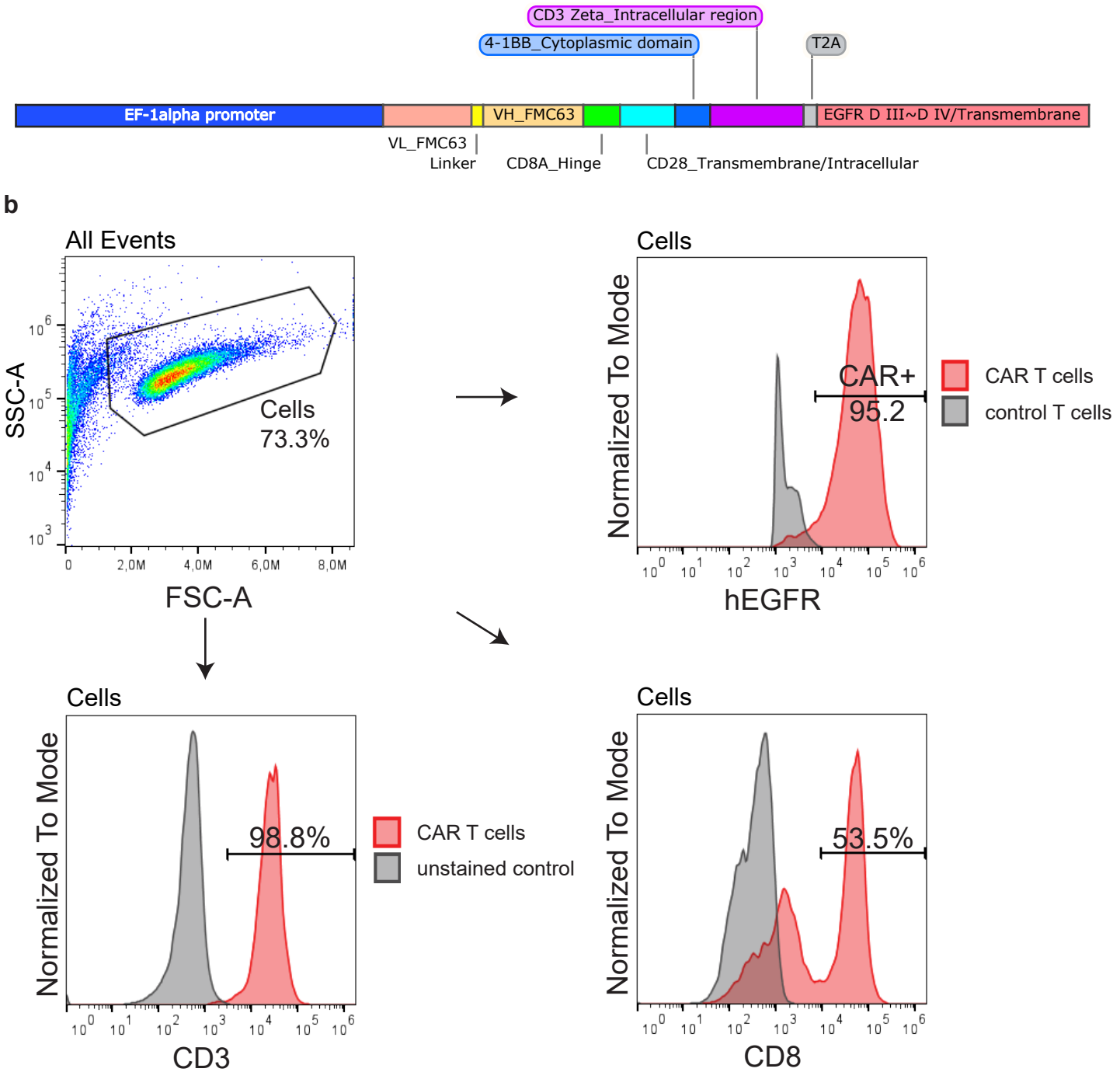
Regulatory programs of B-cell activation and germinal center reaction allow B-ALL escape from CD19 CAR T-cell therapy

