Figure S1. Schematic descriptions of the CAR vectors. (A) pcDNA3-scFv-CD28-HIVPR-YFP vector. (B) pcDNA3-scFv-CD28-HIVPR(Δ4)-YFP vector. (C) pcDNA3-Scissors-CAR-YFP vector. (D) pcDNA3-mCherry-CAR vector. (E) pcDNA3-Signal-CAR-T2A-YFP vector. (F) pHR-PGK-Signal-CAR-T2A-YFP vector for lentivirus packaging. (G) pHR-PGK-Scissors-CAR-T2A-mCherry vector for lentivirus packaging. Red bold lines, promoters; blue bold lines, scFv domains; orange bold lines, CD28 domains; green bold lines, HIVPR protease domains; black bold lines, CD3-ζ domains; pink bold lines, mCherry with or without T2A; yellow bold lines, YFP with or without T2A; and green thin lines, HIVPR recognition peptide sequences. CAR, chimeric antigen receptor.

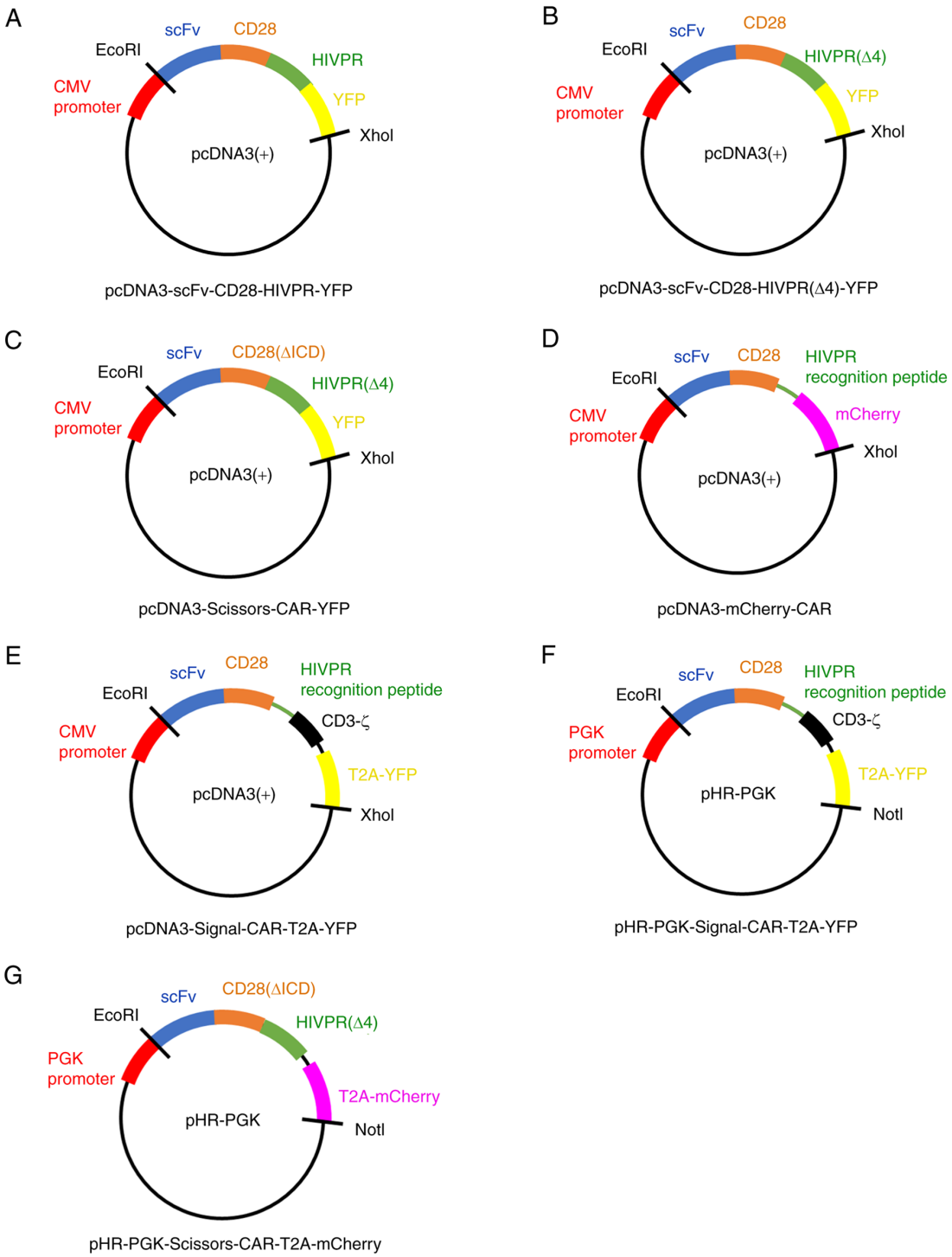

Figure S2. Experiments using 293T cells. Western blot analysis of total cell lysates of 293T cells expressing the indicated CAR constructs. Indicated ratio of anti-CD19-mCherry-CAR and anti-HER2(4D5-3)-Scissors-CAR-T2A-YFP (1:0, 1:0.5, 1:1, 1:2) were transfected into 293T cells as shown at the top. Twenty-four hours after gene transduction, the indicated ratios (top panel 1:0.1, middle panel 1:0.5, bottom panel 1:1) of 293T and Raji cells (indicated at left) were mixed and co-cultivated. After a 24-h co-cultivation, mCherry and YFP proteins were detected with their antibodies, respectively. Arrowheads indicate intact anti-CD19-mCherry-CARs (76 kDa). Asterisks indicate cleaved mCherry proteins (28 kDa). Raji cells expressing only CD19 were used as target cells (left four lanes in each panel); Raji cells expressing both CD19 and HER2 (right four lanes in each panel) were used as target cells. Levels of YFP indicate levels of transduced anti-HER2(4D5-3)-Scissors-CAR-T2A-YFP. Same amounts of anti-CD19-mCherry-CAR were transduced into 293T cells in each transfection. CAR, chimeric antigen receptor.

