Supplementary information for

Single-cell profiling identifies pre-existing CD19-negative subclones in a B-ALL patient with CD19-negative relapse after CAR-T therapy.

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Supplementary Table

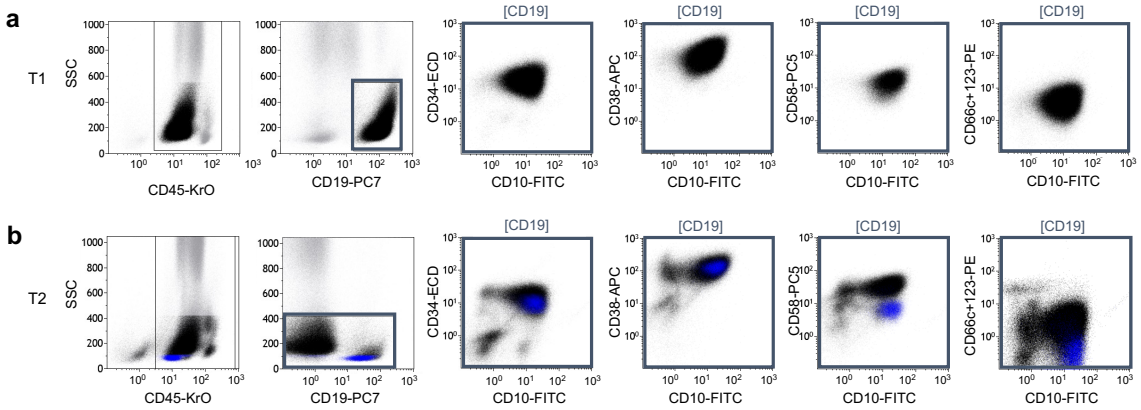
Supplementary Table 1: Oligonucleotide sequences.
All oligonucleotides were synthesized by Integrated DNA Technologies (IDT) Company. For HTO, the specific index sequences are highlighted in red and (*) indicates a phosphorothioate bond. For BC primers, 10X cell identifier barcodes are indicated in green.

	Sequences of HTO
HTO-A	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT AAGTATCGTTTCGCA CCCATATAAGA*A*A
HTO-B	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTTTGTCTTTGTGAG CCCATATAAGA*A*A
HTO-C	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTTGCCGCATGTCAT CCCATATAAGA*A*A
HTO-D	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT AAAGCATTCTTCACG CCCATATAAGA*A*A
	Primers for PCR/nested PCR
P12	CTA CAC GAC GCT CTT CCG ATC T
P10	CCTGGGAGAGCTGGTCCTGCC
P14	CCTCCTCACTGTCAGGTTCCT
P13	CGTACTGGTTCTGGGGCCCGC
P3	AGAGTCTGACCACCATGCC
P4	TTGGGGACTTGAGGAGATCC
P8	CCCTCCACATTGACTGTCC
P9	GGCCGAGCAGTGATCTCCAGG
P33	GAGGCTCTGGTTCAGGCTGT
	BC primers for cell backtracking
BC 2	GATCTCACCACTTCCTTGACC
BC 3	GATCTGTGCGGTTCGAGGTAG
BC 4	GATCTCTAGTGACAATGGTCT
BC 6	GATCTGACACGCAGTCCGGTC
BC 7	GATCTGTTACAGGTTACAGAA
BC 8	GATCTCCTCAGTGTGATGATA
BC 11	GATCTCCGTGGACAGCTGGCT
BC 12	GATCTGTTAAGCTCTAACCGA
BC 16	GATCTAGATCTGGTGAGGCTA
BC 17	GATCTGAATAAGGTGCACTTA
BC 18	GATCTGGACGTCGTAAACACA
BC 19	GATCTTACACGAAGTACGCCC
BC 20	GATCTATTATCCTCAGATAAG
BC 21	GATCTATCATCTGTGGACGAT
BC 22	GATCTGCATGCGGTCGAAAGC
BC 32	GATCTGAACATCAGTGCCAGA
BC 33	GATCTCACACCTGTCGAACAG
BC 34	GATCTGACGTGCTCGGAAATA
BC 35	GATCTGCTTGAACAATGGACG
BC 38	GATCTCGGAGTCGTATGCTTG

