Umbilical cord blood T cells can be isolated and enriched by CD62L selection for use in 'off the shelf' chimeric antigen receptor T-cell therapies to widen transplant options

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[1]UCL Great Ormond Street Institute of Child Health and [2]Great Ormond Street Hospital for Children NHS Trust, London, UK

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[1] UCL Great Ormond Street Institute of Child Health, WC1N 1DZ, London, UK

[2] Great Ormond Street Hospital for Children NHS Trust, WC1N 3JH, London, UK

Supplementary Materials and Methods

Determination of vector copy number (VCN)

CAR19 transgene in transduced cells was assessed by VCN in genomic DNA at end of manufacture of UCB-TT52CAR19 at GOSH NHS Trust. Vector copies were determined by qPCR or droplet digital PCR (ddPCR) targeting HIV-psi or human albumin sequences **(Table S1)**.

Karyotype and Fluorescence in Situ Hybridization (FISH)

UCB-TT52CAR19 cells were cultured with Colcemid overnight in order to arrest cells in metaphase. Karyotype using Giemsa-Banding (G-Banding) and fluorescence in situ hybridization (FISH) analysis was performed at GOSH NHS trust; the latter used a dual colour, break-apart FISH probe (Cytocell TCRAD LPH 047-S; 14q11, red/green fusion) to interrogate interphase nuclei.

Quantification of on- and off-target editing effects and translocations (continued)

On-target and off-target sites informed by previous Digenome-seq studies were captured using Phusion polymerase (New England BioLabs), and the products amplified using TruSeq HT Dual Index primers **(Table S1)**. Libraries were then subjected to paired-end Next Generation Sequencing (NGS) using MiniSeq (Illumina) as previously described. Demultiplexed fastq files were uploaded to Galaxy for quality checks, trimming and alignment. Non-homologous end joining (NHEJ) signatures were analysed using Pindel and figures were created in R.

Vector integration site analysis

Lentiviral integration sites (IS) were mapped by Genwerks (Protagene, Germany) using Shearing Extension Primer Tag Selection Ligation-Mediated PCR (S-EPTS/LM PCR) as previously described [1] and the top 10 most frequent loci tabulated.
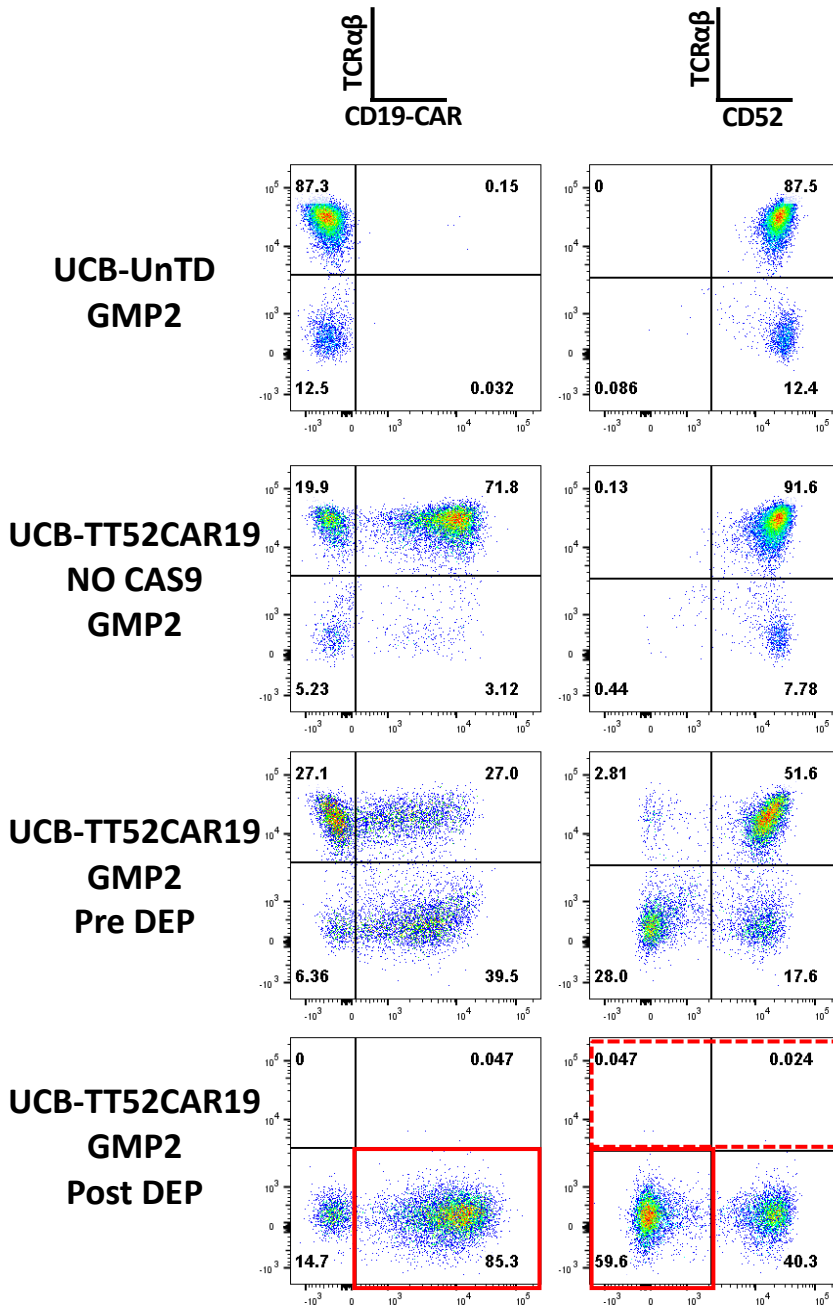
Cytokine Bead Array

Cytokine release was quantified after overnight co-culture of UCB-TT52CAR19 GMP1 (UCB1) and GMP2 (UCB2) or PBL derived batches alongside TT52CAR19 TCR+ or UnTD control cells with CD19+Daudi and CD19+ or CD19- SupT1 cells at a 1:1 ratio using a TH1/TH2/TH17 human bead array kit (Becton Dickinson Biosciences).