Supplementary Data

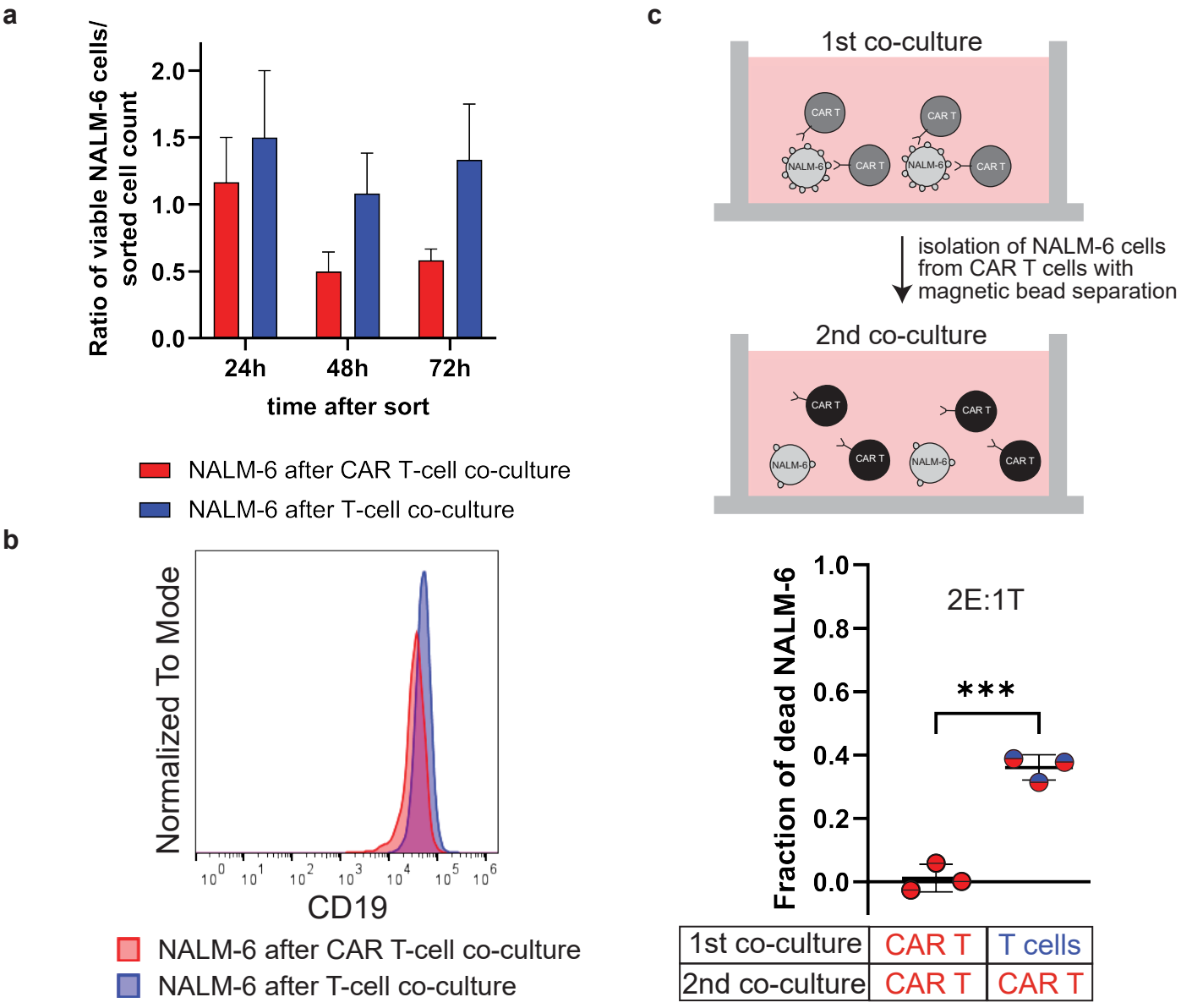
Supplementary Figure S1



Supplementary Figure S1. Properties of CD19 CAR–encoding lentiviral vector and CD19 CAR T cells.

a, A codon-optimized, single-chain variable fragment comprising the variable heavy and variable light chains of the anti-CD19 monoclonal antibody (mAb) that was derived from the FMC63 mouse hybridoma, as has been described previously [66], separated by a (G4S)3 linker, was synthesized (Genewiz) and cloned into the pLVX-CMV100 lentiviral vector (Addgene), where it was fused to a human CD8A hinge and a CD28 transmembrane and intracellular module, followed by a 4-1BB/CD3 zeta signaling module in cis with a T2A element and truncated epidermal growth factor receptor (EGFRt) to be expressed under the control of the EF-1 alpha promoter. **b,** Representative histograms of CD19 CAR T cells after isolation with magnetic beads. Expression of the CAR-construct in CAR T cells (top right). High expression of T-cell markers CD3 (bottom left) and CD8 (bottom right).

