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## Supplemental Information

# Development of CAR T Cells Expressing a Suicide Gene Plus a Chimeric Antigen Receptor Targeting Signaling Lymphocytic-Activation Molecule F7

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# Supplemental Materials

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# Supplemental Figure S1: Memory Phenotype

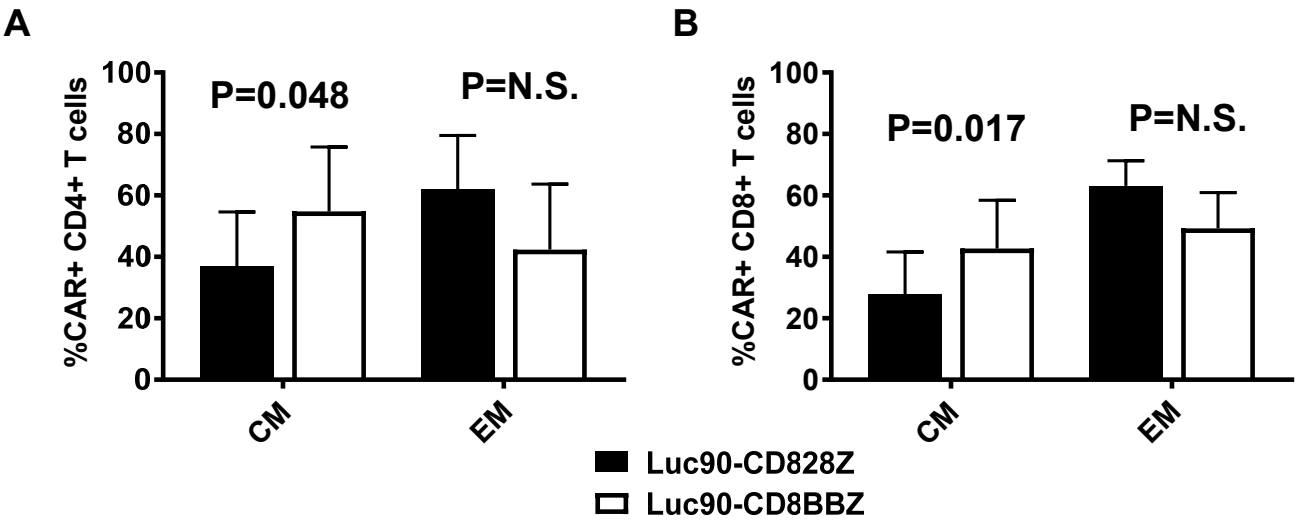



**Figure S1. Memory phenotype of T cells expressing different anti-SLAMF7 CARs.** T cells were transduced with vectors encoding either Luc90-CD828Z or Luc90-CD8BBZ. On day 9 of T-cell culture, cells were stained for detection of CAR, CD45RA, and CCR7. The graphs show percentages of (A) $CD4^+$ and (B) $CD8^+$ CAR T cells that were either CD45RA-negative, $CCR7^+$ central memory cells (CM) or CD45RA-negative, CCR7-negative effector memory cells (EM). N=3 different donors for all groups. For both $CD4^+$ and $CD8^+$ CAR-expressing T cells, the percentage of CM cells was higher for Luc90-CD8BBZ T cells compared with Luc90-CD828Z T cells. There was not a statistically-significant difference in the percentage of EM cells between the two CARs. Bars represent mean+SEM. Statistical testing was done by paired, 2-tailed T tests, and statistical significance was defined as $P<0.05$. N.S. stands for not statistically significant.