References

1. Ottaviano G, Georgiadis C, Gkazi S, et al. Phase 1 clinical trial of CRISPR-engineered CAR19 universal T cells for treatment of children with refractory B cell leukemia. Science translational medicine. 2022;14(668):
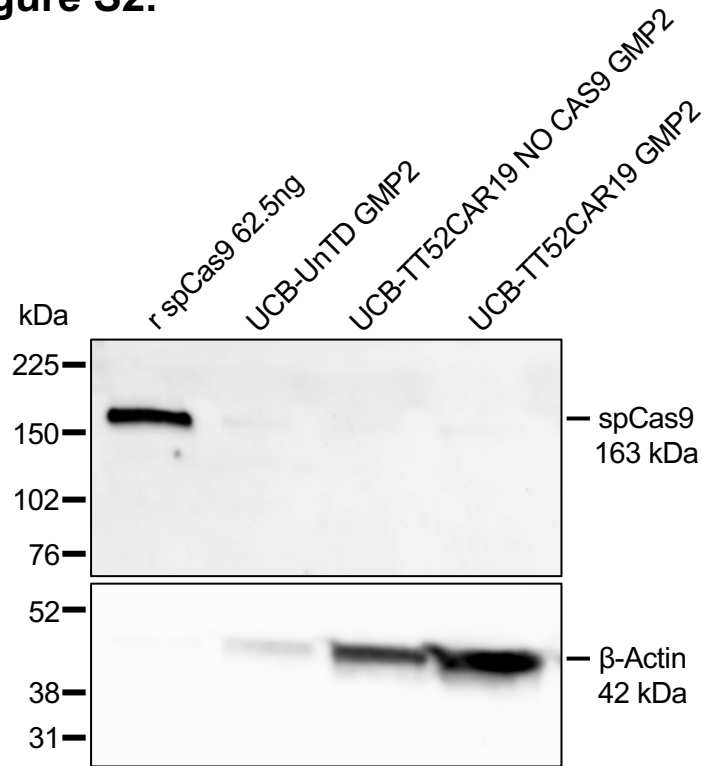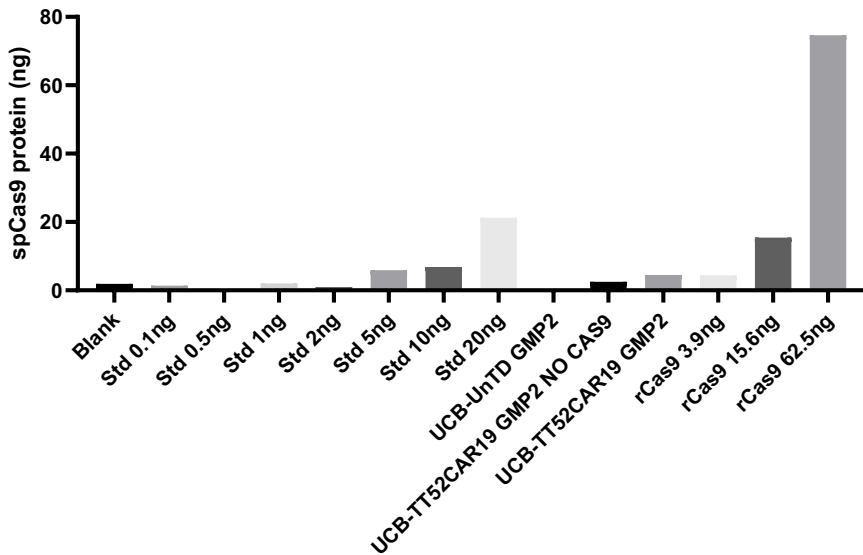
UCB-TT52CAR19-GMP2