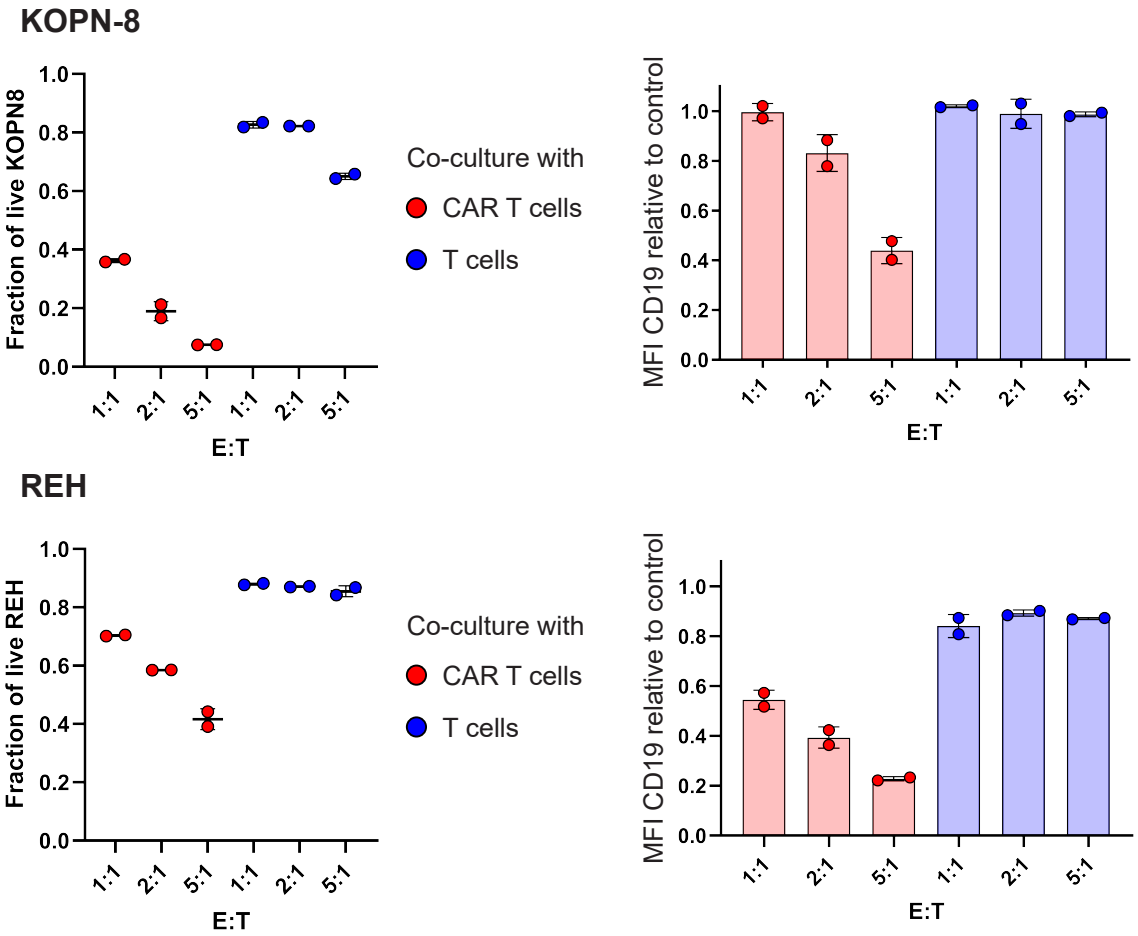
Supplementary Figure S2



Supplementary Figure S2. Remaining NALM-6 cells that survive after co-culture with anti-CD19 CAR T effector cells are viable and refractory to fresh CAR T cells.

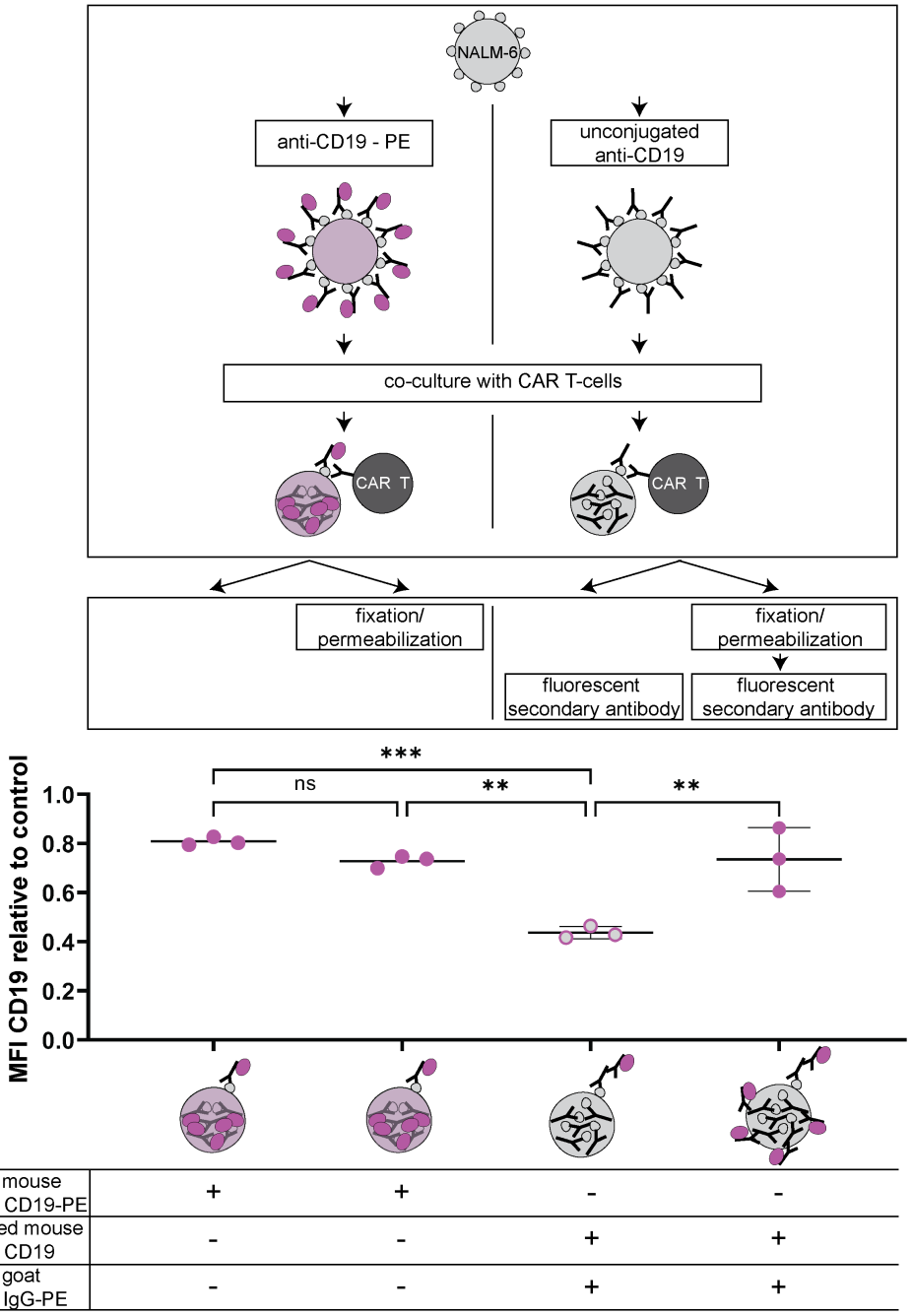
a, NALM-6 cells sorted and isolated after 24h of co-culture with effector cells in 5:1 (E:T) ratio survive over the next 72h. Ratio of viable NALM-6 cells to sorted NALM-6 cell counts shown. Cells were stained with trypan blue and viable cells were counted with a Neubauer cell counting chamber three times. Data are mean ± s.d. **b,** Histogram demonstrating recovered CD19 expression in NALM-6 cells 120h after isolation from co-culture with CD19 CAR T cells (red) relative to NALM-6 isolated from co-culture with uninfected control T cells (blue). **c,** Top, schematic of experimental workflow for two consecutive co-cultures. NALM-6 cells were co-cultured overnight at 2:1 (E:T) ratio with CAR T cells or uninfected T cells. Co-culture with CAR T cells yields CD19low NALM-6 cells, as described in Figure 1. Target cells were then separated from effector cells by magnetic bead selection. After isolating and washing surviving target cells, they were re-exposed to fresh CAR T cells in a second co-culture for 6h at 2:1 (E:T) ratio. Bottom, fraction of dead target cells after re-exposure to fresh CAR T cells. CD19low NALM-6 cells show higher rates of survival after re-exposure to fresh CAR T cells. Fraction of dead cells normalized to cytotoxicity of re-exposure to uninfected T cells. P values determined by two-tailed unpaired t-test, n=3. ***=p<0.001. Data are mean ± s.d.

