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SUPPLEMENTARY INFORMATION

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Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell

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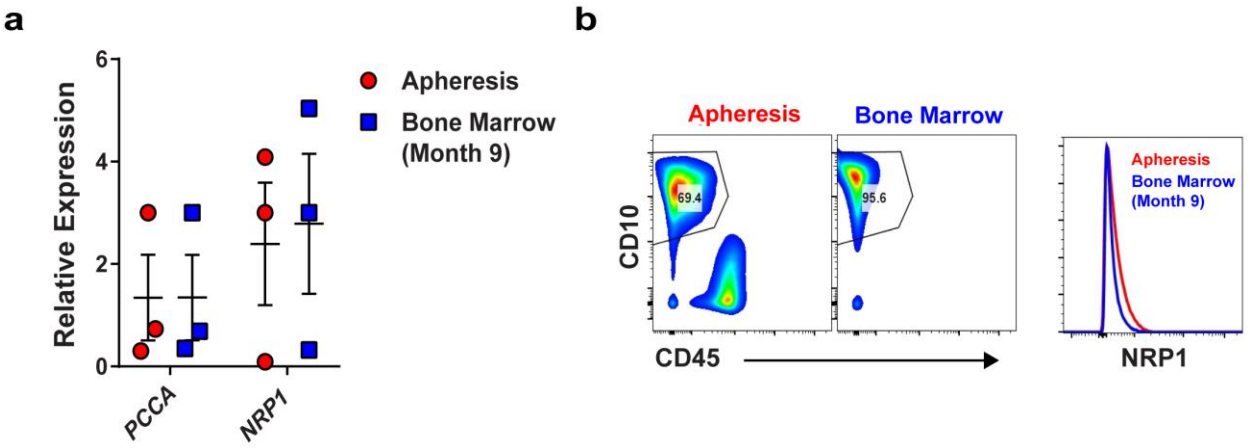
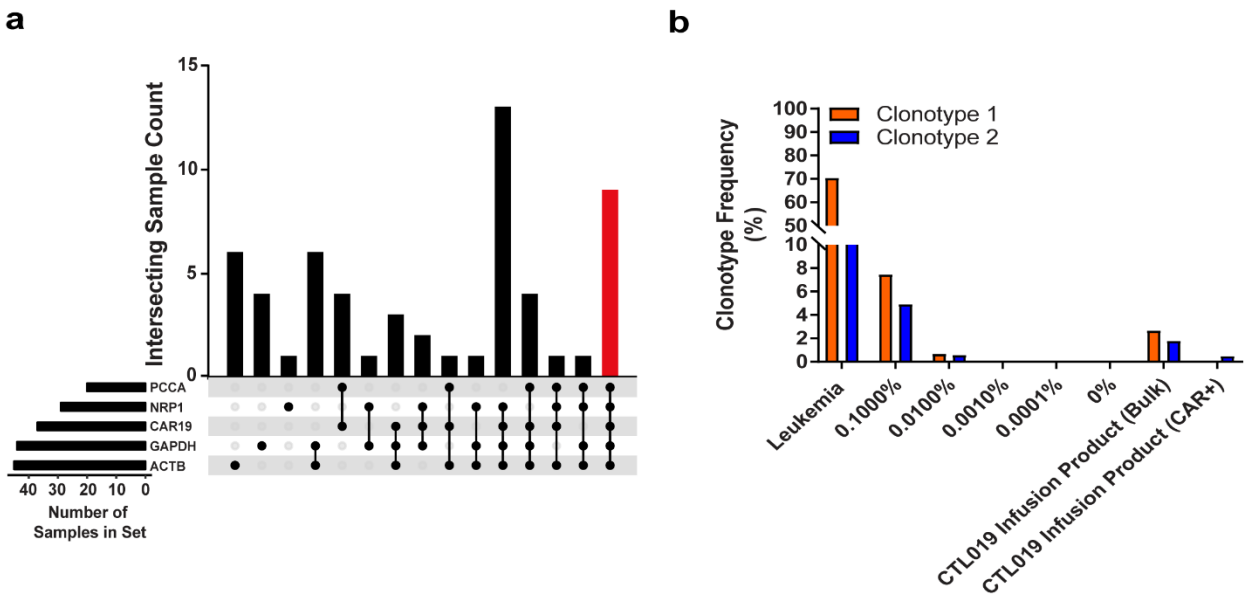
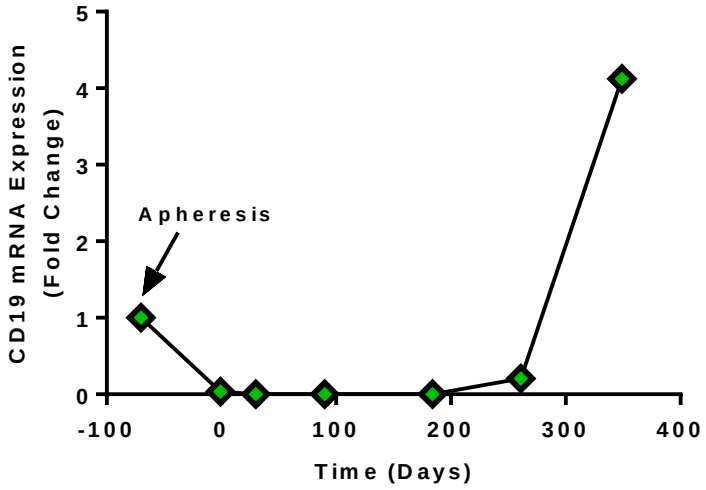
Supplementary Figures



