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Supplemental Information

Long Terminal Repeat CRISPR-CAR-Coupled "Universal" T Cells Mediate Potent Anti-leukemic Effects

Christos Georgiadis, Roland Preece, Lauren Nickolay, Aniekan Etuk, Anastasia Petrova, Dariusz Ladon, Alexandra Danyi, Neil Humphryes-Kirilov, Ayokunmi Ajetunmobi, Daesik Kim, Jin-Soo Kim, and Waseem Qasim

Figure S1

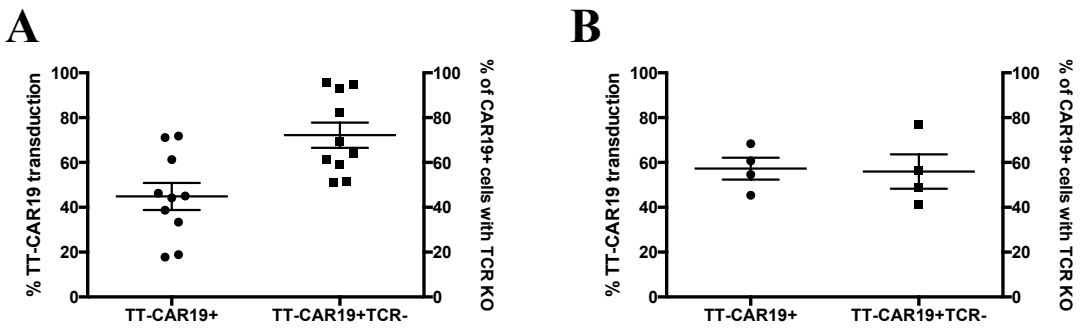
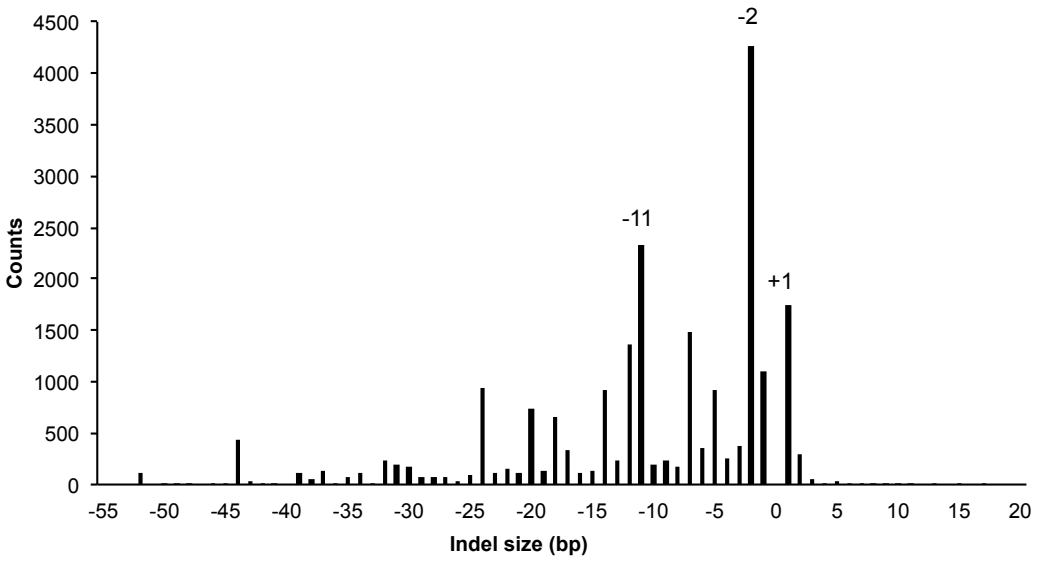


Figure S1. Reproducibility of TT-CAR19 transduction and CRISPR/Cas9-mediated TCR knockout across donors in small and large-scale experiments. (A) Small scale transductions of primary T cells with TT-CAR19 vector ranging between 17.8%-71.8% (Mean 44.9% SEM±6%) in n=10 laboratory experiments on different donors. CRISPR/Cas9-mediated TCR knockout averaged 72.2% SEM±5.6% in TT-CAR19+ transduced cells. **(B)** Large scale T cell manufacture and TT-CAR19 transduction of primary T cells ranged between 45.4-68.4% (Mean 57.3% SEM±4.9% in n=4 experiments on different donors. CRISPR/Cas9-mediated TCR knockout averaged 55.9% SEM±7.8% of the transduced population.

