plasmids pCMVdR8.91 and pMD2.G using Lipofectamine LTX(Life Technologies #15338). Infection medium/supernatant was collected 48 hours after transfection to transduce cells. Twenty-four hours prior to viral transduction, primary human T cells were activated using Dynabeads Human T-Activator CD3/CD28 (Life Technologies #11131D) at a 1:3 cell:bead ratio. Jurkat and K562 cells were split 24~48 hours in advance to ensure that cultures would be in log phase at the time of transduction. Transduced primary T cells were maintained at ~10^6/mL in human T cell medium as previously described (*32*). Transduced Jurkat and K562 cells were cultured for at least 9 days before experiments were conducted. Expression of transgenes was confirmed by either staining with fluorophore-conjugated antibodies or by detecting fluorescent reporter proteins using a BD LSRII flow cytometer.

Verifying CAR expression on T cells

Jurkat or primary human T cells were resuspended in FACS wash buffer (PBS + 0.5% BSA + 0.1% sodium azide) and stained with an Alexa 488- or Alexa 647-conjugated anti-myc antibody (Cell Signaling Technology #2279, #2233). Stained cells were washed three times in wash buffer, fixed in a 1:1 mixture of the wash buffer and BD Cytofix (BD #554655), and processed with a BD LSRII cytometer. FlowJo software (TreeStar) was used to quantify Alexa dye and/or mCherry fluorescence intensities.

Quantifying CD19 antigen ligand expression on target cells

Target cells were pre-treated with human IgG (Thermo Scientific #31154) in FACS wash buffer (PBS + 0.5% BSA + 0.1% sodium azide) and then stained with an anti-human CD19-FITC antibody or isotype control (BD Pharmingen #555412 #555748). Stained cells were washed three times in wash buffer, fixed in a 1:1 mixture of the wash buffer and BD Cytofix (BD #554655), and processed with a BD LSRII cytometer. FlowJo software (TreeStar) was used to quantify FITC fluorescence intensity.

Quantitation of IL-2 and/or IFN-γ production

Jurkat $CD4^+$ T cells or primary $CD4^+$ T cells expressing CARs were mixed with cognate ($CD19^+$) or non-cognate ($mesothelin^+$) K562 target cells at a 1:2 T cell:target cell ratio. The rapalog A/C Heterodimerizer (Clontech Laboratories #635055) was serially diluted in medium and added to reaction mixtures. Gibberellic acid acetoxymethyl ester (Toronto Research Chemicals #G377500) was dissolved in ethanol and added to reaction mixtures. After overnight incubation, supernatants were collected and analyzed with BD OptEIA Human IL-

2 or IFN-γ ELISA Set (BD Biosciences #555190 and #555142). Flow cytometry was performed to quantify NFAT-dependent GFP reporter expression in Jurkat cells as a separate indicator for CAR activity. Data plots were generated using Prism software (GraphPad).

Quantitation of CD69 surface expression

Primary $CD4^+$ T cells expressing CARs were mixed with cognate ($CD19^+$) or non-cognate (mesothelin$^+$) K562 target cells at a 1:2 T cell:target cell ratio in a U-bottom 96-well plate. The rapalog A/C Heterodimerizer (Clontech Laboratories #635055) was serially diluted in medium and added to reaction mixtures. After overnight incubation, cells were pelleted by centrifugation at 400g for 5min. Cells were resuspended in FACS wash buffer (PBS + 0.5% BSA + 0.1% sodium azide) and stained with an Alexa 488 conjugated anti-human CD69 antibody (BioLegend #310916). Stained cells were washed three times in FACS wash buffer, fixed in a 1:1 mixture of the wash buffer and BD Cytofix (BD #554655), and processed with a BD LSRII cytometer. FlowJo software (TreeStar) was used to compare Alexa 488 fluorescence intensities of gated T cells (unique forward/side scatters) in samples. Data plot in Fig. 4D was generated using Prism software (GraphPad).

Quantitation of T cell proliferation

Primary $CD4^+$ T cells expressing the ON-switch CAR were labeled with CellTrace Violet (Life Technologies #C34557) following manufacturer's instructions. K562 target cells expressing either the cognate ligand (CD19) or non-cognate ligand (mesothelin) were treated with 25ug/mL mitomycin C (Molecular Toxicology, Inc. #60-100.20) for 30 minutes at 37°C to render target cells replication-incompetent. T cells and target cells were mixed at 1:2 ratio, and the rapalog A/C Heterodimerizer (Clontech Laboratories #635055) was added to desired final concentrations. Cells were collected daily for flow cytometry analysis (BD LSRII) after incubation for 3, 4, 5 and 6 days. Histograms of CellTrace Violet stained cells were generated using FlowJo software (TreeStar).

Flow cytometry-based re-directed cytotoxicity/cell killing assay

For Figure 4: The cognate ($CD19^+$) and non-cognate (mesothelin$^+$) K562 target cells were engineered with lentivirus to express the mCherry and GFP fluorescent proteins respectively, so that both cell types in a mixture could be simultaneously quantified by flow cytometry. The two target cell lines were mixed at a 1:1 ratio and then co-incubated with resting primary human $CD8^+$ T cells at 5:2 T cell:target cell ratio in a U-bottom 96 well plate. Human IL-2 was added to a final concentration of 100 IU/mL in each reaction well. The hetero-dimerizing rapalog (Clontech Laboratories #635055) was added to concentrations noted in figures. After intended periods of incubation, samples were centrifuged at 400g for 5 minutes. Pelleted cells were resuspended in FACS wash buffer (PBS +

0.5% BSA + 0.1% sodium azide) and fixed with an equal volume of BD Cytofix (BD #554655) prior to flow cytometry. Control samples containing only the target cells were used to set a flow cytometry gate for intact target cells based on forward and side scatter patterns that had been previously confirmed to exclude apoptosed cells. The gate was applied to all reaction samples, and abundance of the two target cell types was quantified. A ratio of the surviving cognate target cells ($mCherry^+$ GFP^-) to non-cognate target cells ($mCherry^-$ GFP^+) was calculated for each sample to enumerate re-directed cytotoxic activities of T cells. Flow cytometry data analysis was performed using FlowJo software (TreeStar). Summary data plots were generated using Prism software (GraphPad).

For Figure S7: When cell-sized beads (Bangs Laboratories, Inc. #580) were used for normalization, 50uL of washed beads (re-suspended in PBS) were added to each sample at the end of incubation period. Samples were stained with a fixable, near-IR dead cell stain (Life Technologies #L10119) and anti-CD19 FITC antibody (BD Pharmingen #555412). Stained cells were washed three times in FACS wash buffer, fixed in a 1:1 mixture of the wash buffer and BD Cytofix (BD #554655), and processed with a BD LSRII cytometer. FlowJo software (TreeStar) was used enumerate target cells ($FITC^+$, dead $stain^-$) and beads following bead manufacturer's instructions.

For Figure S8: The experiment was set up as described above for Figure 4 with modifications. ON-switch CAR T cells and $CD19^+$ target cells were co-cultured at a 1:10 ratio in 24-well plates. Sample aliquots were collected for flow cytometry analysis at designated time points. At the 36 and 72 hour time points, cells were washed 3 times with pre-warmed growth medium to remove metabolites and/or rapalog. Cultures were supplemented with IL-2 at 25 IU/mL initially and after washes. Data plotting and statistical analysis (student's t test) were performed using Prism software (GraphPad).