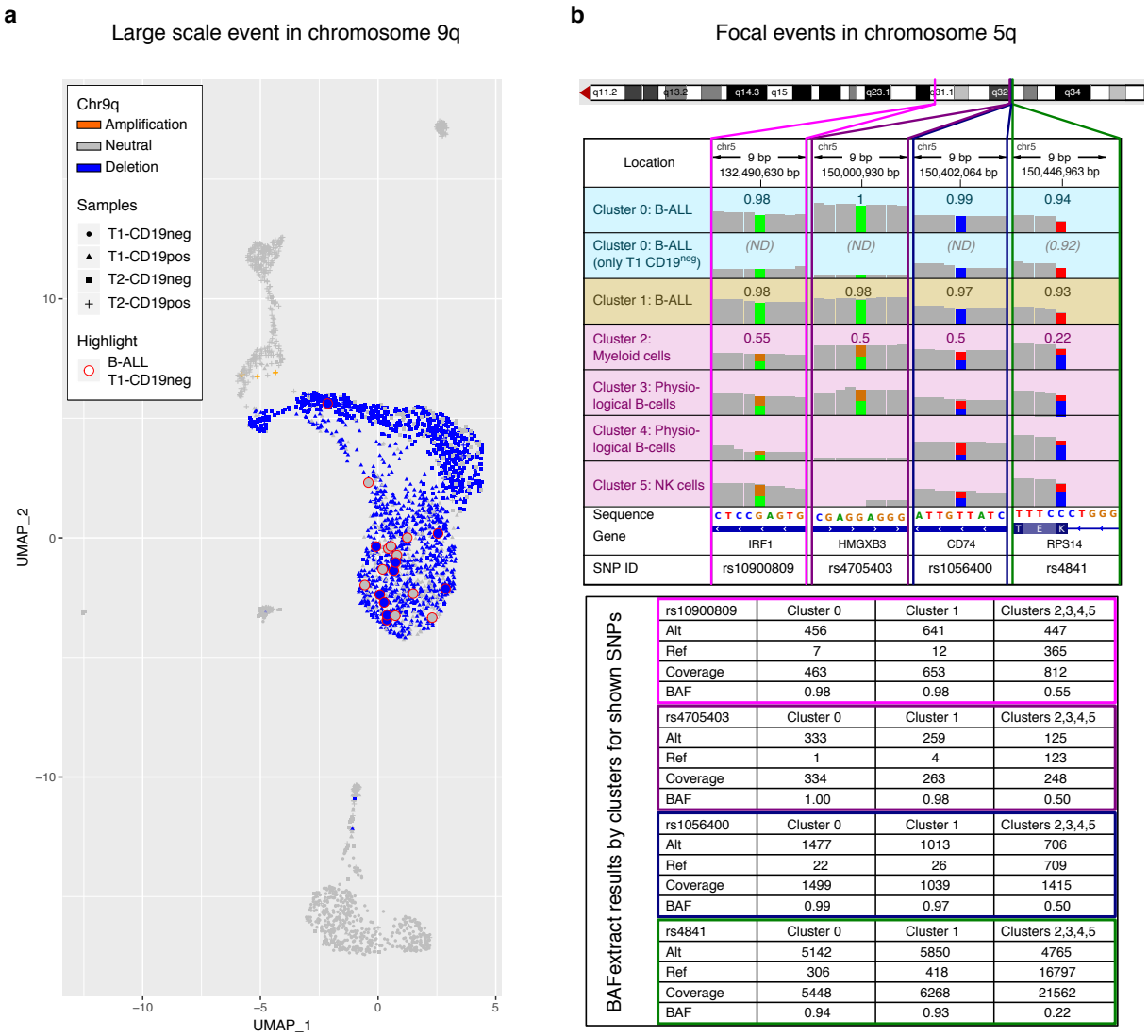
Supplementary Table 2: Antibodies used for flow cytometry.

Antigen	Fluorochrome	Clone	Manufacturer	Catalogue number	Lot number	Final dilution
CD19	PC7	J3-119	Beckman Coulter	IM3628	200148	1/20
CD45	Krome Orange (KrO)	J33	Beckman Coulter	B36294	200070	1/20
CD34	ECD	581	Beckman Coulter	B49202	200025	1/20
CD10	FITC	ALB1	Beckman Coulter	A07759	200043	1/20
CD38	APC	HB7	BD	345807	9113909	1/20
CD58	PC5	AICD58	Beckman Coulter	IM3702	200019	1/20
CD66c	PE	KORSA354	Beckman Coulter	IM2357U	200052	1/20
CD123	PE	9F5	BD	340545	8087759	1/20
CD3	PE	HIT3a	BD	555340	4066560	1/5
CD19	APC	HIB19	Biolegend	302212	B295177	1/20