Figure S2



CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGGTACAC-GGCAGGGTCAGGGTTCTGGATATCTGTGGG	WT
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGGTAC---GGCAGGGTCAGGGTTCTGGATATCTGTGGG	-2
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGGT----------CAGGGTTCTGGATATCTGTGGG	-12
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGGTACACCGGCAGGGTCAGGGTTCTGGATATCTGTGGG	+1
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGG--------CAGGGTCAGGGTTCTGGATATCTGTGGG	-7
CAGACTTGTCACTGGATTTAGAGTCTCTCAGC----------AGGGTCAGGGTTCTGGATATCTGTGGG	-11
CAGACTTGTCACTGGATTTAGAGTCTCTCAG----------GGTCAGGGTTCTGGATATCTGTGGG	-14
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGGTACA--GGCAGGGTCAGGGTTCTGGATATCTGTGGG	-1
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGG----------GTCAGGGTTCTGGATATCTGTGGG	-11
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGG----------ATATCTGTGGG	-24
CAGACTTGTCACTGGATTTAGAGTCTCTCAGCTGGTACA------GGGTCAGGGTTCTGGATATCTGTGGG	-5

Figure S2. Mutation pattern of Indels from NGS output of TT-CAR19+TCR- DNA. Frequency of insertions and deletions from next generation sequencing output detect high number of reads of recurring -2bp and -11bp deletions and +1bp insertions with larger deletions of -44bp also seen. TRAC sgRNA sequence marked (blue) with corresponding PAM site recognized by SpCas9 (red).

Table S1: NGS validation of *in silico* predicted off-targets in TT-CAR19+TCR- DNA.

