FIGURE S1: Design and testing of E01 DARPin CAR. (A) Selection and expansion of CAR-T cells using the truncated CD19 transduction marker. (B) Human EGFR expression in transduced K562 and endogenous $EGFR^+$ solid tumor lines. (C) Design of CARs with various spacer and linker formats.

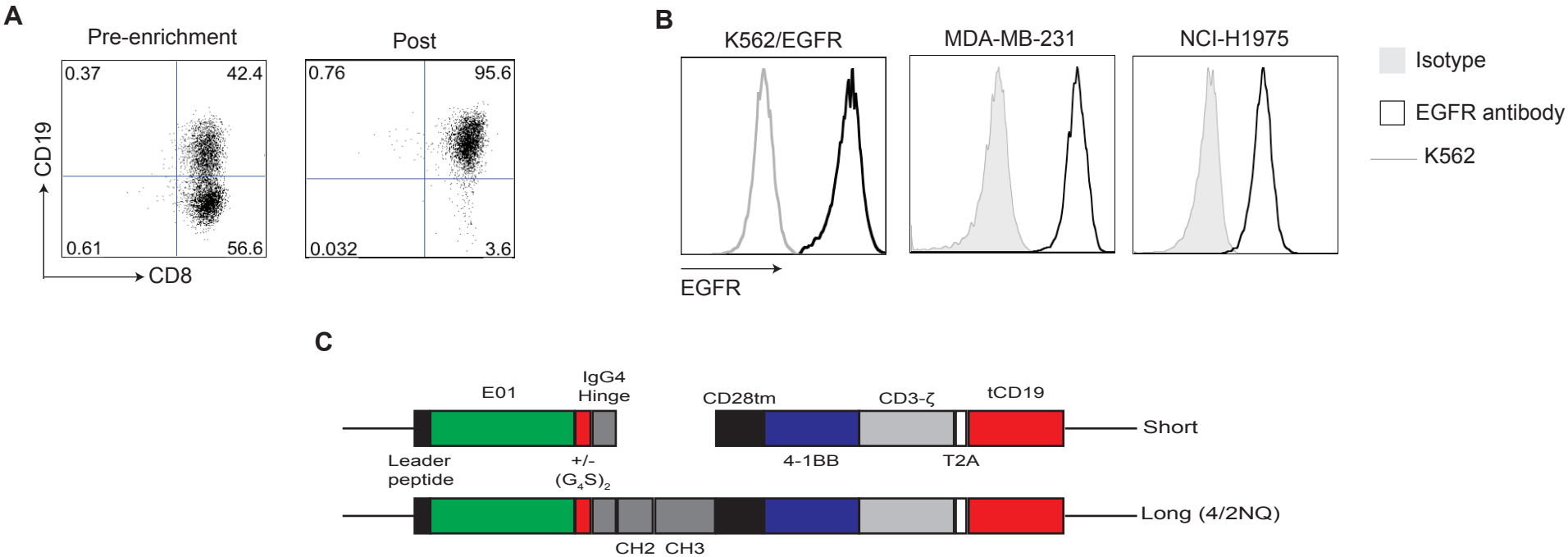
FIGURE S2: In vitro function and in vivo persistence of E01 DARPin CAR-T cells. (A,B) Cytokine secretion and proliferation of $CD4^+$ E01 DARPin CAR with short and long spacer (+/-$(G_4S)_2$) against MDA-MB-231 target cells (n=3). (C) Measurement of activation-induced cell death (AICD) in cetuximab scFv and E01 DARPin specific cells after incubation with MDA-MB-231 target cells (n=3). (D) Frequency of E01 DARPin specific cells with short and long spacer (+/-$(G_4S)_2$) linker in blood 3,7 and 14 days after adoptive transfer (n=4). (E) Frequency of E01 short versus long spacer (+$(G_4S)_2$) linker specific cells at tumor sites 6 days after adoptive transfer (n=4). (**-$p<0.05$, ns-not significant)