Time-lapse microscopy

Primary human $CD8^+$ T cells were isolated, stimulated, and transduced as described above with the two component ON-switch CAR. The two parts were tagged C-terminally with EGFP or mCherry, respectively. Dual positive $CD8^+$ T cells (cells ~ 1 log higher in fluorescence intensity compared to non-transduced controls in both channels) were sorted on a FACS Aria II (BD Biosciences) in the UCSF Laboratory for Cell Analysis. All imaging was performed on a Yokogawa CSU spinning disk confocal mounted on a Nikon Ti-E inverted microscope equipped with temperature control and a Photometric Evolve EMCCD camera controlled with Nikon Elements. A 20x Nikon Plan Apo (0.75 NA) or 60x CFI Apo TIRF Oil (1.49 NA) objective was used for all microscopy. Briefly, imaging was performed in 386 well glass bottom plates (Matrical MGB101-1-2-HG) in imaging buffer (PBS supplemented with 5% human AB serum, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). K562 $CD19^+$ target cells and T cells were added to the well and imaged

as indicated. Cells were treated with 500 nM rapalog or vehicle for 20 minutes prior to imaging. SYTOX Blue (Invitrogen #S11348) was added to the imaging buffer for all experiments at a concentration of 5 nM. For Figure S9: T cell-target cell association was monitored by tracking a stable interaction between the two cell types. K562 cells were immobilized on antibody pre-coated glass slides and sufficiently sparsely seeded for tracking individual T cell-target cell interactions overtime.

Super-resolution microscopy and analysis

The chambered borosilicate cover glasses (8-well, Lab-Tek II, Sigma-Aldrich, #155409) were incubated first with 1 mg/mL biotinylated BSA (Pierce #29130) in T50 buffer (10mM Tris-HCl pH 8.0 and 50mM NaCl) for 5 minutes, 0.2 mg/mL neutravidin (Pierce #31000) for 1 minutes, and biotinylated anti-Myc antibody (Cell Signaling #3946) at a concentration of 10 µg/ml for 5 minutes and washed 3x with imaging buffer. Jurkat T-cells expressing the two chimeric antigen receptor parts fused to the photoactivatable fluorescent proteins PS-CFP2 and PAmCherry1 were allowed to settle and to adhere to the surface for 10 minutes. In the case of drug treated cells, rapalog was added to the medium at a concentration of 500 nM for 20 minutes. All super-resolution microscopy experiments were performed in imaging buffer at room temperature.

Super-resolution microscopy experiments were performed on a custom-built microscope (*36*). The microscope is based on a Nikon Ti–E inverted microscope with the Perfect Focus System. Three activation/imaging lasers (Stradus 405-100, Stradus 488-50, Vortran Laser Technology; Sapphire 561-200-CW, Coherent) are combined using dichroic mirrors, aligned, expanded and focused to the back focal plane of the objective (Olympus ×100 UPlanSApo NA 1.4). The Stradus lasers are controlled directly by the computer whereas the Sapphire 561nm laser is shuttered using an acoustic optical modulator (Crystal Technology). A quadband dichroic mirror (zt405/488/561/640rpc, Chroma) and a band-pass filter (ET595/50m, Chroma) separate the fluorescence emission from the excitation light. For two color imaging, the fluorescence of PS-CFP2 and PAmCherry1 was separated by a two channel split view. The images were recorded at a frame rate of 19 Hz on an electron multiplying CCD camera (Ixon+ DU897E-CS0-BV, Andor). The camera was cooled down to -68 degree Celsius and the amplifying gain was set to 30. To estimate the used power density for the activation and excitation laser, we measured the power in the back focal plane of the microscope and divided it by the illuminated area. The typical power density for the 561 nm excitation was ~ 1 kW/cm2 (power 17 mW), 0.2 kW/cm2 for 488 nm (power 4 mW) and for the 405 nm activation in a range between 0.06W/cm2 to 6 W/cm2 (at a power between 1-100µW). While simultaneous dual-color imaging of PAmCherry1 and PS-CFP2 to track their co-localization was possible, we obtained more PAmCherry1 localization data points and achieved a superior signal-to-noise ratio by consecutive single-color imaging, potentially due to bleaching or de-activation of PAmCherry1 by 488 nm light. The continuous

activation power (405nm) was adjusted over time to achieve a sparse and constant switching rate per frame. Both data acquisition and analysis were performed using custom-written software. During image acquisition, the axial drift of the microscope stage was stabilized by the Perfect Focus System.

A typical super-resolution image was generated from a sequence of 5.000 – 20.000 image frames, recorded at 19 Hz. For each imaging frame, fluorescent spots were identified and fit to an elliptical Gaussian function to determine their centroid positions, intensities, widths and ellipticities. Based on these parameters, peaks too dim, too wide or too elliptical to yield satisfactory localization accuracy were rejected from further analysis. For all localizations the fit-parameters like the x- and y-coordinates, photon number, background photons and frame of appearance were saved in a molecule list for further analysis. Single molecule diffusion traces were generated by linking localizations in consecutive frames that appear within a distance of 4 pixels (620 nm). Due to the low activation power, on average 10 localizations were detected in an area of about 15x15 µm, corresponding to a density of 0.04 localizations per μm^2. Therefore, the error of accidentally linking two localizations from different molecules within a distance of 4 pixels is less than 2%.

For each single-molecule trajectory longer than three consecutive frames, the mean-square displacement was calculated by averaging the squared displacement of all time intervals with length Δt. Error bars depict S.E.M. according to the number of trajectories (*58*), and the cell-cell variability is represented as the standard deviation of averaged mean square displacements from different cells.

I*n vivo* target cell killing

Mice were housed in the UCSF Laboratory Animal Research Center and cared by the UCSF veterinary staff and by the Preclinical Therapeutics Core. All procedures were performed as approved by the Institutional Animal Care and Use Committee.

$CD8^+$ T cells engineered to express anti-CD19 CARs (conventional, ON-switch, or defective ON-switch) were prepared as described in "Lentiviral engineering of T cells". T cells were transduced with lentivirus and cultured for over 10 days prior to *in vivo* use.

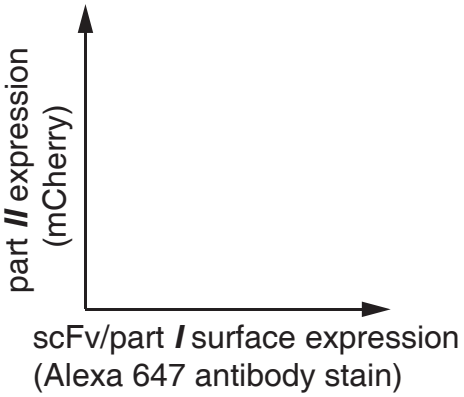
Rapalog (Clontech Laboratories #635055) was dissolved in vehicle solution (16.7% 1,2-propanediol, 22.5% PEG-400, 1.25% Tween-80) to 0.6mg/mL prior to injection. The compound was first dissolved in 100% 1,2-propanediol with rigorous vortexing or sonication and then diluted to the final working concentration using an appropriate mixture of PEG-400, Tween-80 and water. The working stocks of rapalog or vehicle were stored at 4°C between injection

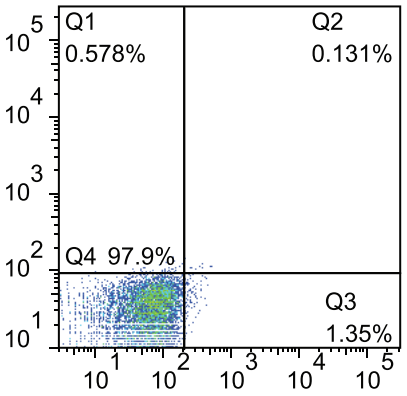
time points. The vehicle components were purchased from Sigma-Aldrich (#82280, #91893, #P4780).

