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Supplemental Information

Function of Novel Anti-CD19 Chimeric Antigen Receptors with Human Variable Regions Is Affected by Hinge and Transmembrane Domains

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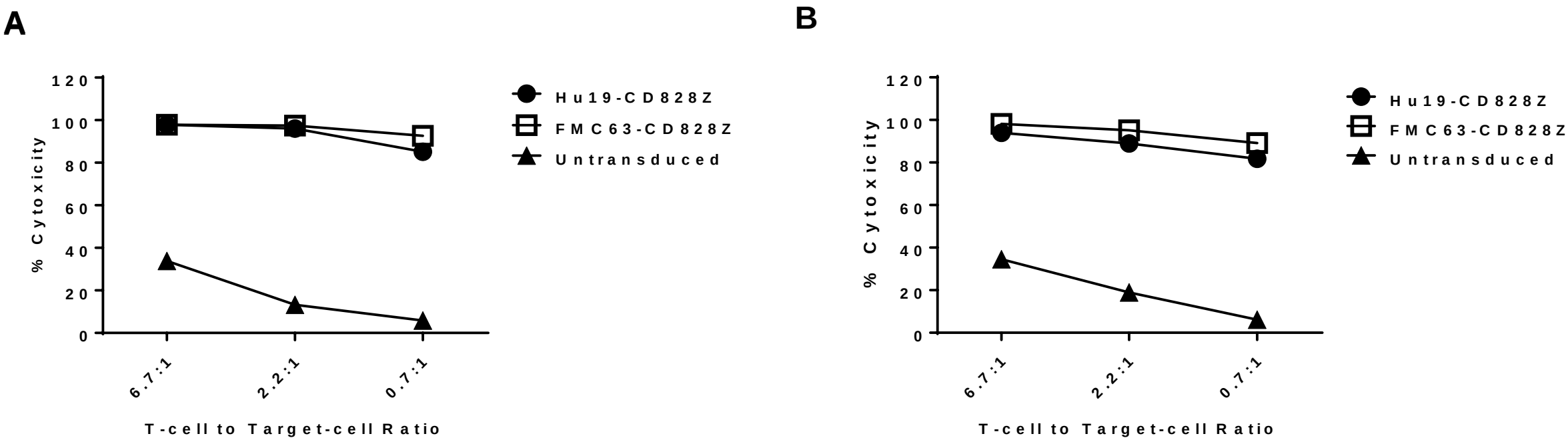
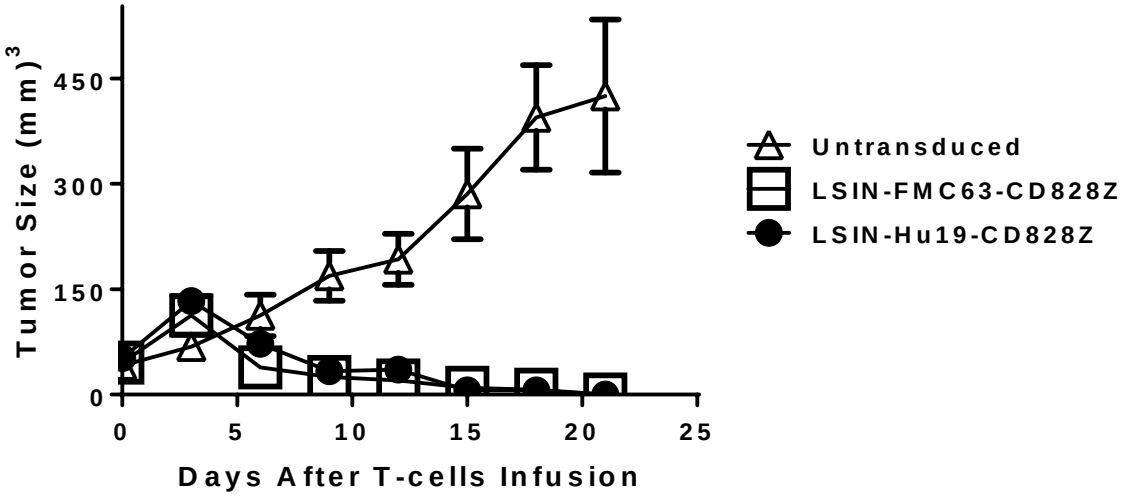
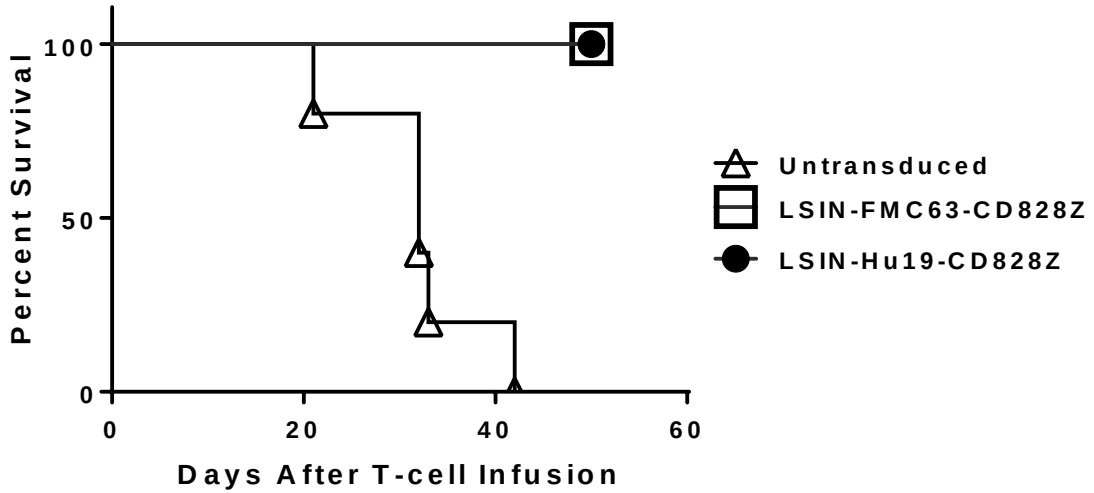
Supplemental Figure 1