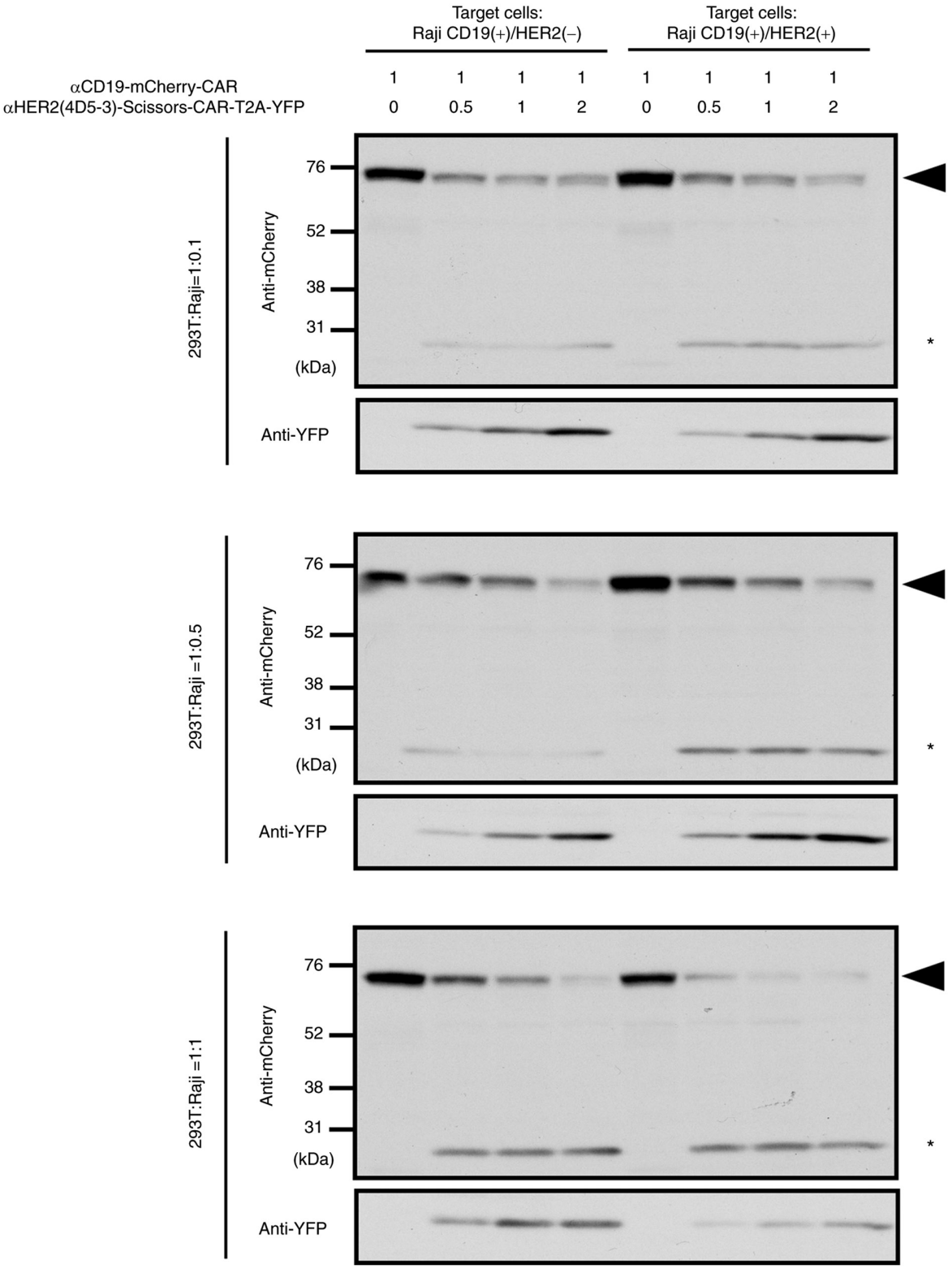

Figure S3. Experiments using Jurkat cells. (A) Flow cytometric analysis of Jurkat cells. Levels of YFP expression are plotted. A gray filled histogram shows data of parental Jurkat cells. A black line histogram shows data of Jurkat cells stably expressing anti-CD19-Signal-CAR-T2A-YFP. (B) Flow cytometric analysis of expression of YFP and mCherry. The x-axis indicates levels of YFP expression. The y-axis indicates levels of mCherry expression. Jurkat cells with anti-CD19-Signal-CAR-T2A-YFP (αCD19) were analyzed (left panel). Middle and right panels show results of cells with both anti-CD19-Signal-CAR-T2A-YFP and anti-HER2-Scissors-CAR-T2A-mCherry. Two types of anti-HER2 scFv, 4D5-3 (middle panel) and 4D5-8 (right panel), were used in the Scissors-CARs. YFP-positive cells (left panel) and cells positive for both YFP and mCherry (middle and right panels) were sorted and used for co-cultivation assays in Fig. 5. (C) Flow cytometric analysis of CD69 expression on Jurkat cells. The x-axis indicates levels of YFP expression. The y-axis indicates levels of CD69 expression. Top panels indicate results of co-cultivation with Jurkat cells expressing only anti-CD19-Signal-CAR-T2A-YFP and indicated target cells. Middle and bottom panels indicate results of