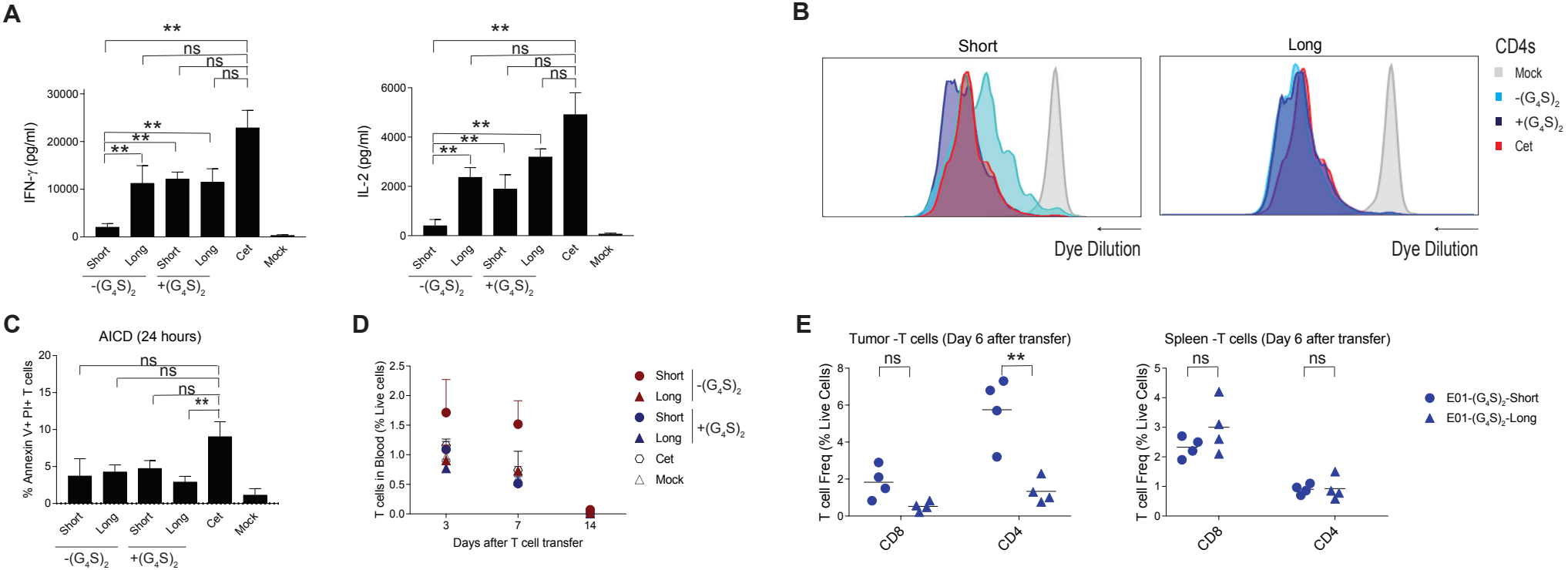
FIGURE S3: Transduced targets for testing of multispecific CARs. (A) Raji target cells were transduced to express a single antigen EGFR or EpCAM or HER2 or express a combination of 2 or all 3 targets antigens. (B) Relative median fluorescence intensity (MFI) of antigen expression on single, dual and triple antigen transfected target cells.