Supplementary Figures

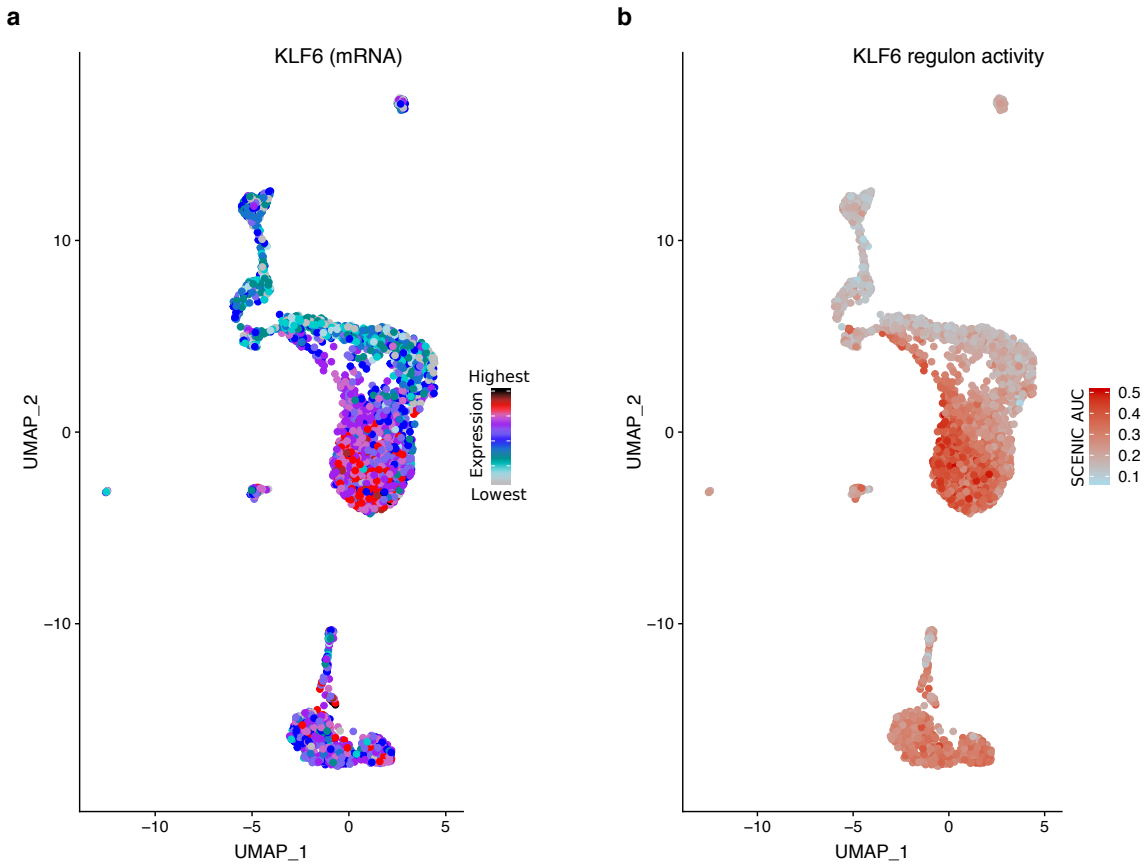


Supplementary Figure 1: Flow cytometry immunophenotyping of leukemic blasts.
Cells were stained with CD45-KrO, CD19-PC7, CD34-ECD, CD10-FITC, CD38-APC, CD58-PC5, CD66c-PE and CD123-PE. Dot plots show the expression of CD45, CD19, CD10, CD34, CD38, CD58 and CD66c+CD123 on blasts cells before CAR-T treatment (**a**) and at relapse (**b**), three months after. (**a**) At T1, blasts cells (in black) are $CD45^{dim}$ and $CD19^{pos}$. Gating on $CD19^{pos}$ cells, blasts are $CD10^{pos}$, $CD34^{pos}$, $CD38^{pos}$, $CD58^{pos}$ and $CD66+CD123^{pos}$. These markers are used as measurable residual disease (MRD) markers. (**b**) At T2, blasts cells are $CD45^{dim}$ but $CD19^{neg}$. They still express the same markers of MRD allowing us to differentiate them from physiological immature B-cells (in blue, around 4% of live cells).

