Genome editing of donor-derived T cells to generate allogeneic chimeric antigen receptor-modified T cells: optimizing αβ T-cell-depleted haploidentical hematopoietic stem cell transplantation

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Supplemental file for

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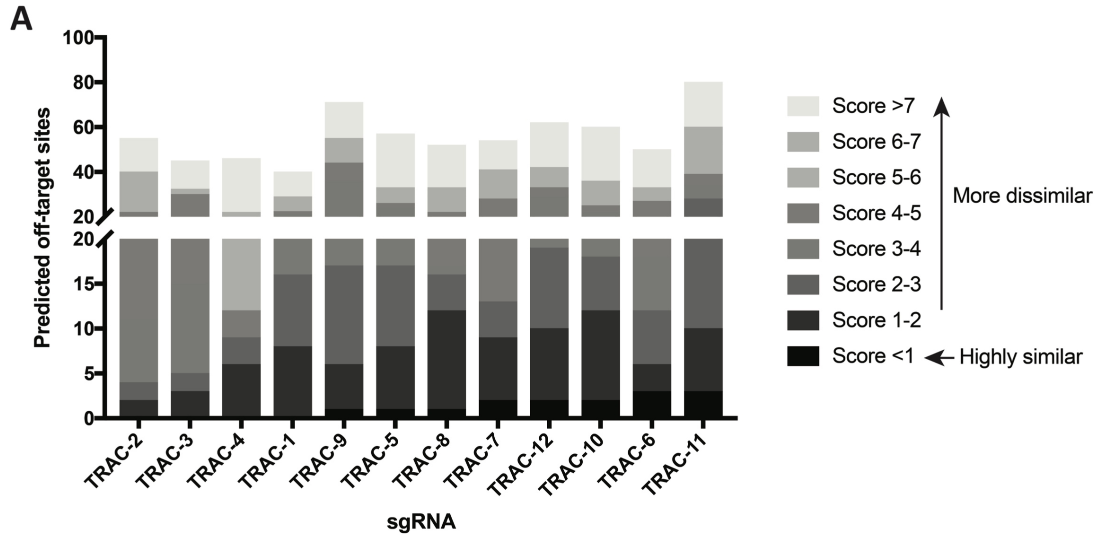
Suppl. Figures

