YMTHE, Volume *28*

Supplemental Information

Allogeneic FLT3 CAR T Cells with an Off-Switch Exhibit Potent Activity against AML and Can Be Depleted to Expedite Bone Marrow Recovery

Cesar Sommer, Hsin-Yuan Cheng, Duy Nguyen, Danielle Dettling, Yik Andy Yeung, Janette Sutton, Moustafa Hamze, Julien Valton, Julianne Smith, Ivana Djuretic, Javier Chaparro-Riggers, and Barbra J. Sasu

SUPPLEMENTAL FIGURES

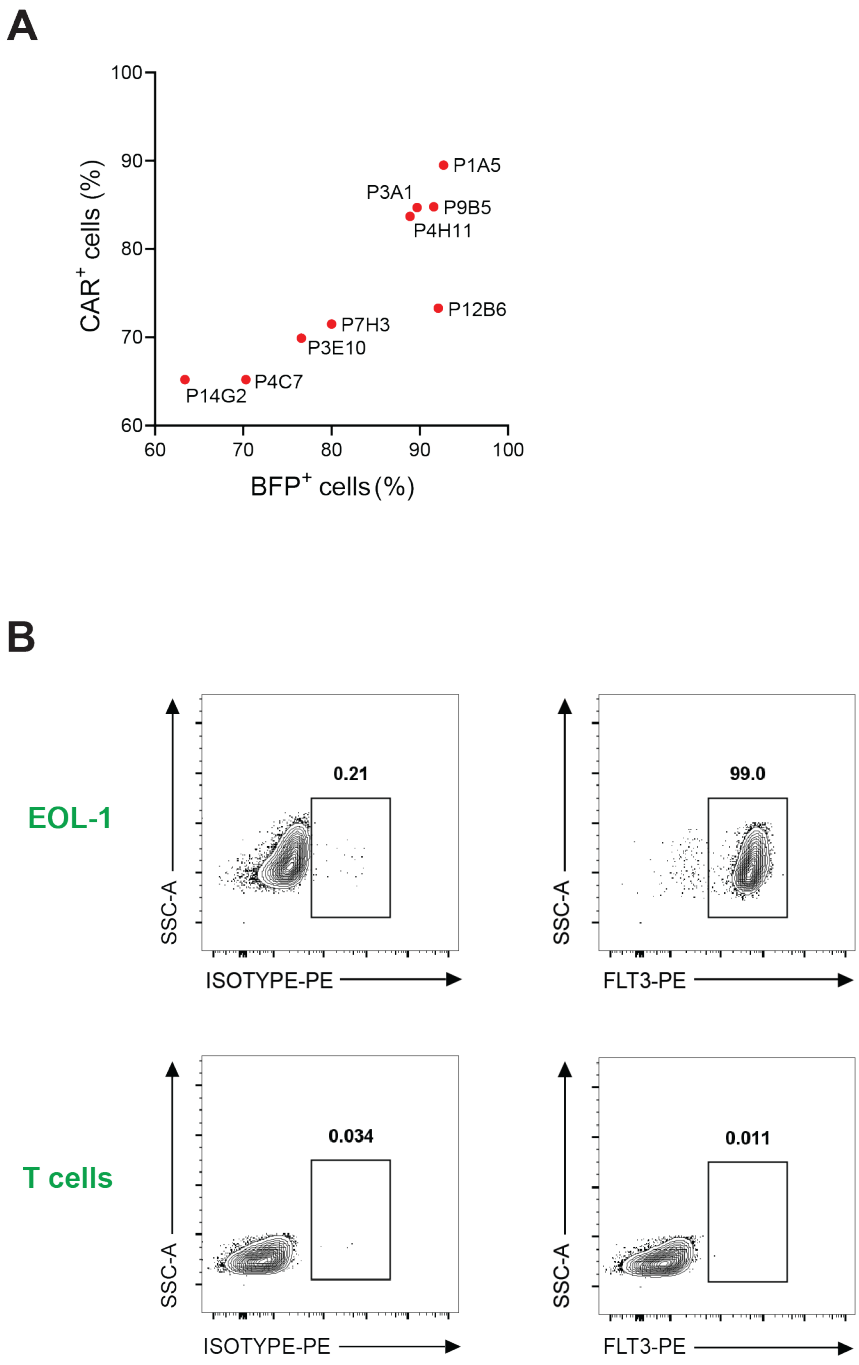


Figure S1: Co-expression of FLT3 CARs and BFP in transduced Jurkat cells and absence of detectable FLT3 expression in activated human T cells. (A) Expression of candidate FLT3 CARs and blue fluorescent protein (BFP) was measured by flow cytometry analysis of Jurkat cells three days post-transduction. **(B)** Human T cells were activated and analyzed for the expression of FLT3 by flow cytometry.