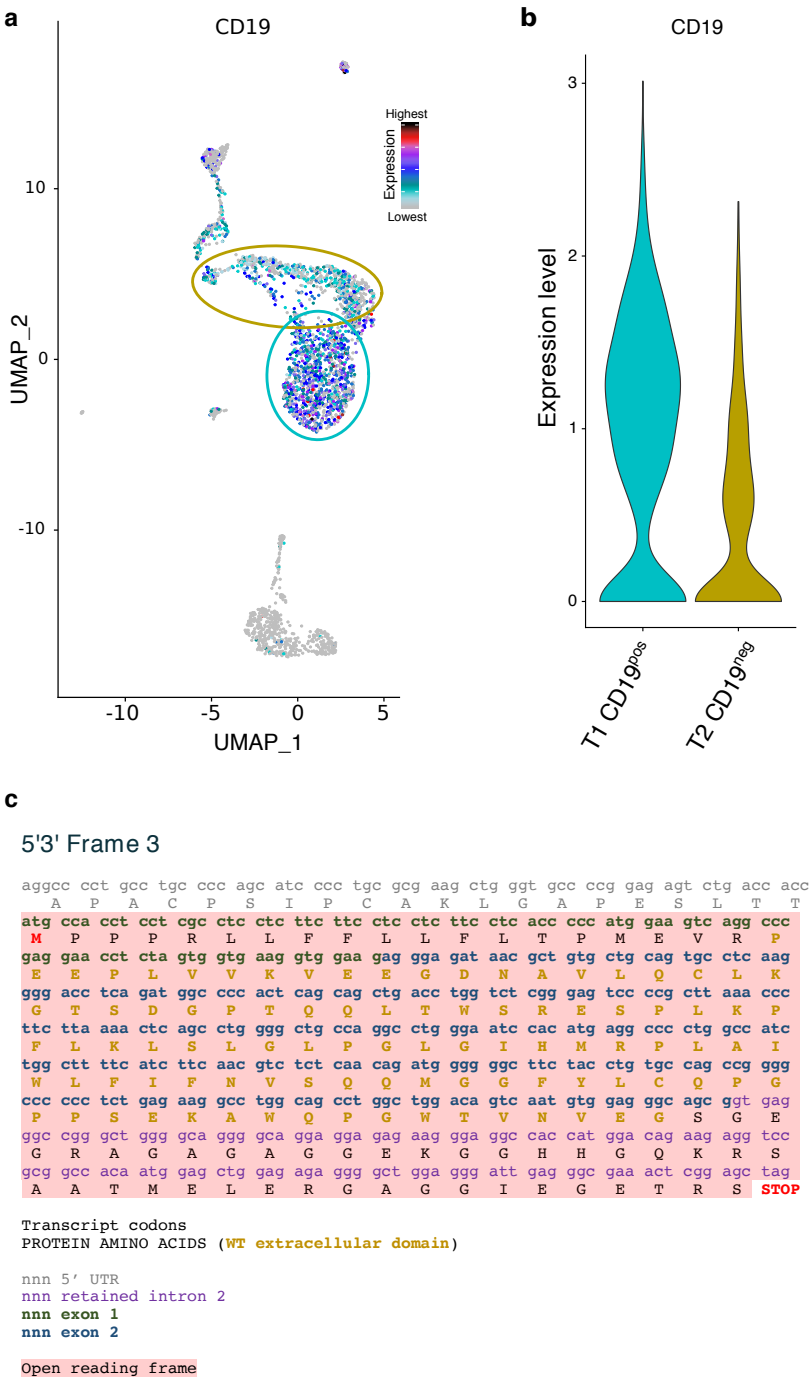


Supplementary Figure 2: Analysis of allelic imbalance using scRNAseq data.
(**a**) UMAP plot displaying large-scale events detected for chromosome 9q by using CaSpER tool. Cells with normal copy number, deletion or amplification of chromosome 9q are respectively colored in grey, blue and orange. The 20 T1 CD19^{neg} cells within B-ALL clusters are represented by larger dots circled in red. In the B-ALL clusters, the majority of the cells appears in blue, thus this CaSpER analysis predicts that one chromosome 9q is deleted in B-ALL cells. (**b**) BAF analysis of SNPs located in chromosome 5q. In the upper panel, IGV (integrative genomics viewer) screenshots show individual SNPs. BAF values of these SNPs in clusters 0, 1 and in physiological cells' clusters (2, 3, 4 & 5) are indicated (clusters are numbered as mentioned in figure 1). Two independent BAF analysis were performed for Cluster 0. The first analysis labelled 'Cluster 0: B-ALL' comprises all cells from cluster 0. The second analysis labelled 'Cluster 0: B-ALL (only T1 CD19^{neg} cells)' comprises only T1 CD19^{neg} cells from cluster 0. Thus for those T1 CD19^{neg} B-ALL cells, BAF were calculated independently. Yet, a BAF value was obtained only for *RPS14*; ND, indicates that the BAF value could not be determined because of a low number of sequencing reads. BAF close to 1 indicates absence of one of the two alleles, while a BAF close to 0.5 indicates the equal presence of both alleles. Green, red, orange and blue bar colors