T cells from the same patient were transduced with either Hu19-CD828Z or FMC63-CD828Z. These T cells were tested in 4 hour cytotoxity assays. T cells were cultured at the indicated T cell to target cell ratios with either NALM6 (A) or Toledo (B) target cells. The mean and standard error of the mean cytotoxicity are shown. No substantial difference in cytotoxicity between the 2 CARs was detected.

Supplemental Figure 2: in vivo comparison of FMC63-CD828Z and Hu19-CD828Z





NALM6 tumors were established in immunocompromised NSG mice. The mice then received infusions of 8 million T cells transduced with LSIN-FMC63-CD828Z or LSIN-Hu19-CD828Z or Untransduced T cells. Tumors were measured every 3 days in a blinded manner. There were 5 mice in each group. The tumor sizes and the survival of the mice are shown.

Supplemental Table 1: Raw IFN-gamma ELISA data from Figure 2

	Nalm-6	NGFR-K562	CAR %
Patient 1 Hu19-28z	12368	111	65
Patient 1 Hu19-828z	3285	151	77
Patient 2 Hu19-28z	11589	44	55
Patient 2 Hu19-828z	3617	123	74
Patient 3 Hu19-28z	7047	29	57
Patient 3 Hu19-828z	2665	68	64
Patient 4 Hu19-28z	3637	25	66
Patient 4 Hu19-828z	1728	36	75
Patient 5 Hu19-28z	12770	189	39
Patient 5 Hu19-828z	5469	203	45

Cultured T cells from the indicated patients were transduced with the indicated CARs and cultured overnight with the indicated target cells. After the overnight incubation, a standard ELISA assay was performed on the culture supernatant. NALM6 is $CD19^{+}$ and NGFR-K562 is CD19-negative. The percentage of the T cells expressing the indicated CAR is given as the CAR %. All values are in pg/mL. The normalized version of these results is in Figure 2G.

Supplemental Table 2: Raw TNFα ELISA data from Figure 2

	Nalm-6	NGFR-K562	CAR %
Patient 1 Hu19-28z	1558	<40	65
Patient 1 Hu19-828z	530	<40	77
Patient 2 Hu19-28z	1663	<40	55
Patient 2 Hu19-828z	894	<40	74
Patient 3 Hu19-28z	2224	0	57
Patient 3 Hu19-828z	840	0	64
Patient 4 Hu19-28z	1797	25	66
Patient 4 Hu19-828z	771	19	75
Patient 5 Hu19-28z	1988	35	39
Patient 5 Hu19-828z	950	35	45

Cultured T cells from the indicated patients were transduced with the indicated CARs and cultured overnight with the indicated target cells. After the overnight incubation, a standard ELISA assay was performed on the culture supernatant. NALM6 is $CD19^{+}$ and NGFR-K562 is CD19-negative. All values are in pg/mL. The percentage of the T cells expressing the indicated CAR is given as the CAR %. The normalized version of these results is in Figure 2H.

Supplemental Table 3: Raw IFN-gamma ELISA data from Figure 3

IFN-gamma			
	CD19-K562	NGFR-K562	CAR %
Patient 1 FMC63-28z	81568	202	75
Patient 1 FMC63-828z	29983	133	89
Patient 2 FMC63-28z	69146	170	79
Patient 2 FMC63-828z	21771	144	93
Patient 3 FMC63-28z	74756	462	73
Patient 3 FMC63-828z	36054	155	73
Patient 4 FMC63-28z	57845	985	84
Patient 4 FMC63-828z	19888	254	93
Patient 5 FMC63-28z	20694	282	71
Patient 5 FMC63-828z	8795	72	87

Cultured T cells from the indicated patients were transduced with the indicated CARs and cultured overnight with the indicated target cells. After the overnight incubation, a standard ELISA assay was performed on the culture supernatant. CD19-K562 is $CD19^{+}$ and NGFR-K562 is CD19-negative. All values are in pg/mL. The percentage of the T cells expressing the indicated CAR is given as the CAR %. The normalized version of these results is in Figure 3E.

Supplemental Table 4: Raw TNF-α ELISA data from Figure 3

	CD19-K562	NGFR-K562	CAR %
Patient 1 FMC63-28z	9671	60	75
Patient 1 FMC63-828z	5510	36	89
Patient 2 FMC63-28z	2732	21	79
Patient 2 FMC63-828z	1552	16	93
Patient 3 FMC63-28z	6243	89	73
Patient 3 FMC63-828z	2354	32	73
Patient 4 FMC63-28z	9865	100	84
Patient 4 FMC63-828z	3238	<40	93
Patient 5 FMC63-28z	15518	671	71
Patient 5 FMC63-828z	7462	53	87

Cultured T cells from the indicated patients were transduced with the indicated CARs and cultured overnight with the indicated target cells. After the overnight incubation, a standard ELISA assay was performed on the culture supernatant. CD19-K562 is $CD19^+$ and NGFR-K562 is CD19-negative. All values are in pg/mL. The percentage of the T cells expressing the indicated CAR is given as the CAR %. The normalized version of these results is in Figure 3F.