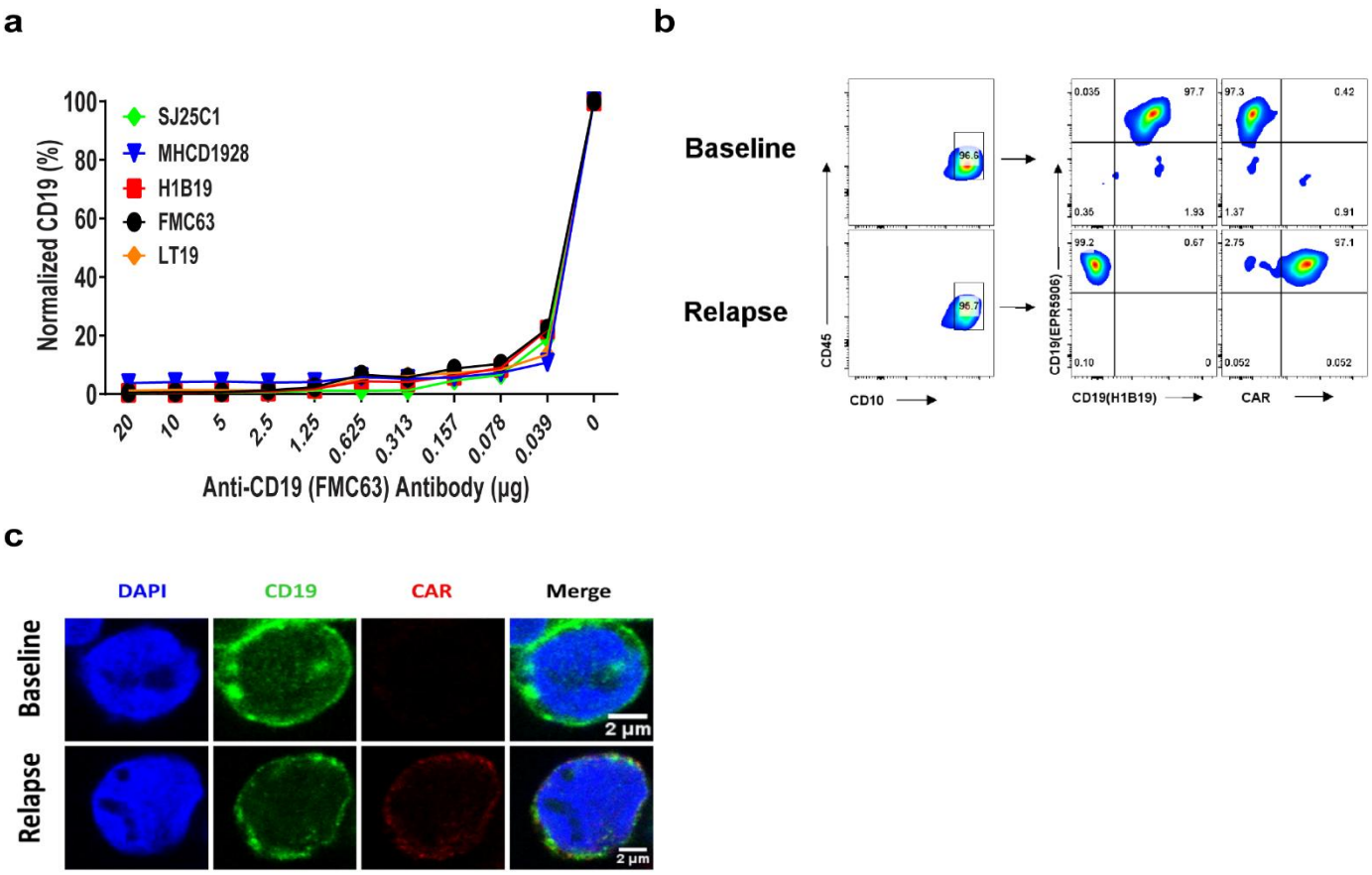
Supplementary Fig. 1 Levels of PCCA and NRP1 in Patient #107 are similar at the time of apheresis and relapse. (a) qRT-PCR analysis of *PCCA* and *NRP1* expression in $CD45^{dim}$ $CD10^{+}$ leukemia cells purified at baseline (apheresis) and at the time of relapse (month 9 bone marrow). n = 3; Center line depicts mean and error bars indicate the SEM. **(b)** NRP1 flow cytometry in apheresis and relapse (month 9 bone marrow) leukemia cells from Patient #107. The cells were derived from apheresis and relapse leukemia cells from Patient #107 and gated on intact, live $CD3^{-}$ cells. Further gating was done on $CD45^{dim}$ $CD10^{+}$ cells. Histograms depicting NRP1 expression in apheresis samples (red) and month 9 bone marrow (blue) are shown. Results are representative of 2 independent experiments.