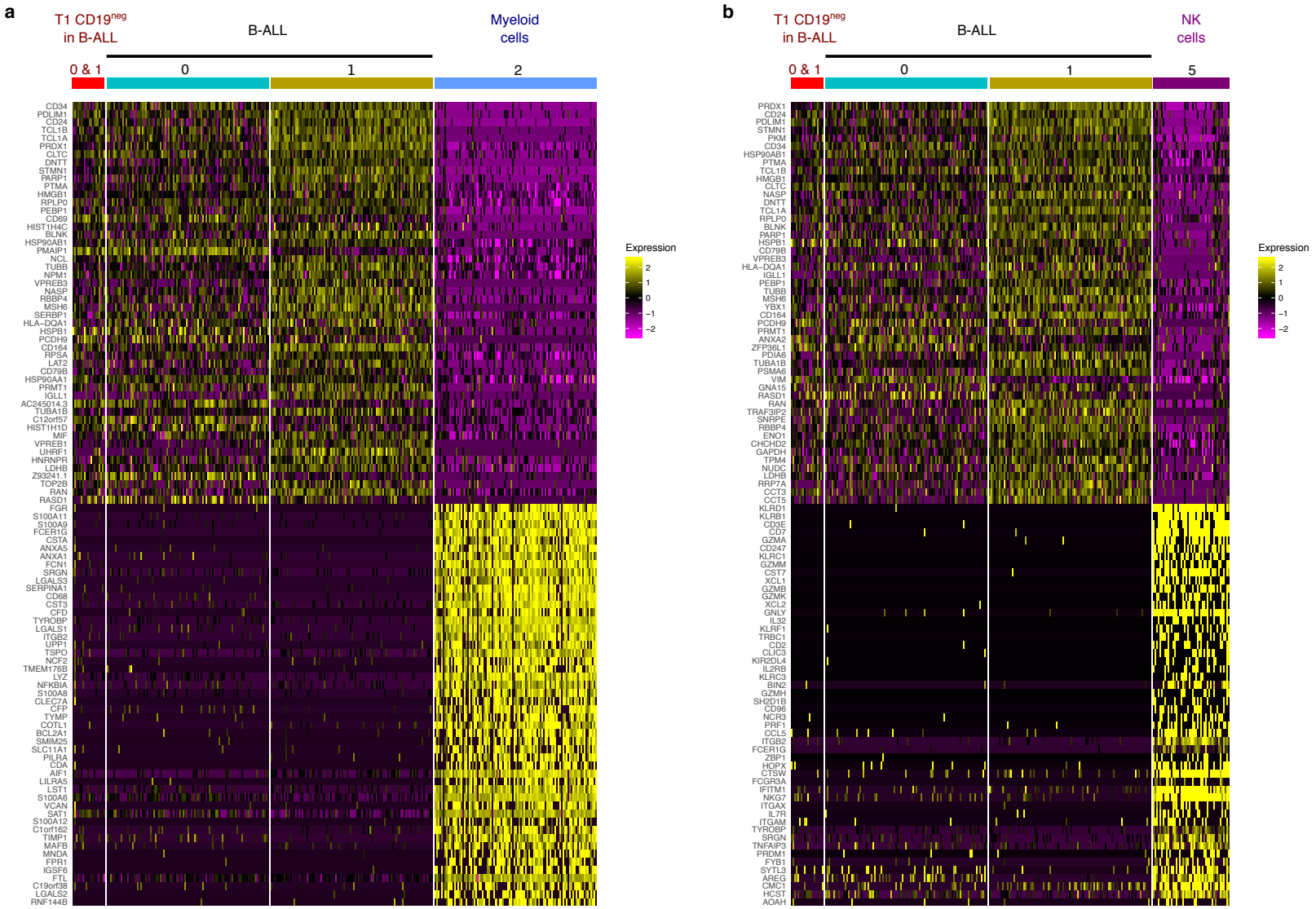
correspond to A, T, G and C nucleotides respectively. In the bottom panel, the table displays numbers of reads which maps to either the reference (Ref) or alternative (Alt) allele at each SNP. Those analyses were focused on chromosomes 9q & 5q as clinical cytogenetic analyses of T1 & T2 B-ALL samples detected alterations within these chromosomes.



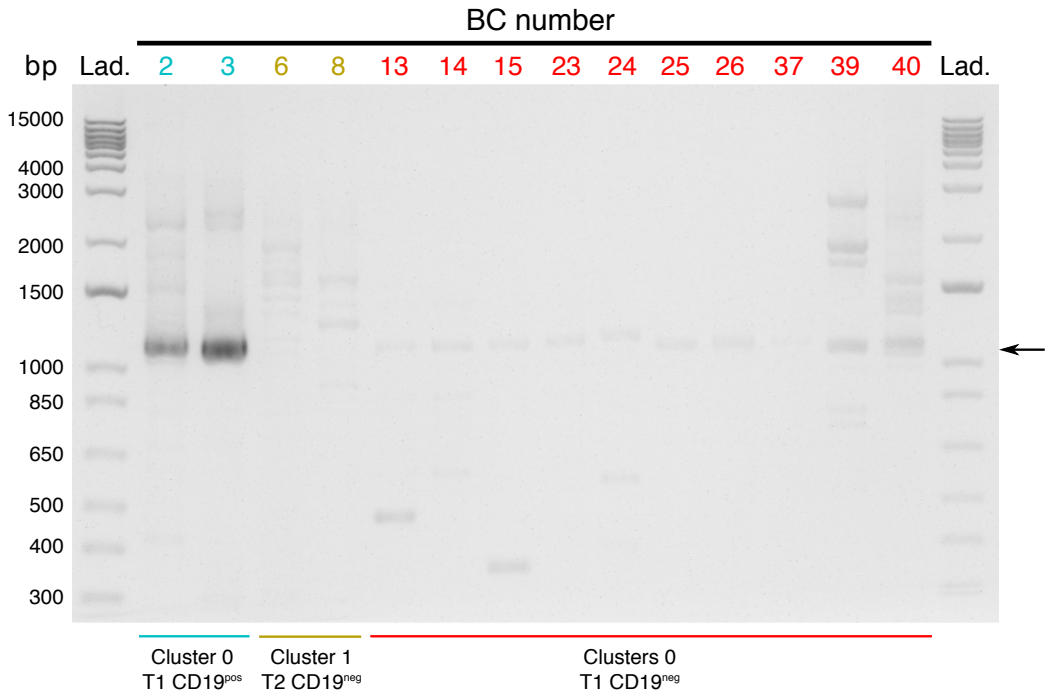
Supplementary Figure 3: KLF6 expression in tumoral clusters.
UMAP visualization of KLF6 mRNA expression (**a**) and regulon activity (**b**) in the 6 main clusters. In cluster 1, down-regulation of KLF6 comes along a decrease of its activity as assessed by its regulon activity using SCENIC.



