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SUPPLEMENTARY INFORMATION

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Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR

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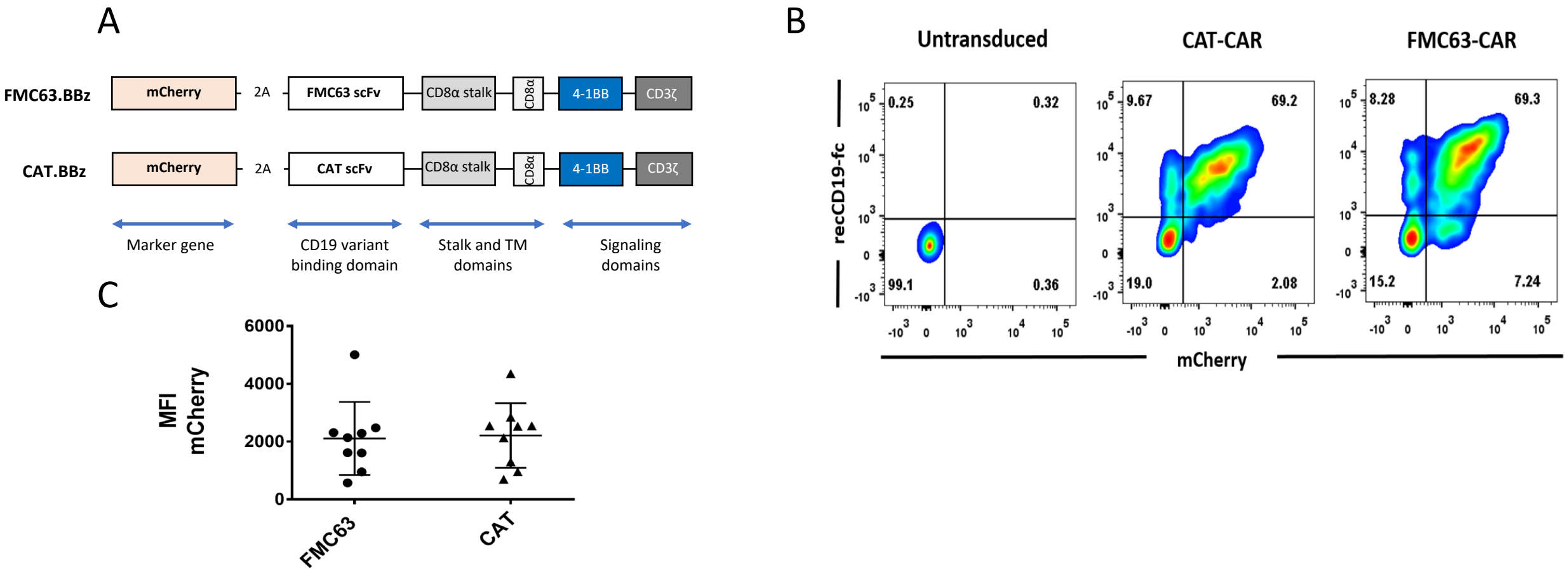
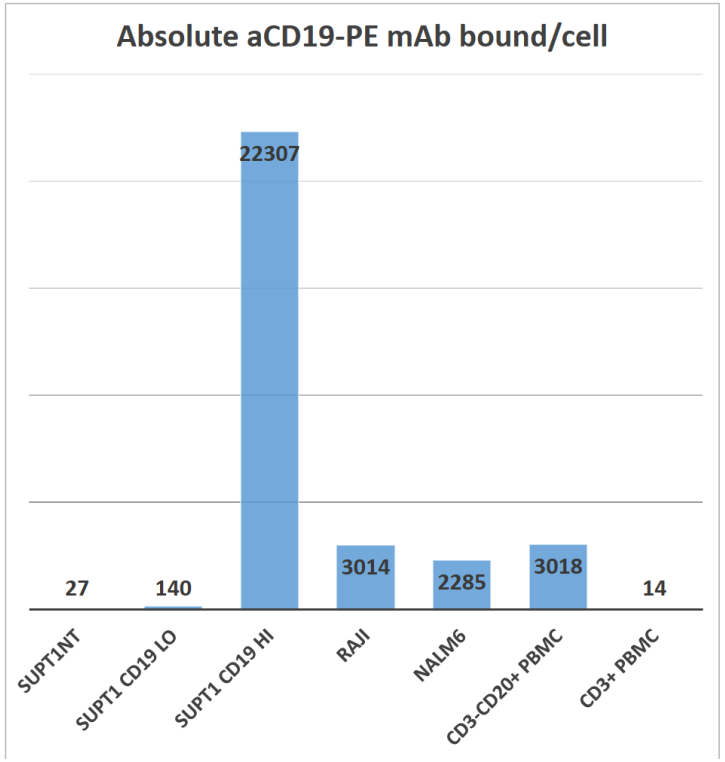
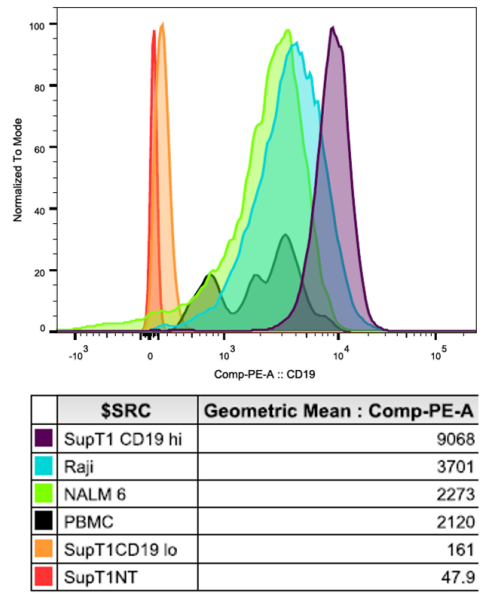
Supplementary Figure 1