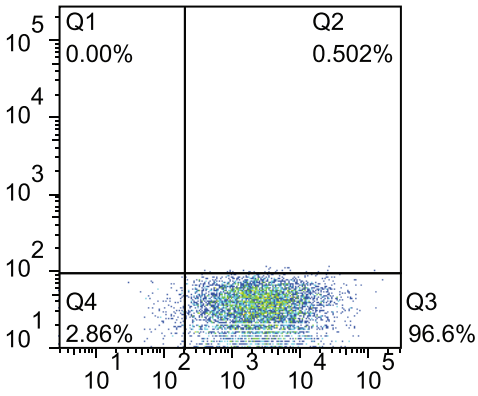
A mixture of cognate ($CD19^+$ $mCherry^+$ GFP^{low}) and non-cognate ($CD19^-$ GFP^{high}) K562 target cells (2×10^7 cells per cell type) resuspended in PBS was injected into the intraperitoneal space (*i.p.*) in each NOD *scid* gamma (NSG) mouse (female, 8~12 weeks old, Jackson Laboratory #005557). 14 hours later, 3×10^7 T cells were injected *i.p.*, followed by injection of rapalog (at 3mg/Kg dosage) or vehicle control using the same administration route. Drug or vehicle injection was repeated three more times at 6 hour intervals. 6 hours after the last drug/vehicle injection, mice were euthanized using carbon dioxide asphyxiation followed by cervical dislocation. Peritoneal lavage was collected based on a procedure described previously (*44*). 1.5mL of cold FACS-wash buffer (PBS + 0.5% BSA + 0.1% sodium azide) was used to re-suspend cells in the peritoneal cavity. The lavage was centrifuged at 400g and room temperature for 5 min, re-suspended in 0.5mL of red blood cell lysis solution at 1X concentration (BD Biosciences #555899) for 15 minutes at room temperature, centrifuged again at 400g for 5 min, and fixed in a 1:1 mixture of the FACS wash buffer and BD Cytofix (BD #554655). Samples were analyzed with a BD LSRII cytometer. Data processing and target cell quantification were performed using FlowJo software (TreeStar) as described for *in vitro* cell killing experiments. Data plotting and statistical analysis (student's t test) were performed using Prism software (GraphPad).

Figure S1. Expression levels of CARs in Jurkat cells.

Cells engineered with lentiviral constructs encoding CAR molecules were analyzed by flow cytometry to quantify fraction of CAR+ cells as well as median fluorescence intensity (MFI) of each molecule.



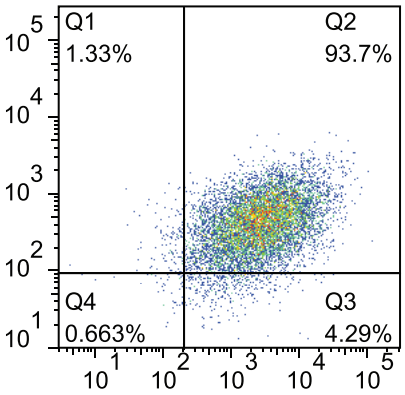




part *I* MFI: 2548

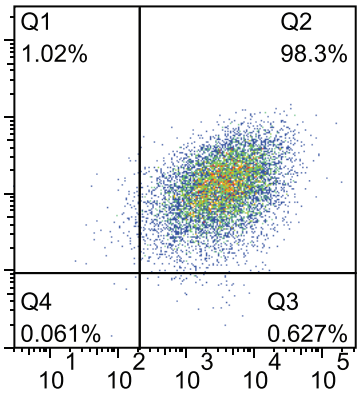
ON-switch CAR variants:

I.a* + *II.a



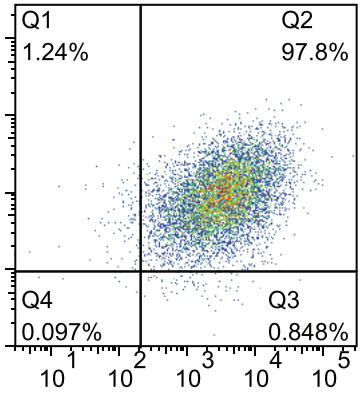
Q2 part *I* MFI: 2869
part *II* MFI: 455

I.a* + *II.b



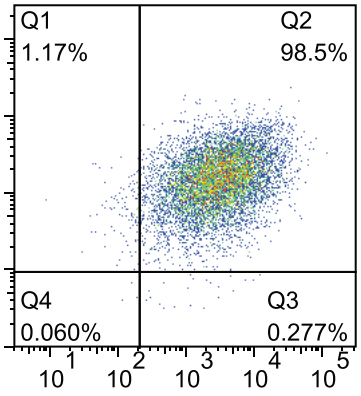
Q2 part *I* MFI: 3454
part *II* MFI: 1274

I.a* + *II.c



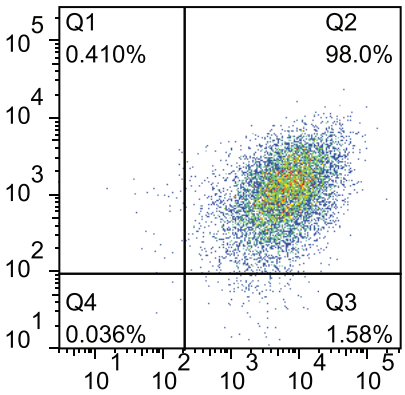
Q2 part *I* MFI: 3371
part *II* MFI: 926

I.a* + *II.d



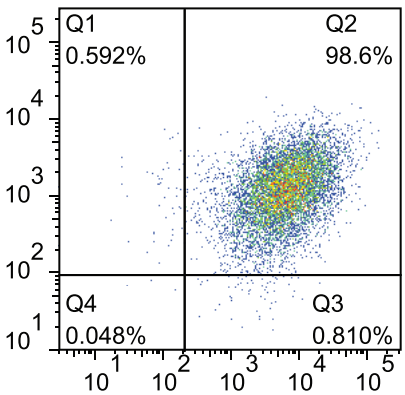
Q2 part *I* MFI: 3034
part *II* MFI: 1566

I.b* + *II.b



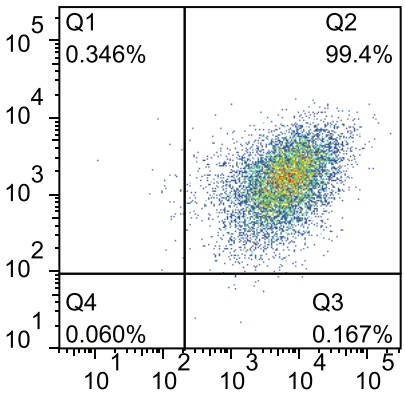
Q2 part *I* MFI: 6218
part *II* MFI: 1174

I.b* + *II.d



Q2 part *I* MFI: 6220
part *II* MFI: 1270

I.b* + *II.e



Q2 part *I* MFI: 6254
part *II* MFI: 1590

Figure S2. NFAT activity in CAR-expressing Jurkat cells induced by cognate ligand in the presence or absence of rapalog.

a After 22hr co-incubation of CAR^{+} Jurkat cells and K562 target cells, samples were analyzed by flow cytometry to examine NFAT-dependent GFP expression in Jurkat cells. Jurkat cells were gated based on lower forward and side scatter intensities than those of K562 target cells. Only singlet Jurkat cells are shown in the histograms below.