# Supplemental Figure S2: Activation-induced Cell Death of CARs with CD28 Versus 4-1BB

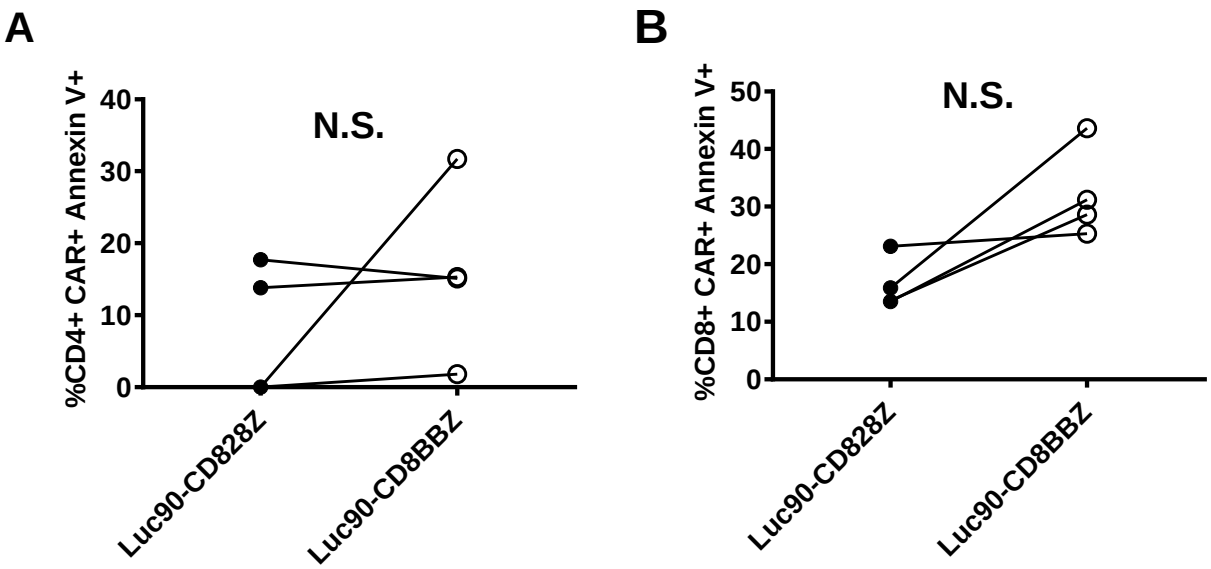



**Figure S2. Apoptosis of CAR-transduced T cells was assessed by Annexin V staining.** $CD4^+$ or $CD8^+$ T cells expressing either Luc90-CD828Z or Luc90-CD8BBZ were cultured overnight with either MM.1S cells ($SLAMF7^+$) or NGFR-K562 cells (SLAMF7-negative). Flow cytometry was then performed to assess Annexin V staining of live (A) $CD4^+$ or (B) $CD8^+$ CAR-expressing T cells. The graphed results show the %Annexin $V^+$ cells with NGFR-K562 stimulation subtracted from the %Annexin $V^+$ cells with MM.1S stimulation. N=4 different donors. Statistical significance was assessed with a 2-tailed, paired T test. $P<0.05$ was considered statistically significant. Lines connect paired results with cells from the same donors. There was no statistically significant difference between Luc90-CD828Z T cells and Luc90-CD8BBZ for either $CD4^+$ or $CD8^+$ T cells, but there was a trend toward lower %Annexin $V^+$ cells in $CD8^+$ T cells expressing Luc90-CD828Z compared with T cells expressing Luc90-CD8BBZ ($P=0.059$). N.S. stands for not statistically significant.

# Supplemental Figure S3: IFNγ Release

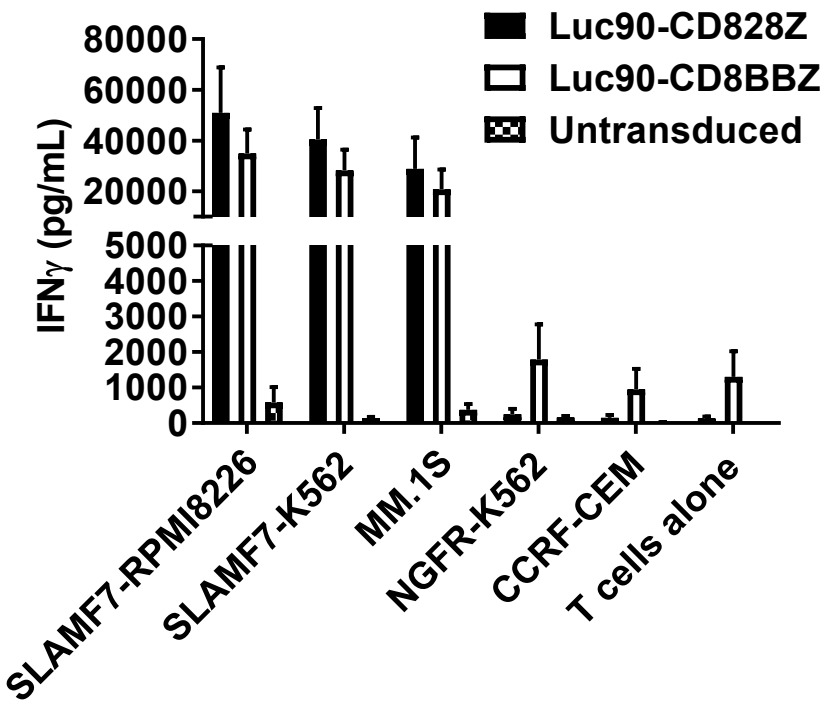



**Figure S3 IFNγ release by Luc90-CD828Z and Luc90-CD8BBZ T cells.** T cells were transduced with either Luc90-CD828Z or Luc90-CD8BBZ and cultured overnight with the indicated target cells. Culture supernatants were then assayed for IFNγ by ELISA. Units are pg/mL. Bars show mean+SEM. SLAMF7-RPMI8226, SLAMF7-K562, and MM.1S were SLAMF7$^{+}$. NGFR-K562, and CCRF-CEM were SLAMF7-negative. N=3 replicates with T cells from different patients for SLAMF7-RPMI8226. N=6 replicates with T cells from different patients for other target cells. Note relatively high nonspecific IFNγ release with SLAMF7-negative targets and T cells alone for Luc90-CD8BBZ T cells.