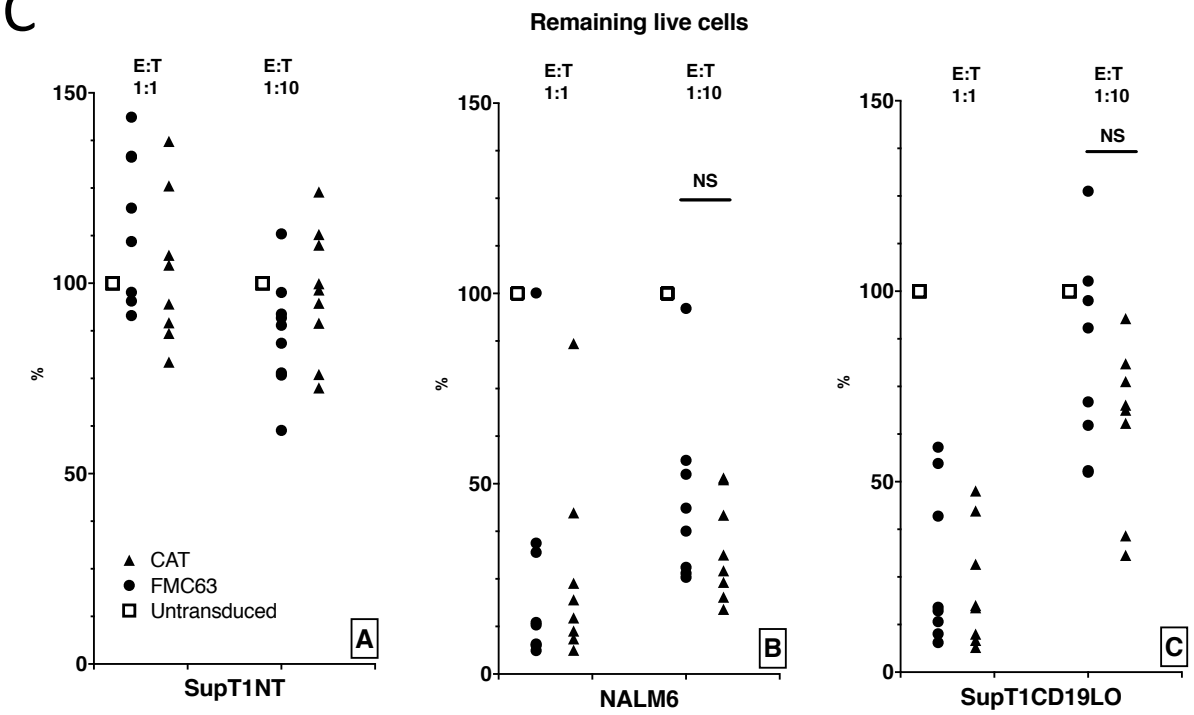


Supplementary Figure 1. Expression of Chimeric Antigen Receptor is identified by transgene expression and binding to recombinant CD19 soluble protein.
(A) Schematic representation of CD19 CARs. Both CAR constructs contain an aCD19 scFv, from either the FMC63 or CAT hybridoma, a CD8 transmembrane domain, a CD137 (4-1BB) costimulatory domain, and a CD3 ζ- signalling domain co-expressed with mCherry as a marker of transduction (B) Transduction efficiency of activated CD3/CD28 bead-expanded human T cells transduced with bi-cistronic lentiviral vectors shown in (A). The panel shows detection of CAR using mCherry transgene expression (x-axis) and a recombinant CD19 protein with an Fc tag construct (y-axis). Data are representative of >20 independent experiments. Similar levels of CAR expression as measured by median fluorescence intensity of mCherry were observed (MFI FMC63 2476, MFI CAT 2132 in representative FACS plot), but a lower MFI for rCD19 in CAT CAR, in accordance with its lower affinity for CD19 (MFI FMC63 7680, MFI CAT 4831); (C) Transgene expression, as measured by MFI of mCherry was determined 7 days-post transduction with bicistronic vectors expressing aCD19 CAR (FMC63 or CAT CAR) and mCherry. Data shown as mean ± SD. No significant difference was noted, *n=9*.

Supplementary Figure 2







Supplementary Figure 2. 24-hr Cytotoxicity assay by flow cytometry at 1:1 and 1:10 E:T ratio to investigate CAR T cell functional avidity