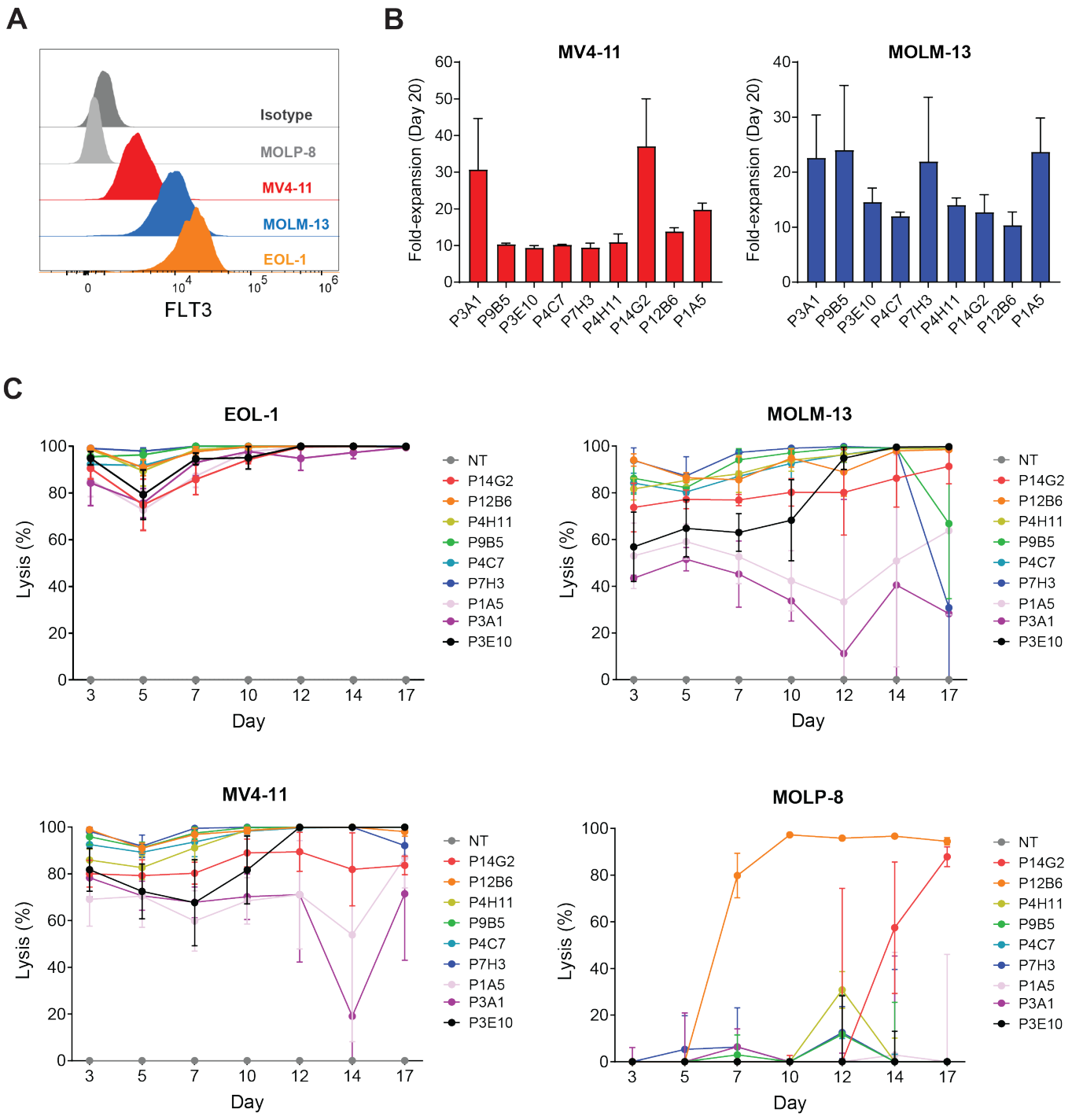


Figure S2: Target-dependent expansion and cytotoxic activity of FLT3 CAR T cells following co-culture with AML cell lines expressing varying levels of FLT3. (A) Cell surface expression of FLT3 in the AML cell lines EOL-1, MOLM-13, and MV4-11, and in the multiple myeloma cell line MOLP-8 was determined by flow cytometry analysis using a commercially available antibody (clone 4G8). A representative histogram for each cell line is shown. **(B)** Expansion of FLT3 CAR T cells upon repeated

exposure to MV4-11 and MOLM-13 cells. The proliferative capacity of FLT3 CAR T cells was estimated by counting viable CAR^+ cells 20 days after co-culture with target cells. Fold-expansion is expressed as mean +/- SEM relative to day 0; n=2 donors. **(C)** CAR T cells maintained high cytolytic activity after multiple encounters with AML cells. Effector and luciferase-labeled target cells were co-cultured at 1:1 E:T ratio followed by periodic addition of fresh target cells and the viability of residual target cells was determined at different timepoints by luminescence assay. The same assay was conducted with the MOLP-8 control cell line to estimate potential for non-specific killing activity. Data is shown as the mean +/- SEM values obtained from triplicate wells for a single experiment, representative of two separate experiments with different donors.