Supplementary Fig. 2 Single-cell lentiviral integration site analysis and quantification of leukemia cell contamination. (a) The number of single CAR^+ leukemia cells that were positive by the various PCR reactions is shown. The matrix indicating which reactions are considered is found below the plot. To the left of the matrix, the horizontal bar graph shows the number of samples contained in each data set. Above the matrix is a bar plot which shows the number of samples with the specific combination of PCR reactions. **(b)** Frequency of leukemia cells (2 different clonotypes) in the CAR T cell infusion product of Patient #107. To establish a standard for quantification, tumor cells were spiked into a cellular product of a healthy donor at final frequencies of 0.1%, 0.01%, 0.001%, and 0.0001%. Results are representative of 2 independent experiments

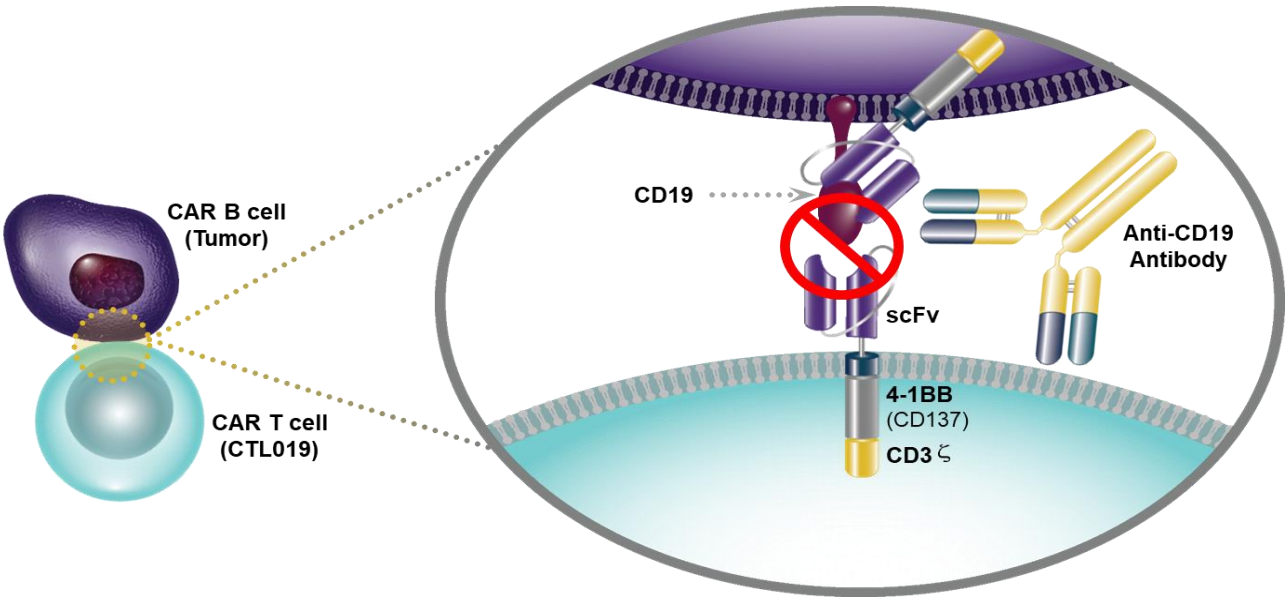


Supplementary Fig. 3 CD19 mRNA expression in Patient #107 over time. CD19 expression levels in the bone marrow over time as determined by qPCR are shown (2 independent experiments).