Supplementary Figure S1. CAR19 and TCRαβ phenotyping of UCB-TT52CAR19 GMP2 bank throughout manufacture. Flow cytometric T cell transduction and knockout analysis of UCB-TT52CAR19 GMP2 (UCB2) cell bank. UCB-TT52CAR19 cells were stained pre and post magnetic TCRαβ depletion alongside untransduced (UnTD) cells or non-edited UCB-TT52CAR19 TCR+ cells. Transduction efficiency was measured by quantifying transgene expression using F(ab')2, and CRISPR/Cas9 mediated protein knockout was determined through staining for TCRαβ and CD52.
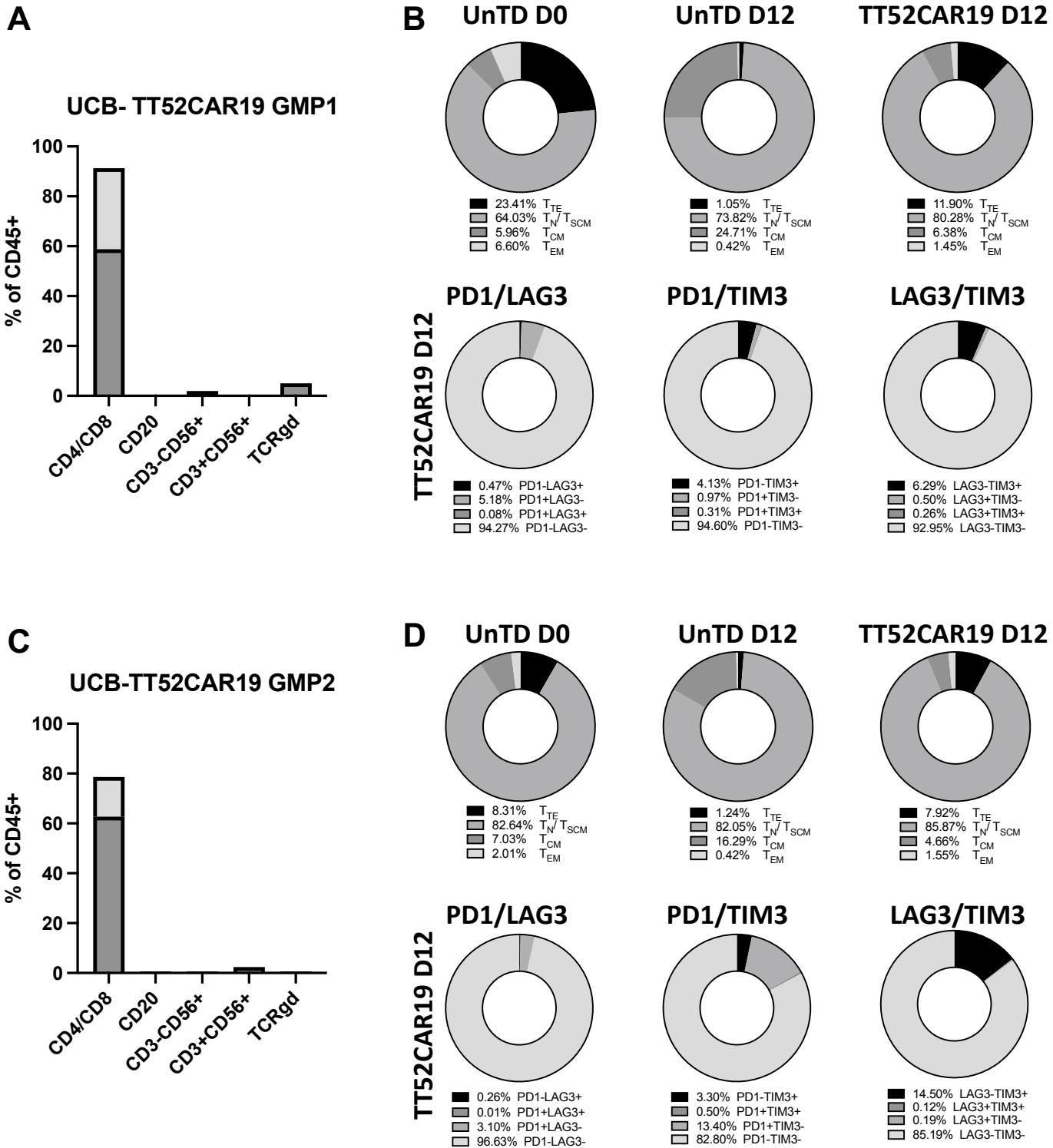
Supplementary Figure S2.