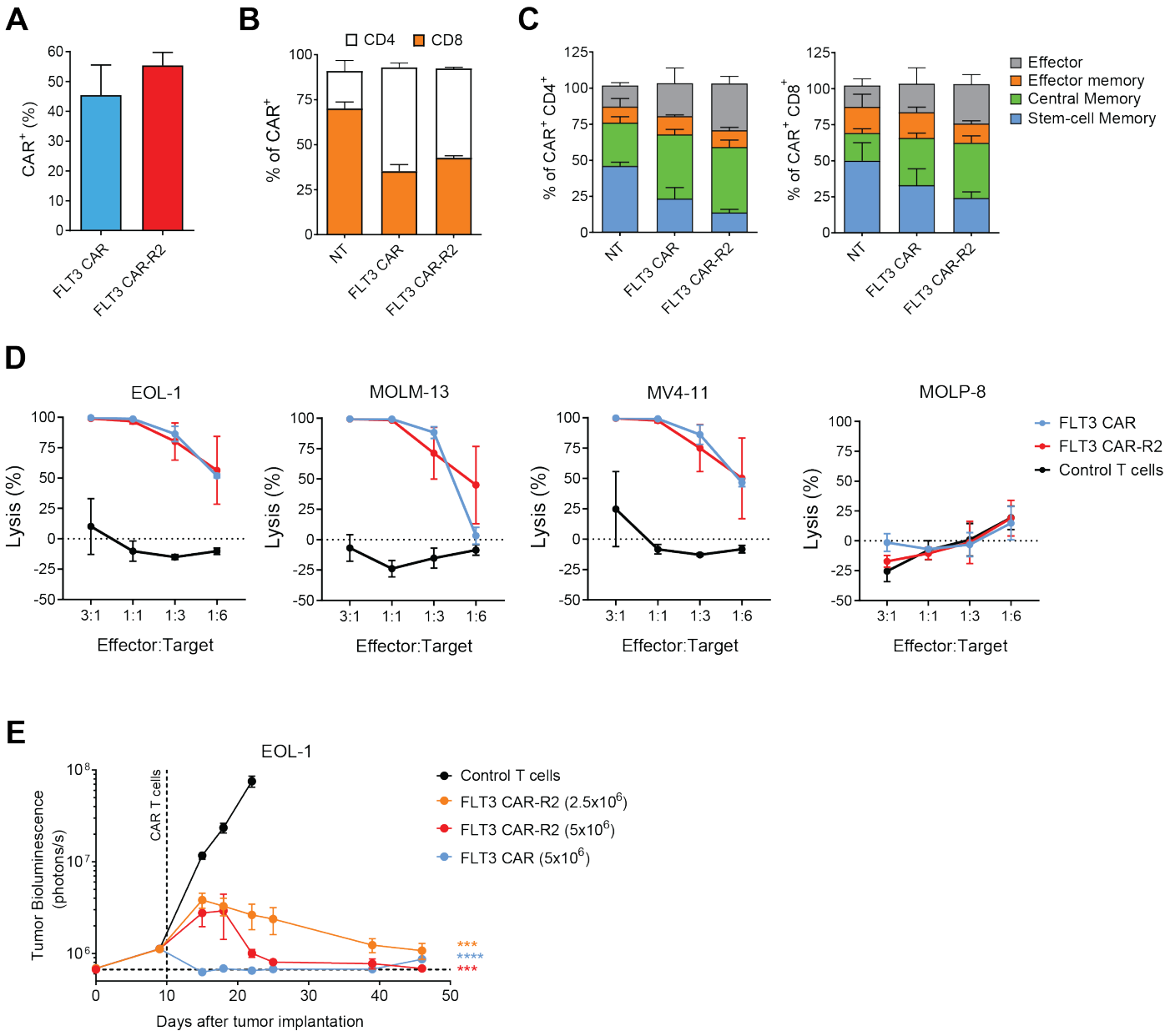


Figure S3: Inclusion of an off-switch into the lead FLT3 CAR P3E10 does not alter CAR T cell phenotype or effector function. (A) T cells from three separate donors were transduced with either the FLT3 CAR construct or with the same construct containing the intra-CAR off-switch (FLT3 CAR-R2). CAR expression was measured by flow cytometry analysis of cells stained with soluble FLT3 three days after transduction. **(B)** CAR T cell populations displayed similar frequencies of $CD4^+$ and $CD8^+$ cells at the end of the expansion phase, as determined by flow cytometry analysis. **(C)** Comparable distribution of T cell differentiation subsets between FLT3 CAR and FLT3 CAR-R2 T cells. CAR T cells were analyzed by flow cytometry 9 days after transduction and phenotypes were determined according to CD62L and CD45RO expression within the CAR^+ cell population: stem cell memory ($CD45RO^-/CD62L^+$), central memory ($CD45RO^+/CD62L^+$), effector memory ($CD45RO^+/CD62L^-$), effector ($CD45RO^-/CD62L^-$) cells