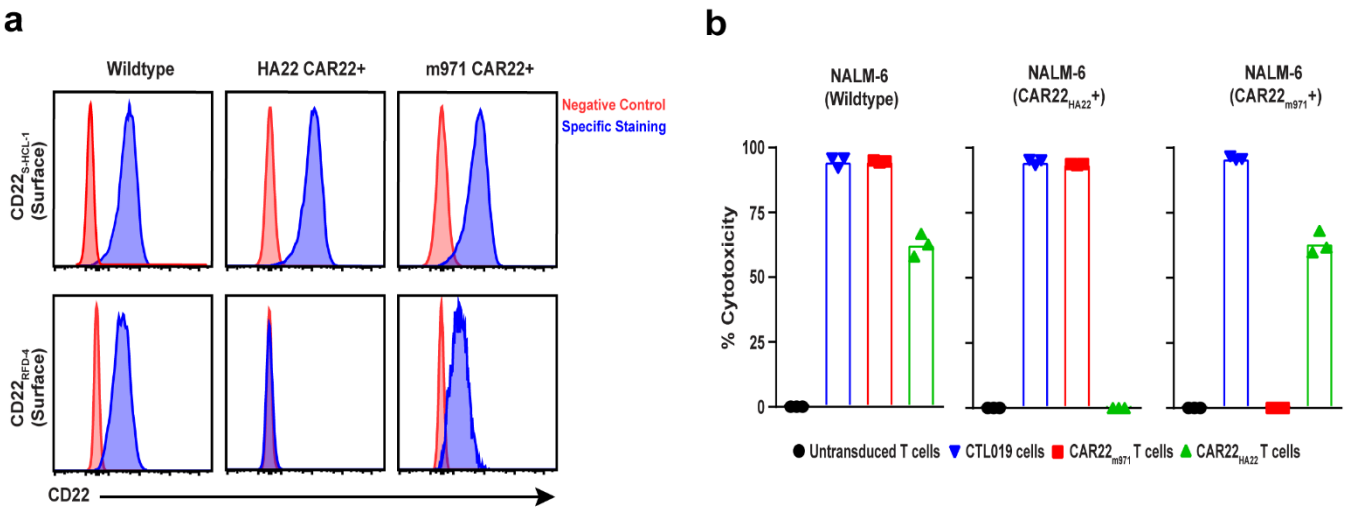


Supplementary Fig. 4 Detection of CD19 and CAR19 in CAR19+ leukemia cells from Patient #107 using multiple anti-CD19 antibody clones. (a) Commercially available anti-CD19 antibodies and the FMC63 clone recognize similar epitopes. K562 cells expressing human CD19 were blocked with different amounts of an anti-CD19 antibody (clone FMC63) for 1 hour at room temperature and then stained with five different fluorescently-labeled monoclonal antibodies against human CD19 (SJ25C1, MHCD1928, H1B19, FMC63 and LT19) for 30 minutes at room temperature. The frequency of $CD19^{+}$ cells is normalized based on no blocking control (n = 3 independent experiments). **(b)** Flow cytometric analysis of Patient #107 leukemia cells at baseline and relapse using anti-CD19 antibodies with intracellular and surface epitope. Leukemia cells at baseline and relapse (bottom) were surface stained with live/dead fixable Aqua, anti-CD45, anti-CD10, anti-CD19 (H1B19) and anti-CAR idiotype mAb, and then permeabilized and stained with rabbit anti-human CD19 (EPR5906) intracellularly. The cells were gated on intact, live $CD45^{dim}$ $CD10^{+}$. Representative flow cytometry from 2 independent experiments are show. **(c)** Confocal imaging of co-localization of CAR19 and CD19 in Patient

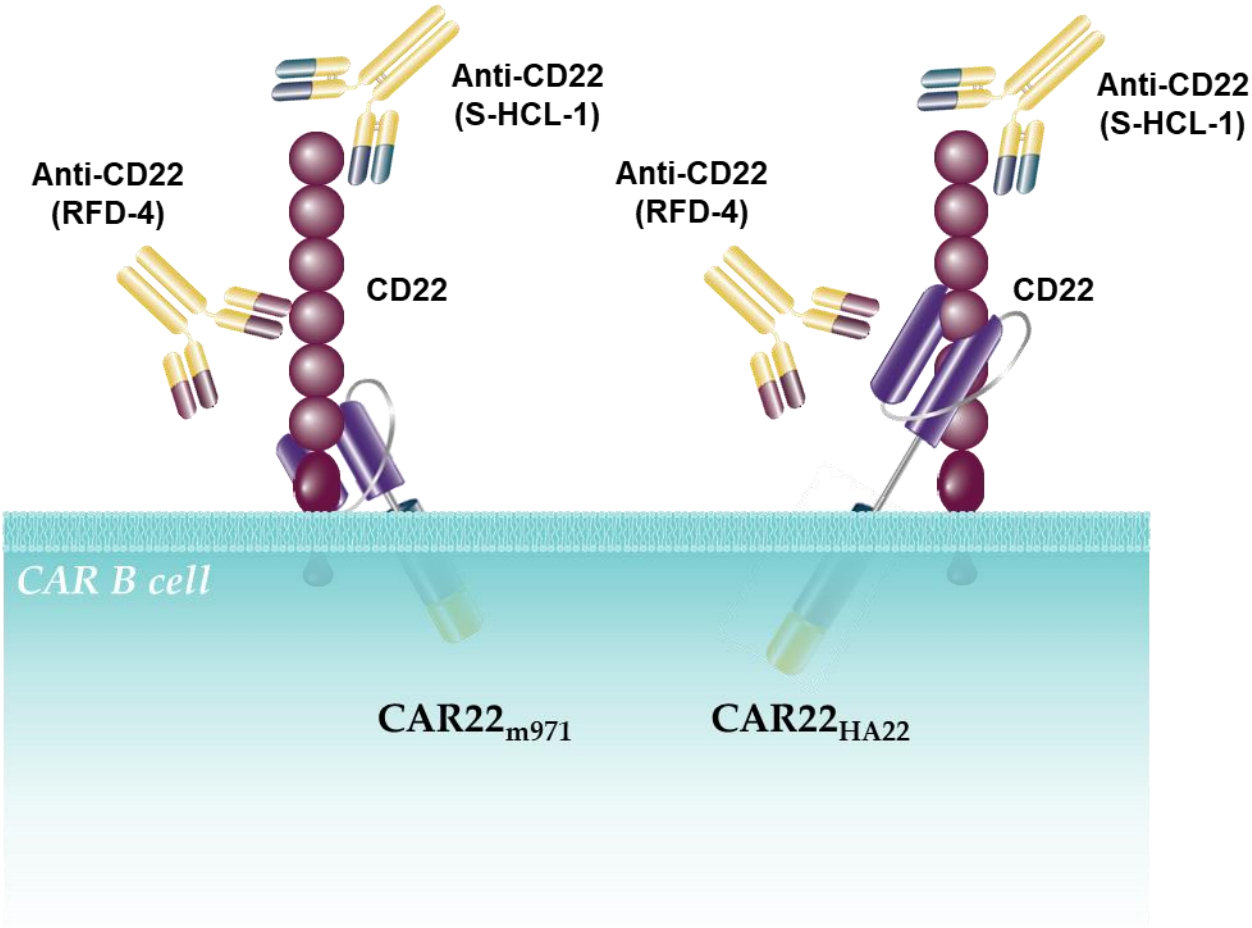
#107 leukemia cells at baseline and relapse. Confocal imaging of Patient #107 blasts at baseline and relapse using rabbit anti-human CD19 monoclonal antibody (EPR5906, green) followed by a secondary antibody Alx488-conjugated goat-anti-rabbit IgG, anti-CAR idiotype mAb followed by Alx647-conjugated goat-anti-human IgG Fcγ (red) and DAPI (blue). Representative images from 2 different experiments with similar results are shown.