## Supplemental Figure S4: Luc90-CD828Z Versus Luc90-CD8BBZ Degranulation

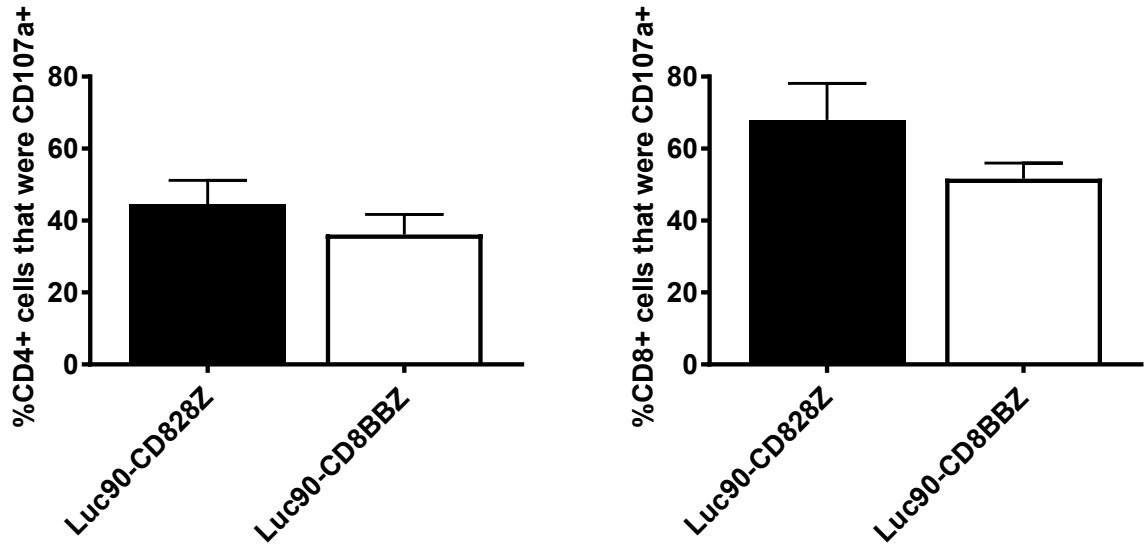



**Figure S4. Degranulation of T cells expressing either Luc90-CD828Z or Luc90-CD8BBZ.** T cells expressing the indicated CARs were cultured 4 hours with either MM.1S or NGFR-K562 cells in the presence of an antibody against CD107a. Cells were then stained with antibodies against CD3, CD4, and CD8; n=3 different donors for both the CD4 and CD8 analyses. Values are $CD107a^+$ events with MM.1S stimulation minus $CD107a^+$ events in a parallel culture with NGFR-K562. The percentage of T cells that degranulated was not statistically different between Luc90-CD828Z and Luc90-CD8BBZ for either $CD4^+$ or $CD8^+$ T cells. The $\%CD107a^+$ events were normalized for CAR expression. Statistical comparison was by 2-tailed paired T test; $P<0.05$ was considered statistically significant.

# Supplemental Figure S5: Tumor Treatment with 4x10$^6$ CAR T-cell Dose

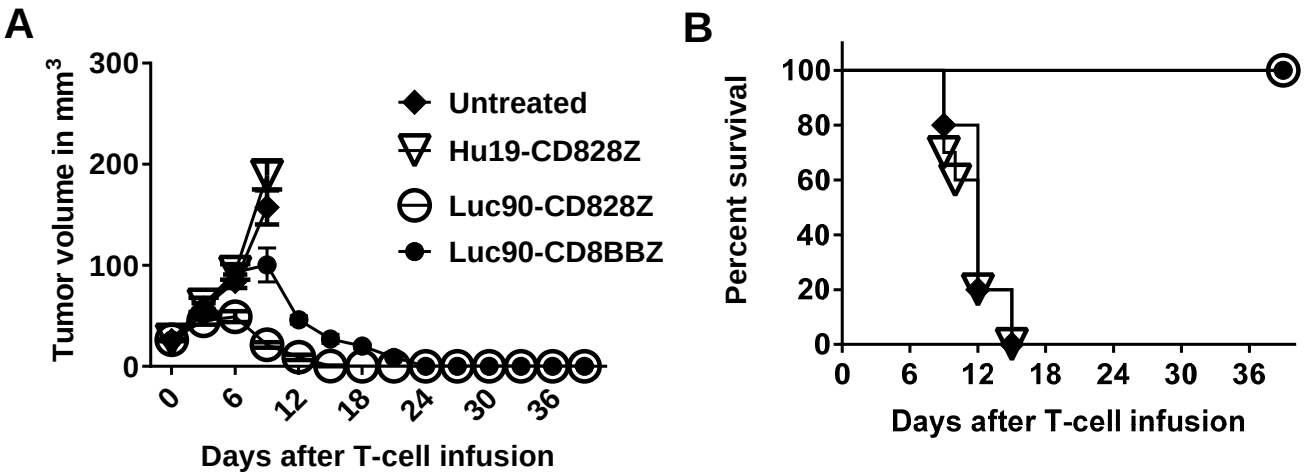



**Figure S5. Both Luc90-CD828Z and Luc90-CD8BBZ CAR T cells eradicated solid tumors at a dose of 4x10$^6$ CAR T cells/mouse.** (A) MM.1S tumors were established in NSG mice. Mice were either left untreated or received infusions of 4x10$^6$ T cells expressing Luc90-CD828Z, Luc90-CD8BBZ, or, as a control, the anti-CD19 CAR Hu19-CD828Z. The graph shows mean tumor volume ±SEM for each time point. The graph includes data from two independent experiments with different donor T cells. There were five mice in each group per experiment, so n=10 total mice for each treatment group. (B) Kaplan-Meier survival plot of the same mice as in A. By log-rank test, there was a statistically-significant survival difference between mice that received T cells expressing Hu19-CD828Z versus Luc90-CD828Z ($P<0.0001$). There was also a statistically-significant survival difference between mice that received T cells expressing Hu19-CD828Z versus Luc90-CD8BBZ ($P<0.0001$). Early tumor control was superior for Luc90-CD828Z T cells versus Luc90-CD8BBZ T cells. For example, on day 9 after CAR T-cell infusion, the mean tumor volume was 21.3 mm$^3$ for Luc90-CD828Z T cells and 100.3 mm$^3$ for Luc90-CD8BBZ T cells ($P=0.0002$ by 2-tailed unpaired T test).