Flow-cytometric quantification of CD19 expression levels on target cells using the PE-QuantiBRITE kit. Cell lines assessed included SupT1NT, a T lymphoblastic cell line which does not express CD19, SupT1 cells transduced to express varying densities of CD19, including SupT1CD19LO cells and SupT1CD19HI, as well as Raji, NALM-6 cell lines and peripheral blood B cells which express CD19 at intermediate levels. Absolute CD19 PE mAb bound/cell SupT1NT 27, SupT1CD19LO 140, NALM-6 2285 depicted as bar chart (A) and as distribution of CD19 expression (B, x-axis = CD19). (C) CAR+ T cells were incubated at 1:1 and 1:10 E:T ratios with non-irradiated target cells (SupT1NT, SupT1CD19LO and NALM6) for 24 hours. The remaining live cell fraction was calculated relative to the live cell fraction in the well co-cultured with non-transduced T cells. At a 1:1 ratio against NALM-6 the mean % remaining live fraction ± SEM was: CAT 26.77 ± 9.47, FMC63 26.82 ± 11.17, *n=8*, *p=0.84*. Against SupT1CD19LO the mean % remaining live fraction ± SEM was: CAT 21.08 ± 5.02, FMC63 28.57± 6.621,*n=8*, *p=0.16*. At a 1:10 ratio against NALM 6 the mean % remaining live fraction ± SEM was: CAT 38.61 ±7.007, FMC63 51.23 ±9.161,*n=8*, *p=0.16*. Against SupT1CD19LO the mean % remaining live fraction ± SEM was: CAT 67.98± 7.29, FMC63 84.23 ± 8.429,*n=8*, *p=0.13*. Comparisons were analysed using two-tailed Wilcoxon matched-pairs signed rank test (non-parametric paired t-test)