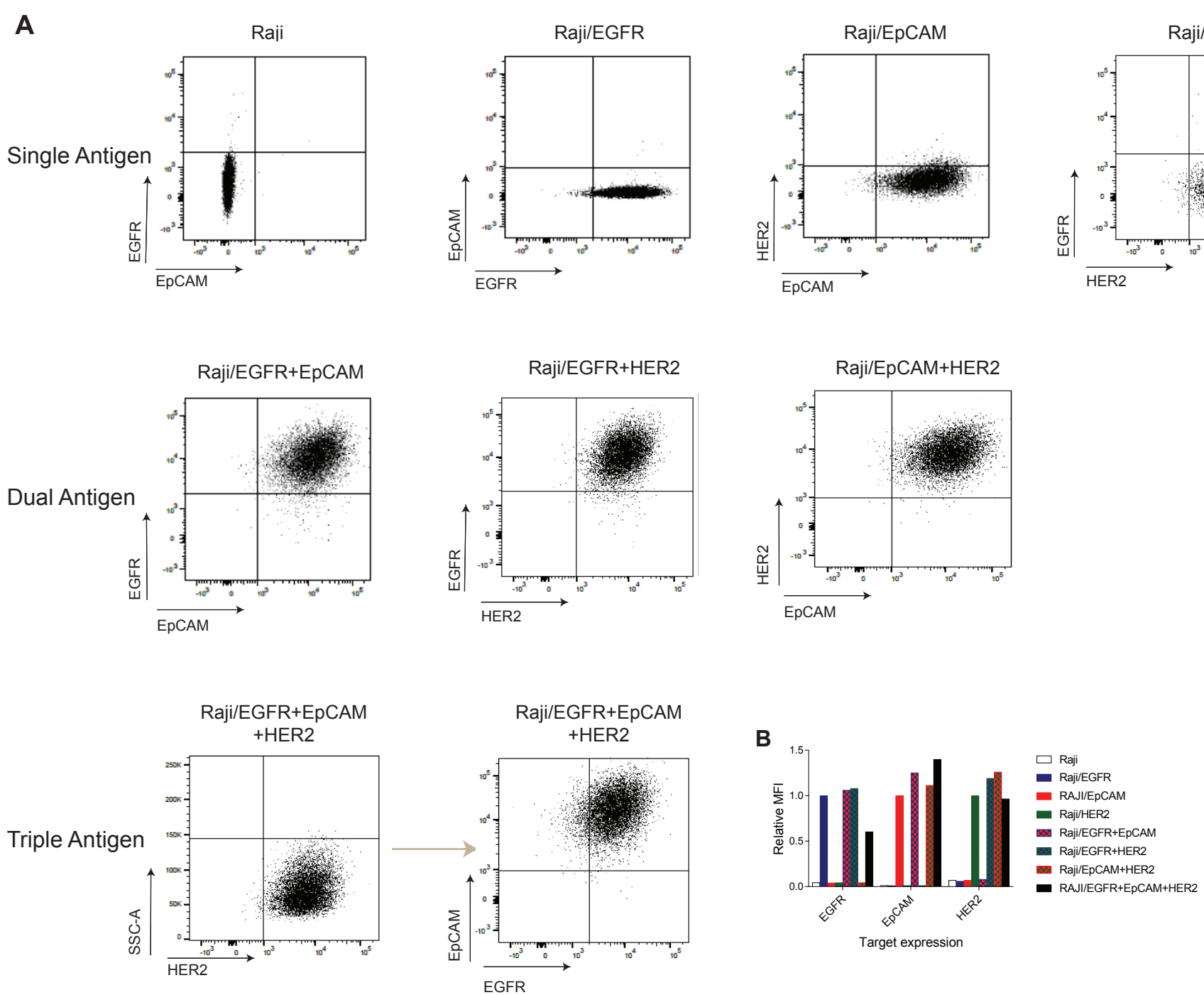
FIGURE S4: Analysis of tonic signaling and function of trispecific and monspecific DARPin CARs. (A) Tonic signaling measured by basal phosphorylation of the CAR CD3ζ chain in monospecific, bispecific and trispecific DARPin CAR T cells. T cells expressing a ROR1-CD28/CD3ζ is shown as a positive control and endogenous CD3ζ as loading controls. (B) PD-1 expression at baseline of monospecific, bispecific and trispecific DARPin CAR T cells. PD-1 expression on CAR-T cells stimulated with MDA-MB-231 targets is shown as a positive control. (C) Cytokine production (IFN-γ) of the bispecific versus trispecific DARPin CAR Tcells against Raji cells expressing 1 or 2 target antigens (n=3). (D) Cytokine production (IFN-γ) of the monospecific, bispecific and trispecific CAR T cells against MDA-MB-231 tumor cells. (**-$p<0.05$, ns-not significant).