# Supplemental Figure S6: Anti-tumor Activity of Anti-CD19 CARs Containing CD28 Versus 4-1BB Domains

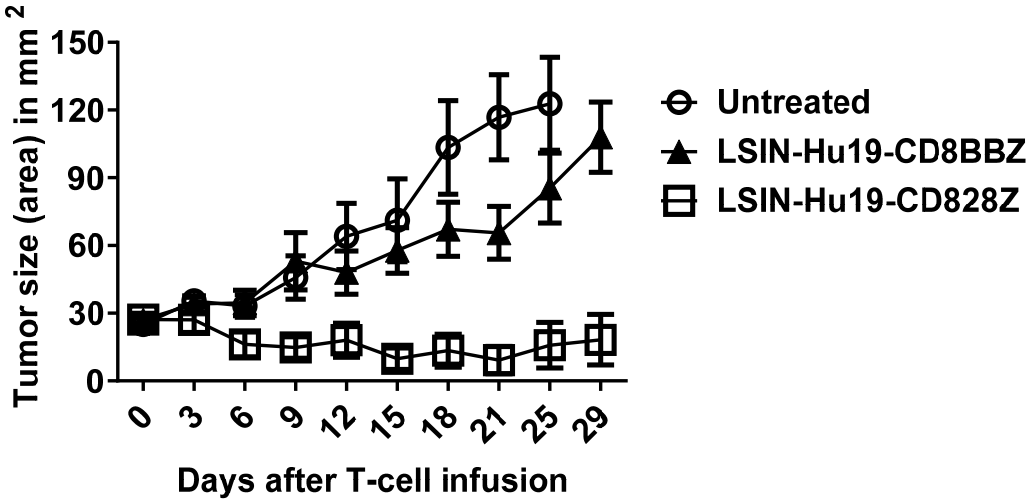



**Figure S6. Comparison of anti-tumor activity of T cells expressing anti-CD19 CARs with 4-1BB versus CD28 costimulatory domains.** NSG mice were injected with 4 million NALM6 cells to form solid tumors. Human donor T cells were transduced with either LSIN-Hu19-CD8BBZ or LSIN-Hu19-CD828Z, and mice received intravenous infusion of 10 million CAR T cells after solid tumors were established. A third group of tumor-bearing mice was left untreated. The day of CAR T-cell infusion was day 0 on the graph. Tumor sizes were monitored by measuring with calipers. The graph shows the mean +/- SEM of tumor sizes of 5 mice per group at each time point. These results are from one of two experiments with similar results. In both of the experiments, tumor growth in mice that received 4-1BB-containing CAR T cells was more similar to tumor growth in untreated mice than to tumor growth in mice that received CD28-containing CAR T cells.

## Supplemental Figure S7: Costimulatory Sequences

**CD28 sequence in all CD28-containing CARs**

| |
|---|
| **RSKRSRLLHSD YMNMTPRRPG PTRKHYQPYA PPRDFAAYRS** |

**4-1BB sequence in Luc90-CD8BBZ**

| |
|---|
| **KRGRKKL LYIFKQPFMR PVQTTQEEDG CSCRFPEEEE GGCEL** |

**4-1BB sequence in Hu19-CD8BBZ**

| |
|---|
| **RFSVVKRGRKKL LYIFKQPFMR PVQTTQEEDG CSCRFPEEEE GGCEL** |

**Figure S7. Sequences of costimulatory domains in CARs.**
The same CD28 sequence was used in all CARs in this report. The anti-SLAMF7 CAR Luc90-CD8BBZ and the anti-CD19 CAR Hu19-CD8BBZ contained the indicated 4-1BB sequences that differed by 5 amino acids at the N-terminus.