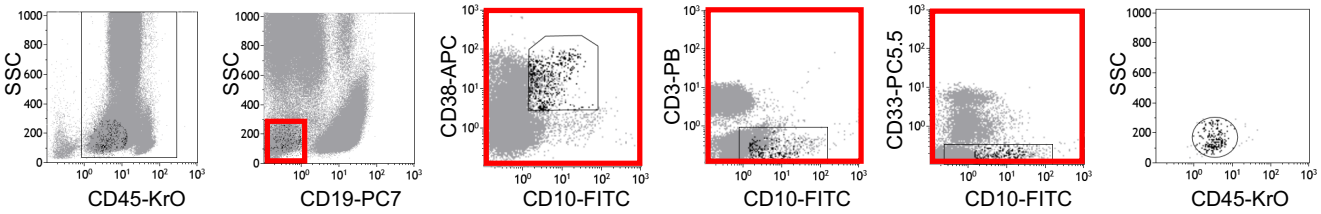
Supplementary Figure 4: Analysis of *CD19* transcript.
UMAP visualization (**a**) and violin plot (**b**) of *CD19* mRNA expression in T1-CD19^{pos} cells (blue) and T2-CD19^{neg} cells (gold). Here mostly the 5' region of *CD19* mRNA is captured since we used 5' 10X genomic approach. **(c)** Non-functional *CD19* mRNA isoform is expressed in leukemic cells. Sanger sequencing of RT-PCR products (Fig. 1e) reveals intron 2 retention. *CD19* transcript codons and their corresponding protein amino acids from 5' UTR region to retained intron 2 (purple) which contained a TAG stop codon leading to a truncated CD19 protein.



Supplementary Figure 5: Differential gene expression between tumoral and physiological cells. Heatmaps showing normalized and scaled expression level of the top 100 differentially expressed genes (50 up- and 50 down-regulated genes) between tumoral cells (clusters 0 & 1 excluding T1-CD19^{neg} cells) and (**a**) myeloid cells (cluster 2) or (**b**) NK cells (cluster 5). Gene expression profiles of T1-CD19^{neg} cells found in clusters 0 & 1 are shown independently from the other B-ALL cells. Clusters 0, 1 & 2 were down-sampled to 100 cells for a better readability