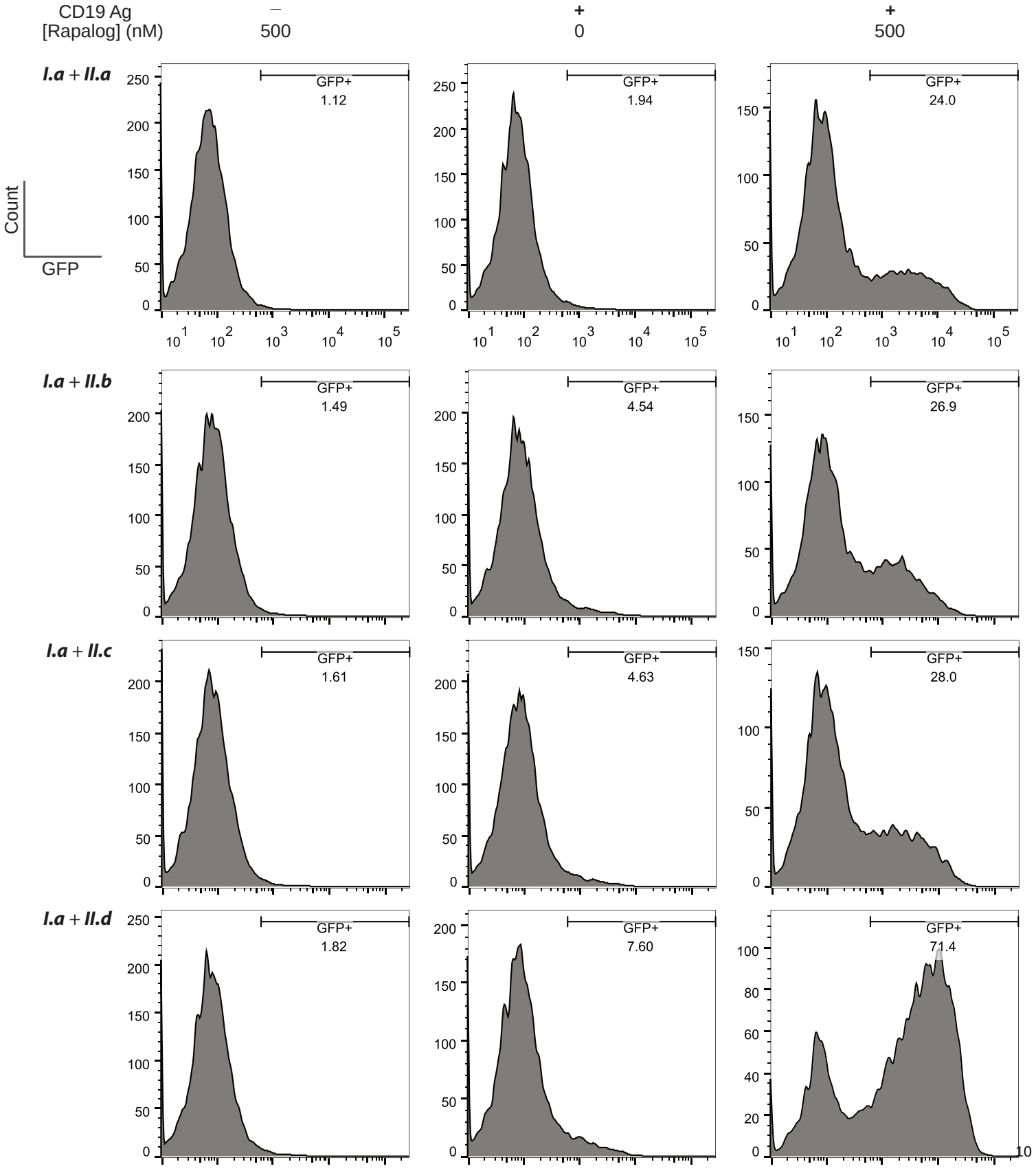


Figure S2a continued.

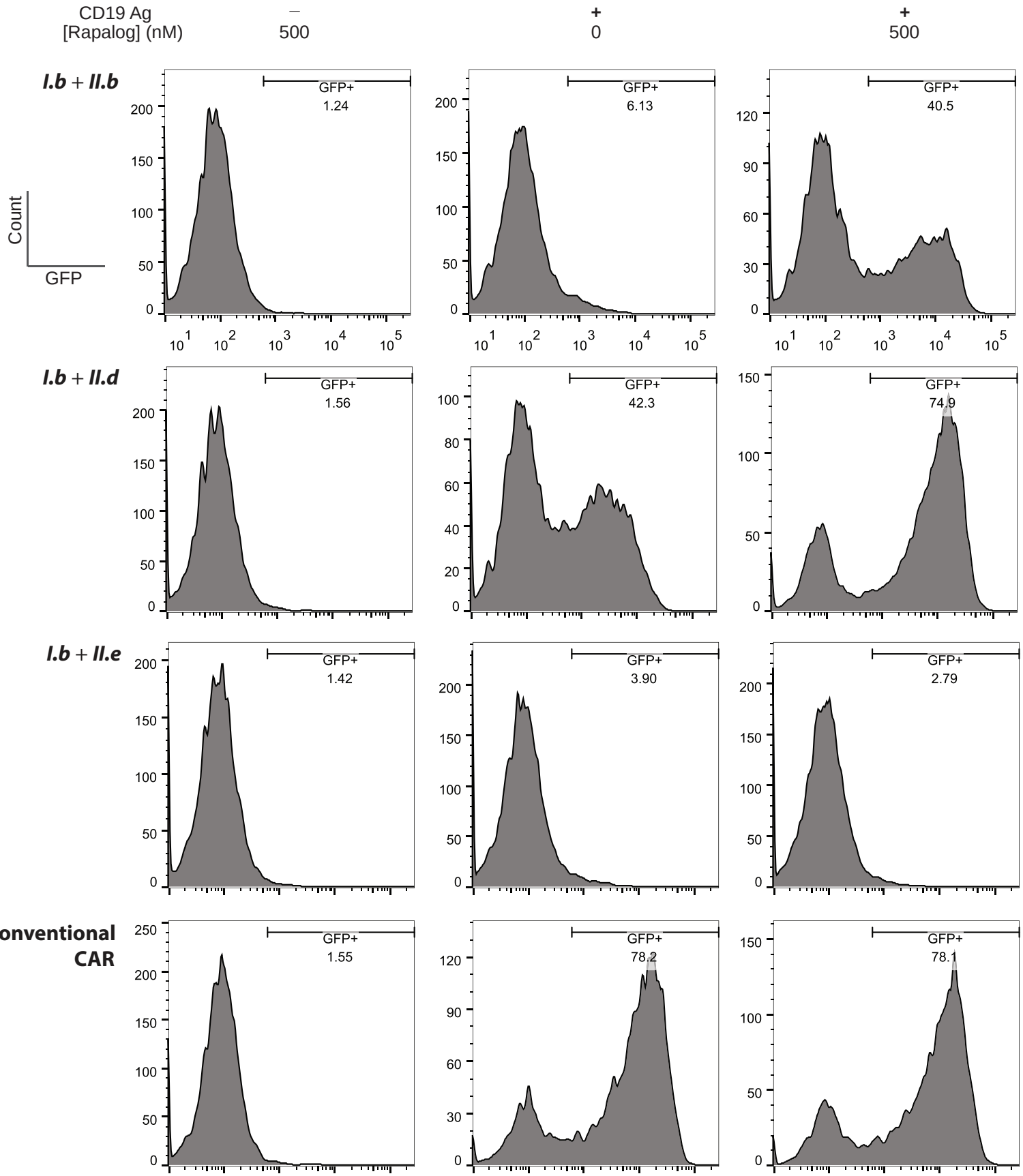


Figure S2 continued.

b Percentages of GFP⁺ Jurkat cells and their median GFP fluorescence quantified from histograms shown in Fig S2a. Average and standard deviation values were caluclated from 3 biological replicates.