co-cultivation with Jurkat cells expressing both anti-CD19-Signal-CAR and anti-HER2-Scissors-CAR. Two types of anti-HER2 scFv, 4D5-3 (middle panels) and 4D5-8 (bottom panels), were used for Scissors-CARs. Circles indicate cells with CD69-positive cells (percentages of positive cells are shown under the circles). (D) Flow cytometric analysis of levels of CD19 and HER2 expression on parental (upper) and the engineered (lower) SK-BR-3 cells. pcDNA3(-)-CD19-Hygro vector was transduced into SK-BR-3 cells. After selection for 4 weeks, the cells were analyzed. The x-axis indicates levels of HER2 expression. The y-axis indicates levels of CD19 expression. (E) Flow cytometric analysis of CD69 expression on Jurkat cells. Jurkat cells expressing both anti-CD19-Signal-CAR and anti-HER2-Scissors-CAR were co-cultivated with the engineered SK-BR-3 cells expressing both CD19 and HER2. Two types of anti-HER2 scFv, 4D5-3 (middle) and 4D5-8 (bottom), were used for the Scissors-CARs. A result of Jurkat cells expressing only anti-CD19-Signal-CAR is shown in the top panel. The x-axis indicates levels of YFP expression. The y-axis indicates levels of CD69 expression. Circles indicate cells with CD69-positive cells (percentages of positive-cells are shown under the circles). CAR, chimeric antigen receptor.