Inclusion criteria

1. Children and young adults (age 24 years or younger) with high risk/relapsed CD19+ haematological malignancy:
    a) Resistant disease (>25% blasts) at end of UKALL 2011 or equivalent induction
    b) ALL with persistent high level MRD at 2nd time point of frontline national protocol (currently > 5 x 10-3 at week 14 UKALL2011 or equivalent)
    c) High risk infant ALL (age < 6 months at diagnosis with MLL gene rearrangement and either presenting white cell count > 300 x 109/L or poor steroid early response (i.e. circulating blast count >1x109/L following 7 day steroid pre-phase of Interfant 06)
    d) Intermediate risk infant ALL with MRD > 10-3 at end of Interfant06 induction
    e) Very early (< 18 months from diagnosis) bone marrow or extramedullary relapse of acute lymphoblastic leukaemia (ALL)
    f) Early (within 6 months of finishing therapy) bone marrow, or combined extramedullary relapse of ALL with bone marrow minimal residual disease (MRD) > 10-3 at end of re-induction
    g) Any on therapy relapse of ALL in patients age 16-24
    h) Any relapse of infant ALL
    i) ALL post ≥ 2nd relapse
    j) Any refractory relapse of ALL
    k) ALL with MRD >10-4 prior to planned stem cell transplant
    l) Any relapse of ALL eligible for stem cell transplant but no available HLA matched donor or other contraindication to transplant
    m) Any relapse of ALL after stem cell transplant
    n) Any relapse of Burkitt's or other CD19+ lymphoma
2. Agreement to have a pregnancy test, use adequate contraception (if applicable)
3. Written informed consent

Exclusion Criteria for registration

1. CD19 negative disease
2. Active hepatitis B, C or HIV infection
3. Oxygen saturation ≤ 90% on air
4. Bilirubin > 3 x upper limit of normal
5. Creatinine > 3 x upper limit of normal
6. Women who are pregnant or lactating
7. Stem Cell Transplant patients only: active significant acute GVHD (overall Grade ≥ II, Seattle criteria) or moderate/severe chronic GVHD (NIH consensus criteria) requiring systemic steroids
8. Inability to tolerate leucapheresis
9. Karnofsky (age ≥ 10 years) or Lansky (age < 10) score ≤ 50%
10. Pre-existing significant neurological disorder (other than CNS involvement of underlying haematological malignancy)

Exclusion criteria for CD19CAR T-cell infusion

1. Severe intercurrent infection at the time of scheduled CD19CAR T-cell infusion
2. Requirement for supplementary oxygen or active pulmonary infiltrates at the time of scheduled CD19CAR T-cell infusion
3. Allogeneic transplant recipients with active significant acute GVHD overall grade >2 or moderate/severe chronic GVHD requiring systemic steroids at the time of scheduled CD19CAR T-cell infusion

Supplementary Table 1. Study inclusion criteria

Baseline characteristics	N (%) N=14
Age, in years	
N	14 (100%)
Median (range)	9.24 (1.35 to 19.28)
Sex	
Female	1 (7%)
Male	13 (93%)
ALL cytogenetics at diagnosis	
Normal	1 (7%)
t(9,22)	1 (7%)
MLL rearrangement	2 (14%)
Other abnormal	9 (64%)
ND	1 (7%)
Status at registration	
1st relapse	1 (7%)
2nd relapse	8 (57%)
>2nd relapse	5 (36%)
Very early relapse	5 (36%)
Relapse post SCT	10 (71%)
CNS disease at relapse	
1^{st} relapse	5 (36%)
2^{nd} relapse	3 (21%)
Post SCT relapse	6 (43%)
Lines of treatment	
Median (range)	4 (2-7)
Prior inotuzumab	2 (14%)
Prior blinatumomab	1 (7%)
Tumour burden prior to lymphodepletion	
Morphological disease	
> 5 % blasts	4 (21%)
≤ 5% blasts	10 (79%)
MRD by IgH PCR/flow cytometry	
$> 10^{-5}$ to $\leq 10^{-4}$	1 (10%)
$> 10^{-4}$ to $\leq 10^{-3}$	3 (30%)
$> 10^{-3}$ to $\leq 10^{-2}$	1 (10%)
$> 10^{-2}$	1 (10%)
Negative	4 (40%)
CNS status at registration	
CNS I	12 (86%)
CNS II-III	2 (14%)