Supplementary Figure S2. End of production verification of absence of residual SpCas9 protein. (A) Whole cell lysate (20µg) from end of production UCB-TT52CAR19 GMP2 (UCB2) cells (lane 4), untransduced (UnTD) (lane 2) or non-edited UCB-TT52CAR19 NO CAS9 GMP2 (lane 3) was subjected to western blot SDS-PAGE and stained with a rabbit polyclonal anti-CRISPR-Cas9 antibody (ab191468, abcam, Cambridge, UK). Absence of specific bands at 163 kDa confirmed the absence of residual SpCas9 protein. Recombinant SpCas9 (rCas9) protein (62.5ng) (lane 1) was used as a positive control. **(B)** Whole cell lysate (5µg) from end of production UCB-TT52CAR19 GMP2 (UCB2) cells, untransduced (UnTD) or non-edited UCB-TT52CAR19 NO CAS9 GMP2 was analysed by EpiQuik CRISPR/Cas9 Assay ELISA Kit (Colorimetric) (EpigenTek, US) against manufacturer's standards (Std) and recombinant SpCas9 (rCas9) protein positive controls. SpCas9 protein levels in end of production UCB samples were comparable to blank control.
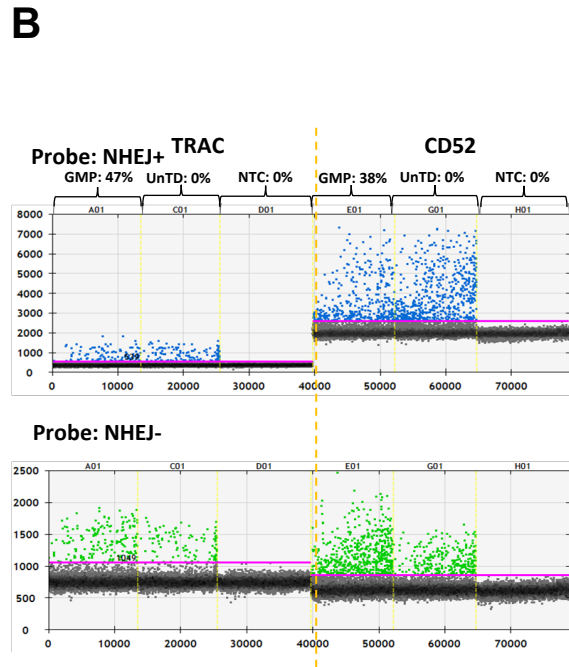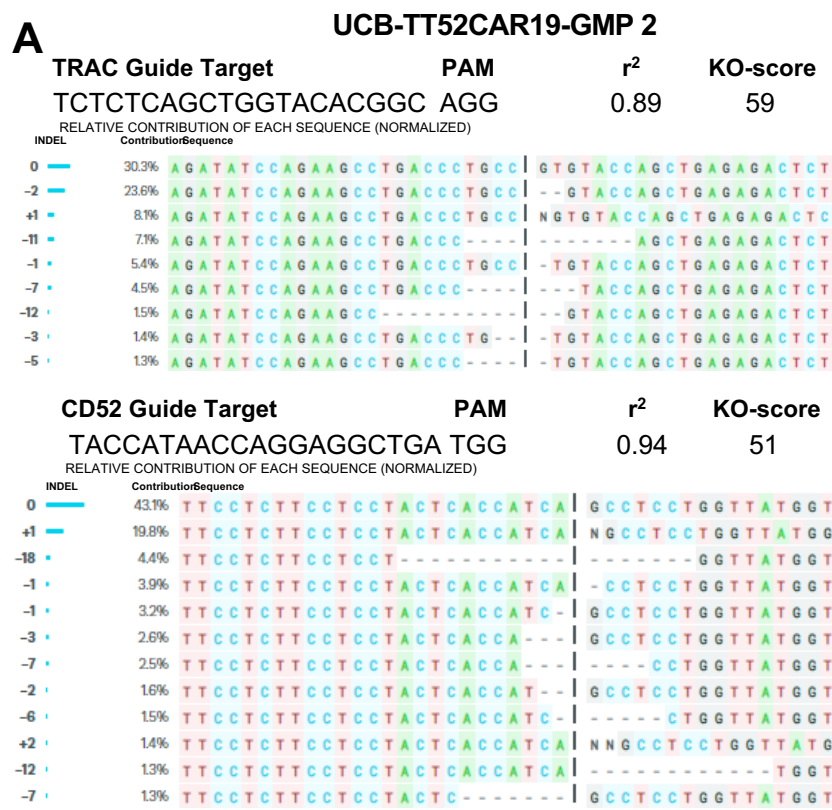
Supplementary Figure S3.

Supplementary Figure S3. Memory phenotype of end of production UCB-TT52CAR19 GMP batches. End of production UCB-TT52CAR19 cells, untransduced (UnTD) cells from day 0 (D0) and day 12 (D12) or UCB-TT52CAR19 cells from GMP batches GMP1 (UCB1) (top) or GMP2 (UCB2) (bottom) were phenotyped by flow cytometry. **(A, C)** Lymphocyte subset distribution of the two cell banks: T cells represented by combined CD4+ cells (dark grey) and CD8+ (light grey); B cells expressing CD20+; NK and T/NK cells (CD3-CD56+ and CD3+CD56+, respectively); TCRγδ T cells; **(B top, D top)** T cell subsets exhibited surface markers CD45RA+CD62L+ attributed to naïve T cells (TN) and memory stem cell T cells (TSCM). Remaining subsets represented differentiated CD45RA-CD62L+ central memory (TCM), CD45RA+CD62L- terminal effectors (TTE) and CD45RA-CD62L- effector memory (TEM) T cells; **(B bottom, D bottom)** Profiling of activation/exhaustion of the three cell banks was investigated using three co-inhibitory markers (PD-1, TIM3, LAG3).

Supplementary Figure S4.

Supplementary Figure S4. Molecular characterisation of on-target events following CRISPR-Cas9 mediated cleavage. (A) ICE analysis of Sanger sequence traces identified indels in end of production UCB-TT52CAR19 GMP2 (UCB2) cells as signatures of NHEJ at the TRAC locus and at the CD52 target site. (B) Droplet Digital PCR (ddPCR) was used for the detection and quantification of indels by ddPCR at both the TRAC and CD52 sites using separate probes, one specific to the predicted NHEJ region (NHEJ+) and a second outside the NHEJ region (NHEJ-).

Supplementary Figure S5.

Top 10 IS Gene Names, Frequencies and Location

Rank		Gene Name	Frequency [%]	Location
Top1		VIPR2	0.185	7+159068479
Top2		NARF	0.077	17+82456875
Top3		TRAF2	0.068	9-136919938
Top4		PTBP2	0.068	1+96752542
Top5		PRRC2B	0.06	9+131411295
Top6		KIAA1715	0.057	2-175988556
Top7		UQCC3	0.057	11-62673431
Top8		MIR548AG1	0.057	4-59547877
Top9		MAML2	0.057	11-96065506
Top10		TNIK	0.057	3-171093835
#All Other mapp. IS		16853	99.257	

Seq Count 10 Strongest	880
Seq Count all other mapp. IS	117583
Total Seq Counts Used	118463

Supplementary Figure S5. Integration site analysis. Ligation-mediated PCR (LMPCR) detection and quantification of vector integration sites (IS) in UCB-TT52CAR19 GMP2 (UCB2) end of production cells where the top 10 most frequent sites comprised <0.1% of integrants.
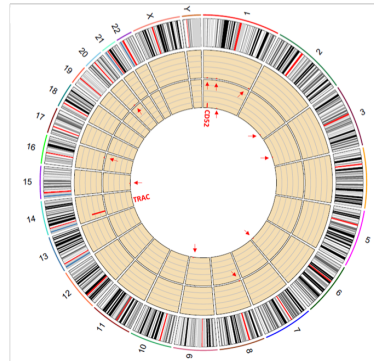
Supplementary Figure S6.