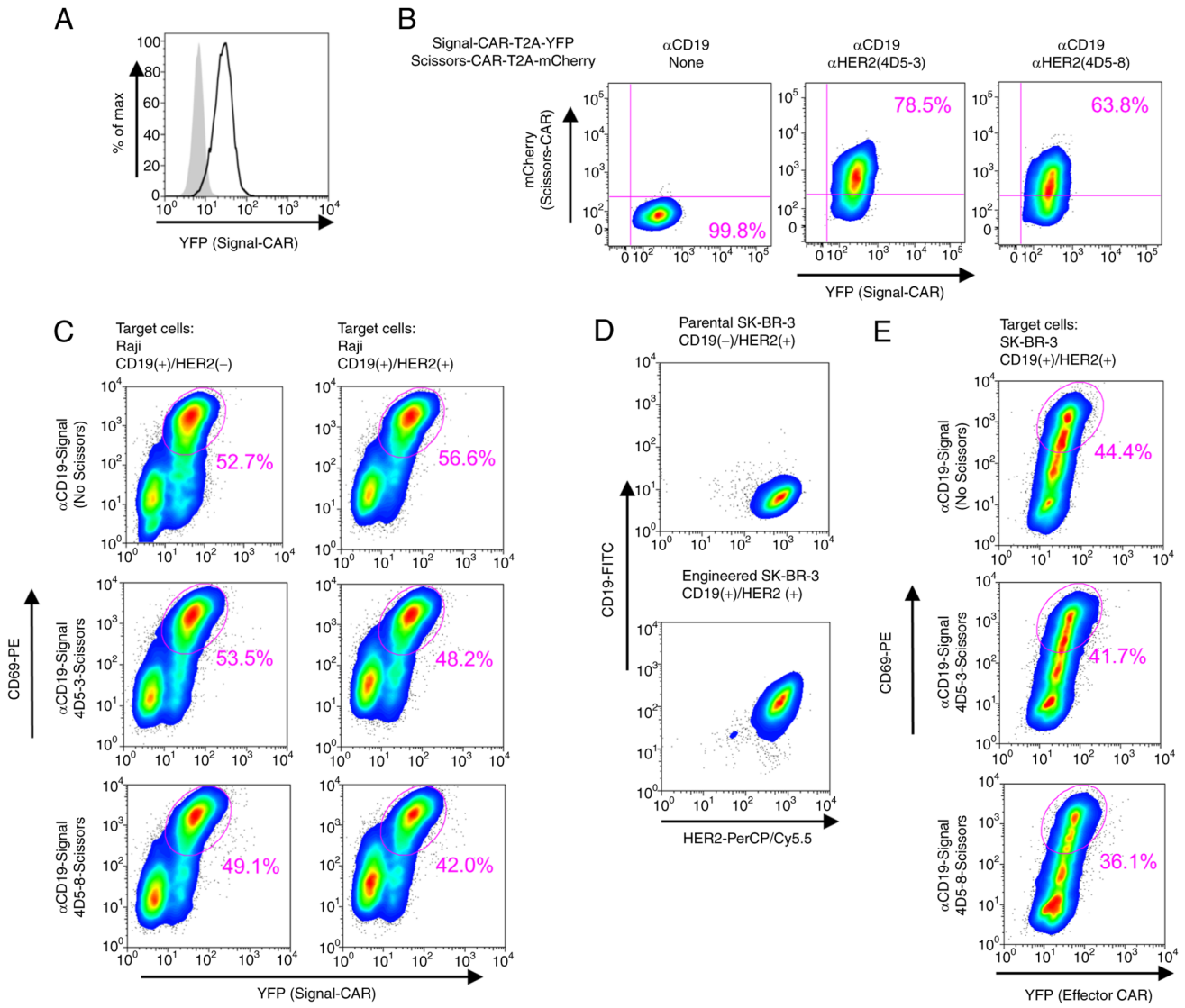

Figure S4. Experiments using primary CAR-T cells. (A) Target-cell-specific cytotoxicity of primary CAR-T cells. Specific cytotoxicity of the CAR-T cells (%) are plotted. Values on the x-axis indicate the ratio of Effector (E) and Target (T) cells. A black broken line indicates results of assays using non-manipulated T cells co-cultivated with K562 cells. A red broken line indicates results of assays using non-manipulated T cells co-cultivated with Raji cells. A black line indicates results of assays using anti-CD19-Signal-CAR-T cells co-cultivated with K562 cells. A red line indicates results of assays using anti-CD19-Signal-CAR-T cells co-cultivated with Raji cells. Mean values ± SD of triplicated experiments are plotted. Data were analyzed using one-way ANOVA with Tukey post-hoc test for multiple comparison as shown in Table SI. (B) Flow cytometric analysis of the levels of YFP and mCherry expressions. The x-axis indicates levels of YFP expression. The y-axis indicates levels of mCherry expression. The left panel shows a result of non-manipulated T cells as a negative control. A second panel from the left shows a result of primary T cells infected by lentivirus with anti-CD19-Signal-CAR-T2A-YFP alone (αCD19). Two panels on the right show results of primary T cells infected by lentivirus with both anti-CD19-Signal-CAR-T2A-YFP and anti-HER2-Scissors-CAR-T2A-mCherry. Two types of anti-HER2 scFv, 4D5-3 (third panel from the left) and 4D5-8 (right panel), were used for the Scissors-CARs. Percentages of double-positive-cells are shown in pink letters (right two panels). Double-positive-cells on the right two panels were sorted and used for cytotoxicity assays in Fig. 6. CAR, chimeric antigen receptor.