# Supplemental Figure S8: Comparison of expression

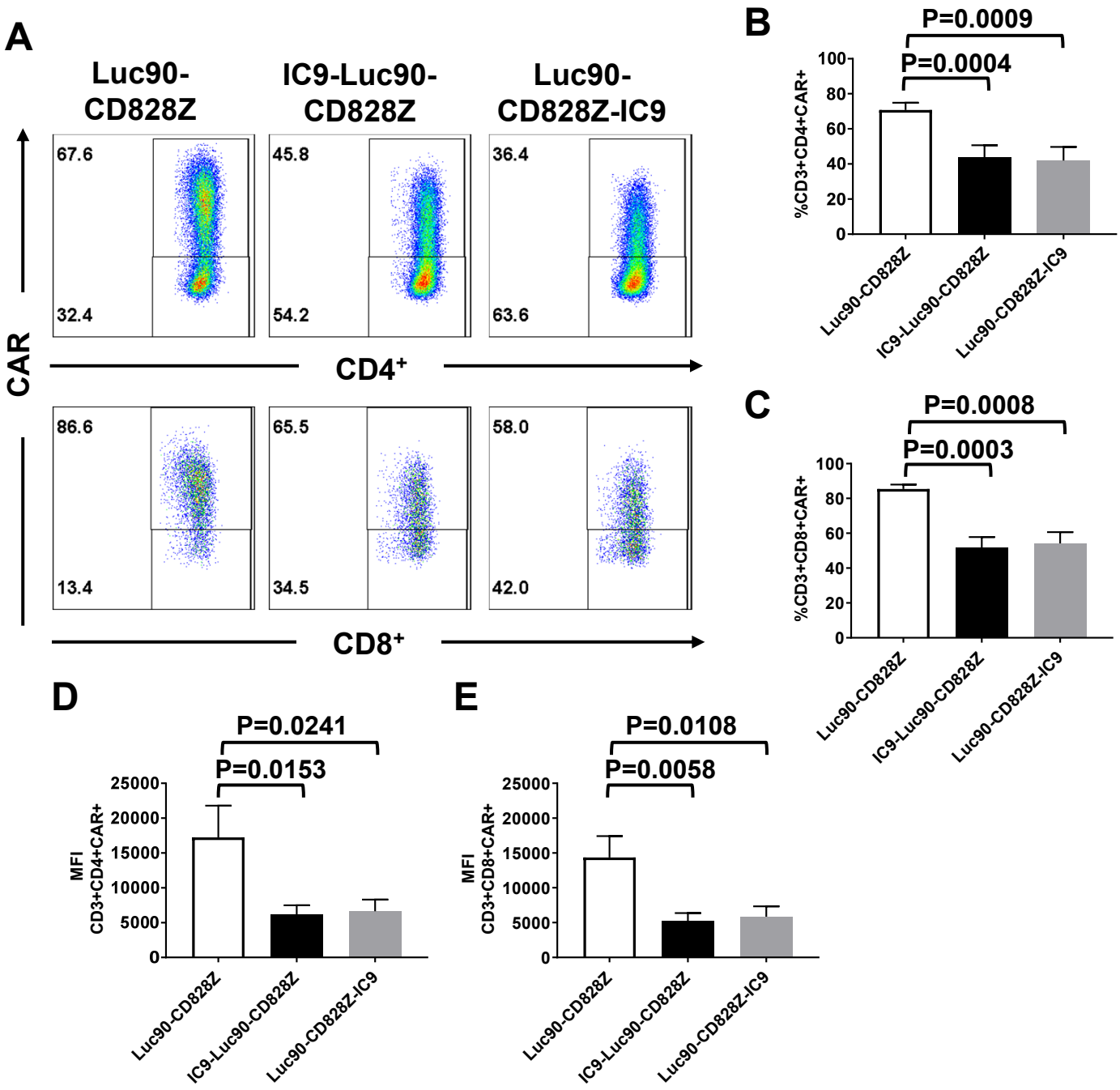



**Figure S8. Comparison of percentage and intensity of CAR expression among T cells transduced with Luc90-CD828Z and IC9-bearing Luc90-CD828Z CAR constructs.** T cells were transduced with vectors encoding either Luc90-CD828Z or IC9-Luc90-CD828Z or Luc90-CD828Z-IC9. On day 7 of T-cell culture, T cell were stained for detection of CAR. (A) The plots are representative examples of the percentages and intensities of CAR expression on $CD4^+$ and $CD8^+$ T cells. (B-C) The graphs show percentages of (B) $CD4^+$ and (C) $CD8^+$ T cells that were $CAR^+$. (D-E) The graphs show median fluorescence intensities (MFI) of CAR expression on (D) $CD4^+$ and (E) $CD8^+$ T cells. For B-E, n=8 different donors for all groups. For both $CD4^+$ and $CD8^+$ CAR-expressing T cells, the percentages of both CAR expression and MFI were statistically higher for Luc90-CD828Z T cells compared with IC9-Luc90-CD828Z and Luc90-CD828Z-IC9 T cells. Bars represent mean+SEM. There was no statistical difference between IC9-Luc90-CD828Z and Luc90-CD828Z-IC9 in either percent CAR expression or MFI. Statistical testing was done by paired, 2-tailed T tests. After correction for multiple comparisons with the Bonferroni method, statistical significance was defined as $P<0.017$.