A

UCB-TT52CAR19-GMP 2
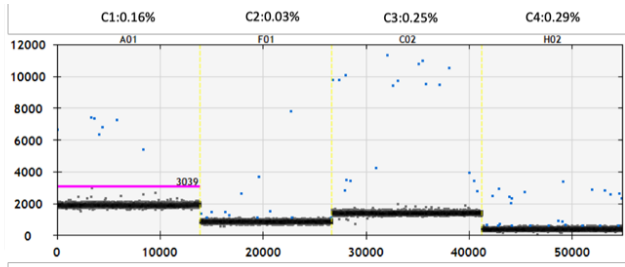
B

TRAC

| Index	Chromosome	ChromStart	ChromEnd	Mutant Frequency
TRAC-01	chr14	23016465	23016487	50.58
TRAC-02	chr8	10627242	10627255	0.02
TRAC-03	chr1	68621798	68621815	0.11
TRAC-04	chr1	19612375	19612394	0.23
TRAC-05	chr1	242872248	242872256	0.02
TRAC-06	chr20	31622178	31622197	0.02
TRAC-07	chr16	66528021	66528030	0.05

CD52

Index	Chromosome	ChromStart	ChromEnd	Mutant Frequency
CD52-01	chr1	26644537	26644559	16.98
CD52-02	chr9	118431471	118431494	0.03
CD52-03	chr15	45497080	45497102	0.02
CD52-04	chr1	95875501	95875523	0.01
CD52-05	chr2	207739023	207739045	0.02
CD52-06	chr3	135905238	135905260	0.02
CD52-07	chr6	156954684	156954706	0.04

Supplementary Figure S6. Molecular characterisation of off-target and predicted translocation events following CRISPR-Cas9 mediated cleavage. (A) ddPCR based quantification of possible translocations after 'on-target' DNA scission. Low frequency translocation events (blue dots) C1-C4 arising between the edited TRAC and CD52 loci. Cumulative events for all four possible events were <1%. **(B)** Circos plot with verification of quantification using targeted NGS across six highest scoring predicted off-target sites. TRAC-01 (solid red line marking locus in outer yellow circle), CD52-01 (solid red line marking inner yellow circle) and at predicted off-target sites TRAC-02-TRAC-07 (red arrows marking outer yellow circle) and CD52-02-CD52-07 (red arrows marking inner yellow circle). Table shows that negligible off-target events were detected for both the TRAC and CD52 guides.