Supplemental Table 5: Raw IL-2 ELISA data from Figure 4

	Nalm-6	NGFR-K562	CAR %
Patient 1 Hu19-28z	373	12	62
Patient 1 Hu19-828z	15	12	74
Patient 2 Hu19-28z	1226	17	34
Patient 2 Hu19-828z	268	20	46
Patient 3 Hu19-28z	197	20	68
Patient 3 Hu19-828z	44	22	80
Patient 4 Hu19-28z	886	126	60
Patient 4 Hu19-828z	714	147	75
Patient 5 Hu19-28z	1024	68	46
Patient 5 Hu19-828z	302	40	50
Patient 6 Hu19-28z	1404	16	57
Patient 6 Hu19-828z	81	16	64

Cultured T cells from the indicated patients were transduced with the indicated CARs and cultured overnight with the indicated target cells. After the overnight incubation, a standard ELISA assay was performed on the culture supernatant. NALM6 is $CD19^{+}$ and NGFR-K562 is CD19-negative. All values are in pg/mL. The percentage of the T cells expressing the indicated CAR is given as the CAR %. The normalized version of these results is in Figure 4E.

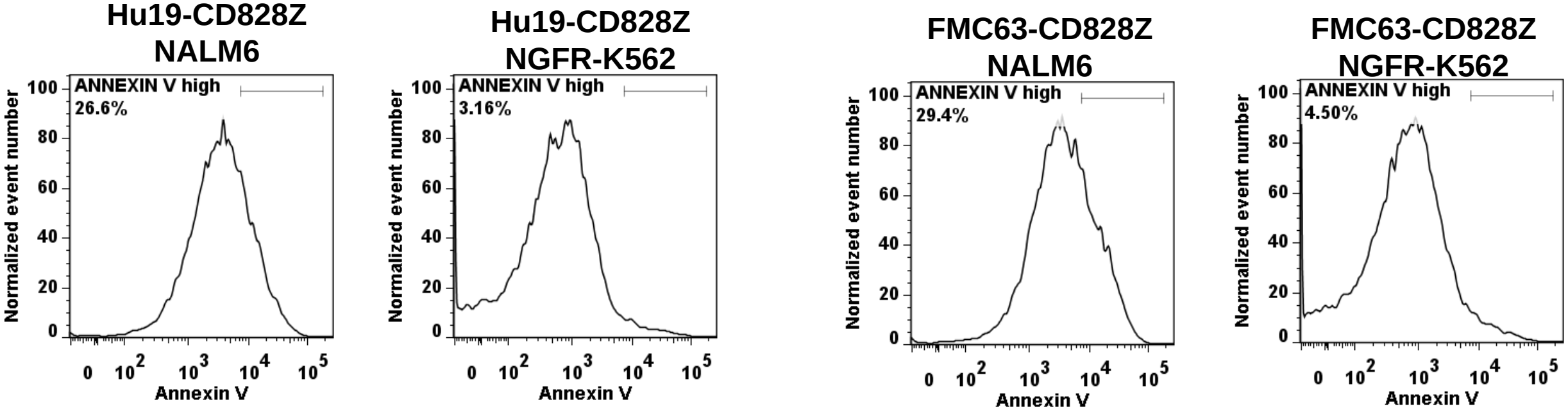
Supplemental Table 6: absolute values of median fluorescent intensity of phosphorylated tyrosine-142 in CD3ζ ITAMs of CAR T cells

Patient	Hu19-CD828Z plus NALM6	Hu19-CD828Z plus NGFR-K562	Hu19-28Z plus NALM6	Hu19-28Z plus NGFR-K562
1	270	245	211	140
2	294	211	235	158
3	348	299	267	190
4	376	347	268	186

All numbers are median fluorescent intensity.