CD19 Ag	–		+		+	
[Rapalog] (nM)	500		0		500	
	% GFP+	MFI	% GFP+	MFI	% GFP+	MFI
I.a* + *II.a	1.07 +/- 0.06	1043 +/- 45.6	1.97 +/- 0.02	1069 +/- 73.1	24.3 +/- 0.36	2217 +/- 174.6
I.a* + *II.b	1.53 +/- 0.16	1087 +/- 172.4	4.92 +/- 0.34	1256 +/- 75.7	26.7 +/- 0.53	2186 +/- 78.4
I.a* + *II.c	1.55 +/- 1.68	1029 +/- 56.3	4.44 +/- 0.21	1266 +/- 36.1	28.17 +/- 0.29	2635 +/- 42.9
I.a* + *II.d	1.80 +/- 0.10	1013 +/- 10.6	7.91 +/- 0.34	1352 +/- 63.8	72.17 +/- 1.08	6364 +/- 195.9
I.b* + *II.b	1.50 +/- 0.25	1039 +/- 88.3	6.03 +/- 0.35	1195 +/- 43.3	40.93 +/- 0.59	6174 +/- 242.3
I.b* + *II.d	1.63 +/- 0.16	981 +/- 3.6	42.73 +/- 0.38	2825 +/- 52.9	74.01 +/- 0.79	11173 +/- 15.6
I.b* + *II.e	1.48 +/- 0.15	1077 +/- 117.0	3.64 +/- 0.28	1148 +/- 56.3	2.81 +/- 0.29	1099 +/- 77.9
Conventional CAR	1.51 +/- 0.04	1050 +/- 74.6	78.07 +/- 0.15	11207 +/- 462.7	78.63 +/- 0.92	12502 +/- 512.0

Figure S3. IL-2 production by Jurkat cells expressing ON-switch and conventional CARs in the presence of cognate ligand.

Samples were prepared as described in Fig. S2. Sample supernatants were collected and analyzed by ELISA.

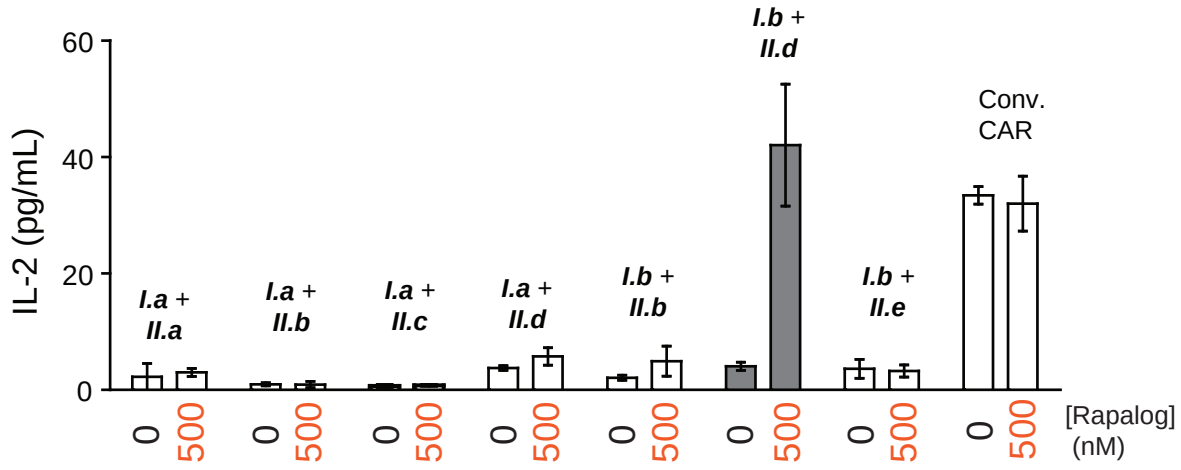
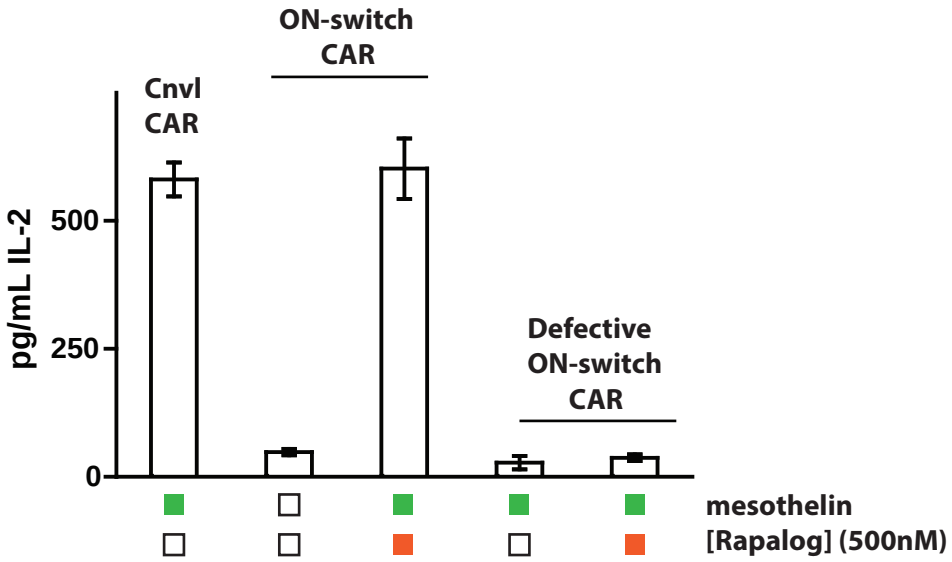


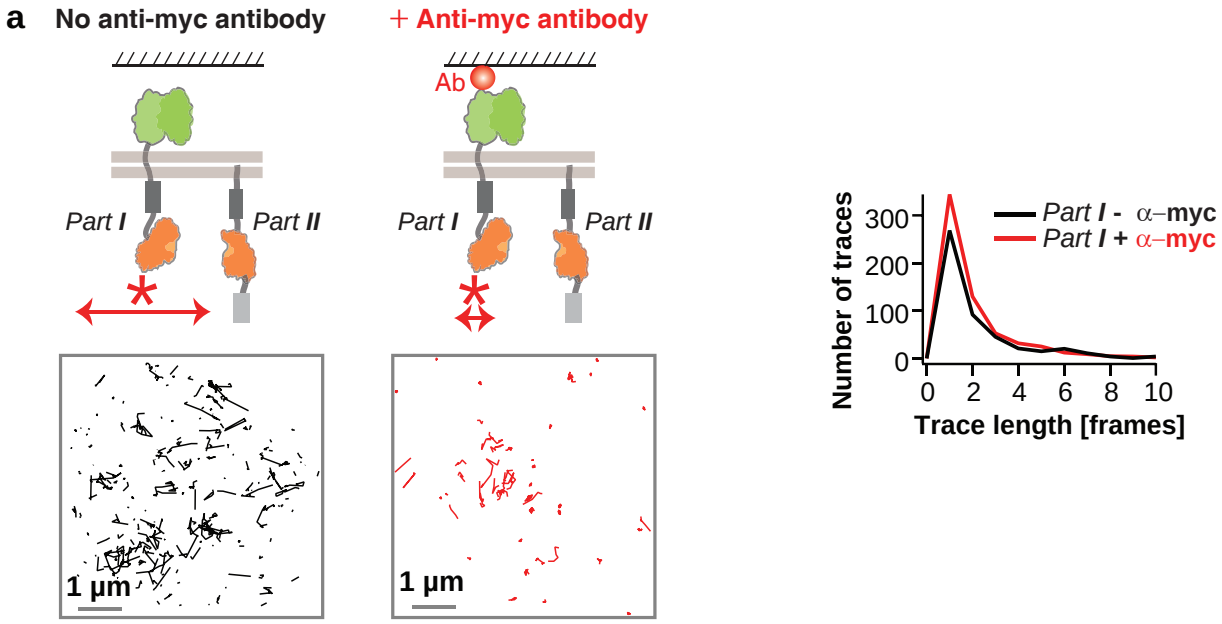
Figure S4. Modularity of the ligand antigen binding region of ON-switch CAR

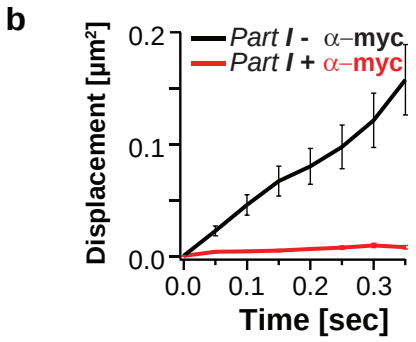


We engineered the ON-swtich CAR with a different ligand binding domain. An anti-mesothelin scFV was used in place of the anti-CD19 scFv. Jurkat cells expressing these CAR constructs (conventional or ON-switch configuration) were co-cultured with mesothelin +/- K562 target cells. Sample supernatants were collected and analyzed by ELISA after an over-night incubation.

