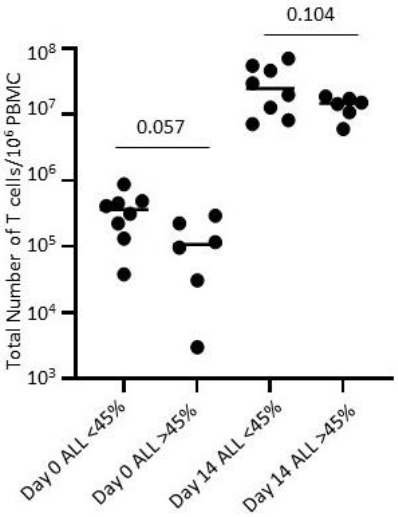
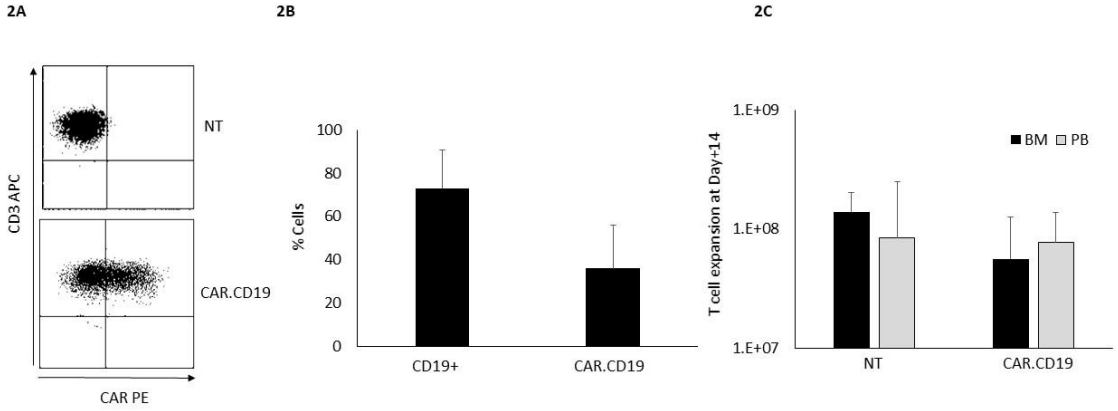
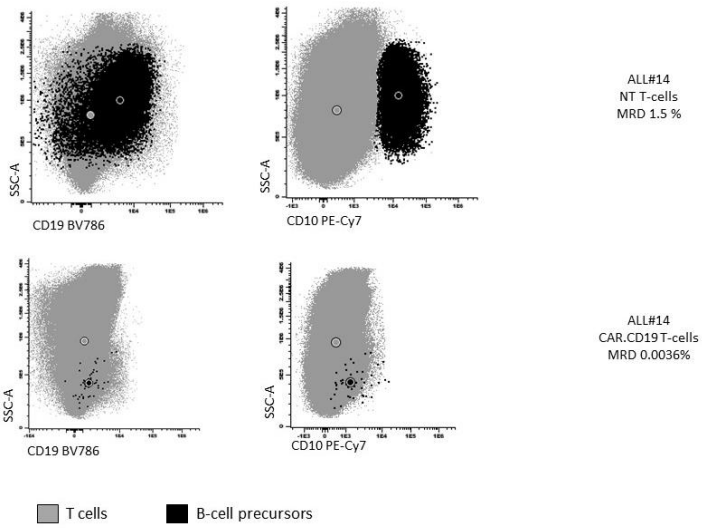
Supplementary Materials



Supplemental Figure 1. T cell numbers in SM and DP. Graph representing the total number of T-cells in the starting material plated for manufacturing (Day 0) and at the end of production of the DP (Day+14) between the two subgroups of patients with either <45% or >45% of CD19+ B cells in SM.