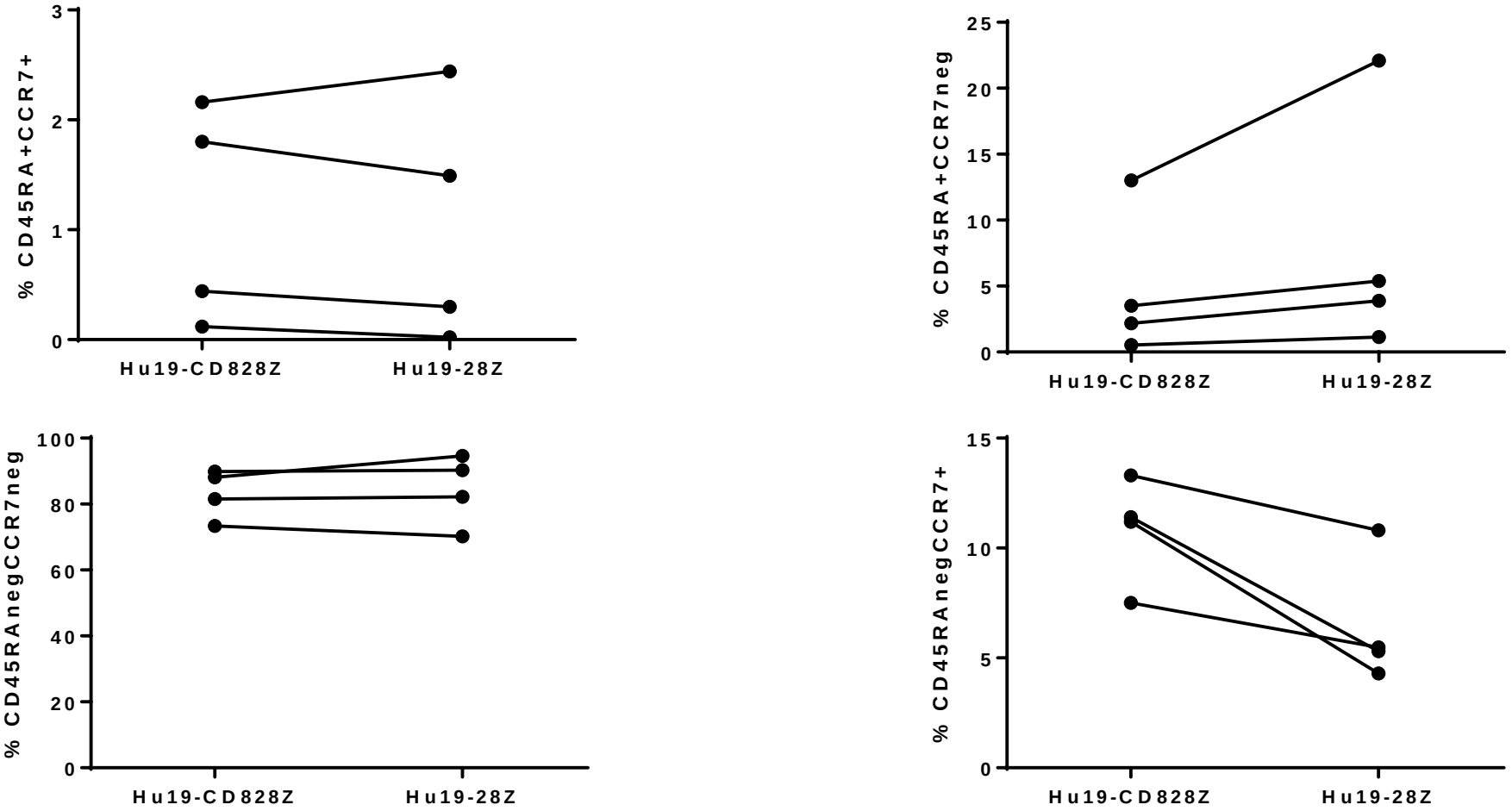
T cells expressing the indicated CARs were cultured with the indicated target cells.

Supplemental Figure 3: Comparison of AICD with Hu19 versus FMC63 scFvs



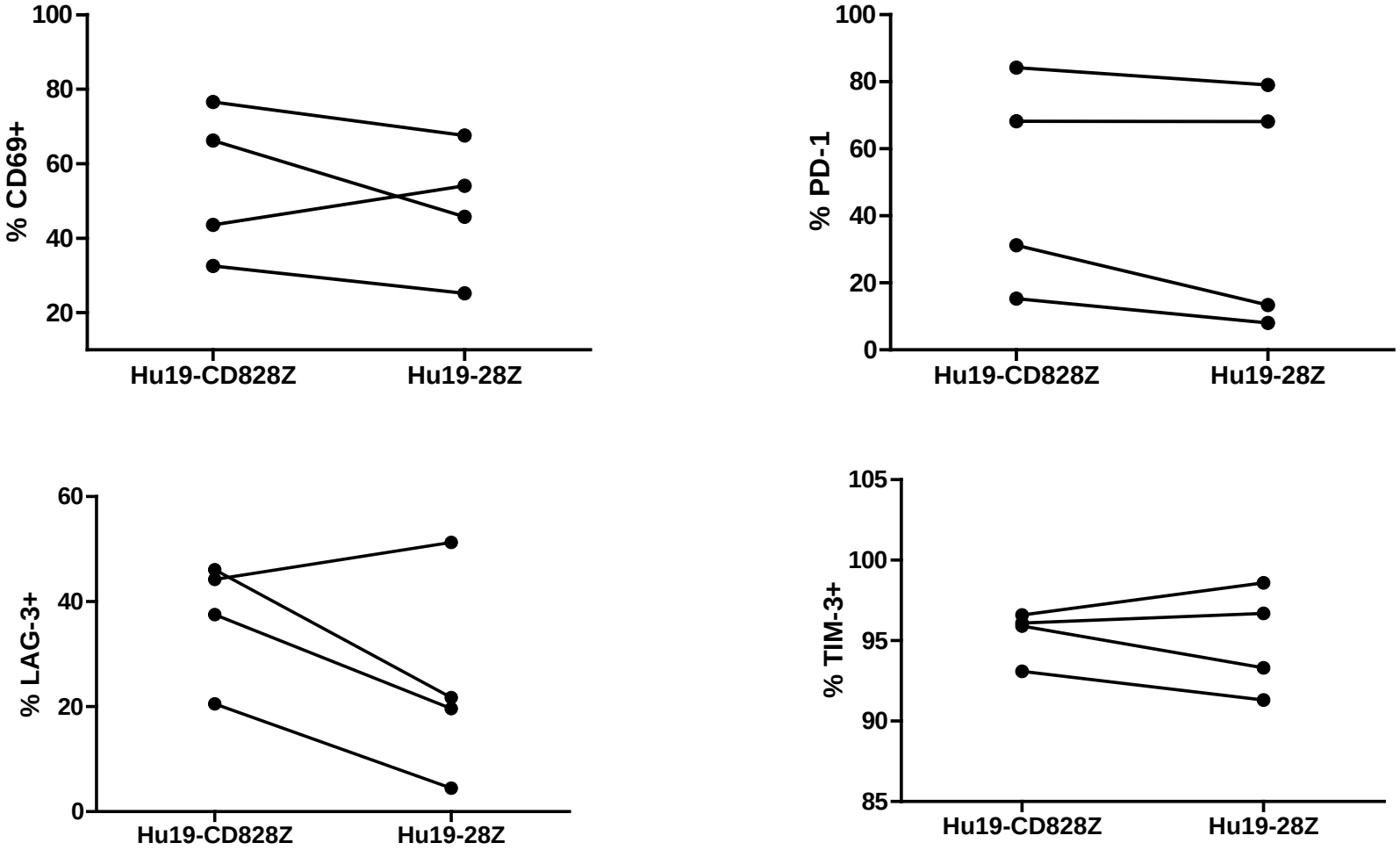
Levels of activation-induced cell death (AICD) were similar in T cells expressing either Hu19-CD828Z or FMC63-CD828Z. T cells from the same donor that were transduced with either Hu19-CD828Z or FMC63-CD828Z were cultured with either $CD19^{+}$ NALM6 cells or CD19-negative NGFR-K562 cells. In the figure, the CAR that T cells were transduced with is on the top line of the label and the target cell is on the bottom line of the label. This is a representative example of 2 different experiments with cells from different donors.