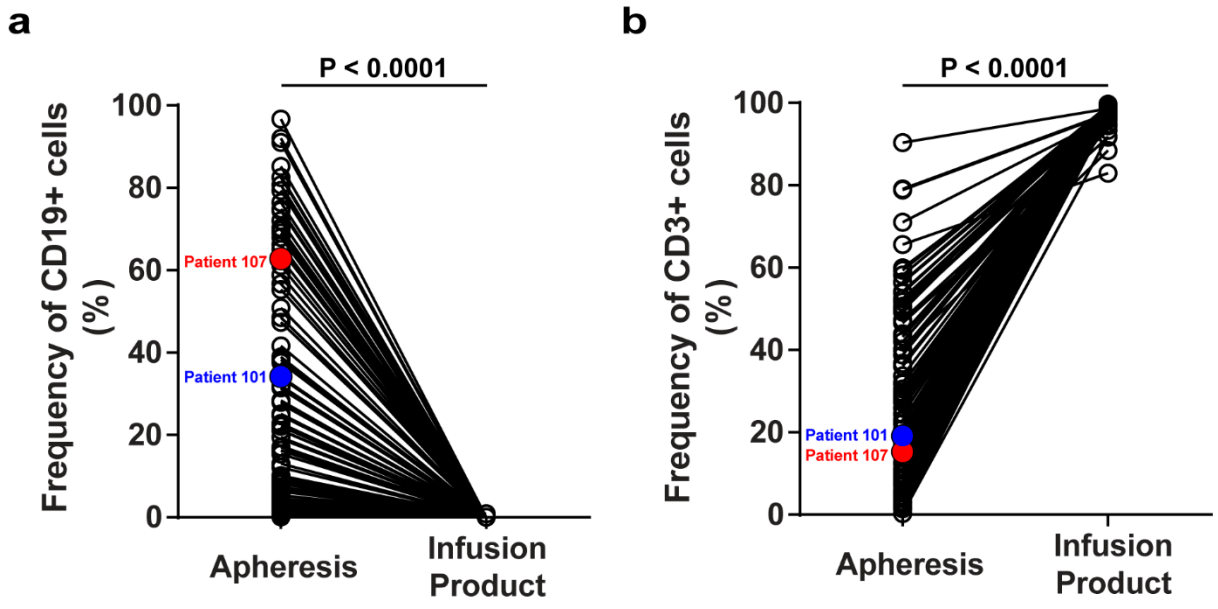
Supplementary Fig. 5 Proposed CAR epitope masking model. The expression of a CAR on target cells leads to the *in cis* binding between the CAR and the antigen resulting in the masking of the specific epitope that is recognized by the cognate CART cells. The target protein (CD19) is still expressed on the surface but its detection by flow cytometry is dependent on the epitope that is recognized by the flow cytometry antibodies. If flow cytometry antibodies and anti-CAR antibodies recognize the same epitope, the leukemic blasts will be detected as negative.



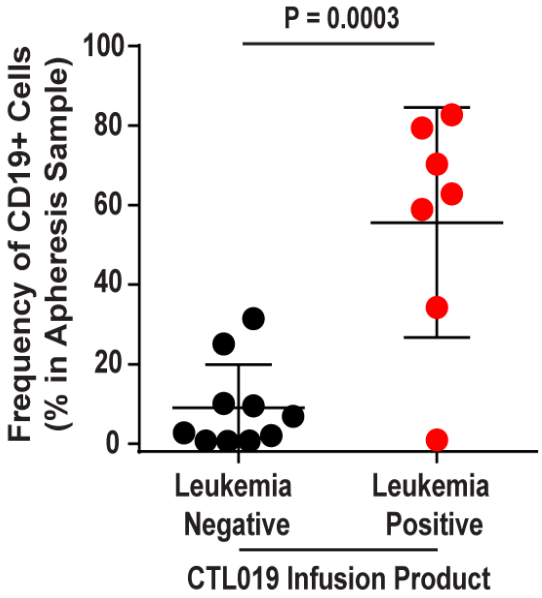
Supplementary Fig. 6 CAR22-expressing NALM-6 cells lose CD22 expression and are resistant to CART22 in an epitope-specific manner. (a) NALM-6 leukemic cells (naturally expressing CD22) were transduced with CARs recognizing different epitopes of the CD22 protein. CAR22 and CD22 expression were detected by flow cytometry antibodies targeting the same epitope used to generate CAR22. NALM-6 transduced with CAR22 (m971 or HA22 scFv), do not lose CD22 expression using the standard S-HCL-1 anti-CD22 antibody. However, no CD22 expression is observed on NALM-6 CAR22 HA22+ cells when stained with an antibody recognizing a similar epitope as the HA22 scFv. **(b)** CAR22 HA22+ T cells can efficiently kill wild-type NALM-6 and NALM-6 expressing CAR22 m971, but not CAR22 HA22-expressing NALM-6 cells. Conversely, CAR22 m971 T cells can efficiently kill wild-type and CAR22 $HA22^{+}$ NALM-6, but not NALM-6 cells engineered to express CAR22 m971 (n = 3; results are representative of 2 independent experiments).