(mean +/- SEM; n=3 donors). **(D)** FLT3 CAR T cells and FLT3 CAR-R2 T cells showed similar cytolytic activity *in vitro*. CAR T cells were co-cultured with luciferase-expressing target cells at the indicated ratios for 24 hours and residual target cell viability was measured by luminescence assay. The FLT3-negative MOLP-8 cell line was used as negative control. **(E)** FLT3 CAR-R2 T cells displayed efficacy in an orthotopic model of AML. NSG mice engrafted with luciferase-labeled EOL-1 cells received a single dose of CAR T cells at the indicated dose 10 days after tumor implantation and tumor burden was monitored by bioluminescence (BLI) over time (n=10 mice/group). Values are expressed as mean ± SEM (****$p<0.0001$, ***$p<0.001$, one-way ANOVA with Dunnett's post-hoc test for multiple comparisons versus non-transduced control T cells).

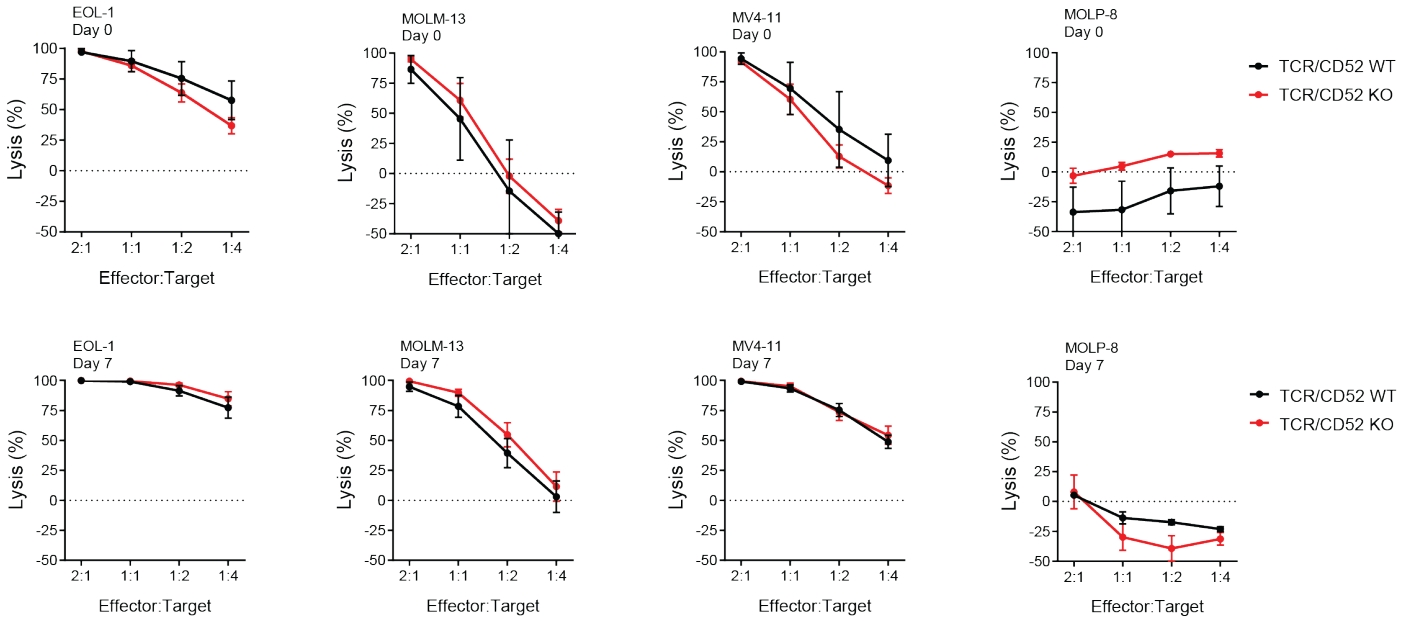


Figure S4: TALEN® treatment does not alter the cytolytic activity of FLT3 CAR-R2 T cells. Gene-edited (TCR/CD52 KO) and non-gene-edited (TCR/CD52 WT) FLT3 CAR-R2 T cells were co-cultured with luciferase-expressing AML cells at the indicated ratios for 24 hours and residual target cell viability was measured by luminescence assay. The FLT3-negative MOLP-8 cell line served as control. Assays were performed with CAR T cells that had not been previously exposed to target cells (day 0, top row) or that had been repeatedly exposed to target cells for 7 days (day 7, bottom row). Values are expressed as mean ± SEM (n=3 donors).