Supplementary Figure S3



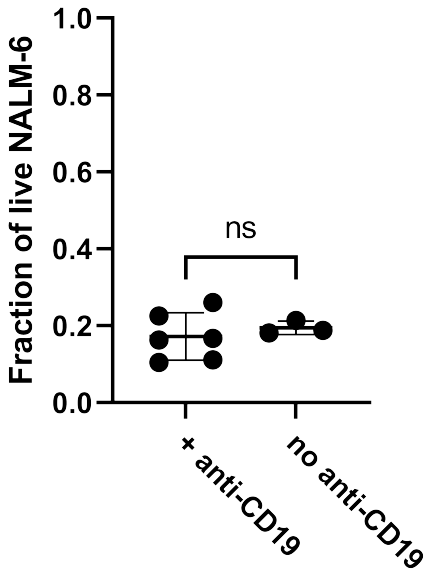
Supplementary Figure S3. CD19 CAR T cells cause reduced CD19 expression and are cytotoxic towards other B-ALL cell lines.
Left, fraction of live KOPN-8 or REH (target) B-ALL cells after 24h of co-culture. Right, mean fluorescence intensity of CD19 expression on remaining live target cells after co-culture. Values normalized to anti-CD19-stained control B-ALL cells that have not been co-cultured with effector cells, n=2, data are mean ± s.d.

Supplementary Figure S4



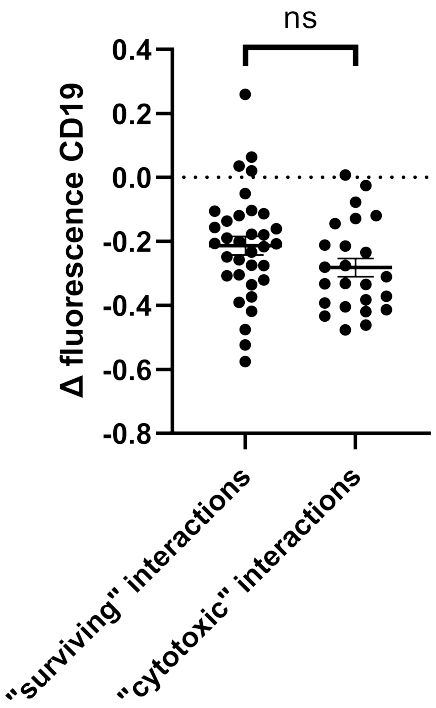
Supplementary Figure S4. Intracellular staining for CD19.
Experimental work-flow used to demonstrate internalization by intracellular flow cytometry. Target cells stained with either anti-CD19 PE or a primary unconjugated anti-CD19 antibody before 16h of co-culture with CAR T cells (construct 2), with subsequent fixation and permeabilization and addition of PE conjugated secondary antibody to selected conditions after 16h of co-culture. Data normalized to triple-stained (CD19-PE, CFSE, 7-AAD) control cells that have not been co-cultured with effector cells, n=3. P values were determined by Tukey's multiple comparisons test. **=p<0.01, ***=p<0.001, ns=not significant. Data are mean ± s.d.