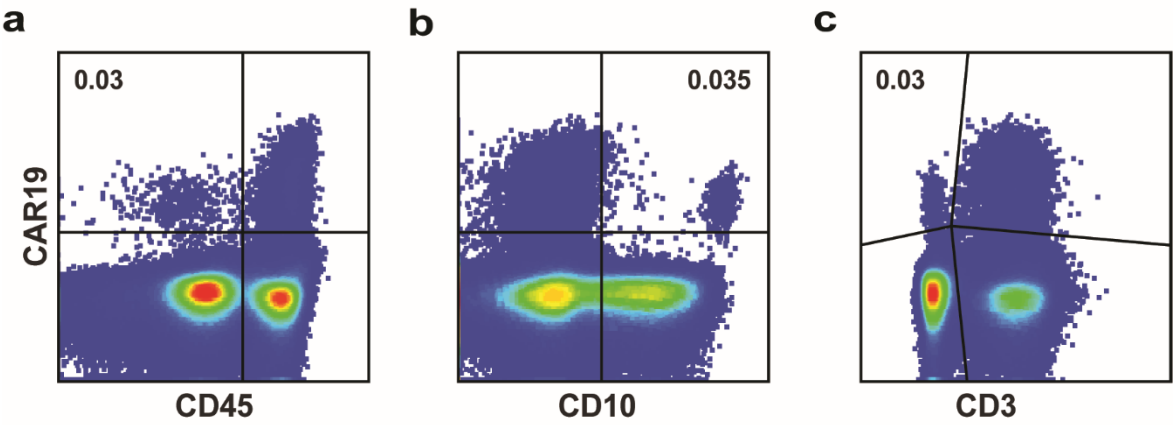
Supplementary Fig. 7 The CAR epitope masking model in the CAR22^{+} leukemia/CART22 model. CD22 expression is detectable using the anti-CD22 mAb S-HCL-1 in both CAR22(m971)-and CAR22(HA22)-transduced leukemia. However, when an anti-CD22 mAb targeting the same epitope recognized by the HA22-CAR is used, no CD22 detection is observed by flow cytometry.



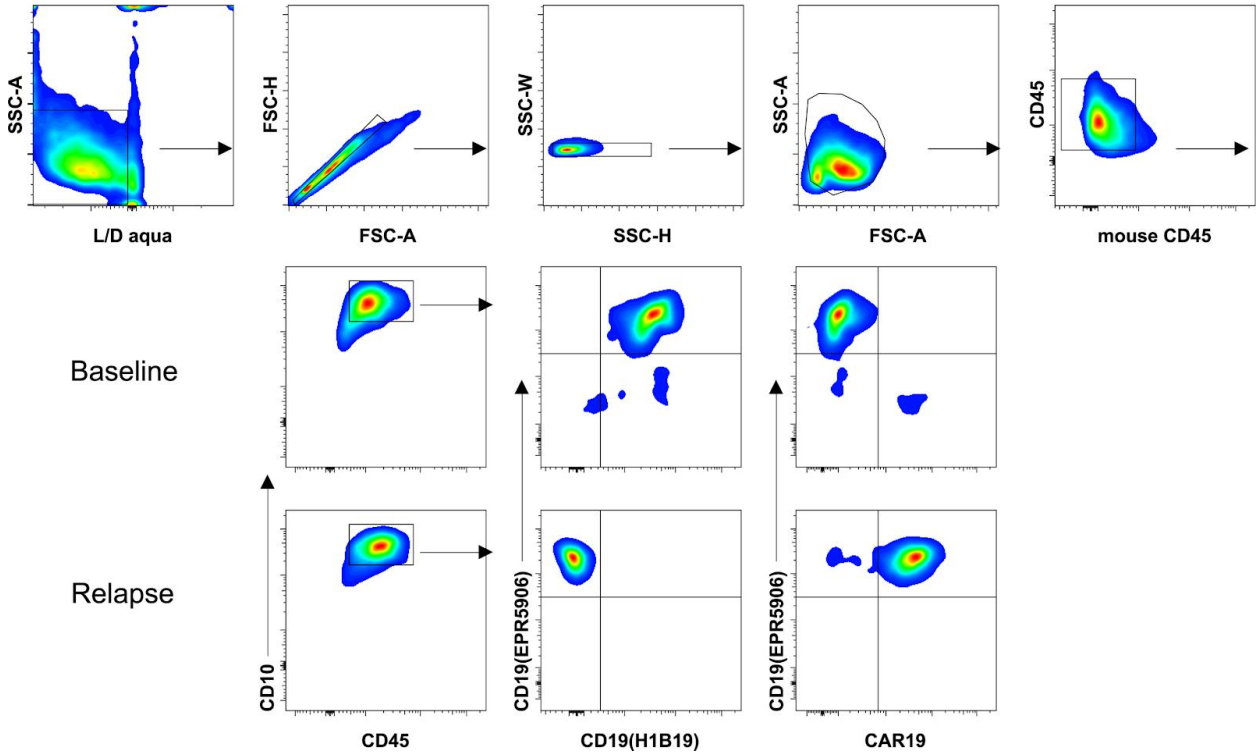
Supplementary Fig. 8 T and B cell composition of apheresis material and CTL019 cellular infusion products. (a) B cells ($CD19^+$) and **(b)** T cells ($CD3^+$) were enumerated in apheresis samples and the infusion products of pediatric and adult patients (*n* = 117) with ALL (red = Patient #107; blue = Patient #101). The leukemia burden in the apheresis was 25.32% (+/- 2.66%). In general, all patients showed a major reduction of the B cell content from apheresis to CTL019 product, as the infused product $CD19^+$ cell content was <0.01% in all patients. Conversely, the percentage of T cells reciprocally increased from apheresis to the infused product. For a-b, P values were determined using a two-sided, paired student's *t*-test.



Supplementary Fig. 9 B-ALL Patient CAR T cell infusion products containing residual leukemia clones. Frequency of $CD19^{+}$ cells in apheresis material in leukemia negative (n = 10) and leukemia positive (n = 7) CTL019 cell infusion products as detected by deep sequencing of immunoglobulin heavy chain rearrangements possessed a significantly (P = 0.0003 as determined using a two-sided, unpaired student's t-test) higher proportion of B cells at the time of apheresis (i.e., beginning of CTL019 cell cultures). Center line depicts mean and error bars indicate the SEM.