# Supplemental Figure S9: Degranulation in Response to Primary Multiple Myeloma Cells

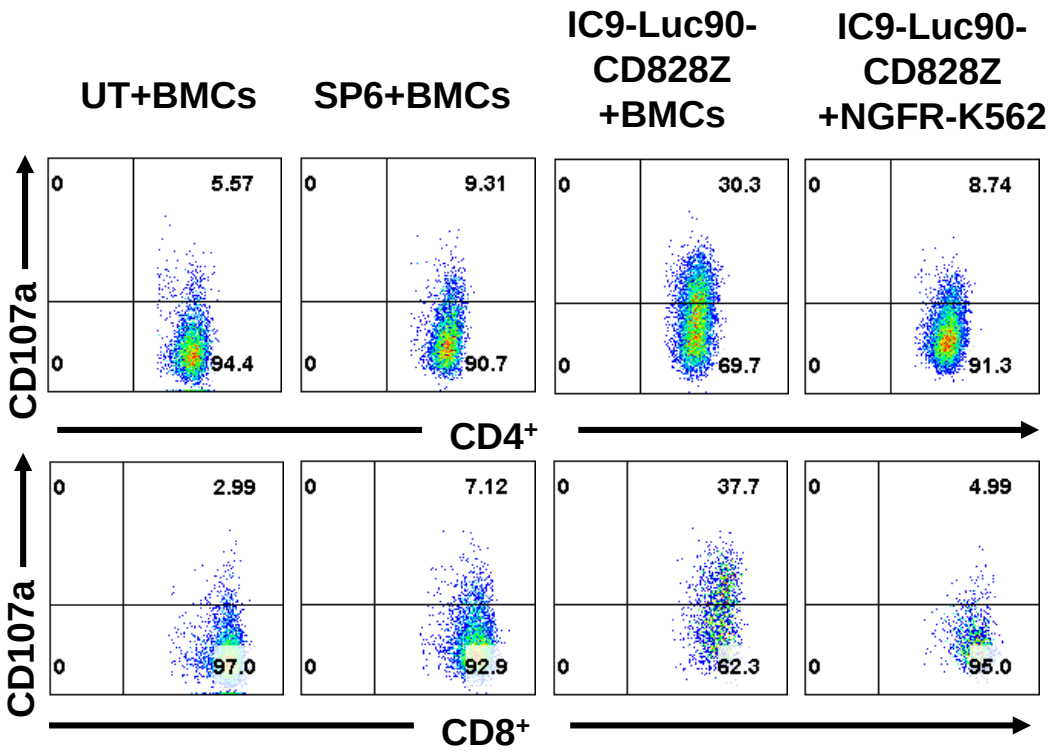
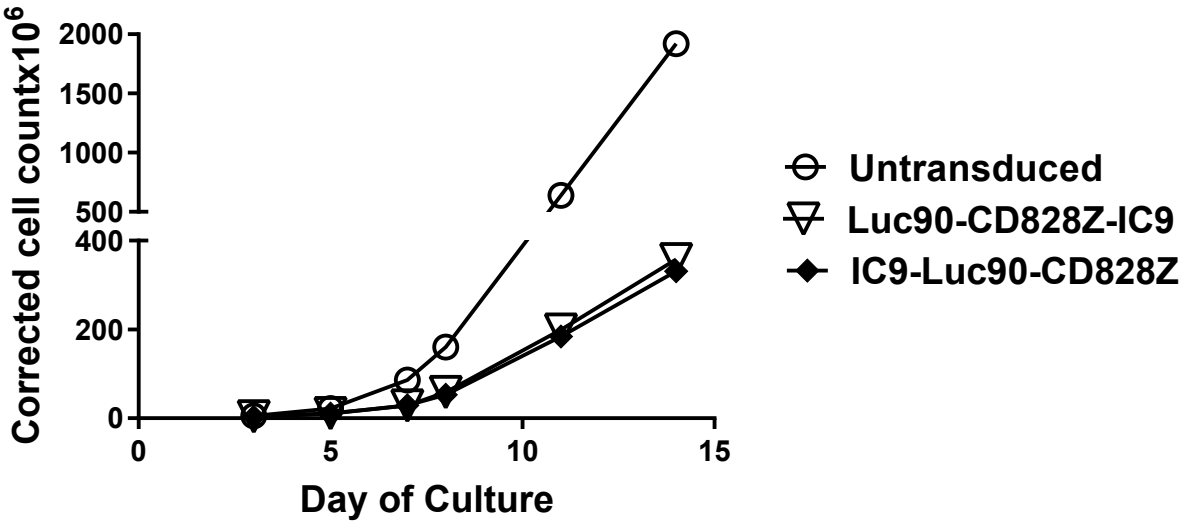



**Figure S9**

**IC9-Luc90-CD828Z CAR T cells specifically degranulate in the presence of primary MM cells.** T cells were transduced with vectors encoding SP6-CD828Z or IC9-Luc90-CD828Z or left untransduced. On day 8 of T cell culture, T cells were cultured with either primary bone marrow cells (BMCs) from a multiple myeloma patient or NGFR-K562 (negative control) for 4-hours in the presence of an antibody against CD107a. Flow cytometry was performed. Plots are gated on live $CD4^+$ or $CD8^+$ T cells. The figure shows one of 2 experiments with similar results performed with cells from different donors.

# Supplemental Figure S10: T-cell Accumulation in Culture




**Supplemental Figure S10.** The cumulative increases in numbers of T cells left untransduced, transduced with the gene for Luc90-CD828Z-IC9, or transduced with the gene for IC9-Luc90-CD828Z are shown. Untransduced T cells accumulated more rapidly than the CAR-transduced T cells, but CAR-transduced T cells did accumulate steadily. One representative example of 4 experiments with cells from 4 different donors is shown.