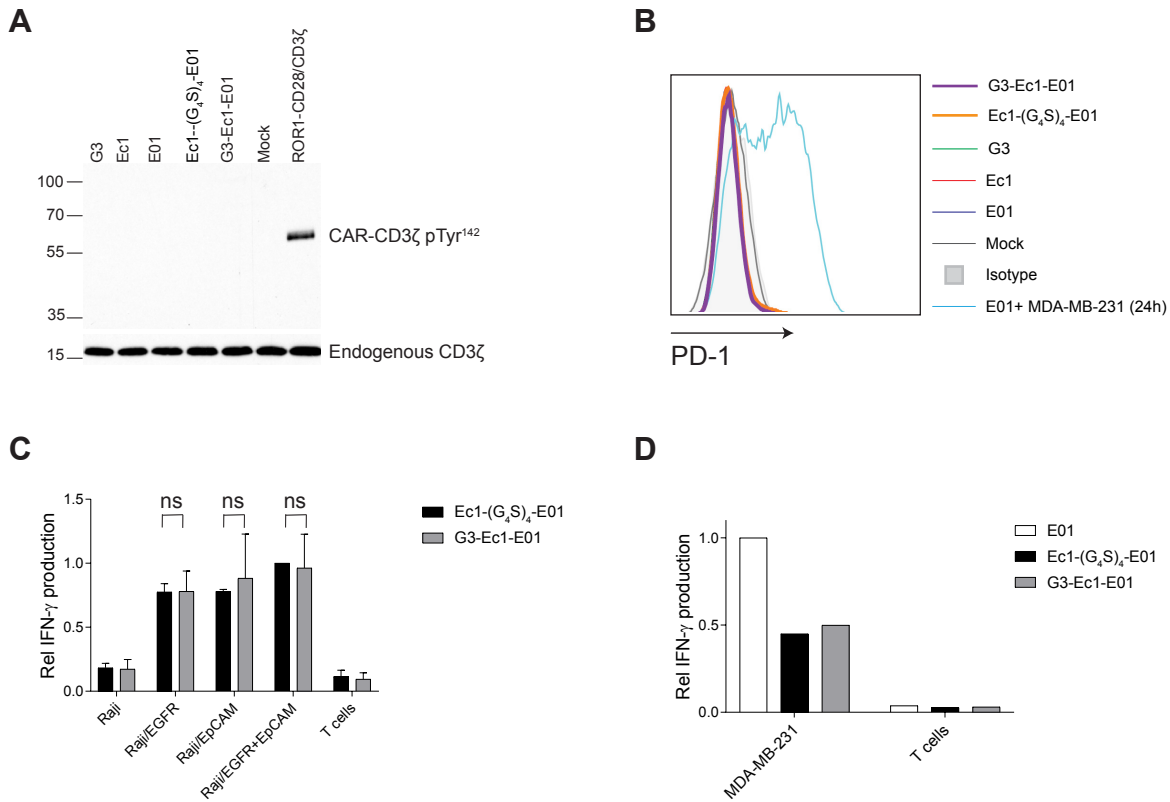
FIGURE S5: Functional assessment of trispecific DARPin CAR T cells against multiple antigens. (A) CAR-T cell expansion of trispecific CAR (G3-Ec1-E01-9 million) in blood of mice with tumors expressing 1 or 2 antigens (n=4). (B) CAR-T cell expansion of trispecific CAR G3-Ec1-E01 (5 million) in blood of mice with tumors expressing 1 or 2 or 3 antigens (n=4). (C) Comparison of trispecific CAR cytotoxicity on expression of single versus multiple antigens on target cells (n=3). (D) Relative Cytokine secretion of trispecific DARPin CAR on incubation with target cells expressing single or multiple antigens (n=3). (E) Proliferation of trispecific DARPin CAR with target cells expressing single or multiple antigens. (F) Trispecific DARPin CAR does not show increased AICD on encountering multiple antigens (n=3). (**-$p<0.05$, ns-not significant)