Supplementary Fig. 10 Detection of CAR19-expressing leukemia in an additional B-ALL relapse patient (Patient #101). Phenotyping of various immune cell populations in Patient #101 at the time of leukemia relapse (month 2 after CTL019 infusion). The frequencies of **(a)** $CAR19^{+}$ $CD45^{-}$; **(b)** $CAR19^{+}$ $CD10^{+}$ and **(c)** $CAR19^{+}$ $CD3^{-}$ cells are shown. Insets indicate the frequency of each cell population in the above gates. Results are representative of 2 independent experiments.



Supplementary Fig. 11 Example flow cytometry gating strategy. Flow cytometric analysis of intracellular CD19 detection in leukemic cells expanded in xenografts (baseline and relapse) is shown. Cells were first gated as live/dead dye negative, followed by singlet exclusion using FSC-H/FSC-A and SSC-W/SSC-H, intact lymphocytes, mouse CD45-negative cells, human CD45-positive cells and CD10-positive.

Supplementary Tables

Supplementary Table 1: Reported cases of CD19 negative relapse in ALL treated with CART19

Clinical Trial Main Sponsor & ClinicalTrials.gov Identifier	CAR Construct	Patient Cohort(s)	Number of Patients Treated Reported	Number of Patients with CD19 Positive Relapse	Number of Patients with CD19 Negative Relapse	Number of Patients with CARB-associated CD19 Negative Relapse	References
University of Pennsylvania NCT01626495	Murine 19scFv-BBz	Pediatric ALL	2 (2013) 30 (2014) 53 (2015) **59 (2016)**	7	13 (2 identified with CD19 mutation and alternative splicing(Sotillo et al. 2015), and 1 identified with CAR+ leukemic cells)	1	(Grupp et al. 2013) (Maude et al. 2014) (Grupp et al. 2015) (Maude et al. 2016)
Memorial Sloan Kettering Cancer Center NCT01044069	Murine 19scFv-28z	Adult ALL	5 (2013) 16 (2014) 44 (2015) **53 (2018)**	9	4 (identified with CD19 loss)	Not reported	(Brentjens et al. 2013) (Davila et al. 2014) (Park et al. 2015) (Park et al. 2018)
UT MDACC NCT00968760, 01497184, 01492036	Murine 19scFv-28z	ALL and NHL	26 (**17** ALL)	Not reported	Not reported	Not reported	(Kebriaei et al. 2016)
National Cancer Institute NCT01593696	Murine 19scFv-28z	Pediatric ALL and NHL	21(2015) **39 (2015)**	Not reported	2 (1 identified with myeloid switch(Jacoby et al. 2016))	Not reported	(Lee, Kochenderfer, et al. 2015) (Lee, Stetler-Stevenson, et al. 2015)
Uppsala University NCT02132624	Murine 19scFv-28-BBz	ALL and lymphoma	11 (**2** ALL)	0	1 (ALL)	Not reported	(Enblad et al. 2015)
The First People's Hospital of Yunnan NCT02968472	Murine 19scFv-28-BB-27z/iCasp9	Pediatric and adult ALL	**50**	Not reported	Not reported	Not reported	(Dong et al. 2015)
Fred Hutchinson Cancer Research Center NCT01865617	Murine 19scFv-BBz	Adult ALL	**29**	6	2 (1 identified with CD19 loss and 1 with myeloid switch)	Not reported	(Turtle et al. 2016)
Seattle Children's Hospital NCT02028455	Murine 19scFv-BBz	Pediatric ALL	**45**	11	7 (5 with CD19 loss and 2 identified with myeloid switch(Gardner et al. 2016))	Not reported	(Gardner et al. 2017)
Novartis Pharmaceuticals NCT02435849	Murine 19scFv-BBz	Pediatric ALL	**75**	1	15 (3 with concomitant CD19+ blasts, and 6 with unknown CD19 status)	Not reported	(Maude et al. 2018)
		Total	**369**	**34 (9.21%)**	**44 (11.92%)**	**1 (0.27%)**	

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Supplementary Table 2: Amino and nucleotide sequences of Patient #107 VDJ rearrangements at the time of apheresis.

V	D	J	CDR3 Amino Acid Sequence	Nucleotide Sequence
IGHV02-70	IGHD03-10	IGHJ03-01*02	CARIPLRGSGAFDIW	CTCCAAGGCCACCTCCAAAAACC AGGTGGTCCTTACAATGACCAACA TGGACCCTGTGGACACAGCCACGT ATTACTGTGCACGGATACCCCTCC GGGGATCAGGTGCTTTTGATATCT GGGGCCAAGGG
IGHV01-14*01	Unresolved	IGHJ04-01*02	Null	AGAAGTTTCAGGGCAGAGTCACC ATGACCAGGGACACATCCACGAG CACAGCCTACATGGAGCTGAGCA GTCAGAGATCTGAGGACATAGATG TGTACTACTGTGCCCCCGAGACTA CTGGGGCCAGGGA

Supplementary Table 3: IgH-seq analysis of CTL019 cellular infusion products from patients enrolled on the CHP959 clinical trial (NCT01626495).