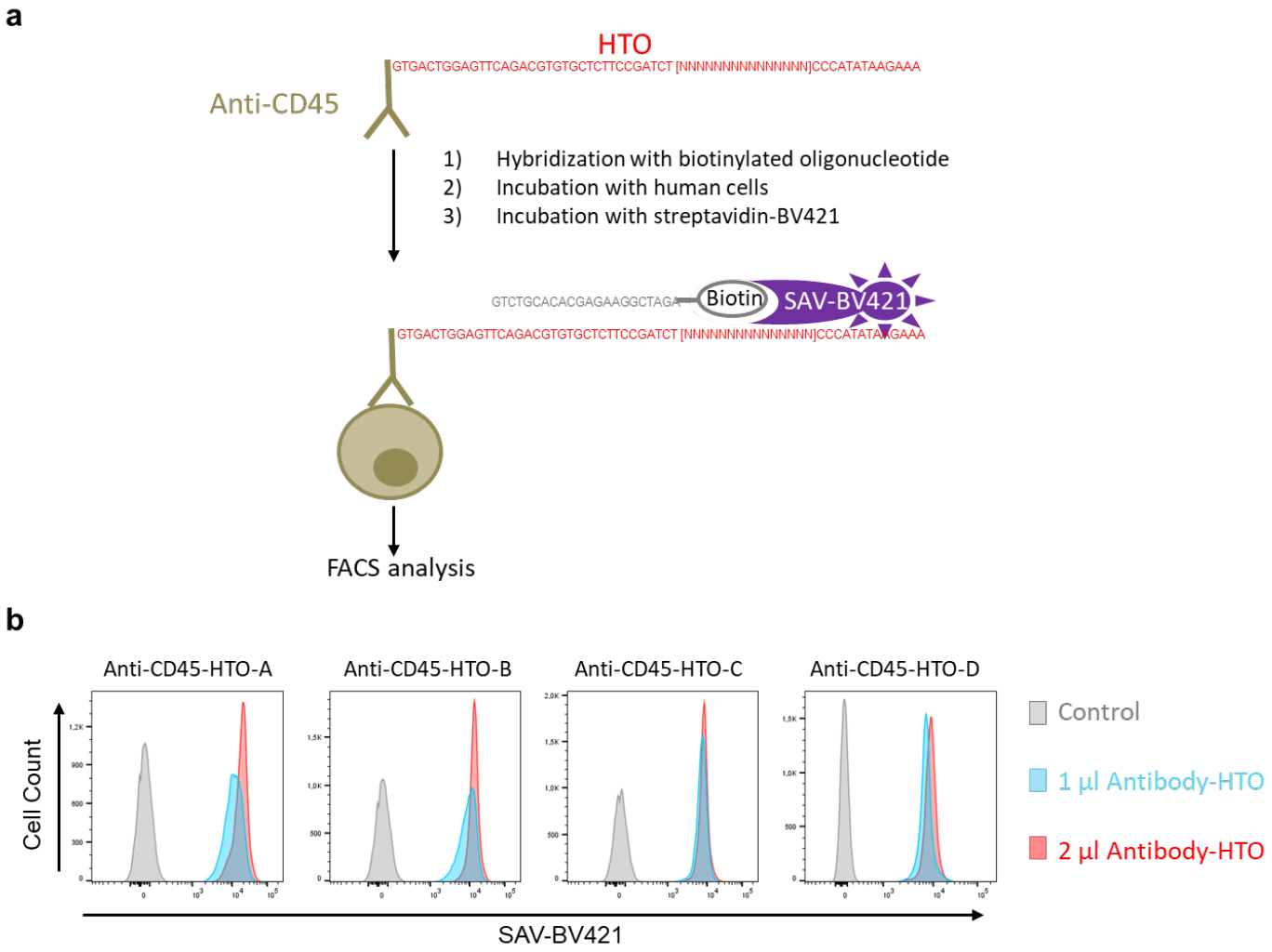


Supplementary Figure 6: Backtracking of CD19^{neg} clones.
Agarose gel of CD19 cDNA amplified by nested-PCR as described in figure 2e. Lanes are labelled according to the cell-BC number for which the sample of origin is indicated at the bottom of the gel. Here are shown putative T1-CD19^{neg} B-ALL clones (cells with BC number: 13, 14, 15, 23, 24, 25, 26, 37, 39 and 40) that were considered ambiguous since a faint band corresponding to *CD19* mRNA (indicated by an arrow) was detected. However, intensities of this band are much lower than in CD19^{pos} cells (BC number 2 and 3), thus we conclude that in accordance with the assignment of FACS-sorting, these T1-CD19neg B-ALL clones are truly CD19-negative.
Cells with BC number 2 and 3 are CD19-positive, while cells with BC number 6 and 8 are CD19-negative cells.



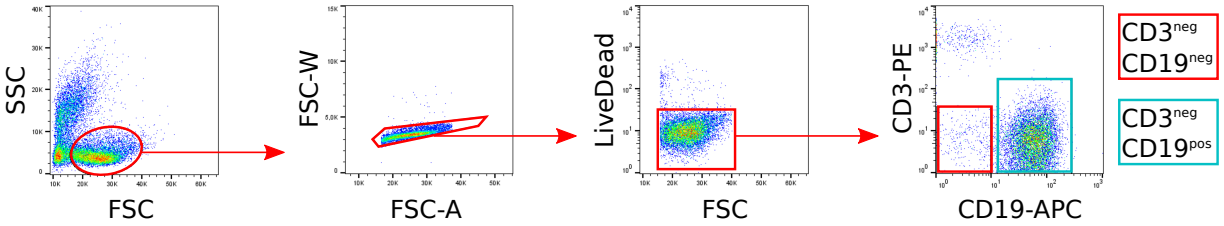
Supplementary Figure 7: FACS analysis of CD19^{neg} cells before CAR-T treatment.

T1 cells were first gated on SSC/CD45 to eliminate cellular debris. Then, CD19^{neg} cells were selected and analyzed according to the positivity of MRD markers (CD38^{pos}, CD10^{neg}) and the negativity of lymphoid T-cell (CD3) and myeloid cell (CD33) markers. The boolean gate shows the cells with the appropriate phenotype.