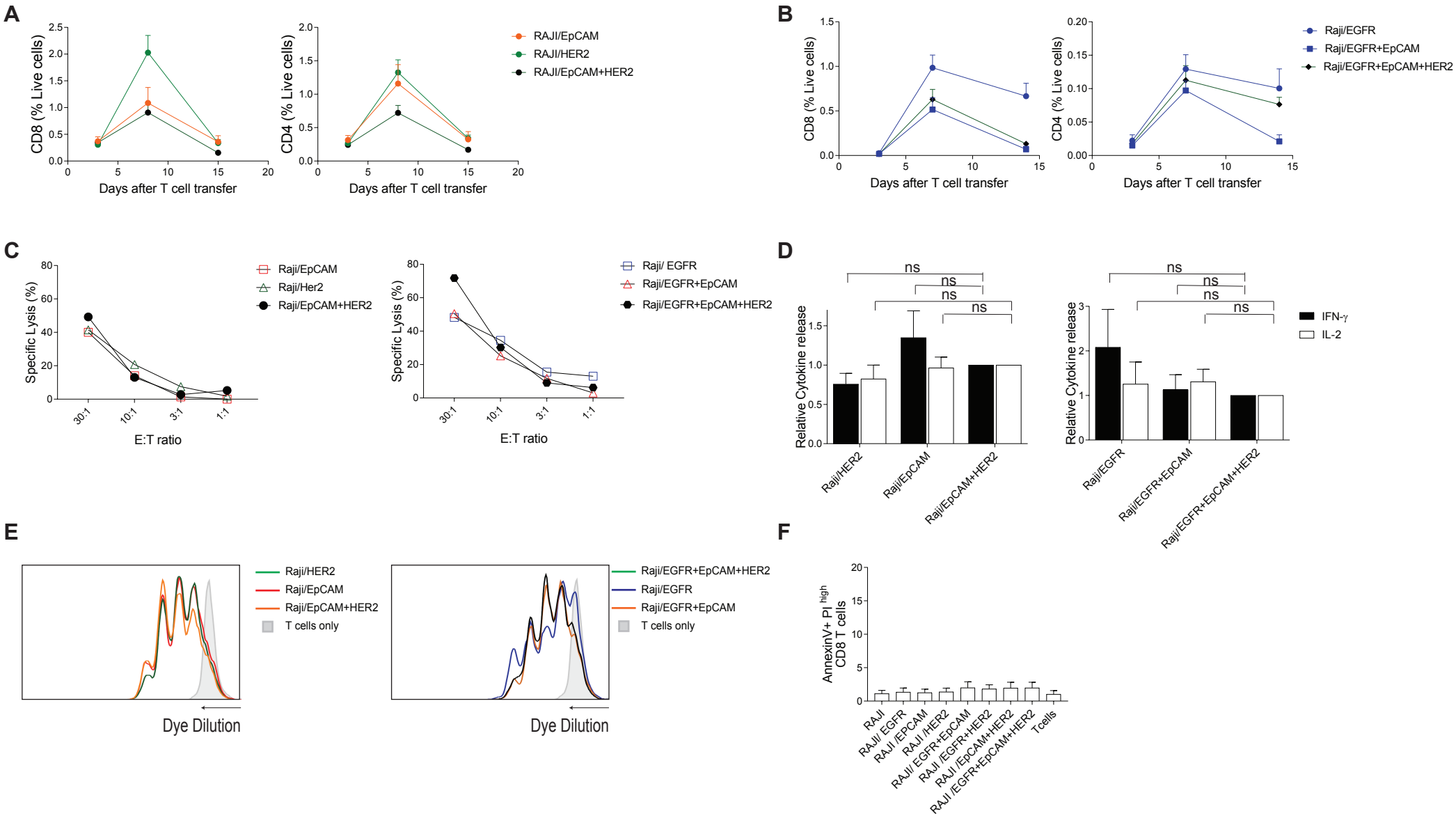
TABLE S1: 9-mer peptides in the scFv and DARPin sequences with predicted binding to the MHC class I supertype with a predicted binding affinity of less than 100 nM.


A
Pre-enrichment
Post
0.37
42.4
0.61
56.6
0.76
95.6
0.032
3.6
CD19
CD8
B
K562/EGFR
MDA-MB-231
NCI-H1975
EGFR
Isotype
EGFR antibody
K562
C
E01
IgG4
Hinge
CD28tm
CD3-ζ
tCD19
Short
Leader
peptide
+/-
(G4S)2
4-1BB
T2A
Long (4/2NQ)
CH2
CH3


A
IFN-γ (pg/ml)
IL-2 (pg/ml)
Short
Long
Cet
Mock
-(G4S)2
+(G4S)2
B
Short
Long
CD4s
Mock
-(G4S)2
+(G4S)2
Cet
Dye Dilution
C
AICD (24 hours)
% Annexin V+ PI+ T cells
D
T cells in Blood (% Live cells)
Days after T cell transfer
E
Tumor -T cells (Day 6 after transfer)
Spleen -T cells (Day 6 after transfer)
T cell Freq (% Live Cells)
CD8
CD4
E01-(G4S)2-Short
E01-(G4S)2-Long