Supplementary Figure S7.
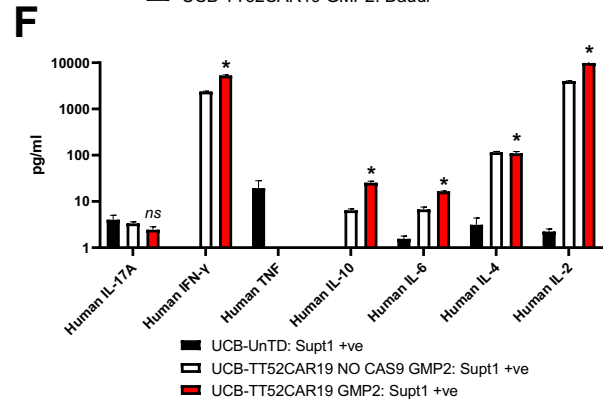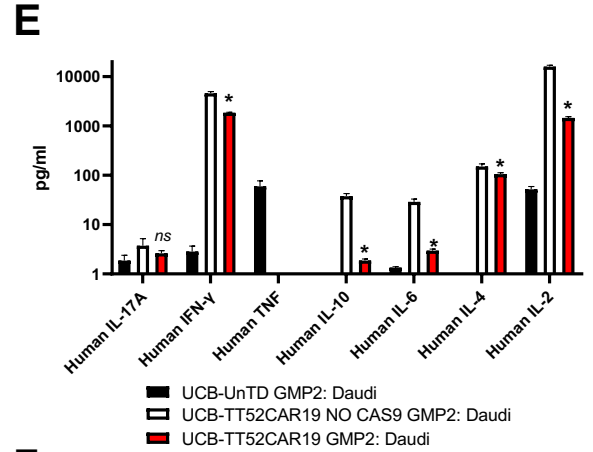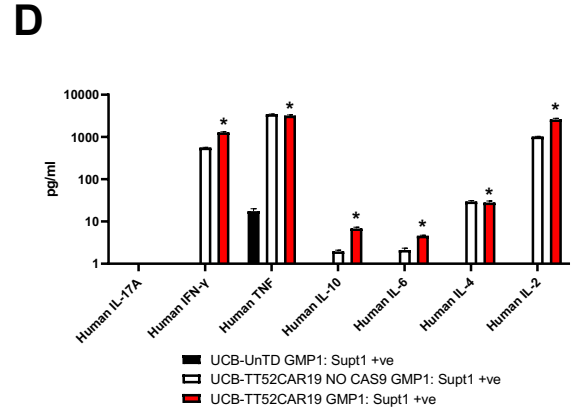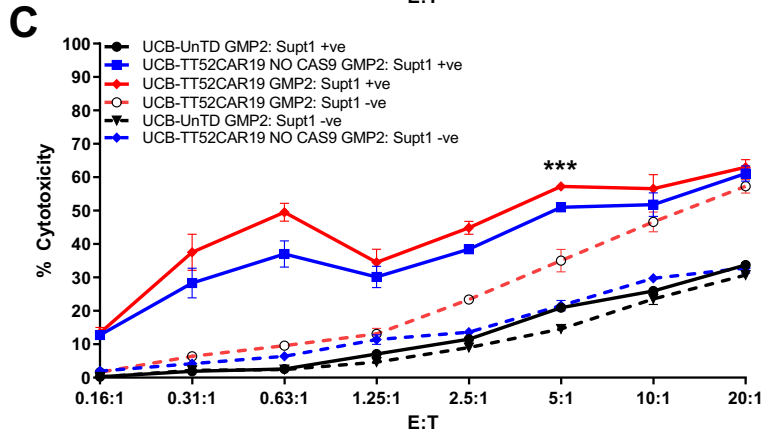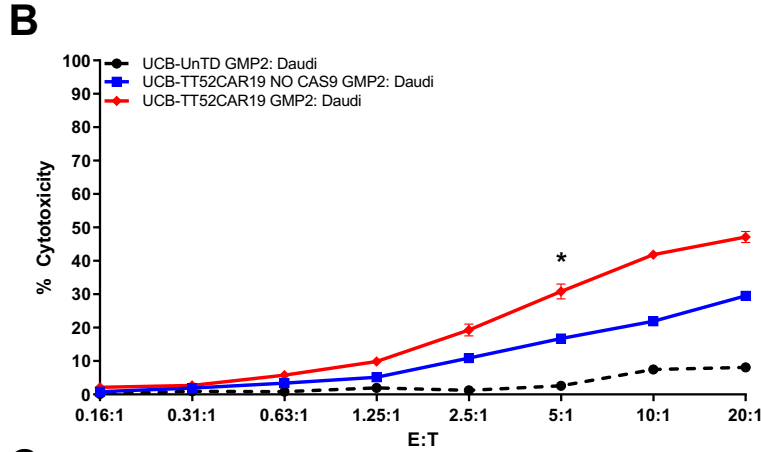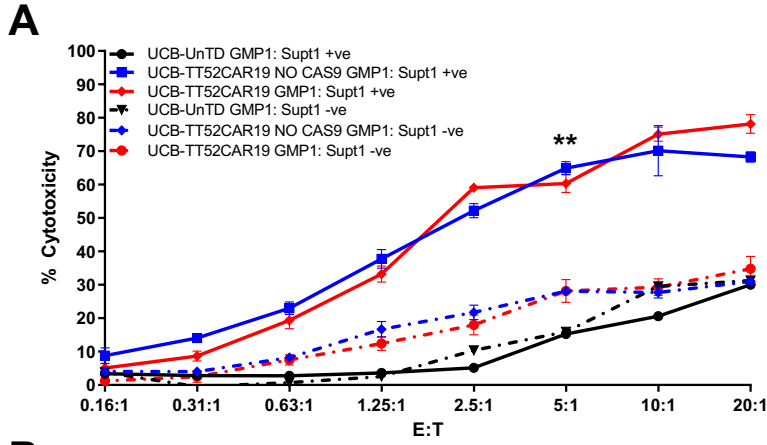
Supplementary Figure S7. *In vitro* **killing potential and cytokine production of UCB-TT52CAR19 cells against CD19+ targets.** In vitro cytotoxicity of UCB-TT52CAR19 cell banks compared to respective UCB-TT52CAR19 transduced but not edited (UCB-TT52CAR19 TCR+) or non-transduced (UnTD) controls when measured by ^{51}Cr chromium release of labelled Supt1 CD19+ or CD19- cells **(A, C)** or CD19+ Daudi **(B)** cells following four hours co-culture at incremental effector (E) – target (T) ratio. Cell co-cultures ranged from $1 \times 10^5 : 5 \times 10^4$ (at E:T of 20:1) to $8 \times 10^2 : 5 \times 10^4$ (at E:T of 0.16:1). Effector responses were considered successful if ≥50% lysis was detected compared to UnTD controls at E:T cell ratios between 1.25:1 and 5:1. Error bars SEM, n=3 replicate/wells. *P<0.05, **P<0.01, ***P<0.001 by t-test. In vitro target specific cytokine secretion of UCB-TT52CAR19 GMP cell banks, and respective UCB-TT52CAR19 TCR+ or non-transduced (UnTD) controls at 1:1 E:T (1.25×10^5 of each effector and target cells) co-culture with Supt1 CD19+ cells **(D, F)** or CD19+ Daudi **(E)** cells overnight. The presence of cytokines in the co-culture supernatant was measured by cytokine bead array and levels >50pg/ml were considered positive responses. Significance was calculated between UCB-TT52CAR19 GMP1 and GMP2 banks and respective UnTD controls. *P<0.05 by t-test. Error bars represent SEM, n=3 replicates.