Supplementary Table 2. Summary of Clinical Characteristics of Infused Patients

BASELINE CHARACTERISTICS				BM STATUS AT LYMPHODEPLETION		BM STATUS AT INFUSION		CNS STATUS	CRS								MAX GRADE NEUROTOXICITY	BEST OUTCOME BY D90	OUTCOME AT LAST FOLLOW UP	DURATION OF F/U (DAYS)	B CELL APLASIA AT LAST F/U	CAR T CELL DETECTION AT LAST F/U (QPCR)
PATIENT	AGE (YRS)	NO OF RELAPSES	PRIOR ALLO SCT	% BLASTS	MRD BY IGH PCR	% BLASTS	MRD BY IGH PCR		MAX CRS GRADE BY LEE CRITERIA	MAX GRADE BY UPENN CRITERIA	HYPOTENSION DUE TO CRS REQUIRING MULTIPLE FLUID BOLUSES	OXYGEN REQUIREMENT (<40% FIO2)	COAGULOPATHY REQUIRING SUPPORT	VENTILATORY SUPPORT	INOTROPES	SOURCE OF SEPSIS DURING CRS						
CPL-01	1.4	2	Yes	1 (FISH)	ND	ND	ND	Ongoing CNS disease	2	2	No	No	No	No	No	Fungal chest infection	None	Stable disease	Death due to progressive (CD19 +) disease	28	Y	Yes
CPL-02	15.8	2	Yes	17	>10-2	88	ND	Clear	2	3	Yes	No	No	No	No	Fungal chest infection	None	Molecular CR/CRi	CD19 - relapse	189	Y	Yes
CPL-04	17.2	2	Yes	3	Negative	1	Negative	Ongoing CNS disease	1	1	N/A	N/A	N/A	No	No	Device related infection	Grade 2 Dysarthria, Dysphasia, Nystagmus	Molecular CR/CRi	Ongoing molecular CR	689	Y	Yes
CPL-05	11.3	2	Yes	81 (flow)	>10-2	40	ND	Clear	2	3	Yes	Yes	Yes	No	No	None	Grade 2 Encephalopathy	Molecular CR/CRi	CD19- relapse	91	Y	Yes
CPL-06	7.9	>2	Yes	3	> 10-4 to 10-3	0	> 10-4 to 10-3	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 1 Ataxia	Molecular CR/CRi	Ongoing molecular CR	728	Y	Yes
CPL-07	7.3	2	Yes	0	> 10-3 to 10-2	0	> 10-3 to 10-2	Clear	1	1	N/A	N/A	N/A	No	No	None	None	Molecular CR/CRi	CD19- relapse	401	N/A	Yes
CPL-08	4.3	1 (very early)	No	50 (flow)	>10-2	1 (flow)	>10-2	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 1 Dysarthria	Molecular CR/CRi	CD19- relapse	366	Y	Yes
CPL-09	6.9	>2	No	35	10-5 to 10-4	58	10-5 to 10-4	Clear	2	3	No	Yes	No	No	No	None	None	Molecular CR/CRi	CD19- relapse	362	N/A	Yes
CPL-10	10.6	2	Yes	0.5	> 10-4 to 10-3	0	10-5 to 10-4	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 1 Tremor	Molecular CR/CRi	CD19+ relapse	359	Y	No
CPL-11	7.4	2	No	0	Negative	0	Negative	Clear	1	1	N/A	N/A	N/A	No	No	None	None	Molecular CR/CRi	Ongoing molecular CR	357	Y	Yes
CPL-12	19.3	>2	Yes	1	Negative	ND	ND	Clear	0	0	N/A	N/A	N/A	No	No	N/A	Grade 4 encephalopathy	Molecular CR/CRi	Death in CR	119	Y	No
CPL-14	13.5	>2	Yes	1	Negative	ND	Negative	Clear	1	1	N/A	N/A	N/A	No	No	None	Grade 2 parasthesiae	Molecular CR/CRi	Ongoing molecular CR	273	Y	Yes
CPL-15	3.3	>2	Yes	0.01 (flow)	> 10-4 to 10-3	0	10-5 to 10-4	Clear	1	1	N/A	N/A	N/A	No	No	None	None	SD	Alive with (CD19+) disease	28	No	No
CPL-17	13.1	2	No	1	10-5 to 10-4	0	Negative	Clear	1	1	N/A	N/A	N/A	No	No	None	None	Molecular CR/CRi	Ongoing molecular CR	119	Y	No

Supplementary Table 3. Characteristics and outcomes of CARPALL study patients.