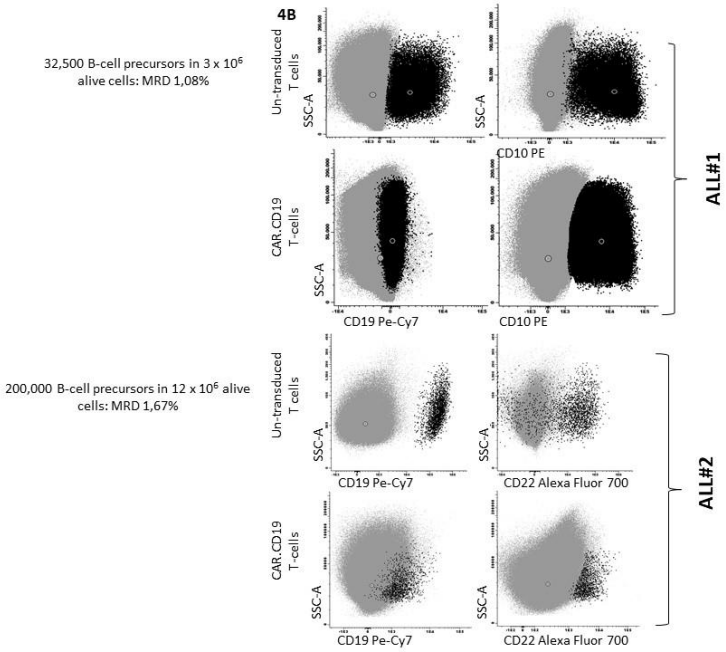
Supplemental Figure 2. BM Patient-derived CAR.CD19 T-cells. (A) Flow-cytometry analysis in a representative DP generated from BM mononuclear cells of a Bcp-ALL patient collected at the time of diagnosis. Upper panel A shows flow-cytometry analysis of CAR+ T-cells in the negative control sample of un-transduced (NT) T-cells, whereas bottom panel shows the analysis in CAR.CD19 genetically modified T-cells. **(B)** Average of the percentage of CD19+ leukemic blasts in the BM derived starting materials considered for CAR.CD19 T-cell manufacturing from Bcp-ALL patients at diagnosis and CAR+ T-cells in DPs generated from BM samples of Bcp-ALL patients (n=10). **(C)** Total cell collection at the end of production of un-transduced (NT) and CAR.CD19 T-cells derived from PBMCs of Bcp-ALL patients (n=15), and BM samples of Bcp-ALL patients (n=10), at the end of production. Data are expressed as average ± SD.



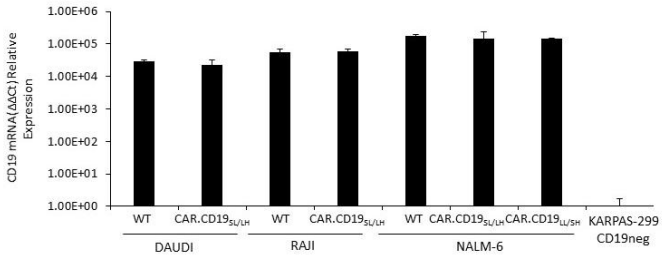
Supplemental Figure 3. Flow-cytometry analysis of control un-transduced T-cells and CAR.CD19 T-cells from one representative Bcp-ALL patient. Upper panels show flow-cytometric analysis of CD19 and CD10 B-cell markers in control un-transduced T-cells from ALL#14 patient revealing 1.5% of leukemic cells, whereas the contamination was significantly reduced in the CAR.CD19 T-cell sample manufactured from the same patient ALL#14 (0.0036% of leukemic cells).

4A

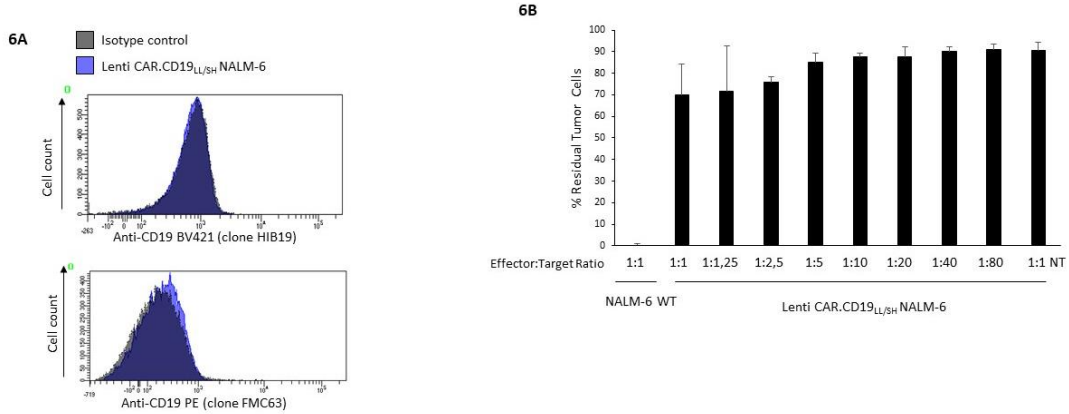
ALL Patients (BM)	%CD19+ Blasts	Sample	MRD#1	MRD#2
ALL#1	80,7	NT	6,03E-03	7,50E-03
		CAR	3,60E-03	5,20E-03
ALL#2	40,5	NT	7,60E-03	5,20E-03
		CAR	9,90E-03	7,00E-03
ALL#3	50,9	NT	2,60E-03	4,20E-03
		CAR	5,40E-02	5,40E-02
ALL#4	75,6	NT	2,80E-03	2,60E-03
		CAR	2,30E-03	1,90E-03
ALL#5	83,9	NT	1,20E-03	**ND**
		CAR	1,20E-03	1,90E-04
ALL#6	83,5	NT	**ND**	**ND**
		CAR	POS<QR	NEG