Figure S5. Single-molecule mobility characterization of ON-switch CAR

(a) Part ***I*** -mEos2 fusion protein shows free diffusion in the absence of anti-Myc antibody but becomes immobile when engaged with the antibody presented on a surface. (b) Mean square displacement quantifies the diffusive behavior in the absence (black) and presence (red) of antibody (upper panel). Both data sets have a similar number of traces and trace length distribution (lower panel) (c) Trace length distribution of the data sets shown in Figure 2b and 2c.





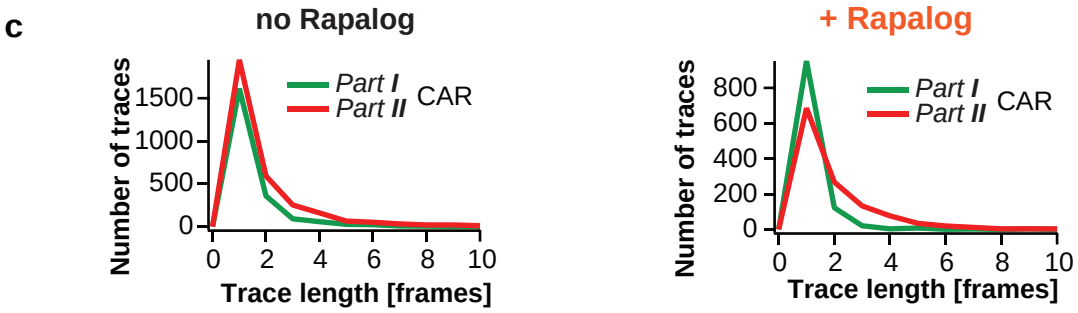
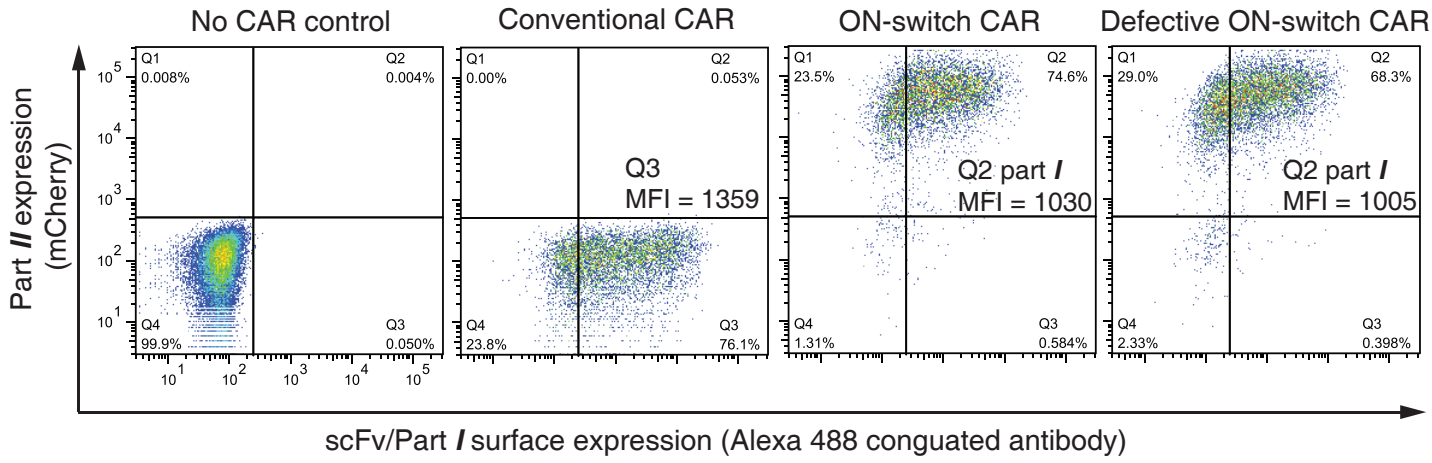


Figure S6. Expression levels of ON-switch and conventional CARs in primary human T cells.

Primary CD4⁺ and CD8⁺ T cells isolated from healthy anonymous donors were engineered with lentivirus and then analyzed as described in Fig. S1.

a

CD4⁺ T cells, 8 days after lentiviral transduction:



b

CD8⁺ T cells, 9 days after lentiviral transduction:

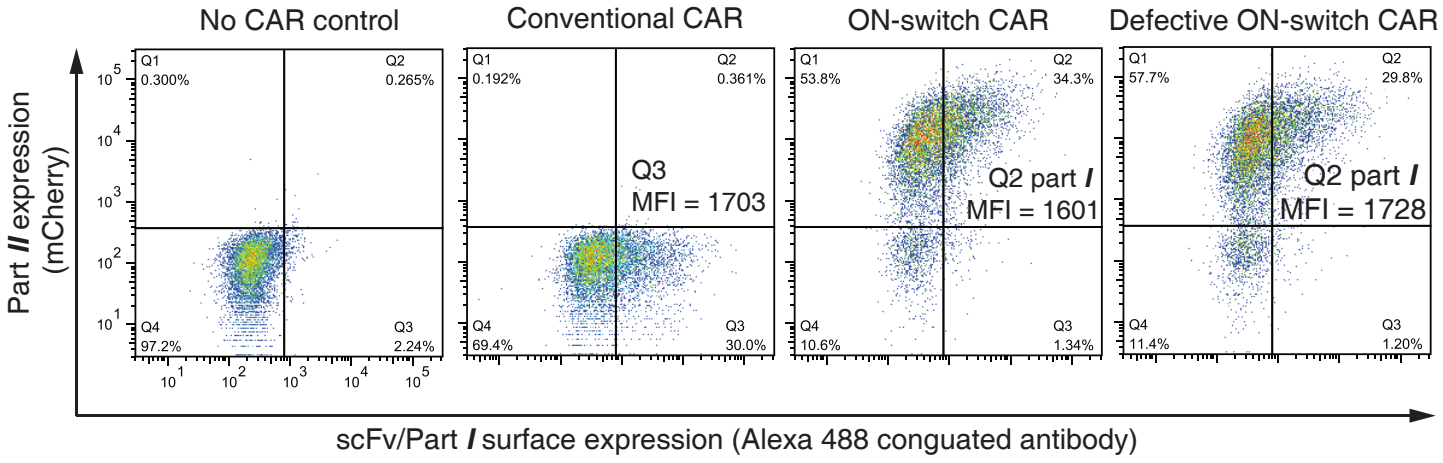


Figure S7. Killing of multiple types of CD19⁺ target cells by primary human CD8⁺ ON-switch CAR T cells