Supplemental Figure 4: memory markers at day 7 after culture initiation



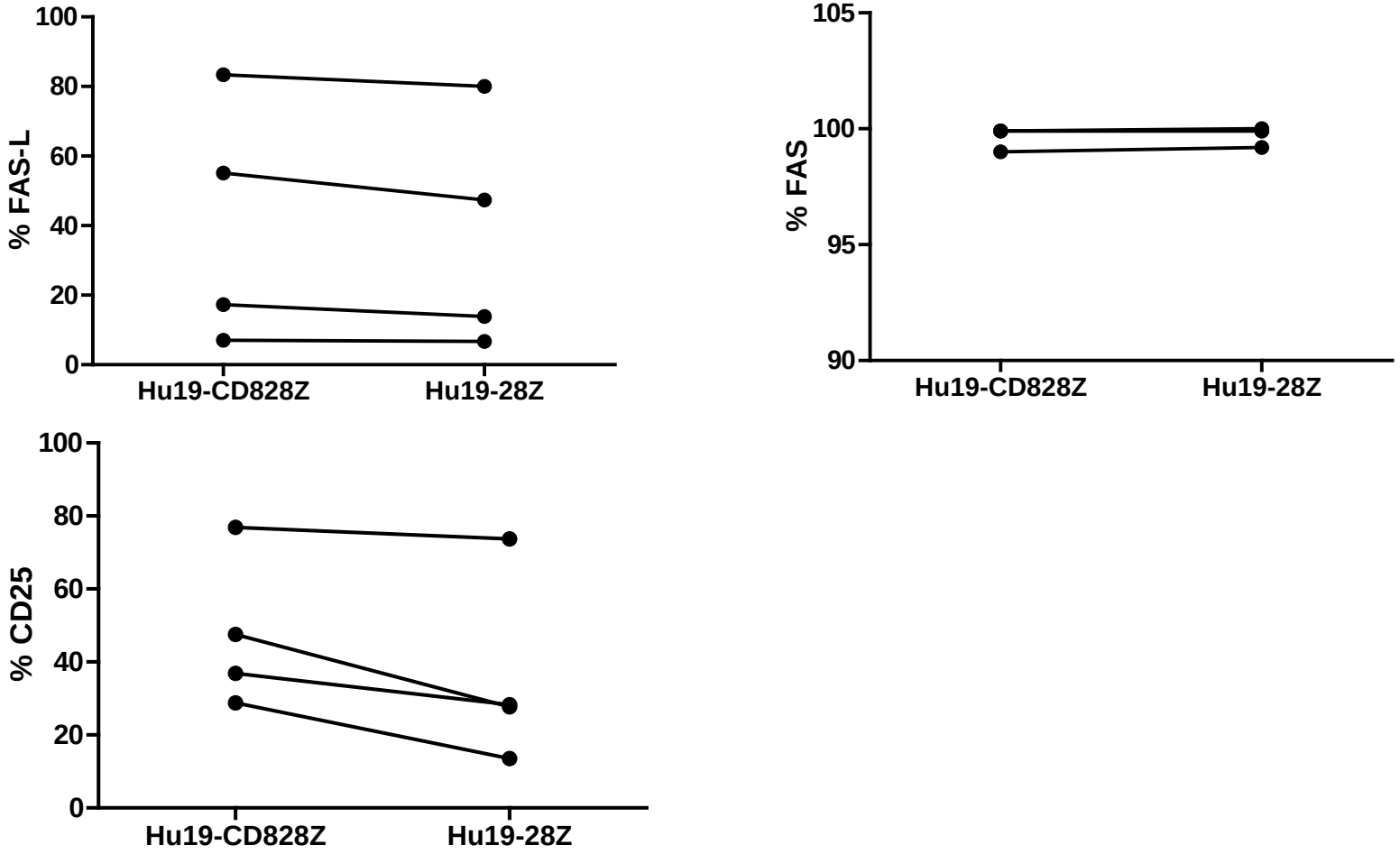
PBMC from 4 patients were activated with an anti-CD3 antibody and transduced with either Hu19-CD828Z or Hu19-28Z. On day 7 after culture initiation, the cells were stained for the indicated markers. Plots of CCR7 versus CD45RA gated on live, $CD3^+$, CAR^+ lymphocytes were analyzed. There was a statistically-significant difference between Hu19-CD828Z and Hu19-28Z only for the %CD45RA-negative, $CCR7^+$ central memory T cells (P=0.039, paired two-tailed t test).