			CAR19-TCR+ (-) sgRNA			CAR19+TCR+ (+) sgRNA			TT-CAR19+TCR- (+) Cas9/sgRNA		
			Number of total reads	Number of mutated reads	Indel frequency(%)	Number of total reads	Number of mutated reads	Indel frequency(%)	Number of total reads	Number of mutated reads	Indel frequency(%)
chr14	23016463	TCTCTCAGCTGGTACACGGCAGG	48396	0	0.00	34931	0	0.00	37810	34887	92.27
chr7	44580723	aCgCTCgcCTGGTACACGGCTGG	55169	0	0.00	39687	2	0.01	42129	0	0.00
chr8	142048510	TCaCaCAGCgGGTACACGGgGAG	49517	14	0.03	33643	14	0.04	43973	26	0.06
chr6	44205360	gaTCaCAGCTGGTACAgGGCAAG	53568	17	0.03	37688	11	0.03	46834	4	0.01
chr16	87840807	gCcCTCAcCTGGgACACGGCAGG	51989	3	0.01	40118	0	0.00	48640	0	0.00
chr16	8807456	gCaCTCAGCTGGTACcCGGCAAG	49822	9	0.02	35907	5	0.01	46413	13	0.03
chr1	186039861	TCcCTCAGCTGGTACAaGGaTGG	53435	6	0.01	39272	2	0.01	50167	3	0.01
chr20	41198892	TtaCTCAGCaGGgACACGGCGGG	45631	0	0.00	33865	2	0.01	43977	0	0.00
chr12	15737994	TtTCTCcGCaGcTACACGGCAGG	58799	2	0.00	42195	0	0.00	58212	5	0.01
chr1	237087837	TCaaTCAGCTGGTgCACGGCTGG	53806	0	0.00	38605	0	0.00	51392	0	0.00
chr22	35253776	TCaCTCtcCTGGTACAtGGCGGG	57646	6	0.01	40544	2	0.00	46592	4	0.01
chr14	45553764	TCcCggAGCTGGaACACGGCTGG	52479	0	0.00	39109	0	0.00	46541	3	0.01
chr3	78045539	aCTCTCAtCTtGTACACGtCAAG	55188	84	0.15	52065	73	0.14	50526	88	0.17
chr1	68621787	cCcCTCAGCaGGTACACaGCCGG	47208	2	0.00	36173	0	0.00	46461	0	0.00
chr7	110391685	cCTCaCAGCTGtaACACGGCAAG	64678	6	0.01	48158	9	0.02	58494	17	0.03
chr14	24475017	TCcCTCAcCTGGgACACGGgGAG	28510	24	0.08	21425	19	0.09	27982	19	0.07
chr18	42501803	TtTCcCAGCTGGaACAaGGCAAG	45923	5	0.01	33283	2	0.01	41427	7	0.02
chr8	10627235	TCTCaCAGCTGGaACACaGCAGG	63425	6	0.01	49785	6	0.01	56721	0	0.00
chrX	53285739	TCaCaCAGCTGGgACAgGGCTGG	34	0	0.00	21	0	0.00	36	0	0.00
chr22	38214973	aCTtgCAGCTGGTcCACGGCCGG	56028	7	0.01	41984	7	0.02	51398	13	0.03
chr3	4075850	gCTCTCAGCaGGcACAtGGCTAG	58439	14	0.02	41068	4	0.01	50454	5	0.01
chr8	134463092	TtTCTCtcCTGGTACACtGCTGG	7324	0	0.00	5374	0	0.00	6976	0	0.00
chr3	76258265	TCTCTaAaCTGtTACAtGGCAAG	62728	27	0.04	47964	12	0.03	59807	16	0.03
chr20	56621614	TCTCaCAcCTtGTACACtGCAGG	51789	0	0.00	36479	0	0.00	50390	0	0.00
chr21	45942609	TgTCTCtGCTGGcACAaGGCGGG	50708	9	0.02	42471	2	0.00	49297	4	0.01
chr12	31673047	TCTCcaAGCTGGTACAtGGaTAG	52351	8	0.02	42940	7	0.02	42907	4	0.01
chrX	140967592	TCTCaCAGCaGGTAgAgGGCAGG	47025	5	0.01	37274	14	0.04	46562	3	0.01
chr1	19612372	gCTCTCAcCTGGTACACaGtGGG	45120	0	0.00	32972	0	0.00	51500	0	0.00
chr1	17335628	TCTCaaAGCTGGaACAgGGCTGG	39829	10	0.03	29388	5	0.02	41912	3	0.01
chr13	113568533	TgTCTCAGCTcGcACAtGGCAAG	53702	14	0.03	40498	10	0.02	51772	3	0.01
chr10	134549621	cCTCTCAGaTGGcACACGaCTGG	50853	0	0.00	38290	2	0.01	49340	3	0.01
chr22	23498145	cCTCaCAGCTGGgACACaGCTGG	52416	0	0.00	38208	0	0.00	49458	0	0.00
chr7	42217710	caTCTCAGCTGGgACACaGCTGG	51656	0	0.00	45518	0	0.00	51372	0	0.00
chr1	226350833	TgTCaCAGCTGGTACAgaGCAAG	52843	0	0.00	46805	0	0.00	44818	0	0.00
chr16	23767191	TCTCcCAGggGGTtCACGGCGAG	42194	0	0.00	35209	0	0.00	41560	5	0.01
chr19	45848028	aCTCTCAGCTGtcACAtGGCCAG	48253	8	0.02	44770	9	0.02	46022	2	0.00
chr17	78117862	TCcCTCAGCTtGTACACGctCAG	44715	0	0.00	39955	0	0.00	41562	2	0.00
chr3	78688933	TCcaTCAGCTGcTgCACGGCAGG	8506	0	0.00	43180	0	0.00	54211	0	0.00
chr19	1043167	TCcCTCAaCTGGTACgaGGCTAG	52018	0	0.00	46120	4	0.01	50928	0	0.00
chr7	19156238	cCTCTCAGgTGGcAgACGGCAGG	56166	0	0.00	42876	3	0.01	54302	7	0.01
chr10	6485209	TCTCTgAGaTGGTACAtGGtAGG	55550	3	0.01	43777	0	0.00	59628	4	0.01
chr22	33002361	TCTgTCAGaTGcTACACaGCAAG	60628	8	0.01	45983	7	0.02	55728	5	0.01
chr1	230006318	TCTCTggGCTGGcACAgGGCTGG	48884	6	0.01	37602	0	0.00	47169	5	0.01
chr5	6738337	cCTCaCAGCTGGggCACGGCCAG	57704	5	0.01	45314	0	0.00	57556	2	0.00
chr20	60294023	TCTtTCAtCTGGTgCAgGGCAGG	47075	18	0.04	38567	12	0.03	45771	8	0.02

Table S1 : 49 *in silico* predicted off-target sites for the TRAC sgRNA were interrogated by next generation sequencing of genomic DNA from CAR19-TCR+ (-) sgRNA, CAR19+TCR+ (+) sgRNA, TT-CAR19+TCR- (+) Cas9/sgRNA samples. Number of mutated reads and Indel frequency (%) calculated for each site across samples. No significance in off-target site cleavage in TT-CAR19+TCR- observed over control samples with cutting detected solely in on-target location. 4 *in silico* predicted off-target sites (highlighted red) also detected by Digenome-seq.

Table S2 : Digenome-seq capture of on- and off-targets using TRAC sgRNA + SpCas9 treated DNA.