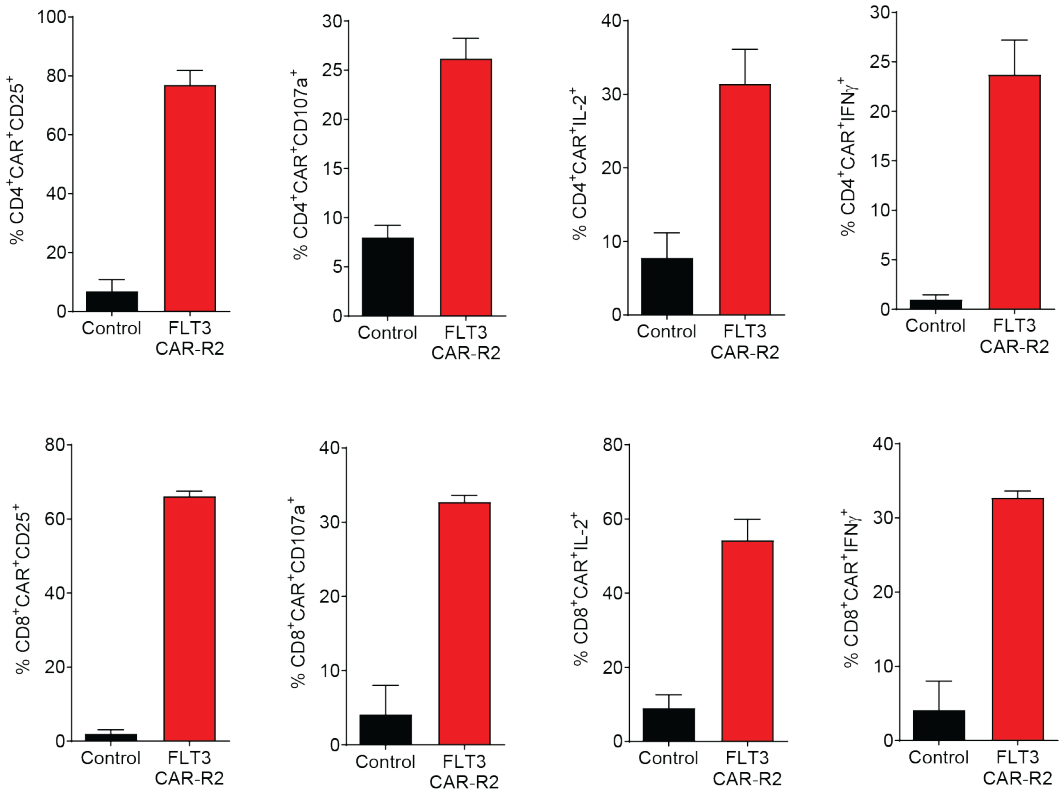
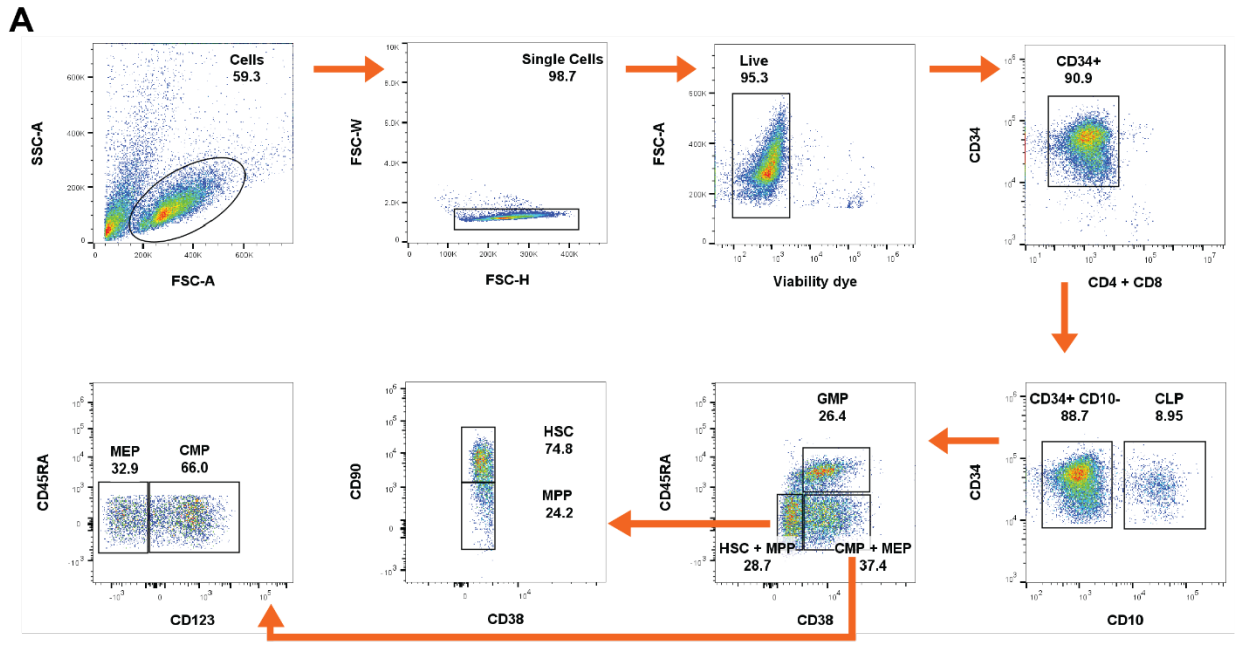
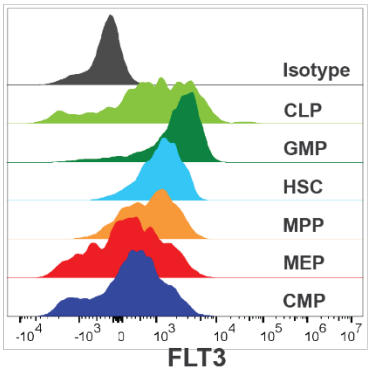