Supplementary Figure S5



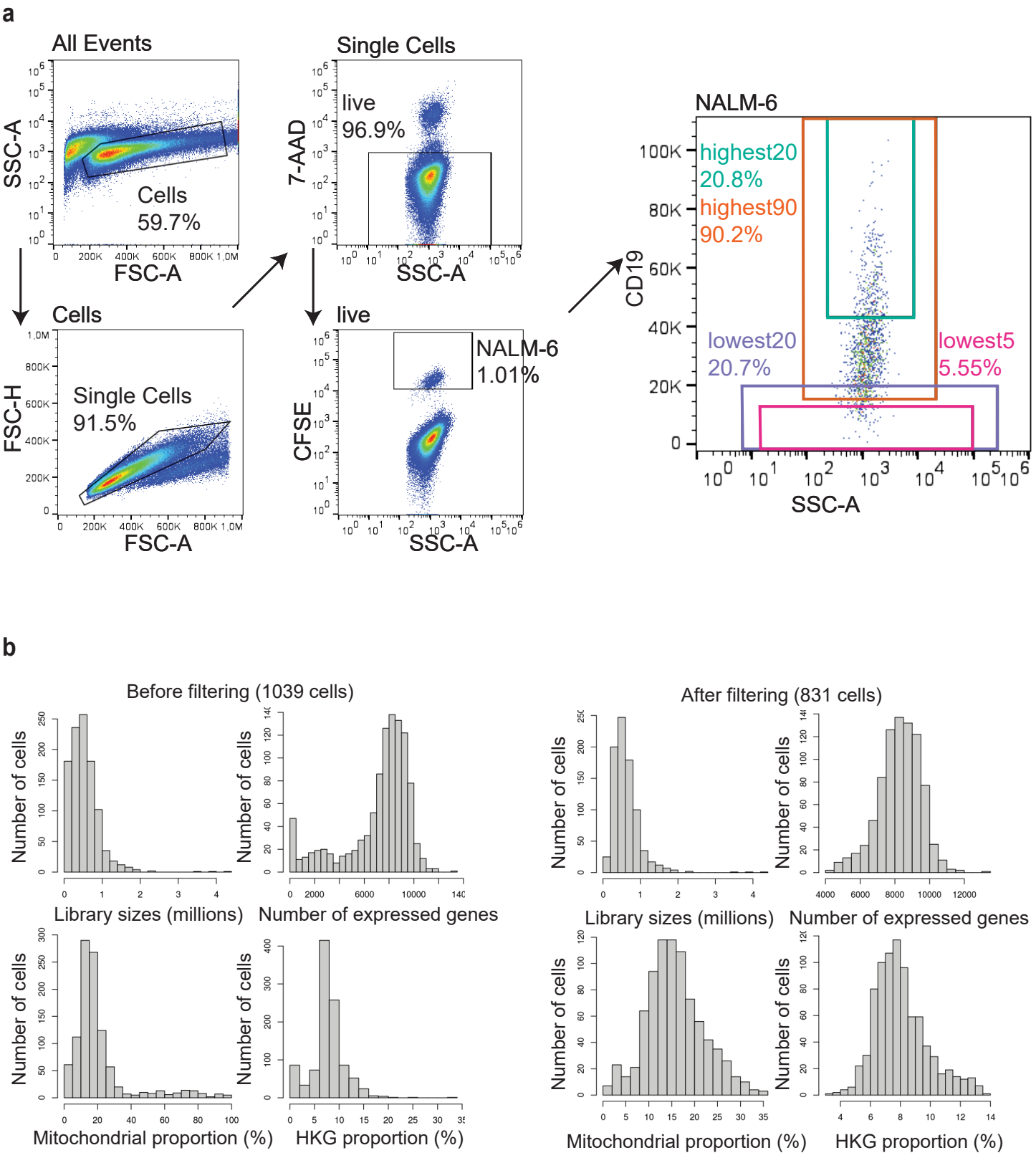
Supplementary Figure S5. Killing efficiency of CAR T cells is independent of presence of anti-CD19.
Fraction of live NALM-6 cells after 24h of co-culture with CAR T cells at 5:1 ratio (E:T). NALM-6 cells were either stained with anti-CD19 antibody prior to co-culture or not. P value determined by two-tailed unpaired t-test, n=3, ns=not significant. Data are mean ± s.d.