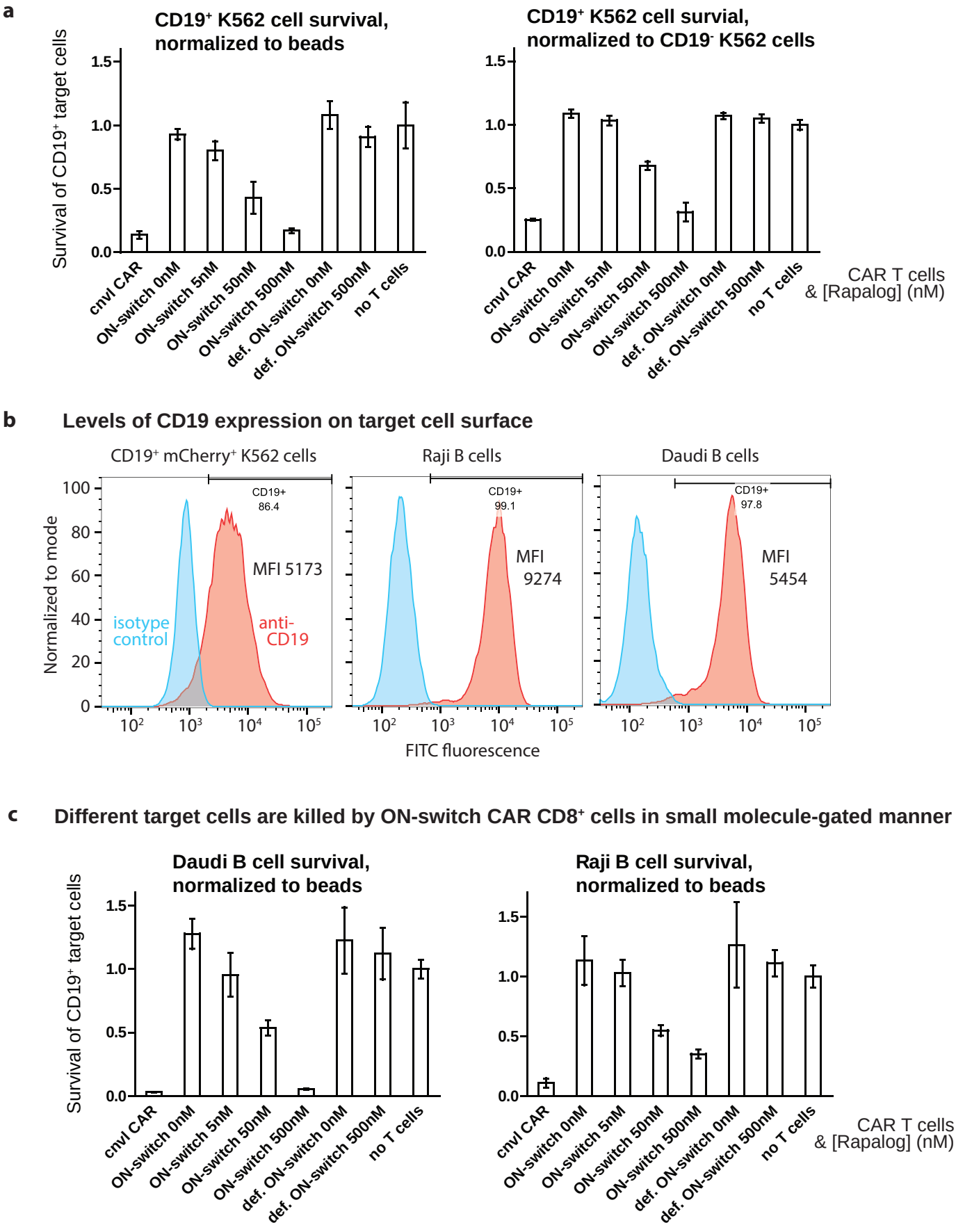


Figure S8. Revesible ON-switch CAR-mediated cytotoxicity by primary CD8⁺ T cells.

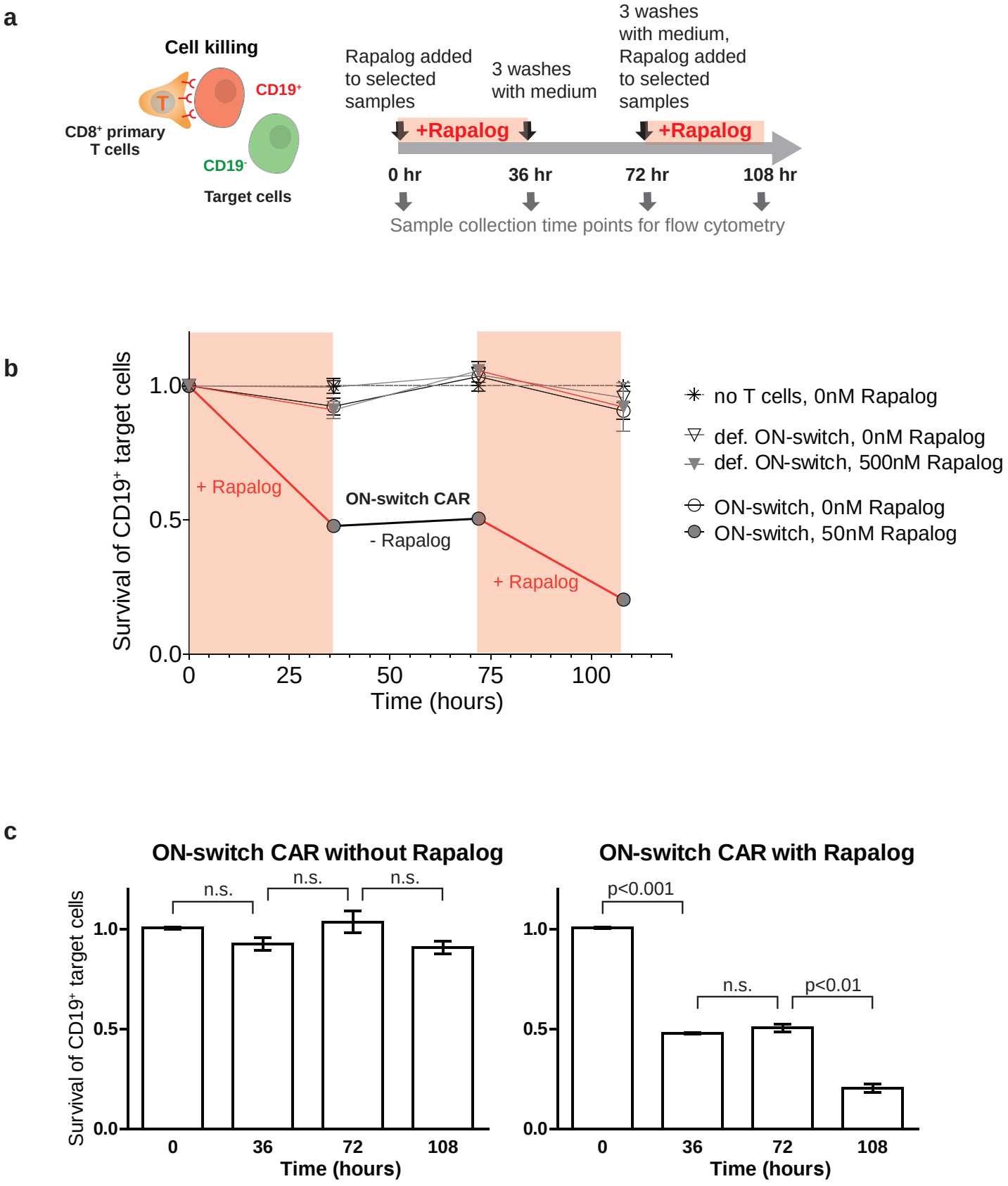
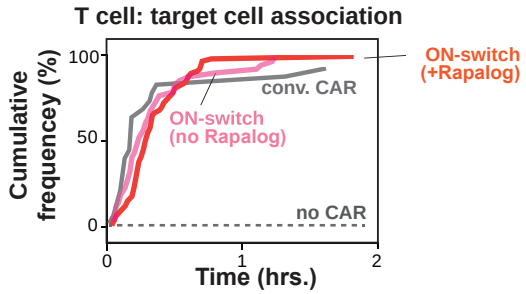