Figure S5: Allogeneic FLT3 CAR-R2 T cells show polyfunctionality in the presence of primary AML cells. (A) The frequencies of $CD4^+$ and $CD8^+$ FLT3 CAR-R2 T cells expressing markers of T cell effector function in response to primary AML cells were determined by flow cytometry analysis 48 hours after co-culture at 1:1 (E:T). Non-transduced T cells were used as control for CAR-independent T cell reactivity. Results are shown as mean +/- SEM of three AML samples.


A
Cells
59.3
SSC-A
FSC-A
Single Cells
98.7
FSC-W
FSC-H
Live
95.3
FSC-A
Viability dye
CD34+
90.9
CD34
CD4 + CD8
CD34+ CD10-
88.7
CLP
8.95
CD34
CD10
GMP
26.4
CD45RA
HSC + MPP
28.7
CMP + MEP
37.4
CD38
HSC
74.8
MPP
24.2
CD90
CD38
MEP
32.9
CMP
66.0
CD45RA
CD123


B
Isotype
CLP
GMP
HSC
MPP
MEP
CMP
FLT3

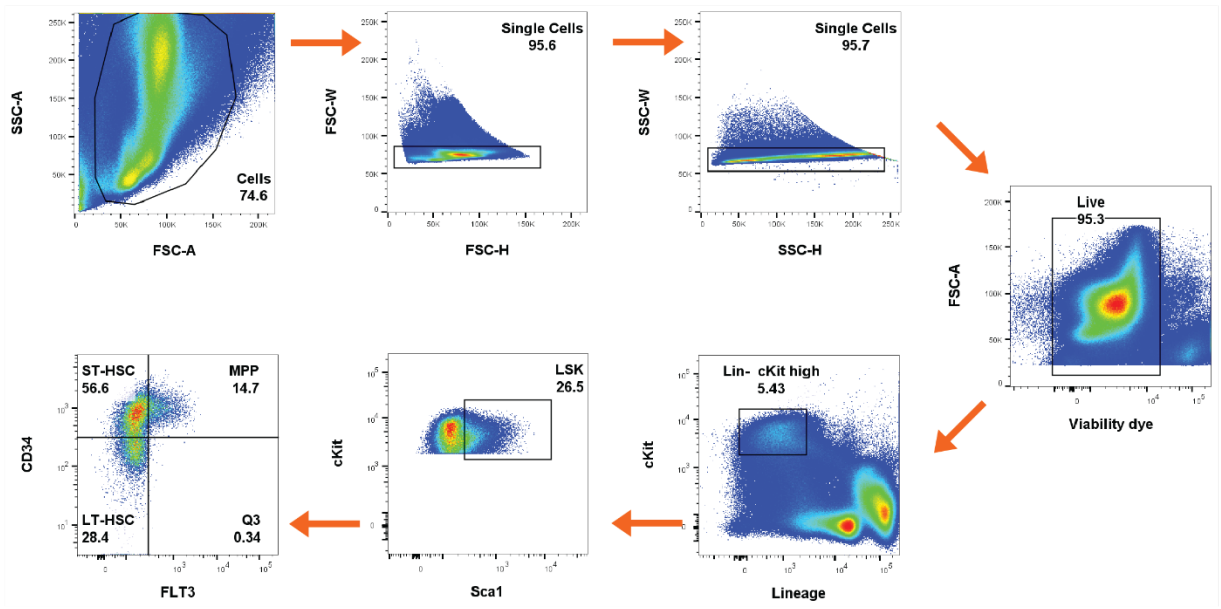

C
SSC-A
Cells
74.6
FSC-A
Single Cells
95.6
FSC-W
FSC-H
Single Cells
95.7
SSC-W
SSC-H
Live
95.3
FSC-A
Viability dye
Lin- cKit high
5.43
cKit
Lineage
LSK
26.5
cKit
Sca1
ST-HSC
56.6
MPP
14.7
LT-HSC
28.4
Q3
0.34
CD34
FLT3