Supplemental Figure 5A: Activation and exhaustion markers at day 7 after culture initiation



PBMC from 4 patients were activated with an anti-CD3 antibody and transduced with either Hu19-CD828Z or Hu19-28Z. On day 7 after culture initiation, the cells were stained for the indicated markers. Plots are gated on live, $CD3^+$, CAR^+ lymphocytes. There was not a statistically-significant difference between Hu19-CD828Z and Hu19-28Z for CD69, PD-1, LAG-3, or TIM-3.

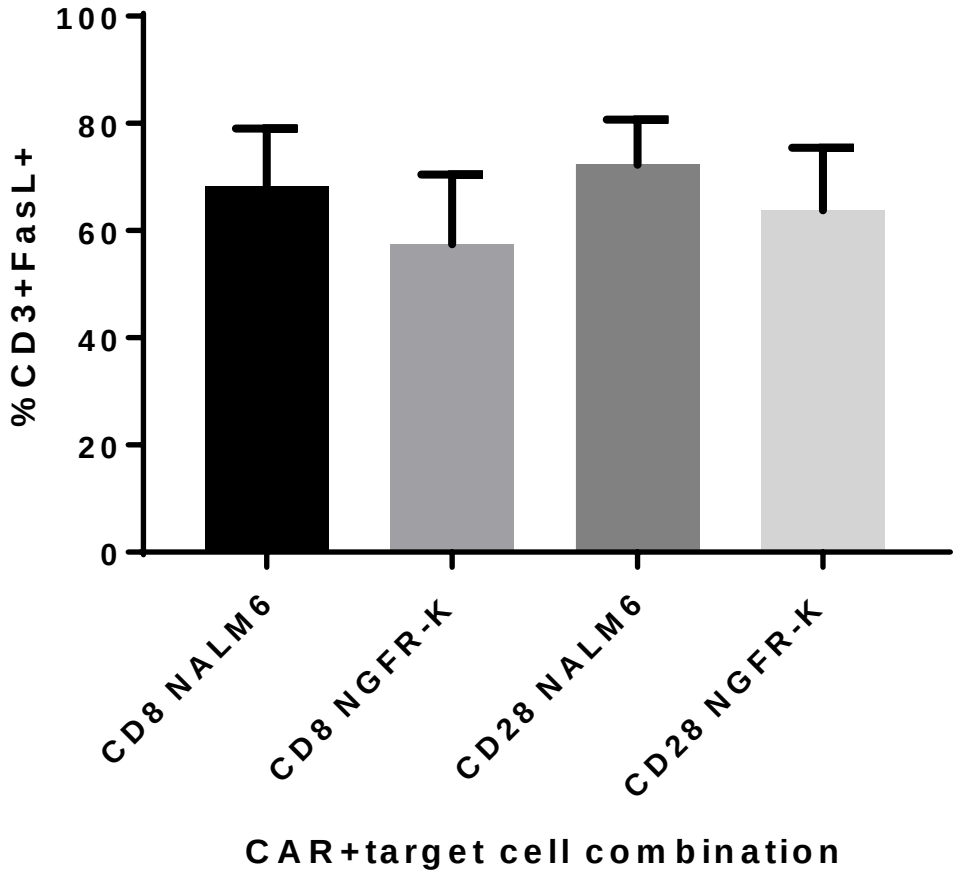
Supplemental Figure 5B: Activation and exhaustion markers at day 7 after culture initiation



PBMC from 4 patients were activated with an anti-CD3 antibody and transduced with either Hu19-CD828Z or Hu19-28Z. On day 7 after culture initiation, the cells were stained for the indicated markers. Plots are gated on live, $CD3^+$, CAR^+ lymphocytes. There was not a statistically-significant difference between Hu19-CD828Z and Hu19-28Z for FAS-L or FAS expression. CD25 expression was slightly lower on Hu19-28Z T cells (P=0.049, paired 2-tailed t test).