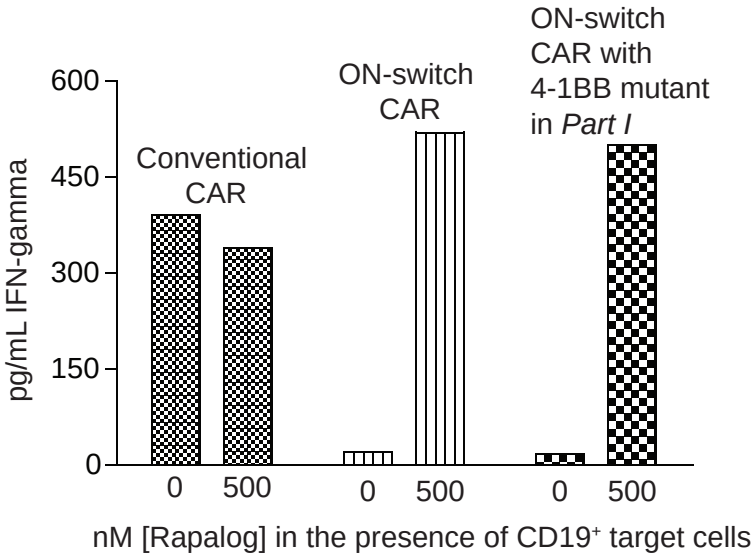
Figure S9. Cumulative frequency of CD8⁺ T cell:K562 target cell interaction shows that ON-switch CAR T cells recognize target cells as efficiently as conventional CAR T cells.



n = 45 for ON-switch CAR w/o Rapalog; n = 70 for ON-switch CAR w/ Rapalog;

n = 21 for conventional CAR; n = 70 for no CAR

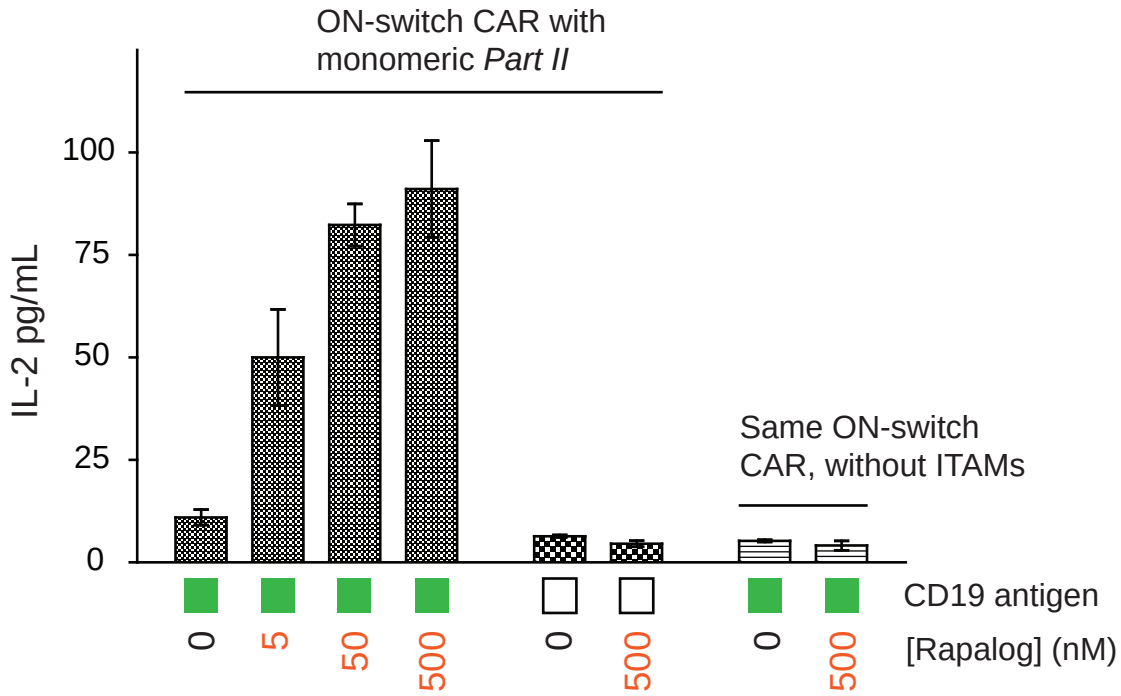
Figure S10. Binding of TRAF proteins to Part I of ON-switch CAR is not necessary for ON-switch CAR activity as measured by IFN-gamma production.



Primary human CD4⁺ T cells were transduced with lentivirus to express the conventional anti-CD19 4-1BB zeta CAR or the indicated ON-switch variants. Cells with comparable CAR expression levels were sorted and stimulated with CD19⁺ K562 target cells overnight under conditions described in Materials and Methods. Amounts of IFN-gamma in supernatants were assessed by ELISA.

The “4-1BB mutant” refers to Alanine subsitutions for residues 234, 236-239, 246-250 in the 4-1BB cytoplasmic chain that are critical for interactions with TRAF proteins, which mediate activation of downstream co-stimulatory signaling. (PMID 10518213, 9464265, 23760533)

Figure S11. Part II of ON-switch CAR can be engineered as a monomeric component as required.



The WT DAP10 extracellular domain used in Part *II* of the ON-switch CAR could dimerize with the same extracellular domain in endogenous DAP10 molecules, which is known to recruit PI3K for co-stimulatory signaling (in most T cells) or cytotoxic responses (in NK cells and subsets of $CD8^+$ T cells). While both consequences are the desirable and intended outcomes of ON-switch CAR activation, we understand that in unique cases, some users might prefer not to engage the endogenous DAP10 molecules.

In this experiment, we used Cys -> Ser substations at the two Cys residues corresponding to the homo-dimerizing residues 39 and 42 in human DAP10 protein in order to abolish dimerization in Part *II* of the ON-switch CAR.

Jurkat cells engineered with the indicated CARs were co-cultured with K562 target cells +/- the cognate CD19 antigen. Sample supernatants were collected and analyzed by ELISA after an over-night incubation.

Reference for DAP10 molecular structure and function: PMID 10426994

Movie S1. Single-molecule diffusion of the ON-switch CAR part *II* in the absence of Rapalog. Most part ***II*** molecules exhibit fast diffusion and are not bound to the immobilized part ***I*** molecules of the ON-switch CAR. Individual trajectories are superimposed on the movie from their time of appearance. Only a small time frame and area of data is displayed in real time. Scale bar = 1 μm.

Movie S2. Single-molecule diffusion of the ON-switch CAR part *II* in the presence of Rapalog. Most part ***II*** molecules are bound to the immobilized part ***I*** molecules of the ON-switch CAR and do not exhibit free diffusion. Individual trajectories are superimposed on the movie from their time of appearance. Only a small time frame and area of data is displayed in real time. Scale bar = 1 μm.

Movie S3. $CD8^+$ cells expressing ON-switch CAR recognize but do not kill $CD19^+$ target cells in the absence of Rapalog. Primary human $CD8^+$ cells expressing the ON-switch CAR were mixed with K562 cells expressing the cognate antigen CD19. The CAR components are labeled with fluorescent proteins: part ***I*** (containing extracellular single chain antibody) is tagged with EGFP, and part ***II*** (containing intracellular CD3ζ ITAMs) is tagged with mCherry. Time-lapse movie was taken over the course of 30 min. The $CD8^+$ cell recognizes the tumor cell, and CAR components are localized to the synapse, but the T cell does not result in killing of the tumor cell.

Movie S4. $CD8^+$ cells expressing ON-switch CAR kill $CD19^+$ target cells in the presence of Rapalog. Primary human $CD8^+$ cells expressing the ON-switch CAR were mixed with K562 cells expressing the cognate ligand CD19. The CAR components are labeled with fluorescent proteins: part ***I*** (containing extracellular single chain antibody) is tagged with EGFP, and part ***II*** (containing intracellular CD3ζ ITAMs) is tagged with mCherry. Time-lapse movie was taken over the course of 45 min. Here, in the presence of 500 nM rapalog, the $CD8^+$ cell recognizes and rapidly kills of the tumor cells, as indicated by target cell blebbing and uptake of Sytox Blue dye. After killing cell 1, the $CD8^+$ cell recognizes cell 2 and re-orients its synapse, immediately resulting in apoptosis of cell 2.