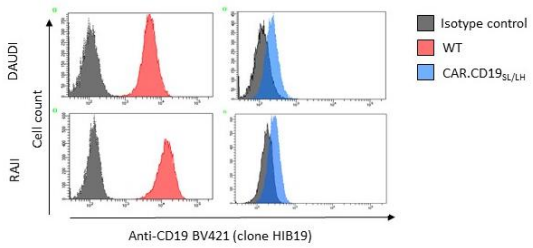
Supplemental Figure 4. MRD analysis of DPs generated from BM raw materials of Bcp-ALL patients highly contaminated by leukaemia cells at diagnosis. (A) Table shows data from each enrolled patient as regarding to the percentage of CD19+ leukaemia cells (MRD=1) in the patient-derived BM mononuclear cells used as starting raw material for the CAR-T cell manufacturing, and the value of MRD for two different Ig markers identified at the time of diagnosis in each single patient. MRD data were reported for both control un-transduced T-cell samples (NT) and CAR.CD19 T-cell samples (CAR). **(C)** Flow-cytometry analysis of B-cell markers in two BM derived DPs from ALL#1 and ALL#2 patients. Panels show the presence of leukaemia cells (black dots).



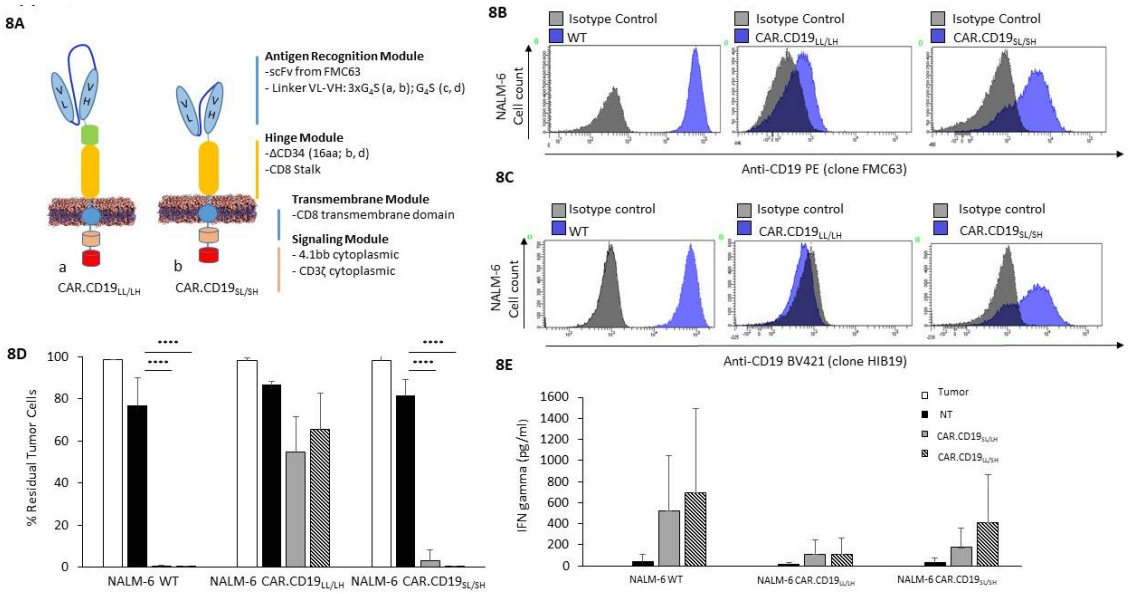
Supplemental Figure 5. CD19 mRNA expression is not modulated in CAR+ tumor cell lines. Quantitative Real-Time PCR (qRT-PCR) of CD19 mRNA expression in WT and CAR.CD19 positive tumor cell lines (WT and CAR.CD19$_{SL/LH}$ DAUDI and RAJI; WT, CAR.CD19$_{SL/LH}$, CAR.CD19$_{LL/SH}$ NALM-6 cell lines). Karpas-299 cell line has been used as negative control. mRNA levels are shown as relative expression of CD19 mRNA versus ACT-B mRNA expression. Reactions were performed in triplicates. Data are represented as mean ± SD.