Supplementary Figure S6



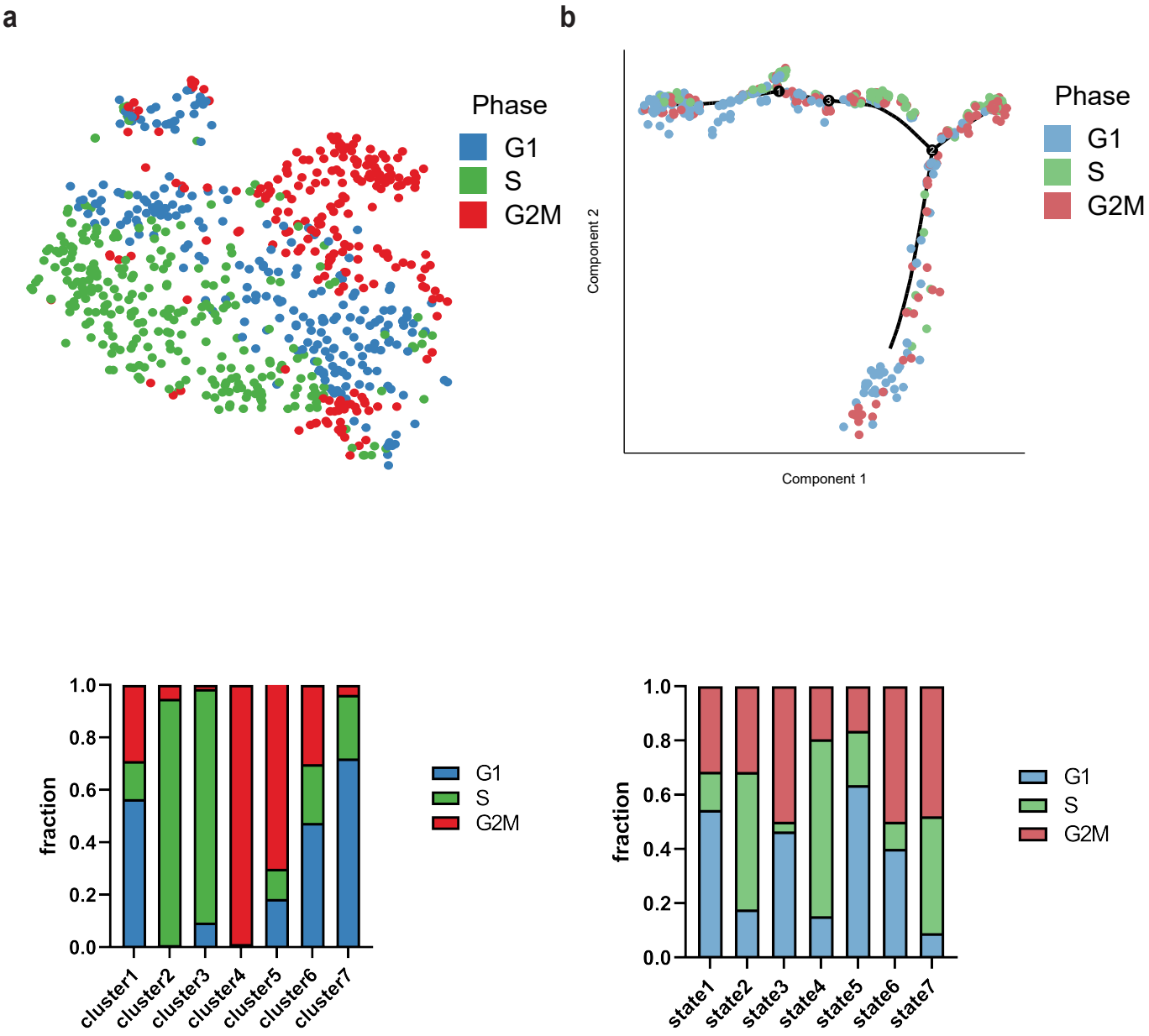
Supplementary Figure S6. Reduced CD19 expression is independent of cytotoxic interactions. Live fluorescence microscopy of NALM-6 and CAR T-cell co-culture using 1:1 ratio. CD19 expression and dead cells determined by anti-CD19 AF647 and SYTOX Blue Dead Cell stain. Delta fluorescence intensity after 6 hours displayed in relation to initial fluorescence intensity. Photobleaching was accounted for by normalizing to controls that did not interact with any effector cell. P-value was determined by two-tailed unpaired t-test. ns= not significant. Data are mean ± s.d.

Supplementary Figure S7



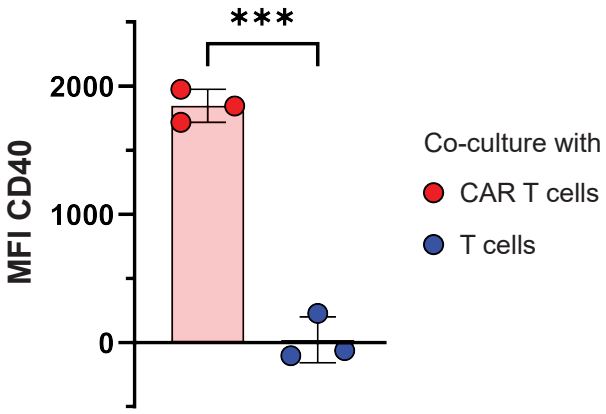
Supplementary Figure S7. ScRNA-seq data processing and quality filtering. **a,** Gating strategy for sorting of single NALM-6 cells after CAR T and T-cell co-cultures. **b,** Quality control filtering of sequenced cells.