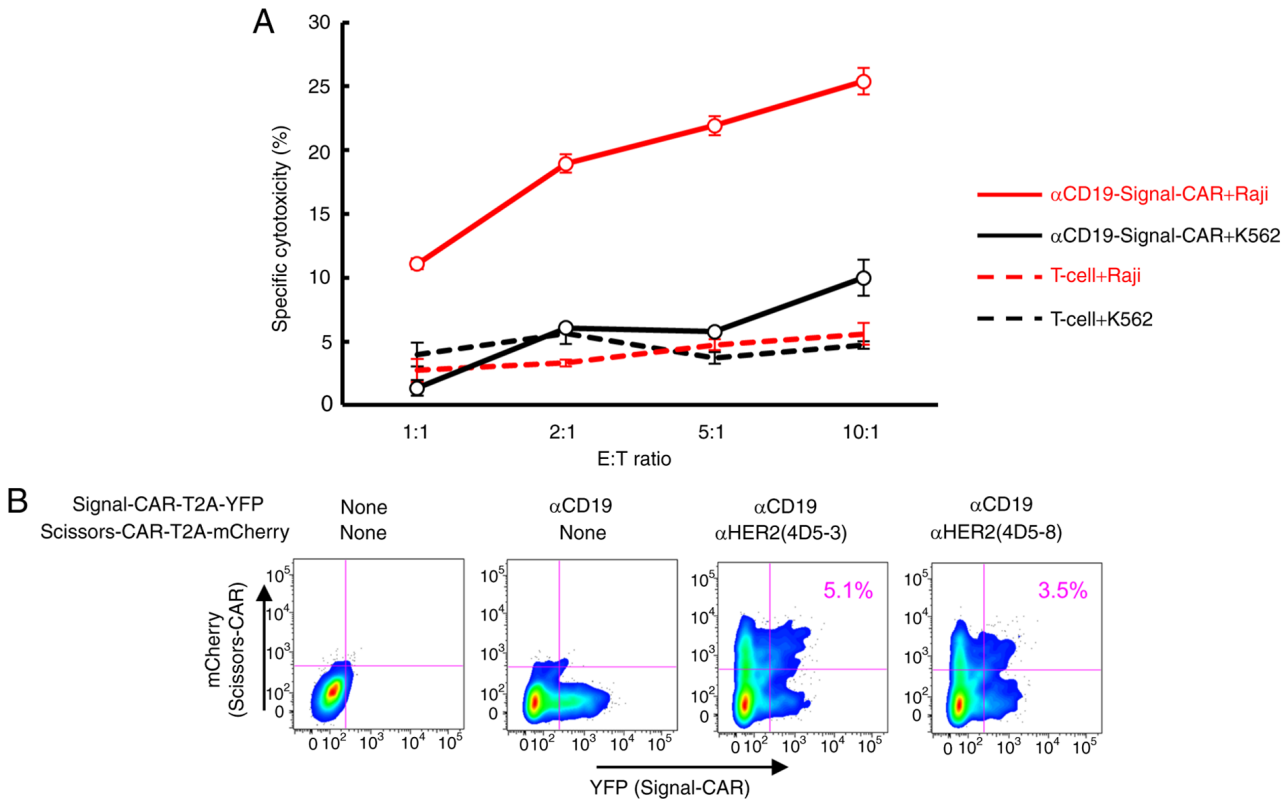

Table SI. Results of the statistical analysis using one-way ANOVA with Tukey post-hoc test for Fig. S4A.

A, Analysis results of Effector:Target (E:T) ratio=1:1 dataset

Comparison group	Compared with	Mean difference	P-value	95% CI	
				Upper limit	Lower limit
T cell + K562	T cell + Raji	-1.23	0.782	2.94	-5.41
	CAR + K562	-2.61	0.265	1.57	-6.78
	CAR + Raji	7.11[a]	0.003	11.29	2.93
T cell + Raji	CAR + K562	1.37	0.725	5.55	-2.80
	CAR + Raji	8.34[a]	0.001	12.52	4.17
CAR + K562	CAR + Raji	9.72[b]	<0.001	13.90	5.54

B, Analysis results of E:T ratio=2:1 dataset

Comparison group	Compared with	Mean difference	P-value	95% CI	
				Upper limit	Lower limit
T cell + K562	T cell + Raji	-2.31	0.150	0.75	-5.37
	CAR + K562	0.45	0.963	3.51	-2.61
	CAR + Raji	13.31[b]	<0.001	16.38	10.26
T cell + Raji	CAR + K562	-2.76	0.077	0.29	-5.83
	CAR + Raji	15.63[b]	<0.001	18.70	12.57
CAR + K562	CAR + Raji	12.86[b]	<0.001	15.92	9.80