Supplementary Figure S8.
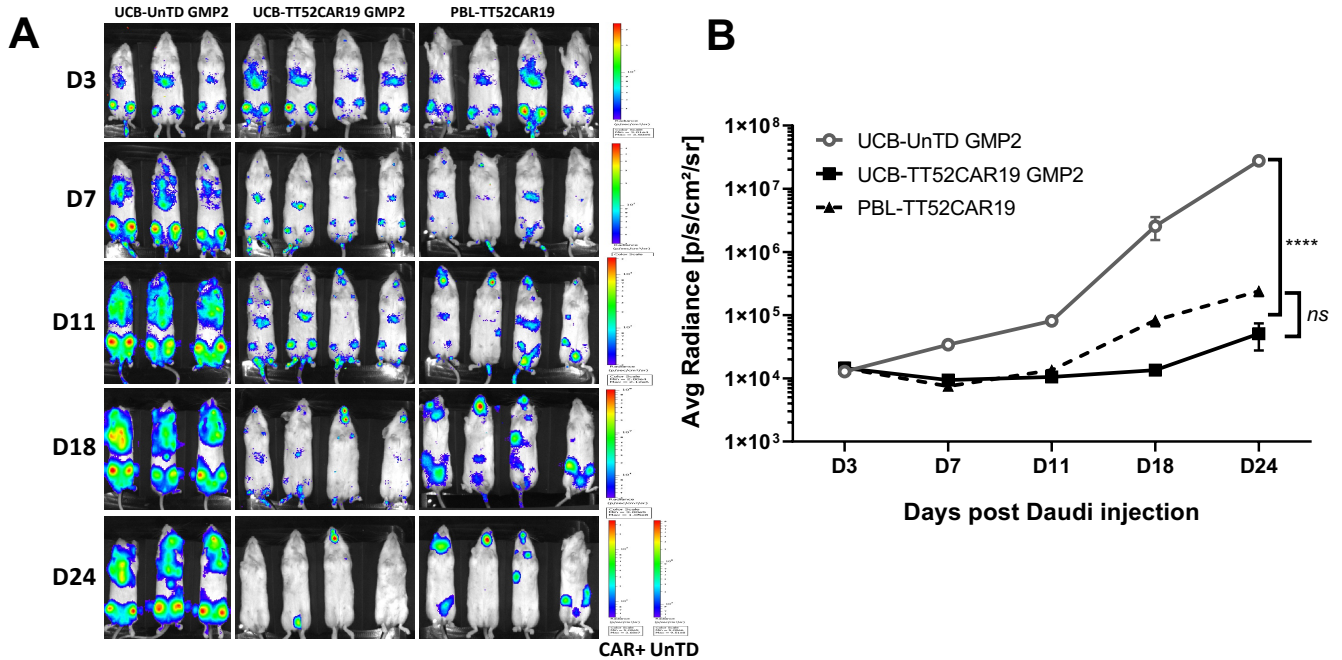
Supplementary Figure S8. *In vivo* **tumour clearance in human:murine xenograft model of CD19+ disease.**

Timeline of *in vivo* human:murine xenograft modelling indicating target and effector intravenous injection days and bioluminescent imaging (BLI) timepoints **(A)**. Serial measurement of bioluminescence of Daudi CD19+ B cell disease in immunodeficient mice NOD/SCID/γc (NSG) mice (n=12) infused with 5 x 10^5 GFP/luciferase expressing Daudi CD19+ B cells were treated on day 4 with either umbilical 5 x 10^6 cord blood (UCB) UCB-TT52CAR19 GMP2 (UCB2) (n=4) or peripheral blood lymphocyte (PBL) PBL-TT52CAR19 (n=4) and were monitored over a 4-week period **(B, C)** Non-transduced (UnTD) (n=3) T cells were used as controls. Error bars represent SEM. Significance compared by area under the curve using one-way ANOVA (F-value 10.63) with Tukey multiple comparison post-hoc; not significant (ns) P ≥ 0.05; ****P < 0.0001.

Table S1.

UCB GMP1

HLA	A		B		Bw	C
Allele	02 : 01:01		07 : 02:01			03 : 03:01
	24 : 02:01		15 : 01:01			07 : 02:01
HLA	DRB1	DRB3	DRB4	DRB5		DQB1
Allele	01 : 02:01			01 : 01:01		05 : 01:01
	15 : 01:01					06 : 02:01
HLA	DPA1		DPB1			DQA1
Allele	01 : 03:01		04 : 01:01			01 : 01:02
	02 : 01:01		11 : 01:01			01 : 02:01

UCB GMP2

HLA	A		B		Bw	C
Allele	01 : 01:01		07 : 02:01			07 : 01:01
	25 : 01:01		08 : 01:01			07 : 02:01
HLA	DRB1	DRB3	DRB4	DRB5		DQB1
Allele	03 : 01:01	01 : 01:02	01 : 03:01			02 : 01:01
	04 : 01:01					03 : 02:01
HLA	DPA1		DPB1			DQA1
Allele	01 : 03:01		04 : 01:01			03 : 01:01
	:		:			05 : 01:01

Table S1. Umbilical Cord Blood unit tissue typing.
High-resolution Next Generation Sequencing tissue typing of UCB GMP1 and GMP2 batches.

Table S2.