Supplemental Figure 6

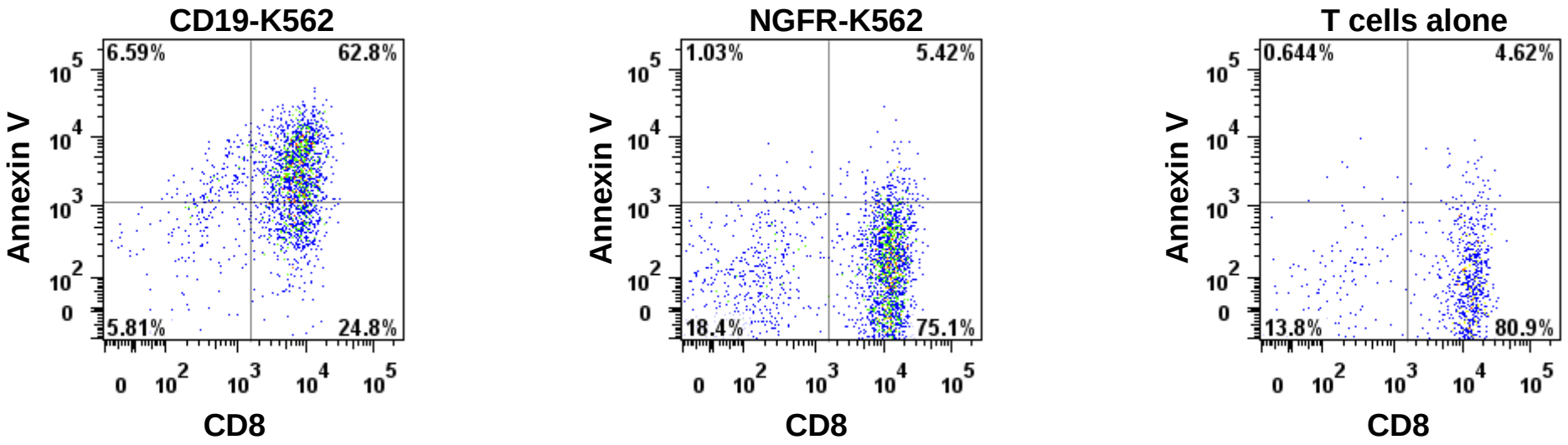
Fas ligand



T cells from 4 patients were transduced with either Hu19-CD828Z (CD8 on x-axis label) or Hu19-28Z (28 on x-axis label). The T cells were stimulated with irradiated CD19-K562 cells on day 7 and day 10 after initiation of the cultures. On day 12 after culture initiation, the T cells were cultured overnight with either $CD19^+$ NALM6 cells (NALM6 on x-axis label) or CD19-negative NGFR-K562 cells (NGFR-K on x-axis label). FasL (Fas ligand) staining was conducted. The mean (+ standard error of mean) percentage of $CD3^+$ CAR^+ cells that expressed FasL is shown. There was not a statistically significant difference between the different groups.