Supplementary Figure S8



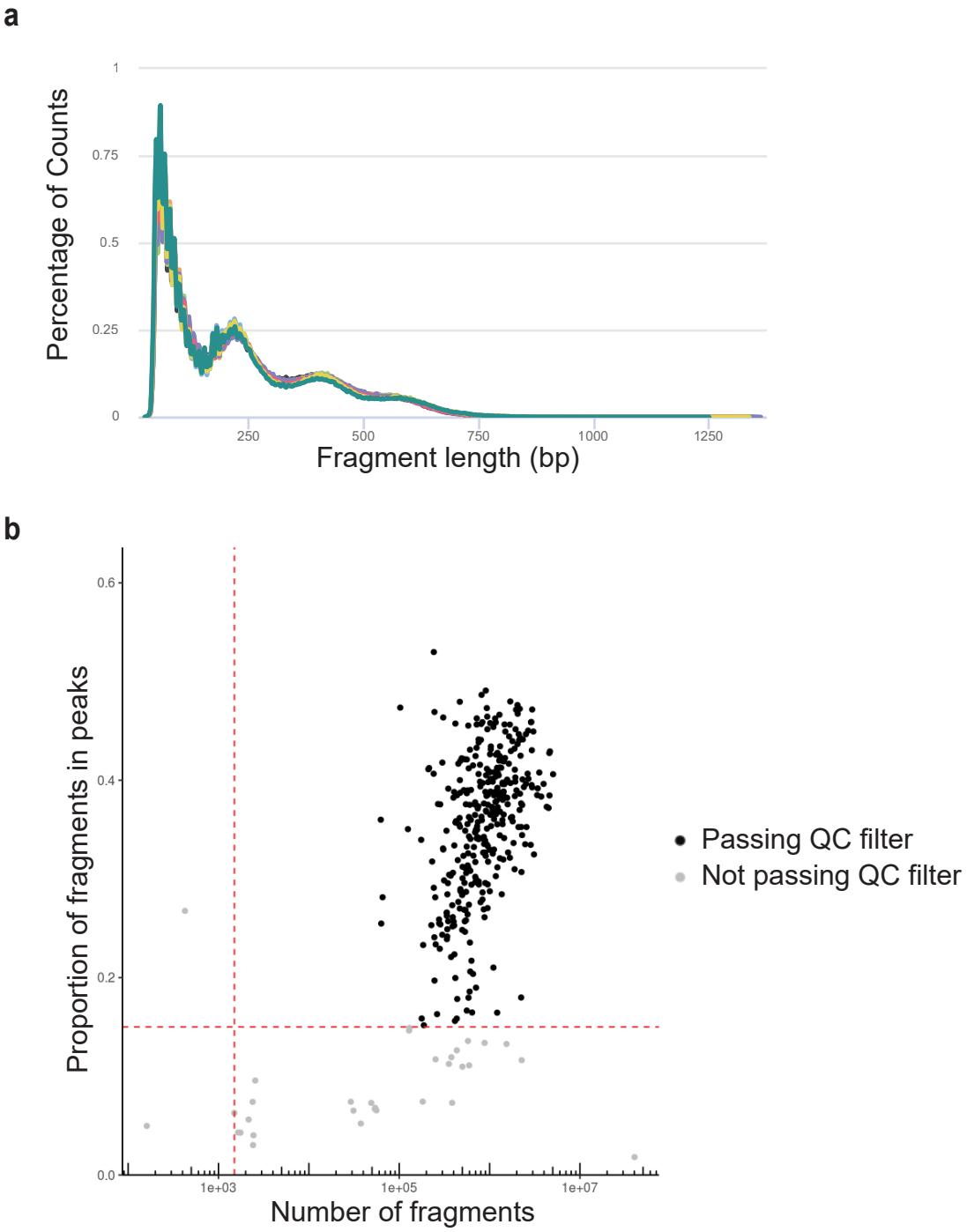
Supplementary Figure S8. Cell cycle phase prediction in CAR T-exposed B-ALL cells. **a,** Top, projection of cell cycle phases on single cell RNA sequencing tSNE plot. Bottom, fraction of cell cycle phases in PAGODA2 clusters. **b,** Top, projection of cell cycle phases on monocle2 pseudotime plot. Bottom, fraction of cell cycle phases in monocle2 states.