A
Single Antigen
Raji
Raji/EGFR
Raji/EpCAM
Raji/HER2
EGFR
EpCAM
HER2
Dual Antigen
Raji/EGFR+EpCAM
Raji/EGFR+HER2
Raji/EpCAM+HER2
Triple Antigen
Raji/EGFR+EpCAM +HER2
SSC-A
B
Relative MFI
Target expression
Raji
Raji/EGFR
RAJI/EpCAM
Raji/HER2
Raji/EGFR+EpCAM
Raji/EGFR+HER2
Raji/EpCAM+HER2
RAJI/EGFR+EpCAM+HER2


A
G3
Ec1
E01
Ec1-(G4S)4-E01
G3-Ec1-E01
Mock
ROR1-CD28/CD3ζ
100
70
55
35
15
CAR-CD3ζ pTyr142
Endogenous CD3ζ
B
G3-Ec1-E01
Ec1-(G4S)4-E01
G3
Ec1
E01
Mock
Isotype
E01+ MDA-MB-231 (24h)
PD-1
C
Rel IFN-γ production
1.5
1.0
0.5
0.0
ns
ns
ns
Raji
Raji/EGFR
Raji/EpCAM
Raji/EGFR+EpCAM
T cells
Ec1-(G4S)4-E01
G3-Ec1-E01
D
Rel IFN-γ production
1.0
0.5
0.0
MDA-MB-231
T cells
E01
Ec1-(G4S)4-E01
G3-Ec1-E01


A
CD8 (% Live cells)
CD4 (% Live cells)
Days after T cell transfer
RAJI/EpCAM
RAJI/HER2
RAJI/EpCAM+HER2
B
CD8 (% Live cells)
CD4 (% Live cells)
Days after T cell transfer
Raji/EGFR
Raji/EGFR+EpCAM
Raji/EGFR+EpCAM+HER2
C
Specific Lysis (%)
E:T ratio
30:1
10:1
3:1
1:1
Raji/EpCAM
Raji/Her2
Raji/EpCAM+HER2
Raji/ EGFR
Raji/EGFR+EpCAM
Raji/EGFR+EpCAM+HER2
D
Relative Cytokine release
ns
IFN-γ
IL-2
Raji/HER2
Raji/EpCAM
Raji/EpCAM+HER2
Raji/EGFR
Raji/EGFR+EpCAM
Raji/EGFR+EpCAM+HER2
E
Dye Dilution
Raji/HER2
Raji/EpCAM
Raji/EpCAM+HER2
T cells only
Raji/EGFR+EpCAM+HER2
Raji/EGFR
Raji/EGFR+EpCAM
T cells only
F
AnnexinV+ PI high CD8 T cells
RAJI
RAJI/ EGFR
RAJI /EPCAM
RAJI /HER2
RAJI/ EGFR+EpCAM
RAJI /EGFR+HER2
RAJI /EpCAM+HER2
RAJI /EGFR+EpCAM+HER2
Tcells

DARPin/scFV	Position	Peptide	Affinity (nM)	Peptide hit (Human proteome)
Cetuximab	135	ILLTQSPVI	51.93	No Hits
Cetuximab	138	TQSPVILSV	86.36	No Hits
E01	19	ILMANGADV	38.9	100% to GABPB1
E01	97	YIGDTPLHL	82.91	100% to NFKB2
Ec1	116	VLLKNVADV	64.38	No Hits
Ec1	83	VLLKYGADV	96.29	89% to GABPB1
G3	19	ILMANGADV	38.9	100% to GABPB1
G3-Ec1-E01	344	ILMANGADV	38.9	100% to GABPB1
G3-Ec1-E01	18	ILMANGADV	38.9	100% to GABPB1
G3-Ec1-E01	261	VLLKNVADV	64.38	89% to GABPB1
G3-Ec1-E01	422	YIGDTPLHL	82.91	100% to NFKB2
G3-Ec1-E01	228	VLLKYGADV	96.29	89% to GABPB1