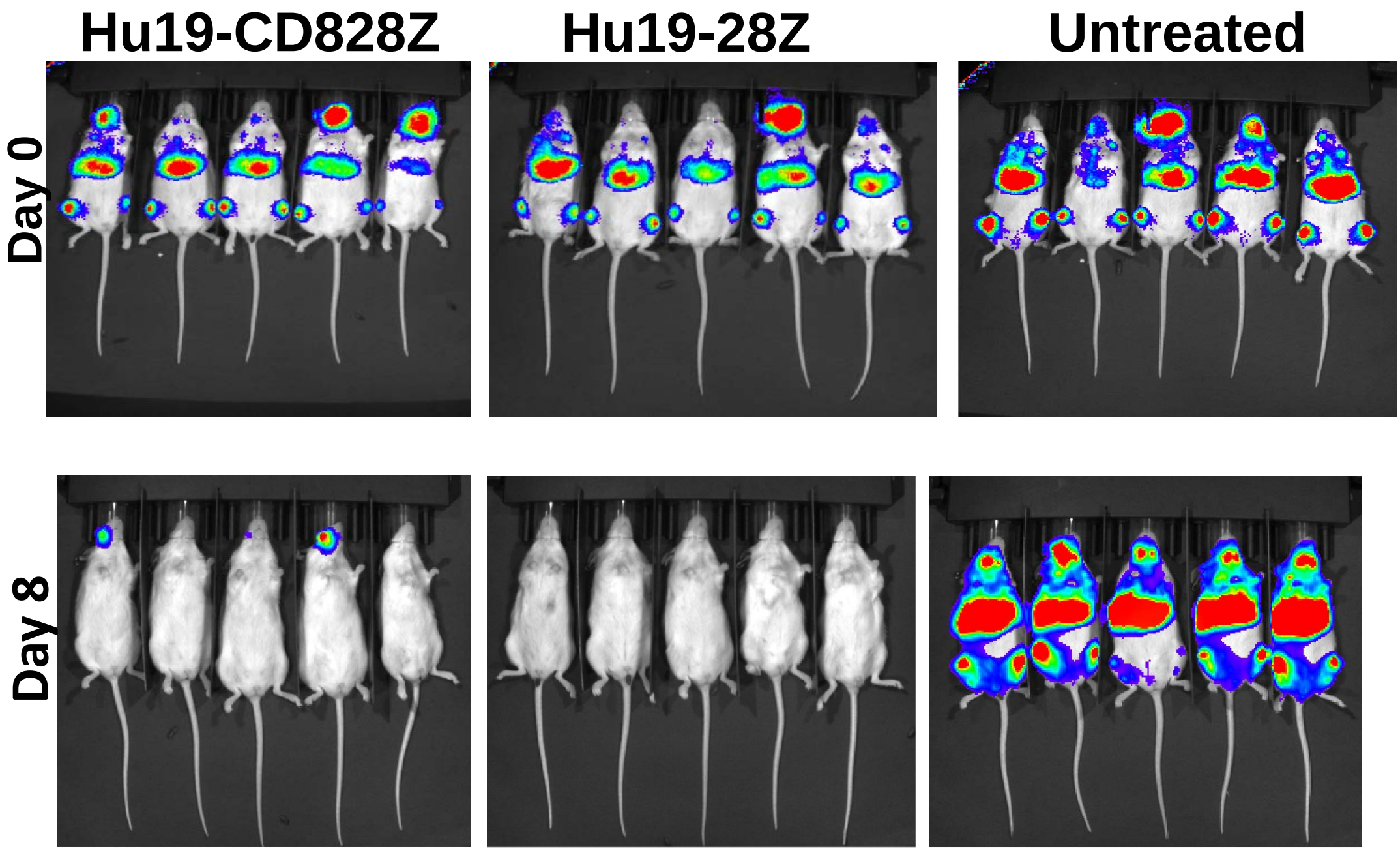
Supplemental Figure 7

T cells expressing FMC63-28Z underwent activation-induced cell death in vitro



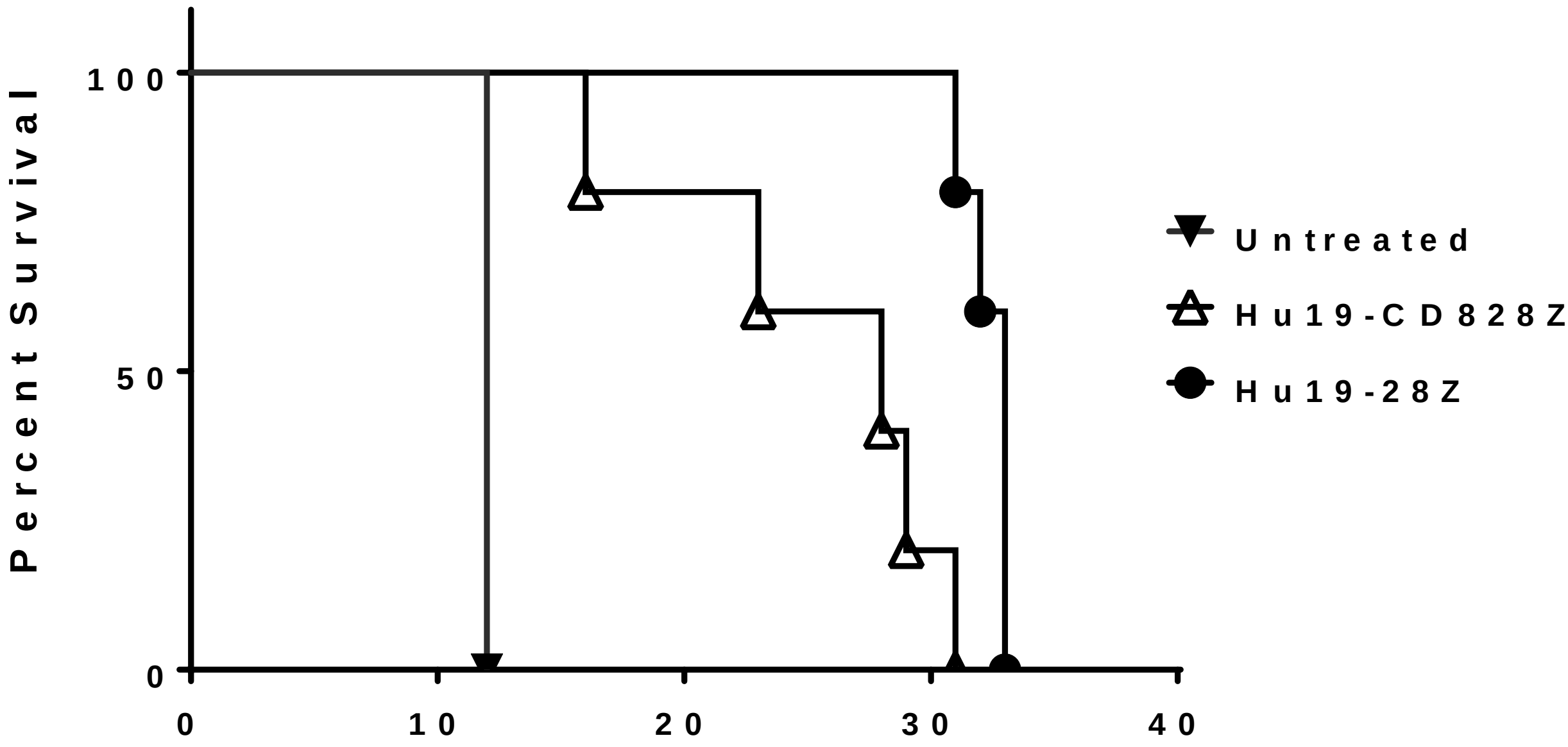
T cells expressing FMC63-28Z were cultured overnight with $CD19^+$ CD19-K562 cells or CD19-negative NGFR-K562 cells. CAR T cells were also cultured alone. At the end of the culture period, cells were stained with annexin V to detect apoptotic cells. Plots are gated on live $CD3^+$, CAR^+ cells. CAR T cells were detected by anti-Fab staining. The percentage of CAR^+ cells undergoing apoptosis increased in a CD19-specific manner.

Supplemental Figure 8



Bioluminescent images of mice bearing NALM6-GL. The groups were either left untreated or infused with $4x10^6$ T cells expressing either Hu19-CD828Z or Hu19-28Z. Images shown were captured on days 0, 8, 16, and 24 after CAR T-cell infusion. One of 2 experiments with very similar results is shown. Units are in photons/second/square centimeter/steradian $x10^8$ at days 8, 16 and 24 and $x10^6$ at day 0. Red is the color of most intense bioluminescence, and blue is the color of least intense bioluminescence.

Supplemental Figure 9



Survival of NALM6-GL bearing mice after treatment with Hu19-28Z or Hu19-CD828Z or no treatment (n = 5 mice for all groups). *P* = 0.0027 for the comparison of Hu19-28Z vs. untreated; *P* = 0.0027 for the comparison of Hu19-CD828Z vs. untreated; *P* = 0.0044 for the comparison of Hu19-CD828Z vs. Hu19-28Z. Comparisons made by the log-rank test. Very similar results were obtained in 2 separate experiments with T cells from different donors.