				Digenome-seq		CAR19-TCR+ (-) sgRNA			CAR19+TCR+ (+) sgRNA			TT-CAR19+TCR- (+) Cas9/sgRNA		
	Chromosome	location	DNA sequence at cleavage site	DNA cleavage score	Bulge	Number of total reads	Number of mutated reads	Indel frequency (%)	Number of total reads	Number of mutated reads	Indel frequency (%)	Number of total reads	Number of mutated reads	Indel frequency (%)
TRAC_On-target	chr14	23016470	TCTCTCAGCTGGTACACGGCAGG	82.9	No	31144	0	0.00	31492	2	0.01	23722	21913	92.37
TRAC_Digenome-1	chr7	97862071	TCTCagAGCTGGTACACaGCAGG	15.5	No	24511	2	0.01	28084	0	0.00	29151	2	0.01
TRAC_Digenome-2	chr15	101405791	TCTCatAGCTGGTACAtGGCGGG	6.9	No	43042	11	0.03	46871	9	0.02	48842	9	0.02
TRAC_Digenome-3	chr8	10627254	TCTCaCAGCTGGaACACaGCAGG	3.6	No	63425	6	0.01	49785	6	0.01	56721	0	0.00
TRAC_Digenome-4	chr1	68621798	cCcCTCAGCaGGTACACaGCCGG	1.9	No	47208	2	0.00	36173	0	0.00	46461	0	0.00
TRAC_Digenome-5	chr1	12543242	agcCagAGCTGGTACACGGCTGG	1.9	No	46416	7	0.02	49604	6	0.01	52010	3	0.01
TRAC_Digenome-6	chr7	111507871	ctgCTCAGCTGGTACACaGaAGG	1.3	No	46485	4	0.01	49867	4	0.01	53449	7	0.01
TRAC_Digenome-7	chr1	19612391	gCTCTCAcCTGGTACACaGtGGG	0.7	No	45120	0	0.00	32972	0	0.00	51500	0	0.00
TRAC_Digenome-8	chr12	133393014	ggaCTCAGaTGGcACACGGCAGG	0.5	No	39279	7	0.02	42692	7	0.02	45349	5	0.01
TRAC_Digenome-9	chr1	248086928	TcTCTCAGCTGGTACAtGGaGGG	0.4	No	40479	6	0.01	44404	10	0.02	46741	9	0.02
TRAC_Digenome-10	chr10	134549632	cCTCTCAGaTGGcACACGaCTGG	0.2	No	50853	0	0.00	38290	2	0.01	49340	3	0.01
TRAC_Digenome-11	chr16	8735339	gggCaCAGCTGGTACACaGCAGG	0.1	No	41172	0	0.00	43686	0	0.00	45707	2	0.00
TRAC_Digenome-12	chr11	92706925	gCTCTCAGCaGGTACACGGtCCAG	0.1	DNA bulge	35734	15	0.04	38548	17	0.04	37600	20	0.05

Table S2: *In vitro* genomic DNA cleavage of CAR19-TCR+ DNA by Cas9 and off-target site capture using Digenome-seq platform. 12 sites identified with DNA cleavage score >0.1. Validation of Digenome-seq captured sites by next generation sequencing of CAR19-TCR+, CAR19+TCR+ and TT-CAR19+TCR- samples was unable to detect off-target cleavage. Verification of Digenome-seq captured on-target cleavage of the TRAC locus by NGS. 4 Digenome-seq captured sites (highlighted red) were also predicted *in silico*.

Supplemental Methods and Materials

Cas9 mRNA

CleanCap Cas9 mRNA (SF370, TriLink biotechnologies, San Diego, USA) expressed *Streptococcus pyogenes* Cas9 and incorporated nuclear localisation signals at both N and C terminus. mRNA was delivered by electroporation using the Neon transfection system (ThermoFisher Scientific), Lonza 4D (Lonza, London, UK) or the AgilePulse waveform electroporation system (BTX, Holliston, USA) device in accordance with manufacturers' instructions. Cells were incubated at 30°C overnight after electroporation before restoration to 37°C.

Quantification of vector copy number

Customized primers and probe directed against vector WPRE region (FWD: AGGAGTTGTGGCCCGTTGT; REV: TGACAGGTGGTGGCAATGC) were used to quantify vector copy number (VCN) in genomic DNA extracted from transduced cells as previously described. [15] Reactions were performed in triplicate using 2x Absolute qPCR master mix + Rox (AB-1138, ThermoFisher Scientific) and results were normalized for albumin expression (FWD: GCTGCTATCTCTTGTGGGCTGT; REV: ACTCATGGGAGCTGCTGGTTC) and plotted against plasmid generated standard curves.