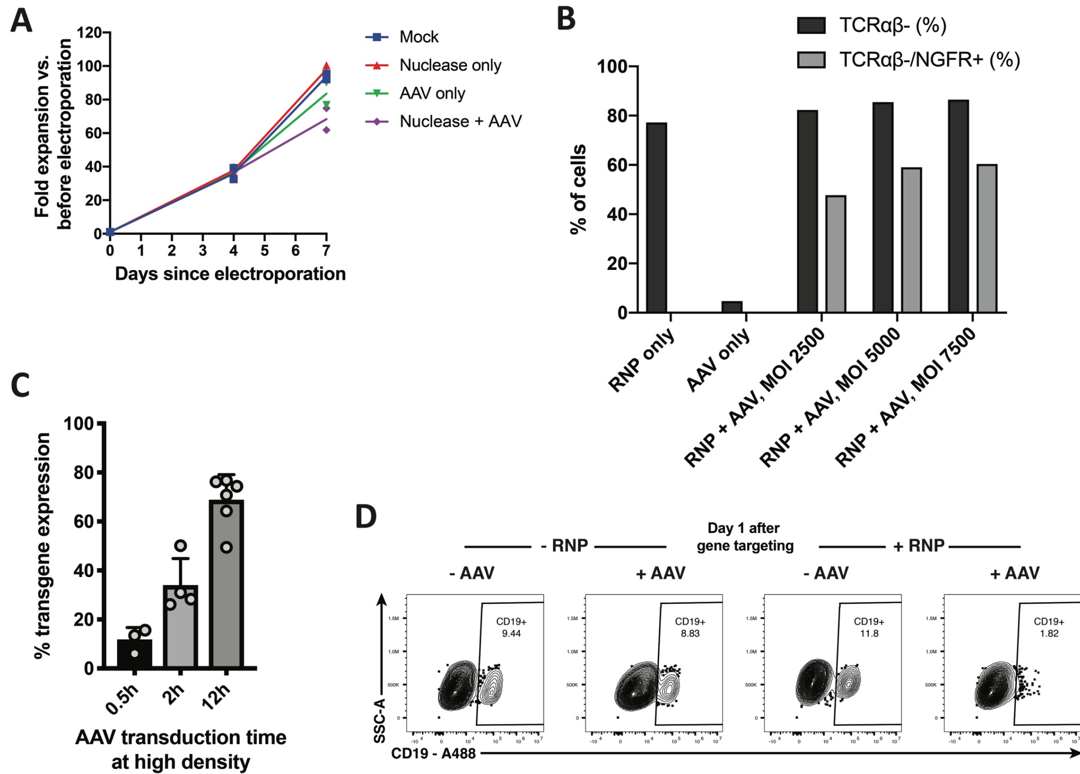


B

Symbol	Target sequence + PAM	Specificity score (MIT)	COSMID total predicted off-target sites	COSMID highly similar off-target sites (score <1)
TRAC-1	GAGAATCAAAATCGGTGAAT AGG	82	40	0
TRAC-2	TCTCTCAGCTGGTACACGGC AGG	85	55	0
TRAC-3	CTCTCAGCTGGTACACGGCA GGG	83	45	0
TRAC-4	TGGATTTAGAGTCTCTCAGC TGG	79	46	0
TRAC-5	TCAGGGTTCTGGATATCTGT GGG	74	57	1
TRAC-6	TAGGCAGACAGACTTGTCAC TGG	71	50	3
TRAC-7	ACACGGCAGGGTCAGGGTTC TGG	66	54	2
TRAC-8	GCTGGTACACGGCAGGGTCA GGG	65	52	1
TRAC-9	GTCAGGGTTCTGGATATCTG TGG	64	71	1
TRAC-10	AGCTGGTACACGGCAGGGTC AGG	62	60	2
TRAC-11	AGAGTCTCTCAGCTGGTACA CGG	59	80	3
TRAC-12	ATTTGTTTGAGAATCAAAAT CGG	49	62	2

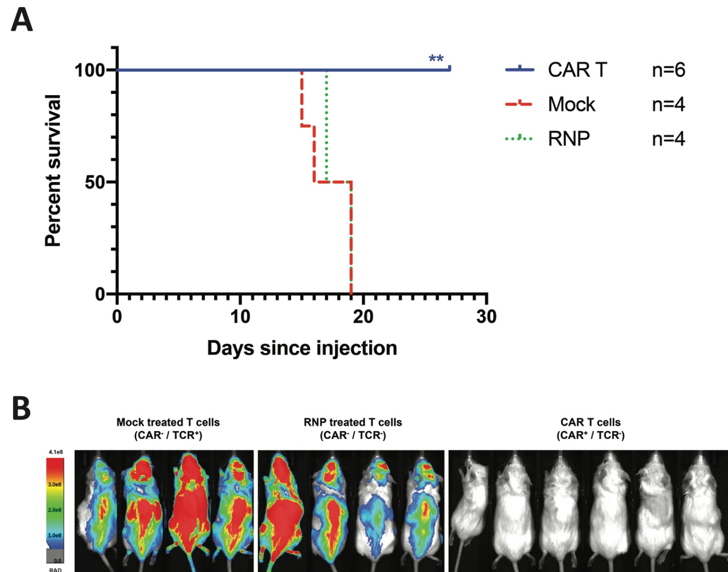
Suppl. figure 1 – Off-target prediction for sgRNAs at exon 1 of the TRAC locus.

A) Counts of COSMID-predicted off-target sites for sgRNAs in exon 1 of the TRAC locus, ranked from left to right by increasing counts of predicted sites, prioritizing sgRNAs with low numbers of highly similar off-target sites (low COSMID scores). B) Overview over sgRNAs in exon 1 of TRAC with specificity scores calculated by CRISPOR (MIT algorithm) and COSMID. sgRNA = single guide RNA, PAM = protospacer-adjacent motif.



Suppl. figure 2 – Optimization of T cell targeting conditions and B cell detection.

A) Expansion after electroporation of CAR T cells or control cells treated with the indicated conditions, relative to their count before electroporation. Cells originated from 2 different donors. B) Titration of different rAAV6 MOIs shows a slight increase in the frequency of targeted cells between 2500 and 5000 vg/cell but no further increase beyond that. C) A strong determinant of targeting frequencies is the duration of transduction at high density (>$5x10^6$ cells per ml) before dilution with medium to the target density for expansion ($5x10^5$ cells per ml). Transduction was performed at the MOI of 5000 vg/cell. D) Detection of B cells 24h after gene targeting shows early disappearance of $CD19^+$ cells for the condition that leads to CAR expression but not in the control conditions (mock electroporation, RNP electroporation only, AAV transduction only). AAV = adeno-associated virus, RNP = ribonucleoprotein, MOI = multiplicity of infection, NGFR = nerve-growth-factor receptor.