# Supplemental Table S1: SLAMF7 Immunohistochemistry

| Organs stained for SLAMF7 by immunohistochemistry and found to not have SLAMF7 expression | |
|---|---|
| adrenal | lung |
| bladder | ovary |
| bone | pancreas |
| breast | parathyroid |
| cerebellum | pituitary |
| cerebral cortex | placenta |
| eye | prostate |
| fallopian tube | skin |
| esophagus | spinal cord |
| stomach | spleen |
| small intestine | skeletal muscle |
| colon | testis |
| rectum | thymus |
| heart | thyroid |
| kidney | tonsil |
| liver | uterine cervix |
| | uterine endometrium |

**Table S1**

SLAMF7 is not expressed on non-hematopoietic tissues from various organs. A paraffin-fixed normal tissue microarray (Pantomics, number MN0661) was stained with anti-SLAMF7 (clone OTI3B3, LSBio number LS-C340266). The table includes tissues where SLAMF7 protein expression was not detected by IHC except on infiltrating plasma cells, some macrophages and some lymphocytes.

# Supplemental Table S2: Soluble SLAMF7

| | SLAMF7-positive targets | | | | SLAMF7-negative targets | | | | T cells only | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **SLAMF7 (ng/mL)** | **0** | **100** | **200** | **400** | **0** | **100** | **200** | **400** | **0** | **100** | **200** | **400** |
| | **SLAMF7-K562** | **SLAMF7-K562** | **SLAMF7-K562** | **SLAM7-K562** | **NFGR-K562** | **NFGR-K562** | **NFGR-K562** | **NFGR-K562** | **T cells** | **T cells** | **T cells** | **T cells** |
| **UT** | 38.6 | 42.5 | 39.7 | 37.3 | 51.4 | 46.9 | 49.9 | 44.7 | 24.4 | 23.0 | 28.0 | 25.5 |
| **MSGV-Hu19-CD828Z** | 35.2 | 35.0 | 34.5 | 38.3 | 40.1 | 35.6 | 38.3 | 40.5 | 31.1 | 32.1 | 30.6 | 31.6 |
| **MSGV-Luc90-CD828Z** | 12553.6 | 10969.2 | 9486.7 | 13422.8 | 17.2 | 16.2 | 12.8 | 13.1 | 19.5 | 16.1 | 13.6 | 13.3 |

**Table S2. Soluble SLAMF7 protein does not interfere with anti-SLAMF7 CAR T-cell target recognition.** T cells were transduced with vectors encoding Hu19-CD828Z or Luc90-CD828Z or left untransduced. Seven days after transduction, T cells were co-cultured with SLAMF7-K562 or NGFR-K562 target cells or were cultured alone. The co-cultures included either vehicle or 100ng/mL, 200ng/mL or 400ng/mL of soluble SLAMF7 protein. After overnight culture, supernatant was harvested and IFN$\gamma$ was evaluated by ELISA.

# Supplemental Table S3: IFNγ Release by Anti-CD19 CAR T Cells

| | CD19-K562 | CLL | CCRF-CEM | T Cells alone | % CAR+ |
|---|---|---|---|---|---|
| **LSIN-Hu19-CD828Z** | **32645** | **2003** | **64** | **61** | **60.3** |
| **LSIN-Hu19-CD8BBZ** | **25240** | **3548** | **495** | **504** | **51.2** |
| **Untransduced** | **112** | **<12** | **239** | **<12** | **0** |

**Table S3. T cells expressing either Hu19-CD828Z or Hu19-CD8BBZ produced IFNγ in an antigen-specific manner in vitro.** Human T cells from the same donor were left untransduced or transduced with LSIN-Hu19-CD828Z or LSIN-Hu19-CD8BBZ. The T cells were cultured overnight with the target cells indicated on the top row. CD19-K562 and primary CLL cells were $CD19^+$. CCRF-CEM was CD19-negative. After the overnight culture, an IFNγ ELISA was performed. The percentage of the T cells that were $CAR^+$ is indicated. All values are the mean of duplicate wells. Units are pg/mL of IFNγ.

# Table S4: Specificity of IC9-Luc90-CD828Z T cells

| | SLAMF7-K562 | A549 | SAOS2 | COLO205 | U251 | 624 | TC71 | A431-H9 | Panc 10.5 | CCRF-CEM | T cells only |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Untransduced** | <12 | 30.3 | 33.6 | 19.2 | 28.8 | 30.2 | <12 | <12 | <12 | 22.6 | <12 |
| **Hu19-CD828Z** | <12 | 27.6 | 37.7 | 37.7 | 28.1 | 18.7 | 13.4 | 0.4 | <12 | 32.7 | <12 |
| **IC9-Luc90-CD828Z** | 45231.4 | 15.6 | 29.5 | 34.2 | 19.0 | 121.9 | 12.3 | 31.8 | 12.3 | 24.0 | 30.3 |

**Table S4. IC9-Luc90-CD828Z does not recognize SLAMF7-negative cell lines.** T cells from the same donor were transduced with vectors encoding the anti-CD19 CAR Hu19-CD828Z or IC9-Luc90-CD828Z or left untransduced. The T cells were cultured overnight with the target cells listed on the top row. All targets were SLAMF7-negative except for SLAMF7-K562. After the overnight culture, an ELISA for IFNγ was performed. All values are pg/mL of IFNγ.