Cytokine secretion

Effector cells (CAR19+TCR+/ CAR19+TCR-) were thawed in Roswell Park Memorial Institute (RPMI) 1640 medium (A1451701, Gibco, Massachusetts, USA) supplemented with 10% foetal calf serum (FCS). Cells were re-suspended in a 1:1 ratio with CD19+ target cells (CD19+ SupT1 cells) and control non target cells (CD19- SupT1 cells) in a 24 well format. After incubation at 37°C for 24 hours, supernatant was filtered and cryopreserved for subsequent analysis using a $T_H1/T_H2/T_H17$ cytometric bead array (CBA) kit as per manufacturer's instructions (560484, BD Biosciences). All samples were run in triplicate.

CD107a degranulation and intracellular cytokine staining assay

$1x10^5$ effector cells (CAR19+TCR+ or CAR19+TCR-) were incubated in a 96-well U-bottom plate at 1:1 ratio with Daudi cells (CD19+ target cells), in the presence of anti-human CD107a APC antibody (328620, BioLegend, London, UK). Cells were cultured for 4 hours (37°C, 5% CO_2), with addition of 0.13µl GolgiStop (554724, BD Biosciences) after 1 hour. Expression of CAR19 was assessed by staining with Biotin-SP (long spacer) AffiniPure F(ab') Fragment Goat Anti-Mouse IgG, F(ab') Fragment Specific antibody (Stratech Scientific Limited) followed by Streptavidin-PE (130-106-789, Miltenyi Biotec). These cells were subsequently stained with zombie violet fixable viability dye (423113 Biolegend), anti-human HLA-DR BV510 (307646, Biolegend) and anti-human CD25 PerCP/Cy5.5 (356112, Biolegend). Cells were then fixed and permeabilised according to manufacturer protocols (554714, BD Biosciences) and stained with anti-human IFNγ AF488 (502515, Biolegend). As a positive control, CAR19+TCR- cells were incubated alone with PMA (P8139, Sigma-Aldrich, Dorset, UK) and ionomycin (I0634, Sigma-Aldrich) to a final concentration of 10ng/ml and 1µg/ml respectively. All samples were run in triplicate.

Detection of Cas9 protein

Cell pellets ($1x10^6$ cells) were acquired at 6, 12, 24, 48, 72 and 168 hours post electroporation with Cas9 mRNA (100µg/mL). Total protein was quantified by a bicinchoninic acid (BCA) assay. Western blot was run using 25µg total protein per sample. Membrane was blotted with mouse anti-CRISPR-Cas9 antibody (ab191468, abcam, Cambridge, UK) at a 1:1000 dilution in 3% milk overnight at 4°C before incubation with secondary HRP-linked sheep anti mouse (NA931-1ML, GE Healthcare Life Sciences, Buckinghamshire, UK) at a 1:3000 dilution in 5% milk for 1 hour at room temperature. Protein was visualized by chemiluminescence using Pierce ECL western blotting substrate (32106, ThermoFisher Scientific).

Quantitative PCR of Guide RNA Expression

sgRNA extractions were performed using the mirVANA miRNA Isolation Kit (AM1560, ThermoFisher Scientific) with subsequent DNA removal by DNA-free removal kit (AM1906, ThermoFisher Scientific). Synthesis of cDNA used 1µg of RNA and Superscript III reverse transcriptase (Invitrogen). The CFX96 Real-Time System (BIO RAD) was used for Real-time PCR, using SYBR Green Master Mix (Applied Biosystems). A common reverse primer was used for all

reactions (Scaffold REV: AAAAAAGCACCGACTCGGTGCCACT). The reverse primer (Scaffold REV: AAAAAAGCACCGACTCGGTGCCACT) and forward primer (TRAC FWD: TCTCTCAGCTGGTACACGGC) were used. Amplification of sgRNA was normalized to amplification of GAPDH from the same sample.

Detection of non-homologous end joining (NHEJ) events

Genomic DNA extraction was performed using the DNeasy Blood and Tissue Kit (69504, QIAGEN, Hilden, Germany) and a PCR based amplification of 700-800bp around sites of predicted Cas9 scission was carried out. Primers were TRAC forward: TTGATAGCTTGTGCCTGTCCC, TRAC reverse: GGCAAACAGTCTGAGCAAAGG and reactions used Q5 High-Fidelity DNA Polymerase (M0491S, New England BioLabs, Hitchin, UK) on an Alpha Cycler 4 (PCRmax, Staffordshire, UK). PCR products were discriminated by 1% agarose gel electrophoresis. PCR amplicons of genomic DNA were sequenced and analyzed using TIDE protocols (https://tide.nki.nl/)[1].

Supplemental References

1. Brinkman EK, Chen T, Amendola M, van Steensel B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res*. 2014;42(22):e168.