C, Analysis results of E:T ratio=5:1 dataset

Comparison group	Compared with	Mean difference	P-value	95% CI	
				Upper limit	Lower limit
T cell + K562	T cell + Raji	1.03	0.644	3.78	-1.72
	CAR + K562	2.09	0.148	4.84	-0.66
	CAR + Raji	18.21[b]	<0.001	20.96	15.46
T cell + Raji	CAR + K562	-1.05	0.626	1.69	-3.81
	CAR + Raji	17.18[b]	<0.001	19.93	14.43
CAR + K562	CAR + Raji	16.12[b]	<0.001	18.88	13.38

D, Analysis results of E:T ratio=10:1 dataset

Comparison group	Compared with	Mean difference	P-value	95% CI	
				Upper limit	Lower limit
T cell + K562	T-cell + Raji	0.88	0.953	6.38	-4.61
	CAR + K562	5.28	0.060	10.77	-0.22
	CAR + Raji	20.68[b]	<0.001	26.18	15.18
T cell + Raji	CAR + K562	-4.39	0.124	1.10	-9.89
	CAR + Raji	19.80[b]	<0.001	25.29	14.30
CAR + K562	CAR + Raji	15.40[b]	<0.001	20.90	9.91

CI, confidence interval; CAR, chimeric antigen receptor (In Fig. S4A, indicated as 'αCD19-Signal-CAR'). [a]P<0.01 and [b]P<0.001 indicate significant differences.