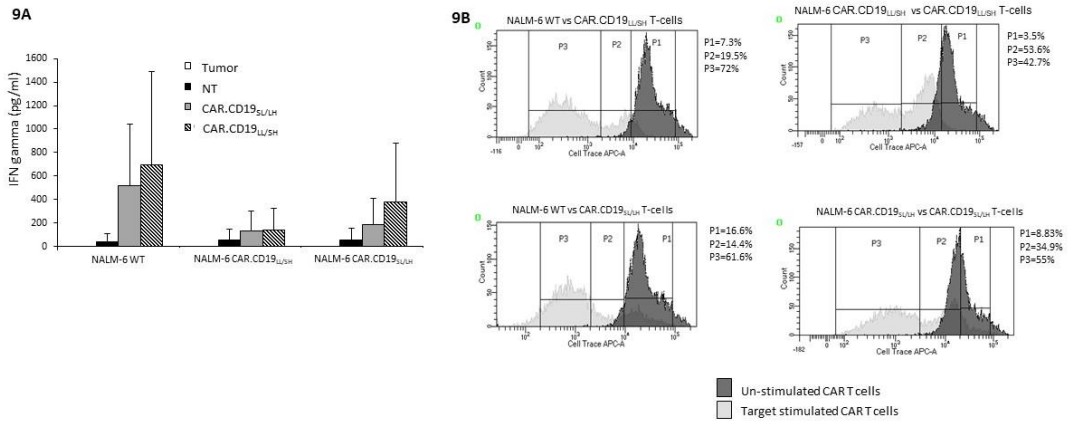
Supplemental Figure 6. NALM-6 genetically modified with a lentiviral vector carrying CAR.CD19$_{LL/SH}$. **(A)** CD19 expression detected by flow-cytometry in NALM-6 cells genetically modified by lentiviral CAR.CD19$_{LL/SH}$ construct. Matched isotype staining is shown by grey histograms, whereas CD19 staining with anti-CD19 PE (clone FMC63) (top panel) or anti-CD19 BV421 (clone HIB19) (bottom panel) is shown by blue histograms. **(B)** 7 days co-culture assays of NALM-6 WT (white bar) and NALM-6 cells genetically modified by lentiviral CAR.CD19$_{LL/SH}$ construct (black bars) with CAR.CD19$_{LL/SH}$ T-cells. NALM-6 WT were used as control at 1:1 effector:target ratio while NALM-6 CAR+ cells were plated at the indicated effector:target ratios from 1:1 to 1:80. Data are expressed as average ± SD. * p-value=<0.05, ** p-value=<0.01, *** p-value=<0.001. n=6



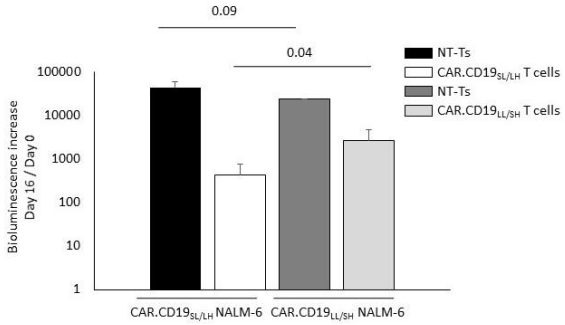
Supplemental Figure 7. CD19 expression detected by flow-cytometry in DAUDI and RAJI tumor cell lines, WT or genetically modified by CAR.CD19. Matched isotype staining is shown in grey histograms, whereas the specific CD19 staining (Clone HIB19) is shown by red histograms for WT cells and blue histograms for CAR.CD19$_{SL/LH}$ cells.