Sample ID	CAR Sorted yes/no [1]	Response to CTL019 [2]	Cell Equivalent [3]	Leukemic Clonotype 1 [4]	Leukemic Clonotype 2 [4]	Clonotype 1, % of all Sequences [5]	Clonotype 2, % of all Sequences [5]	Clone frequency in IP (from titration experiment)
CHP959-107	**No**	**CD19-neg REL MO 8.6**	**156,984**	**1,168**	**777**	**2.65%**	**1.77%**	
	Yes		**190,476**	**ND**	**254**		**0.48%**	**<0.10%**
CHP959-110	Yes	CD19-pos REL MO 2.6	190,476	**58**		0.12%		0.00%
CHP959-112	Yes	CR	190,476	**314**		0.31%		
CHP959-121	Yes	CD19-neg REL MO29	190,476	**33**		0.07%		0.00%
CHP959-138	Yes	CD19-neg REL MO 8.8	134,286	**576**	**585**	0.30%	0.30%	
CHP959-148	Yes	CD19-neg REL MO 6.3	149,048	**155**	ND	0.24%		<0.01%
CHP959-101	Yes	CD19-neg REL MO2.1	101,270	ND	ND			
CHP959-125	Yes	CD19-neg REL MO34	190,476	ND	ND			
CHP959-127	Yes	CD19-neg REL MO 8.8	85,238	ND	ND			
CHP959-133	Yes	CD19-neg REL MO 1.9	190,476	ND	ND			
CHP959-136	Yes	CD19-neg REL MO 2.8	190,476	ND				
CHP959-113	Yes	CD19-neg REL MO 1.4	187,460	ND	ND			
CHP959-120	Yes	BMT in CR at MO8.4; CD19-neg REL MO23	190,476	ND	ND			
CHP959-105	Yes	CD19-pos REL MO8.0	190,476	ND				
CHP959-139	Yes	CR	190,476	ND				
CHP959-146	Yes	CD19-neg REL MO 4.6	174,286	ND	ND			
CHP959-149	Yes	CD19-neg REL MO 5.5	112,540	ND				

Aliquots from the indicated patients were subjected to next-generation immunoglobulin heavy chain deep sequencing (IgH-seq) to assess persistence of the leukemic cells throughout the 9-day manufacturing process. Sequencing data were compared to baseline samples to identify the leukemia, which carried one or two rearranged IgH alleles. [1] Live CAR-expressing cells were purified by FACS prior to IgH-seq. For patient 107 also the unfractionated sample is shown; [2], CR, complete remission, REL, relapse, MO, month. [3], Cell equivalent, calculated by dividing the amount of input DNA by 0.0063, which is the amount of DNA (in ng) single human cells hold; [4], Copy number of the leukemic clonotypes present in the indicated specimen. [5], Calculated by dividing the leukemic clonotype copy number by all IgH rearrangements identified in the same specimen. [6], Frequency of the leukemic clone was determined by performing a log-titration of purified leukemic cells from the indicated patient in normal donor CAR T cells and subjecting those specimens to NGIS. ND, none detected; REL, relapse; IP, infusion product; MO, month

Supplementary Table 4: Patient #107 integration site data summary.

Cell Type	Time Point	Inferred Cell Number	Unique Sites	Gini Coefficient	Shannon Index	UC_{50}	Sites in Transcription Units
Infusion Product (Bulk)	Day 0	3116	2924	0.058	7.96	1367	2379
Infusion Product (CAR^+)	Day 0	7293	6798	0.064	8.80	3152	5724
Peripheral Blood	Day 14	44	37	0.136	3.55	16	31
Peripheral Blood	Day 28	13	12	0.071	2.46	6	10
Bone Marrow	Month 9	1603	47	0.931	0.91	1	22
Bone Marrow (CAR^+)	Month 9	1441	35	0.923	0.86	1	20
Peripheral Blood	Month 20	1279	66	0.920	1.08	2	38

Results for each sample processed by integration site analysis are shown. The inferred cell number is calculated by summing the number of unique fragment lengths present for each integration site. The counts of integration sites mapping to a single location on the reference genome (hg38) are reported as Unique Sites. The Gini coefficient is a measure of inequality across the clonal abundance of a sample, zero indicating complete equality among the abundance of all clones and one indicating a single clone dominating the sample. The Shannon index is a widely-used metric for diversity and considers both the abundance and evenness of clones. The UC_{50} is the number of unique clones, which make up the top 50% of the sample abundance.

Supplementary Table 5: Analysis of factors possibly linked with CD19-negative relapse.

	CD21 (CR2)	CD81	CD225 (IFITM1)	CD200	CD19 insertions and/or mutations (%)
DNA-sequencing					
Patient #107 R*	Wild-type	Wild-type	Wild-type	Wild-type	0
RNA-sequencing					
Patient #107 R*	Wild-type	Wild-type	Wild-type	Wild-type	–

Patient #107 R*	Frequency of Exon 2 Skipping (%)	Frequency of Exon 5-6 Skipping (%)
	0.01265823	0.016

**Relapse time-point*

Supplementary Table 6: PCR primer sequences used for CAR lentiviral integration site detection.

	Primer Sequences	
Target	**Forward**	**Reverse**
ACTB	GAAGATGACCCAGGTGAGTG	CAGGTCAGAGAAGAGAGTCCTA
GAPDH	AGAACGGGAAGCTTGTCATC	GAGCCACACCATCCTAGTTG
CAR19 Vector	CCTTCTCCTGTCACTGGTTATC	GCCATCTTCCTCTTGAGTAGTT
NRP1 Integration Site	GAAGGGCTAATTCACTCCCAA	TTTGGTGTTCAGGCAGGTAG
PCCA Integration Site	GAAGGGCTAATTCACTCCCAA	CCGGTATTGTTGAACGGGTA