Figure S6: Gating strategies used to identify subsets of human and mouse HSPCs. (A) Flow cytometry analysis of $CD34^+$ cells isolated from bone marrow mononuclear cells (n=6 donors). The different populations of HSPCs were identified using antibodies against CD34, CD10, CD38, CD45RA, CD90, and CD123. The gating strategy for a representative donor is shown. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-monocyte progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor. **(B)** Cell surface expression of FLT3 on normal HSPCs was assessed by flow cytometry using an anti-FLT3 antibody (4G8) or an isotype control antibody. Representative histograms from one donor are shown. **(C)** Gating strategy used to identify HSPCs in mouse bone marrow cell suspensions, defined as the lineage $(Lin)^-$, $Sca1^+$, c-kit^+ (LSK) population. Expression of CD34 and FLT3 within LSK cells was used to distinguish long-term (LT)-HSC, short-term (ST)-HSC, and multipotent progenitors (MPP).

Table S1: Binding properties of anti-FLT3 antibodies

ScFv	FLT3 domain	hFLT3 K_D (nM)	mFLT3 K_D (nM)
P4C7	**1**	7.7	400
P3A1	**2**	64	>1000
P9B5	**3**	11	1.5
P3E10	**4**	106	>1000
P1A5	**4**	0.19	>1000
P4H11	**4**	233	>1000
P7H3	**4**	2	0.9
P12B6	**5**	84	>1000
P14G2	**5**	8	>1000

Selected scFvs were reformatted as human IgG molecules and their affinity to human FLT3 (hFLT3) and mouse FLT3 (mFTL3) protein was determined at 37°C by surface plasmon resonance. K_D: equilibrium dissociation constant.

SUPPLEMENTAL MATERIALS AND METHODS

Cells and cell culture conditions

Cell lines and primary cells were cultured in a humidified incubator at 37ºC and 5% CO_2. The cell lines HEK293T (CRL-3216) and MV4-11 (CRL-9591) were acquired from ATCC (Manassas, VA). MOLM-13 (ACC-554), MOLP-8 (ACC-569) and EOL-1 (ACC-386) cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Cell lines were engineered to express Luciferase and GFP using Luc2AGFP lentivirus (AMSbio, Cambridge, MA). Peripheral blood mononuclear cells (PBMC) were sourced from Stanford Blood Center (Palo Alto, CA) or Stemcell Technologies (Vancouver, BC, Canada) and T cells were isolated using the human Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA).

Protein Reagents

The extracellular domain of the human FLT3 protein (NP_004110.2) fused to C-terminal polyhistidine-tag (His-Tag) and Avi-Tag was expressed in Expi293 cells and purified from culture supernatants. For use as a detection reagent, recombinant soluble FLT3 was biotinylated using the Bulk BirA ligase reaction kit (Avidity, Aurora, CO). Rituximab and anti-CD52 antibodies were produced in recombinant form using sequences obtained from public sources. Rituximab was labeled with R-Phycoerythrin (R-PE) using sulfhydryl-maleimide reaction chemistry (Prozyme, Hayward, CA) for use in flow cytometry assays at 1 μg/mL.

Lentiviral vector production

HEK293T cells were transfected with lentiviral transfer vectors, psPAX2, and pMD.2G (École Polytechnique Fédérale de Lausanne, France) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). Twenty-four hours after transfection, the medium was replaced with X-Vivo 15 medium (Lonza, Basel, Switzerland) supplemented with 10% FCS (GE Healthcare, Pittsburgh, PA). Virus-containing supernatants were harvested 24 hours later, filtered through a 0.45 μm filter and immediately used for transduction.

Determination of the binding kinetics of anti-FLT3 antibodies

Binding affinities of selected anti-FLT3 antibodies to human FLT3 were determined by Surface Plasmon Resonance (SPR) as described previously.[1] An anti-human IgG Fc capture chip was prepared by amine-coupling of goat anti-human IgG Fc (Southern Biotech, Birmingham, AL) to a Biacore Series S sensor chip CM4 (GE Lifesciences, Marlborough, MA) surface at 25°C. Kinetic assays were performed at 37°C in running buffer HBST+ supplemented with 1 mg/ml bovine serum albumin. IgG-containing cell culture supernatants were injected for 2 minutes at 10 μl/min onto a downstream flowcell (resulting in different IgGs being immobilized in flowcell 2, 3 or 4). In all experiments, flowcell 1 was used as a reference surface. Human FLT3 was diluted into running buffer at concentrations of 5 and 25 nM, injected as analyte for two minutes at 30 μl/min and dissociation was monitored for 20 minutes. The anti-human IgG Fc surfaces were regenerated using three 60-second injections of 75 mM phosphoric acid between each analyte binding cycle. All sensorgrams were double-referenced and fit to a 1:1 Langmuir binding with mass transport model using Biacore T200 Evaluation Software (Version 2.0).