FISH – Fluorescence in situ hybridization, N/D not done, N/A not applicable, CR complete remission, CRi complete remission with incomplete hematological recovery, SD stable disease

Adverse events	Any time point N (%) N=14	Within 60 days N (%) N=14	After 60 days N (%) N=12
Any AE of any grade	14 (100%)	14 (100%)	9 (75%)
Related to ATIMP	14 (100%)	14 (100%)	7 (58%)
Any grade 3-5 AE	14 (100%)	14 (100%)	9 (75%)
Related to ATIMP	11 (79%)	11 (79%)	7 (58%)

Supplementary Table 4. Summary of adverse events by severity and relation to CAR T cell infusion

Patient	CPL-01	CPL-02	CPL-04	CPL-05	CPL-06	CPL-07	CPL-08	CPL-09	CPL-10	CPL-11	CPL-12	CPL-14	CPL-15	CPL-17
EGF	10	35.92	46.44	63.4	6.32	5.8	6.68	5.8	5.8	5.8	5.8	5.8	5.8	8.8
Eotaxin	41.16	78.04	45.48	78.48	120.2	57.76	105.88	205.2	51.76	57.76	137.2	151.12	48.72	80.16
FGF-basic	28.48	25.64	6320	28.48	40.12	33.64	43.56	28.64	33.64	408.4	23.64	314.64	43.56	534.2
G-CSF/CSF-3	35188	2705.72	1289.2	442.84	121.2	218.56	218.56	218.56	218.56	218.56	698.84	294.08	218.56	218.56
GM-CSF	10	35.92	46.44	63.4	6.32	5.8	6.68	5.8	5.8	5.8	5.8	5.8	5.8	8.8
HGF	2013.2	1389.12	133320	2086	321.2	976.56	371.72	2196.48	574.4	4064	493.12	7288	655.56	9068
IFN-a	59.36	17.92	1842	20	17.92	52.72	68.12	280	52.72	126.72	25.68	907.88	52.72	472
IFN-g	7.52	28.56	13.04	62.2	7.52	7.88	8.96	16.16	12.12	7.88	7.88	8.96	7.88	8
IL-10	110.8	183.28	159.16	392.48	30.2	80.04	299.2	383.52	87.04	30.16	19.8	87.04	43.8	48.96
IL-12	30.36	26.2	8100	71.96	107.64	247	223.6	102.96	206	155.84	123.4	438.6	310.4	592.2
IL-13	32.96	32.96	73	32.96	32.96	32.96	32.96	27.36	27.36	31.12	19.96	38.56	31.12	101.04
IL-15	583.08	351.28	5824	140.56	36.84	93.04	93.04	385.04	70.72	103.08	191	1000.12	53.12	458.88
IL-17A	458.88	36.44	36.44	36.44	36.44	30.84	30.84	30.84	30.84	30.84	30.84	30.84	30.84	30.84
IL-1beta	26.24	26.24	57.16	26.24	26.24	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4
IL-1RA	173.84	992.28	28368	2180.8	115.36	495.52	246.96	1031.32	744.12	2765.16	68.32	1392.64	186.8	1046.12
IL-2	22.6	42.44	7896	12.6	12.6	20.16	22.88	91.12	22.88	76.12	12.64	898.92	17.52	419.32
IL-2R	8240	11828	11736	24112	519.64	736.96	5164	5456	11440	833	978.2	1221.48	953.92	881.28
IL-4	109.64	109.64	15004	109.64	109.64	107.2	107.2	107.2	107.2	358.32	107.2	312.8	107.2	349.2
IL-5	27.8	27.8	27.8	88.6	28.96	28.96	28.96	28.96	28.96	28.96	28.96	28.96	28.96	28.96
IL-6	927.88	12280	15228	1772.76	38.52	87.36	372	497.56	136.44	33.44	433.76	38.36	29.56	47.48
IL-7	71.6	29.04	60.68	41	27.28	53.56	53.56	46.88	4.32	53.56	40.32	60.28	53.56	117.56
IL-8	3435.6	2330	346	2022.84	44.32	184.12	233.48	659.44	154	134.64	335.64	165.16	43.6	90.08
IP-10	102.24	236.68	121.8	247.32	16	74.44	89.6	191.16	199.4	22.6	39.72	63	41	57.4
MCP-1	15228	16544	31584	10612	2217.76	3745.04	6568	15068	4652	6080	18408	4840	2114.52	5060
MIG	397.08	1062.72	646.4	1310.08	216.8	175.72	983.72	1463.08	1843.92	209.68	261.8	297.08	192.6	332.64
MIP-1alpha	302.56	124.64	13844	112.08	61.44	101.32	130.92	174.56	130.92	793.68	244.96	1082.4	71.6	987.72
MIP1-beta	431.04	776.36	18676	648.12	154.48	335.84	723.44	1581.84	188.6	689.6	1581.84	4680	335.84	2720.92
RANTES	19108	17960	18328	19992	24204	17552	26288	13608	28868	24944	2718.88	27188	19328	31688
TNF-a	5.24	4.84	69.32	5.04	4.84	9	9	9	9	9.92	9	18.52	9	9.92
VEGF	3.36	2.48	10.16	0.84	5.84	10.48	14.36	2.08	4.24	1.92	1.72	1.72	3.56	10.96