## Supplemental Methods

### Real-time qPCR to Quantify SLAMF7 Expression

SLAMF7 cDNA copies were quantitated in samples of cDNA from human tissues included in the Human Major Tissue qPCR Panel II (Origene, Rockville, MD) by performing qPCR with a SLAMF7-specific primer and probe set (Applied Biosystems, Foster City, CA). Plasmacytoma cells of a patient with advanced multiple myeloma served as a positive control for SLAMF7 expression. A standard curve for the SLAMF7 qPCR was created by amplifying dilutions of a plasmid that encoded the full-length cDNA of SLAMF7 (Origene, Rockville, MD). β-actin cDNA copy numbers were quantified in the same tissues with a Taqman β-actin primer and probe kit (Applied Biosystems, Foster City, CA) and used to normalize SLAMF7 copy numbers. PCR was conducted on a Roche LightCycler480.

### Cell Lines

MM.1S is an SLAMF7$^+$ MM cell line from ATCC (Manassas, VA). SLAMF7-K562 and SLAMF7-RPMI8226 are cell lines transduced with the SLAMF7 gene in our laboratory. The following CD19-negative human cell lines from ATCC were used: A549 (lung carcinoma, CCRF-CEM (T cell leukemia), Saos2 (osteosarcoma), Panc10.05 (pancreatic carcinoma), COLO205 (colon carcinoma). U251 (glioblastoma) and H431-H9 (epidermoid carcinoma) cell lines were gifts from Dr. Robert Somerville (NIH). TC71 (Ewing's sarcoma were a gift of Dr. M. Tsokos, (NIH). The 624 melanoma cell line was a gift from Dr. Steven A. Rosenberg, NIH. SLAMF7-negative, NGFR-K562 were transduced with the gene for low-affinity nerve growth factor in our laboratory. CD19$^+$

NALM6 cells (acute lymphoid leukemia from DSMZ, Braunschweig, Germany) were used.

**Antibodies Used in Flow Cytometry**

For T-cell phenotyping, cells were stained with the following antibodies: CD3 APC-Cy7 (Clone UCHT1, BD Biosciences, San Jose, CA), CD4 FITC or BV510 (Clone RPA-T4, BD Biosciences, or Biolegend, San Diego, CA), CD8 PE-Cy7 or eFluor450 (Clone RPA-T8, BD Biosciences or Thermo Scientific). For memory T-cell phenotyping, the following antibodies were included: CD45RA FITC (Clone HI100, BD Biosciences) and CCR7 APC (Clone 150503, BD Biosciences).

**Real-time qPCR for quantifying $CAR^+$ Cell in Tumors**

In murine experiments, the percentages of infused T cells that were $CAR^+$ were determined by flow cytometry. These percentages were used in making $CAR^+$ T cell standard curves. Tumor cells were mechanically disaggregated and treated with a 1:1 mix of ACK lysing buffer to PBS. The cells were counted, and DNA was extracted from the lysates using a Qiagen (Valencia, CA) DNeasy kit. DNA from the tumor was amplified in duplicate with a primer and probe set (IDT, Coralville, IA) that was specific for the MSGV1 retroviral vector. Real-time PCR was carried out with a Roche (Basel, Switzerland) Light Cycler 96 real-time PCR system. Similar to an approach used previously, we made serial 1:5 dilutions of DNA from the infused T cells of each experimental group into untransduced T-cell DNA, and we made standard curves by performing qPCR on this DNA [1-3] To determine the percentage of total tumor cells that contained the CAR gene, the qPCR results obtained with each tumor's DNA were compared to the qPCR results obtained from each experimental group's $CAR^+$ T-cell

standard curve. Note that total tumor cells included MM.1S cells and infiltrating human T cells.

PCR results were normalized to tumor β-actin. For the actin PCR, an actin plasmid (Origene plasmid ACTB NM_001101) was amplified in duplicate with a primer and probe set (Bio-rad ACTB qHsaCEP0036280, Hercules, CA) to generate a standard curve. This standard curve was used to determine actin copy numbers in tumor DNA samples amplified with the same actin primer and probe set.

After the percentages of $CAR^+$ cells were determined by qPCR, the absolute number of $CAR^+$ T cells in each tumor was calculated by multiplying the percentage of $CAR^+$ T cells by the total number of cells in the tumor. PCR results showed very similar efficiencies in reactions with DNA from Luc90-CD828Z-transduced T cells versus Luc90-CD8BBZ-transduced T cells. All tested tumors had levels of CAR DNA that were above the lower limit of detection for the PCR reactions.

## References


1. Warren, EH, Fujii, N, Akatsuka, Y, Chaney, CN, Mito, JK, Loeb, KR*, et al.* (2010). Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood* **115**: 3869-3878.
2. Morgan, RA, Yang, JC, Kitano, M, Dudley, ME, Laurencot, CM, and Rosenberg, SA (2010). Case report of a serious adverse event following the administration of t cells transduced with a chimeric antigen receptor recognizing ERBB2. *Molecular Therapy* **18**: 843-851.
3. Brudno, JN, Lam, N, Vanasse, D, Shen, YW, Rose, JJ, Rossi, J*, et al.* (2020). Safety and feasibility of anti-CD19 CAR T cells with fully human binding domains in patients with B-cell lymphoma. *Nat Med* **26**: 270-280.