Detection of human T cells in mouse tissues by immunohistochemistry (IHC)

Sections were de-paraffinized, and heat-induced epitope retrieval was performed in a pressure cooker with citrate buffer. Endogenous peroxidase and protein blocking steps were applied, and slides were incubated with rabbit monoclonal CD3 antibody, clone EP41 (Biocare Medical, CME 324) at a concentration of 0.085 µg/ml. The antibody was detected using an anti-rabbit polymer and visualized with DAB. All slides were counterstained with Mayer's Hematoxylin.

Lists of antibodies used

CAR T cell phenotyping and characterization

Antibody	Fluorophore	Clone	Manufacturer
Anti-human CD4	BV785	OKT4	BioLegend
Anti-human CD8	BV510	RPA-T8	BioLegend
Anti-human CD52	FITC	HI86	Thermo Fisher
Anti-human TCRαβ	PE	BW242/412	Miltenyi
Anti-human CD3ε	APC	BW264/56	Miltenyi
Anti-human CD45RO	PerCP-Cy5.5	UCHL1	BioLegend
Anti-human CD62L	BUV395	SK11	BD Biosciences
Anti-human CD137	APC	4B4-1	BioLegend
Rituximab	PE		In-house

Detection of CAR T cells in mouse blood

Antibody	Fluorophore	Clone	Manufacturer
Anti-mouse CD45	APC-Cy7	30-F11	BioLegend
Anti-human CD45	BV510	HI30	BD Biosciences
Anti-human CD4	BV785	OKT4	BioLegend
Anti-human CD8	APC	RPA-T8	BioLegend
Rituximab	PE		In-house

Mouse bone marrow analysis

Antibody	Fluorophore	Clone	Manufacturer
Anti-mouse Sca-1	FITC	D7	Thermo Fisher
Anti-mouse c-Kit	PE-Cy7	2B8	Thermo Fisher
Anti-mouse CD34	AF700	RAM34	Thermo Fisher
Anti-mouse FLT3	APC	A2F10	Thermo Fisher
Anti-mouse Gr1	PE	RB6-8C5	Thermo Fisher
Anti-mouse CD11c	PE	N418	Thermo Fisher
Anti-mouse CD11b	PE	M1/70	Thermo Fisher
Anti-mouse NK1.1	PE	PK136	Thermo Fisher
Anti-mouse B220	PE	RA3-6B2	Thermo Fisher
Anti-mouse CD19	PE	1D3	Thermo Fisher
Anti-mouse CD3	PE	145-2C11	Thermo Fisher
Anti-mouse Ter-119	PE	TER-119	Thermo Fisher

Human HSPC and primary AML cell analysis

Antibody	Fluorophore	Clone	Manufacturer
Anti-human CD4	PerCP-Cy5.5	SK3	BioLegend
Anti-human CD8	PerCP-Cy5.5	HIT8a	BioLegend
Anti-human CD34	BUV395	8G12	BioLegend
Anti-human CD38	BV605	HB-7	BD Biosciences
Anti-human CD90	APC	5E10	BD Biosciences
Anti-human CD45RA	APC-H7	HI100	BD Biosciences
Anti-human CD123	BV510	9F5	BD Biosciences
Anti-human CD10	FITC	HI10a	BioLegend
Anti-human FLT3	PE	4G8	BD Biosciences
Anti-human CD45	FITC	HI30	BD Biosciences

Detection of functional markers in CAR T cells

Antibody	Fluorophore	Clone	Manufacturer
Anti-human CD25	BV711	BC96	BioLegend
Anti-human CD107a	FITC	H4A3	BioLegend
Anti-human TNFα	PE	Mab11	BioLegend
Anti-human IL2	PE-Cy7	MQ1-17H12	BioLegend
Anti-human IFNγ	PerCP-Cy5.5	B27	BioLegend

Detection of human T cells by IHC

Antibody	Fluorophore	Clone	Manufacturer
Anti-human CD3	N/A	EP41	Biocare Medical

References

1. Yeung, Y. A., Krishnamoorthy, V., Dettling, D., Sommer, C., Poulsen, K., Ni, I., et al. (2020). An optimized full-length FLT3/CD3 bispecific antibody demonstrates potent anti-leukemia activity and reversible hematological toxicity. Mol. Ther. *28*, 889-900.