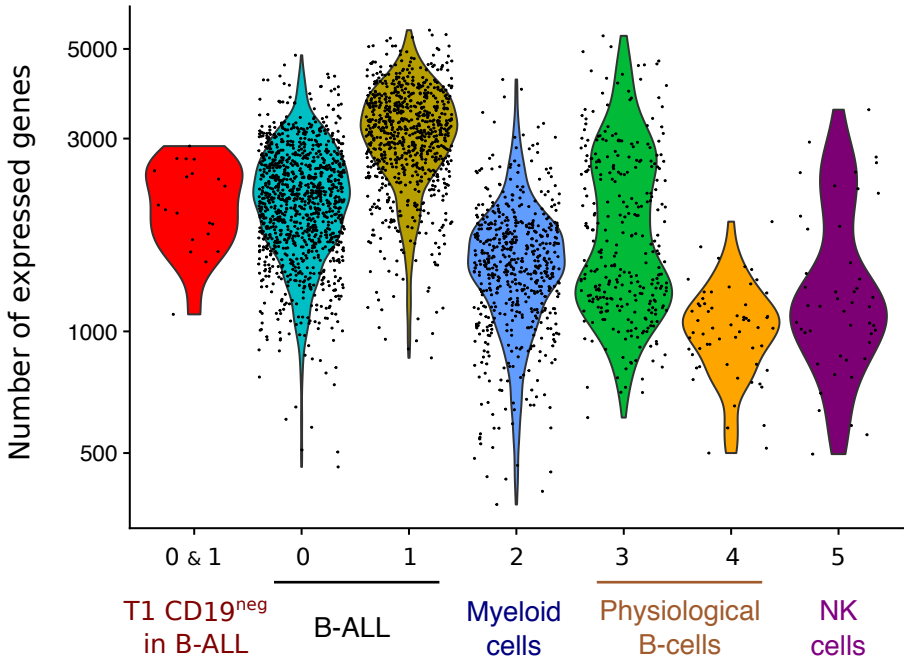


Supplementary Figure 8: Functional validation of conjugated anti-CD45-HTO antibodies

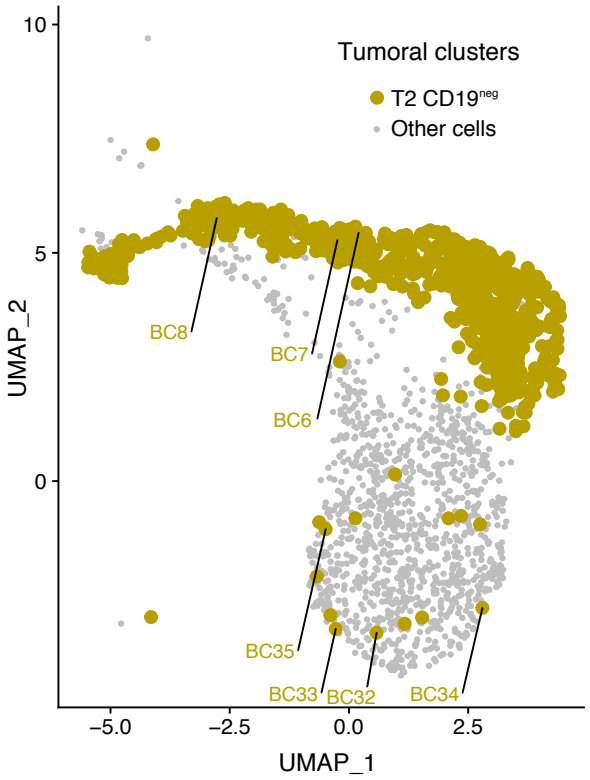
(**a**) Validation strategy. HTO-Antibodies were first hybridized with complementary biotinylated oligonucleotide (Biotin-AG ATC GGA AGA GCA CAC GTC TG). Then, they were incubated with human Jurkat cell line (1.10^6 cells) in 100 µl of FACS buffer (PBS, 2 % FCS, 1 mM EDTA) (30 min at 4°C). After a wash in FACS buffer, 10 µg of streptavidine (SAV) coupled to BV421 (BD Biosciences) were added and samples were further incubated 10 min at 4°C, then they were washed with FACS buffer and finally analyzed on a CANTO II cytometer (BD). Data were analyzed using FlowJo (10.5.3) software. (**b**) Typical histograms obtained for anti-CD45 antibodies coupled to HTO-A, HTO-B, HTO-C and HTO-D. The assays were performed with 1 µl or 2 µl of Antibody-HTO stock solution (estimated concentration of 0,5 µg/µl). Control corresponds to the assay performed in absence of antibody.



Supplementary Figure 9: The gating strategy used for cell sorting.
Forward versus Side Scatter (FSC vs SSC) was used to include lymphocytes and exclude any red blood cells, apoptotic cells, or debris. Also, a forward scatter width (FSC-W) versus forward scatter area (FSC-A) density plot was used to exclude doublet cells. Then, LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells. Finally, live cells were sorted according to CD3 and CD19 expression.



Supplementary Figure 10: Number of expressed genes per cell.
Violin plot showing the number of expressed genes in cells within the 6 clusters (0 to 5). Each dot represents a single cell. The 20 T1-CD19^{neg} cells found in clusters 0 & 1 were analyzed separately. Those T1-CD19^{neg} cells express sufficient genes (around 2090 genes/cell) to reliably assess their identity.



Supplementary Figure 11 (related to figure 2e): Localizations of backtracked T2-CD19^{neg} B-ALL cells.

UMAP plot focused on tumoral cells (clusters 0 and 1). T2-CD19^{neg} cells are highlighted in gold (all others cells are in grey). Cells identified by their BC number and tested by nested-PCR (Fig. 2e) are indicated. Cells with BC 6, 7 & 8 are localized in cluster 1, while cells with BC 32 to 35 are localized in cluster 0.