Supplemental Figure 8. CAR.CD19 structure affects CD19 antigen *in cis* binding. (A) Cartoons representing CAR.CD19$_{LL/LH}$ (*a*) and CAR.CD19$_{SL/SH}$ (*b*). **(B-C)** CD19 expression detected by flow-cytometry in NALM-6 cells, genetically modified by different CAR.CD19 constructs. Matched isotype staining is shown by grey histograms, whereas CD19 staining with anti-CD19 PE (clone FMC63) **(B)** or anti-CD19 BV421 (clone HIB19) **(C)** is shown by blue histograms for each CAR.CD19 modified NALM-6 cell lines. **(D)** 7 days co-culture assay of NALM-6 WT and CAR.CD19 genetically modified NALM-6 with NT (black bars), CAR.CD19$_{LL/LH}$ (grey bars) and CAR.CD19$_{SL/SH}$ (dotted bars) T-cells. Tumor alone is represented by white histograms. n=6. * p-value=<0.05, ** p-value=<0.01, *** p-value=<0.001, **** p-value=<0.0001. (**E**) IFN-γ production was measured after 24h of co-culture. Data from 6 HDs are expressed as mean ± SD.



Supplemental Figure 9. CAR.CD19 T-cells activation profile is similar beside the CAR configuration. (A) IFN-γ production was measured after 24h of co-culture of effector T-cells and NALM-6 WT, or NALM-6 genetically modified with CAR.CD19 constructs. Data from 6 different CAR T products generated from HDs are expressed as mean ± SD. **(B)** CFSE Proliferation analysis representing the overlays of CAR T-cells unstimulated (dark grey) and stimulated with WT or CAR.CD19 modified NALM-6 cells (light grey).



Supplemental Figure 10. CAR.CD19$_{SL/LH}$ NALM-6 cells are controlled better than CAR.CD19$_{LL/SH}$ NALM-6 in the *in vivo* setting. Histograms representing tumor bioluminescence differences at Day 16 (endpoint of *in vivo* experiment) between mice bearing CAR.CD19$_{SL/LH}$ and CAR.CD19$_{LL/SH}$ NALM-6 cells. Data are shown as increment Mean ± SD of bioluminescence values of the two mice cohorts at Day 16 compared to Day0.