Suppl. figure 3 – CAR T cell efficacy in vivo using two different control groups.

A) Survival graph for Nalm6 xenografts in NSG mice after transplantation of mock treated T cells (Mock), TRAC RNP treated T cells (RNP) or RNP + AAV treated T cells (CAR T) at 5 million cells per mouse. Groups were compared by log-rank test and statistical significance indicated in the graph. B) BLI imaging result on day 15 after injection comparing the 3 groups.

Suppl. methods

AAV production

rAAV6 was produced as previously described[1] or acquired from Vigene Biosciences Inc. The absolute concentration of ITR copy numbers was determined by Droplet Digital PCR (Bio-rad) using previously reported primer and probe sets[2].

T cell culture and genome editing

The TCRαβ$^+$/CD19$^+$ cell fraction (non-target fraction from the graft manipulation procedure) was used fresh or cryopreserved. Cells were cultured in X-VIVO 15 (Lonza) supplemented with 5% human AB serum (Sigma) and 100 IU/ml recombinant human IL-2 (Peprotech). Medium changes were performed every 2-4 days and T cells maintained at a target density of $5x10^5$ cells/ml unless otherwise indicated.

T cells were activated with Dynabeads Human T cell Activator (Gibco) for 3 days and beads removed before electroporation. For gene editing, electroporation was performed as previously described[1]. HPLC-purified sgRNA with 2'-O-methyl-3'-phosphorothioate modifications at the three terminal nucleotides on both ends[3] (Synthego) was complexed with high-fidelity spCas9 protein[4] (IDT) at a molar ration of 2.5:1 (sgRNA : protein) and electroporated in buffer P3 (Lonza) into activated T cells using a 4D-Nucleofector (Lonza) in 16-cuvette strips. 1E6 activated T cells were used per electroporation using program EO-115. The cells were resuspended directly after electroporation in 80µl of complete T cell medium and then diluted to the target density. For gene targeting, cells were incubated within 15 minutes after electroporation with rAAV6 for transduction at a multiplicity of infection (MOI) of >5000 vg/cell unless otherwise stated. After

the specified transduction time, the suspension was diluted with complete medium to reach the target cell concentration as indicated.

In vitro cytokine measurement and cytotoxicity assay

$CD19^+$ Nalm6-GL cells stably expressing GFP and Firefly Luciferase (FLuc)[5] and $CD19^+$ Raji cells, also stably expressing GFP and Firefly Luciferase, were gifts from Crystal Mackall (Stanford) and used in co-culture assays with the CAR T cells or control T cells at different effector:target ratios for 20 hours. Concentrations of IL-2 and IFN-γ in supernatant were measured with the respective ELISA kits (Biolegend). For cytotoxicity assays, the cells were co-cultured and the absolute number of GFP^+ cells in culture determined by high-throughput flow cytometry on a CytoFLEX (Beckman Coulter) after adding a specified number of CountBright Absolute Counting Beads (Thermo Fisher Scientific) to the cell suspension. The number of GFP^+ cells killed were estimated relative to control samples in which target cells were cultured without effector cells with the formula $(100 - ((GFP^+count_{Control} - GFP^+count_{sample}) / GFP^+count_{Control}) \times 100)$.

Off-target analysis

COSMID (crispr.bme.gatech.edu[6]) was used to identify potential off-target sites in the human genome (hg38) allowing up to 3 mismatches or 1 bp deletion/insertion and 1 mismatch in the 19 PAM-proximal bases.

For empirical analysis of predicted off-targets, T cells from 6 different donors were electroporated with Cas9 RNP targeting TRAC or mock electroporated and genomic DNA extracted using the Qiagen Blood and Tissue kit. Primers for amplification of all predicted sites were designed by the COSMID program. All genomic loci were amplified by specific PCR, barcoded in a second round

of PCR, pooled at equimolar ratios and sequenced using an Illumina MiSeq with 250bp paired end reads as described previously[7]. The resulting data was analyzed with the script indelQuantificationFromFastqPaired-1.0.1.pl[8].

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