Supplementary Table 5. Maximum serum cytokine levels (in pg/ml) noted between day 0 (pre) and day 14 post CAR T cell infusion

Adverse events	Maximum grade reported N=14	
	Grade 3-5	
	All AEs	Related to ATIMP
Abnormal laboratory parameters		
Anemia	5 (36%)	2 (14%)
Lymphocyte count decreased	8 (57%)	5 (36%)
Neutrophil count decreased	13 (93%)	9 (64%)
Platelet count decreased	5 (36%)	4 (29%)
White blood cell decreased	7 (50%)	3 (21%)
Alanine aminotransferase increased	2 (14%)	
Blood bilirubin increased	2 (14%)	1 (7%)
Bone marrow hypocellular	1 (7%)	1 (7%)
Hypernatremia	1 (7%)	
Hypokalemia	1 (7%)	
Blood and lymphatic system disorders		
Febrile neutropenia	8 (57%)	7 (50%)
Cardiac disorders		
Supraventricular tachycardia	1 (7%)	
Hypotension	3 (21%)	3 (21%)
Electrocardiogram QT corrected interval prolonged	1 (7%)	
General disorders and administration site conditions		
Fatigue	1 (7%)	
Fever	4 (29%)	4 (29%)
Infections and infestations		
Device related infection	2 (14%)	1 (7%)
Lung infection	2 (14%)	1 (7%)
Periorbital infection	1 (7%)	1 (7%)
Sepsis	2 (14%)	1 (7%)
Upper respiratory infection	1 (7%)	
Other infections	1 (7%)	
Injury, poisoning and procedural complications		
Generalized muscle weakness	1 (7%)	
Nervous system disorders		
Dysarthria	1 (7%)	
Encephalopathy	1 (7%)	1 (7%)
Paresthesia	1 (7%)	
Somnolence	1 (7%)	
Respiratory, thoracic and mediastinal disorders		
Bronchopulmonary hemorrhage	1 (7%)	1 (7%)
Hypoxia	2 (14%)	2 (14%)
Skin and subcutaneous tissue disorders		
Rash maculo-papular	1 (7%)	1 (7%)
Any adverse event		
Any adverse event	**14 (100%)**	**11 (79%)**

Supplementary Table 6. Summary of severe (grade 3-5) adverse events by system and relation to CAR T cell infusion

Patient	%MRD at CD19neg relapse	Nucleotide position (chromosome 16)	Location of mutation in CD19 gene	Mutation type	Effect	VAF at diagnosis	VAF CD19 neg relapse
CPL02	99%	g.28932553C>CG	Exon 2	INS	Frameshift (LOF)	0	0.18
		g.28933331C>CA	Exon 4	INS	Frameshift (LOF)	0	0.14
CPL05	100%	g.28932411T>C	Exon 2	SNV	Missense	0	0.37
		g. 28932413G>A	Exon 2	SNV	Stop gain (LOF)	0	0.28
		g.28932439T>C	Exon 2	SNV	Missense	0	0.03
		g.28933043G>T	Exon 3	SNV	Missense	0	0.30
		g.28936557G>A	Exon 7	SNV	Synonymous	0	0.04
CPL07	20%	g.28933075C>G	Exon 3	SNV	Missense	0.42	0.85
CPL08	50%	g.28933074T>TGTAAG	Exon 3	INS	Frameshift (LOF)	0	0.15
CPL09	6%	g.28933086G>A	Exon 3	SNV	Synonymous	0	0.05
		g.28938895G>A	Exon 14	SNV	Missense	0	0.03

Supplementary Table 7. Summary of mutations associated with CD19- relapse