Patient	IG/TR	SR	ASO primer	RQ primer		TaqMan Probe	
ALL#1	IGH VH4/JH5	1,00E-05	GTCCGCAATTTTTCATTGGTAGTA	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	IGK VK2Kde	1,00E-05	GCAAGCTACACAATTAAAGGAGAAGATAGT	kde rp2	ATATGGCAAAAATGCAGCTGC	kde tp1	AGCCCAGGGCGACTCCTCATGAGT
ALL#2	IGH VH3/JH6	1,00E-04	TAGAGATCCGGCCTTTTAACTGGAACT	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA
	IGH VH3/JH5	1,00E-05	GCAGCACCCCCTCAAGCA	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
ALL#3	IGH VH3/JH4	1,00E-05	TGTGCGAAAGATCTTTTTTTTATGGTGTATGCTATTTCTT	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	TRD VD2/DD3	1,00E-05	CGTATCCCCCCCCCACA	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
ALL#4	TRB VB20/JB2.7	1,00E-05	GCCCCGGACTAGCTAGTTTACGA	jb2.7 rp	GCTGGAAGGTGGGGAGA	jb2.7 tp	CGGGCACCAGGCTCACGGTC
	IGH VH3/JH5	1,00E-04	ACTGTCCCCGAGGTTGTACTAATG	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
ALL#5	IGH VH3/JH6	1,00E-04	TGCTATACCGGGCGGGTG	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA
	TRD VD2/DD3	ND	CCCAGTAAGGTCGGTGGAGTC	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
ALL#6	TRA VD2/JA29	1,00E-04	CGTATCCCCCAGGAGAAGCA	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
	IGH VH1/JH4	1,00E-04	ATAGATGTGTACTACTGTGCGAGCGTACTA	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
ALL#7	IGH VH4/JH4	1,00E-04	TCCGGTTGGTATCACCTATCCCCTAA	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	TRD VD2/DD3	1,00E-04	TGTGCGTATCCCCCAGAGACA	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
ALL#8	IGH DH1/JH5	5,00E-04	TGGGTATAACTGGAACTACGGCTGGTT	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
ALL#9	IGH VH1/JH4	1,00E-04	CGGATTTAACTGGGGATCTCCCCTTA	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	TRD VD2/DD3	1,00E-04	CCCCTCCACTCCCCCG	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
ALL#10	IGK VK2Kde	5,00E-04	CAAGGTACACACTGGCTGGGAA	kde rp2	ATATGGCAAAAATGCAGCTGC	kde tp1	AGCCCAGGGCGACTCCTCATGAGT
	IGH VH3/JH6	1,00E-04	CTGCCGACCCACTACATGGA	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA
ALL#11	IGH VH3/JH6	1,00E-04	TATAACAGCTCTACTTCTACCACACGACCTA	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA
	TRD DD2/DD3	1,00E-04	CTACGTGGAACCGTGAGGCT	dd3 rp1	TTTGCCCCTGCAGTTTTTGT	dd3 tp1	CGCACAGTGCTACAAAACCTACAGAGACCTG
ALL#12	TRD VD2/DD3	1,00E-05	CGTATCCCCCAGTCGCACA	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
	IGH VH3/JH4	1,00E-05	CGGAGGGTAAATTACTATGATAGTAGTGG_TTT	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
ALL#13	IGH VH3/JH4	1,00E-05	AAAAGGGTCTTGGGCGTTTAGGA	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	TRD VD2/DD3	1,00E-04	GTCCGTACCCCTTGCCG	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
ALL#14	IGH VH2/JH4	1,00E-04	AGTTCCTATCCGAGACCTCCAATT	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	TRA VD2/JA29	1,00E-04	AATTCGGGAGTCGGGGGTAT	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA
ALL#15	IGH VH4/JH6	1,00E-05	AGAGAGGAGAGCCTAGGGATATTTTGA	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA
ALL#16	IGH VH1/JH4	1,00E-05	GCGAGCAACAACTGGATTTTGA	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
	TRG VG9/JG1.3	1,00E-05	GAACCAACCTCCGAGGCCT	vg9 fp	GGCATTCCGTCAGGCAAA	vg9 tp	TAGGATACCTGAAACGTCTACATCCACTCTCACC
ALL#17	IGH VH2/JH3	1,00E-04	GTTAATATGGGGCCATCTGGG	jh3 rp	AGGCAGAAGGAAAGCCATCTTAC	jh3 tp	CAAGGGACAATGGTCACCGTCTCTTCA
ALL#18	IGH VH1/JH3	1,00E-04	AGAGGGGGCTCCCCTATGG	jh3 rp	AGGCAGAAGGAAAGCCATCTTAC	jh3 tp	CAAGGGACAATGGTCACCGTCTCTTCA
	IGH VH3/JH4	1,00E-05	AGCAGTGGCATGCCATTGA	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG
ALL#19	TRD VD2/DD3	1,00E-05	CCTGCCCCCCGCTACAA	dd3 rp3	CTGCTTGCTGTGTTTGTCTCCT	dd3 tp2	ATATCCTCACCCTGGGTCCCATGCC
Gene	**Forward primer**			**Reverse primer**		**TaqMan Probe**	
iC9	5'-ACCAGCTGGATGCCATCTC-3'			5'-CAGCTGCCTGACTTTGGATC-3'		5'-5HEX/AGCCTGCCC/ZEN/ACACCTTCTGACAT/3IABkFQ-3'	

IG/TR= Immunoglobulin/TCR; SR= Sensitive Range; ASO=allele-specific oligonucleotide; RQ primer: Reverse primer.

Supplemental Table 1. List of primers used in MRD detection by qRT-PCR.

IG/TR=Immunoglobulin/TCR; SR=Sensitive Range; ASO=Allele-Specific Oligonuclotide.

Peptide	Binding Alleles	CAR Construct
VTVSSPAPR	HLA-A11:01, HLA-A33:03	Short Hinge no CD34
SVTVSSPAPR	HLA-A33:03	Short Hinge no CD34
GSELPTQGTF	HLA-B40:01	Long Hinge including CD34
STNVSPAPR	HLA-A11:01, HLA-A33:03	Long Hinge including CD34

Supplemental Table 2. List of immunogenic peptides in CAR constructs including CD34 domain. Peptides predicted to be immunogenic are reported in the first column. The amino acids in bold and plain text are derived from different regions in the construct. In column 2, we show all the alleles that are predicted to present such peptides, with a rank score of 0.5 or less. The source construct of the peptide is reported in column 3.