Supplementary Figure S9



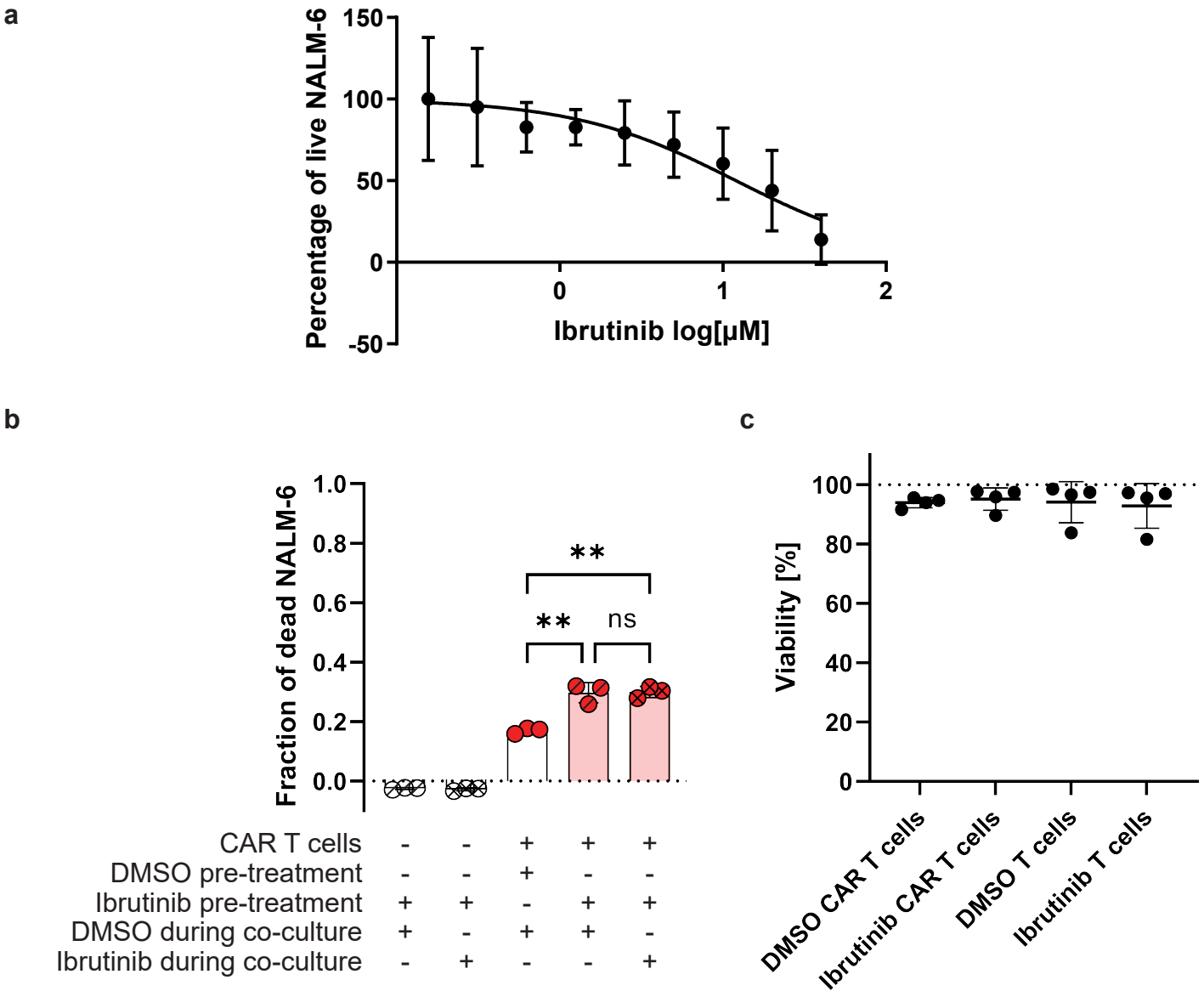
Supplementary Figure S9. Expression of the activation marker CD40 in NALM-6 cells after CAR T-cell co-culture.
MFI of CD40 expression on live leukemia cells co-cultured with CD4+ CAR T cells for 72h at 5:1 ratio. Values normalized to triple-stained (CD40-PE, CFSE, 7-AAD) leukemia cells that have not been co-cultured with effector cells. P value determined by two-tailed unpaired t-test, n=3, ***=p<0.001. Data are mean ± s.d.

Supplementary Figure S10



Supplementary Figure S10. scATAC-seq data processing and filtering.
a, Insert size frequencies of scATAC sequencing reads of NALM-6 B-ALL cells that have been co-cultured with anti-CD19 CAR T cells for 24h in a 5:1 (E:T) ratio. **b,** Quality control filtering of sequenced leukemia cells with a minimum of 1500 fragments and a minimum proportion of 0.15 of fragments in peaks (black = passing QC filter, gray not passing QC filter; see methods).

Supplementary Figure S11



Supplementary Figure S11. Increased cytotoxicity after ibrutinib pre-treatment independent of ibrutinib effect on CAR T cells.

a, Ibrutinib IC10 determined by CellTiter-Glo luminescent cell viability assay after 72h, testing concentrations between 156.25nM to 40 µM, n=3. **b,** Cytotoxic efficacy of CD19 CAR T cells after 6h of co-culture with NALM-6 cells that were pre-treated with ibrutinib for 72h at IC10 concentration of 0.963µM. NALM-6 cells were ficolled and washed before co-culture to remove ibrutinib. Fraction of dead cells normalized to baseline cytotoxicity of uninfected T-cells. P values determined by Tukey's multiple comparisons test, n=3. **c,** High viability of CAR T cells in the presence of ibrutinib at IC10 concentration of 0.963µM after 6h. Viability was determined by negativity of 7-AAD stain. n=4 from two separate experiments, ns=not significant,*=p<0.05, **=p<0.01. Data are mean ± s.d.