Name	Application	Sequence	Primer/Probe
TRAC-on F	On- & off- targets NGS	CATGAGACCGTGACTTGCCA	Primer
TRAC-on R	On- & off- targets NGS	ACACATCAGAATCCTTACTTTGTGA	Primer
TRAC-02 F	On- & off- targets NGS	CAACTCTTGCTGCAACCTGA	Primer
TRAC-02 R	On- & off- targets NGS	TGCATCAGTCAACTTAGGTGAG	Primer
TRAC-03 F	On- & off- targets NGS	CCAAGATGGCAGAAGGGAAT	Primer
TRAC-03 R	On- & off- targets NGS	GAGGTCTTGCAAATTCAGGCT	Primer
TRAC-04 F	On- & off- targets NGS	AGCTTGAATGGCATCCTGAG	Primer
TRAC-04 R	On- & off- targets NGS	GCTTGGCCTCTCCAACTATG	Primer
TRAC-05 F	On- & off- targets NGS	CTGGAGGAAAGAAACAGACAGTAC	Primer
TRAC-05 R	On- & off- targets NGS	AAAAGGAGCCTGGCCATATTTC	Primer
TRAC-06 F	On- & off- targets NGS	CCGTCATCACACTGAACTTTGT	Primer
TRAC-06 R	On- & off- targets NGS	TCCCCATAACCTTTTCTGACCA	Primer
TRAC-07 F	On- & off- targets NGS	CACCCCAGCACCCACTATAC	Primer
TRAC-07 R	On- & off- targets NGS	GCCTCACAAGCAAGCCATAG	Primer
TRAC F	ddPCR NHEJ	TTGTCCATCACTGGCATC	Primer
TRAC R	ddPCR NHEJ	TGTGACACATTTGTTTGAGAATC	Primer
TRAC/NHEJ-	ddPCR NHEJ	TCATGTCCTAACCCTGATCCTCTTGT	Probe
TRAC/NHEJ+	ddPCR NHEJ	ACCCTGCCGTGTACCAGCT	Probe
CD52-on F	On- & off- targets NGS	CAAGACAGCCACGAAGAT	Primer
CD52-on R	On- & off- targets NGS	AGGAGAGAAGGCTGGGTC	Primer
CD52-02 F	On- & off- targets NGS	AGCCAGCCTACTTGCCAA	Primer
CD52-02 R	On- & off- targets NGS	CCCAGCTTCTTAGGAGTGTC	Primer
CD52-03 F	On- & off- targets NGS	GTGTGTAGACTTCAAAGGGCA	Primer
CD52-03 R	On- & off- targets NGS	ACTGGGTAATTCTGAGTTGTGG	Primer
CD52-04 F	On- & off- targets NGS	CTGGTTTCCTTGGAGCCA	Primer
CD52-04 R	On- & off- targets NGS	GTAGACCTGAGCCACCTGAC	Primer
CD52-05 F	On- & off- targets NGS	TTGCCTTACCACTGAGCT	Primer
CD52-05 R	On- & off- targets NGS	AGACAAGTGCTGCCTTACCA	Primer
CD52-06 F	On- & off- targets NGS	CTAGATATCCATGGGTGATTGG	Primer
CD52-06 R	On- & off- targets NGS	GACCCAGTTCACTTCCTGCT	Primer
CD52-07 F	On- & off- targets NGS	ATGCAAGAGTGGCCCAAAT	Primer
CD52-07 R	On- & off- targets NGS	TCACTTGTTCTCCCCTGACC	Primer
CD52 F	ddPCR NHEJ	CAAGACAGCCACGAAGAT	Primer
CD52 R	ddPCR NHEJ	AGGAGAGAAGGCTGGGTC	Primer
CD52/NHEJ-	ddPCR NHEJ	CCAAAGTTGCTTGGCATGGA	Probe
CD52/NHEJ+	ddPCR NHEJ	CATCAGCCTCCTGGTTATGGTACA	Probe
TRAC F + CD52 R	ddPCR Translocations	TTGTCCATCACTGGCATC	Primer F
TRAC F + CD52 R	ddPCR Translocations	GCCAGGCGTTGCTCTTAC	Primer R
CD52 F + TRAC R	ddPCR Translocations	CAAGACAGCCACGAAGAT	Primer F
CD52 F + TRAC R	ddPCR Translocations	TGTGACACATTTGTTTGAGAATC	Primer R
TRAC R + CD52 R	ddPCR Translocations	TCAGAATCCTTACTTTGTGACAC	Primer F
TRAC R + CD52 R	ddPCR Translocations	GCCAGGCGTTGCTCTTAC	Primer R
CD52 F + TRAC F	ddPCR Translocations	CAAGACAGCCACGAAGAT	Primer F
CD52 F + TRAC F	ddPCR Translocations	ATCACTGGCATCTGGACTC	Primer R
TRAC F + CD52 R	ddPCR Translocations	TCATGTCCTAACCCTGATCCTCTTGT	Probe
CD52 F + TRAC R	ddPCR Translocations	AGTCTGTCTGCCTATTCACCGATT	Probe
TRAC R + CD52 R	ddPCR Translocations	TCGGTGAATAGGCAGACAGACTTGT	Probe
CD52 F + TRAC F	ddPCR Translocations	TGGGACAAGAGGATCAGGGT	Probe
Albumin F	ddPCR Translocations	GCTGCTATCTCTTGTGGGCTGT	Primer
Albumin R	ddPCR Translocations	ACTCATGGGAGCTGCTGGTTC	Primer
Albumin	ddPCR Translocations	CCTGTCATGCCCACACAAATCTCTCC	Probe

Table S2. On-, off-target and translocation primer and probe sets.
Primer and probe sets used for quantification of 'on-target' and 'off-target' NHEJ events or possible translocations after 'on-target' DNA scission in UCB-TT52CAR19 Good Manufacturing Process (GMP) batches GMP1 (UCB1) and GMP2 (UCB2).

Table S3.

	UCB1	UCB2
Cell counts in fresh UCB unit		
Total WBC	1234×10^6	822×10^6
Lymphocyte	438×10^6	418×10^6
Cell counts post CD62L$^+$ selection		
Total WBC	184×10^6	118×10^6
Lymphocyte	133×10^6	96×10^6

Table S3. Umbilical Cord Blood (UCB) cell counts pre or post CD62L+ selection. White blood cell (WBC) and lymphocyte Sysmex counts in fresh UCB units pre and post machine-based CD62L+ selection.