Supplemental Methods

Isolation of peripheral blood mononuclear cells (PBMCs) and T-cell activation

PBMCs were isolated from peripheral blood of healthy donors by density gradient centrifugation (Biocoll, Biochrom, Berlin, Germany). To avoid interference of magnetic beads attached to the T cell during electroporation, we chose an untouched T-cell isolation using EasySep Human T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) according to the supplier's information. T cells were activated using T Cell TransAct, human (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's information. T cells were cultured in TexMACS GMP medium (Miltenyi Biotec, Bergisch Gladbach, Germany)/ 2.5% human AB serum (Institute for Clinical Transfusion Medicine, Ulm, Germany) + 12.5 ng/ml human IL-7 and IL-15, premium grade (Miltenyi Biotec, Bergisch Gladbach, Germany) and splitted every two to three days.

Flow cytometry

Cellular composition, transduction, KO rate and phenotype of the final product was determined by flow cytometry using anti-human CD56-BV421, 7-AAD Viability Staining Solution, TCR gamma/delta-BV421, TCR alpha/beta-PE, CD20-APC, CD45RO-PE (Biolegend, San Diego, California, USA), LAG-3-PE (R&D Systems, Minneapolis, Minnesota, USA), CD56-APC (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), CD4-VioGreen, c-myc-FITC, CD19-PE, CD3-PE/Vio770, CD14-APC, CD8-APC/Vio770, CD14-APC, CD16-APC, CD62L-VioBlue, CD95-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). The co-inhibitory, co-stimulatory, activation and exhaustion profile of T cells was stained using anti-human LAG-3-PE, VISTA-APC (R&D Systems, Minneapolis, Minnesota, USA), TIGIT-PE (eBioscience/ Thermo Fisher Scientific, Waltham, Massachusetts, USA), CD45RO-PE/Vio770, OX40-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), CD62L-APC/Cy7, 2B4-APC, CD95-BV785, PD-1-BV650, TIM-3-BV421, 4-1BB-BV421, BTLA-BV650 (Biolegend, San Diego, California, USA), CD56-BUV737, CD8-BUV496, CD3-BUV395, CD28-APC and CD69-BV650 (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The phenotype was defined as follows: naïve T cells (T_n) are $CD62L^+$, $CD45RO^-$, $CD95^-$, stem cell memory like T cells (T_{scm}) are $CD62L^+$, $CD45RO^-$, $CD95^+$, central memory T cells (T_{cm}) are $CD62L^+$, $CD45RO^+$, $CD95^+$, effector memory T cells (T_{em}) are $CD62L^-$, $CD45RO^+$, $CD95^+$ and effector T cells (T_{eff}) are $CD62L^-$, $CD45RO^-$, $CD95^+$. Intracellular stains were performed with anti-human IFN-γ-PE (Becton, Dickinson and Company, Franklin Lakes, New Jersey,

USA) and TNF-α-Pacific Blue (Biolegend, San Diego, California, USA) using Fix & Perm Cell Permeabilization Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's information. Detection of T cells in peripheral blood, spleen and bone marrow was performed using c-myc-APC (Abcam, Cambridge, UK), CD45-BV785, CD3-BUV737, CD8-BUV496, CD4-BUV395 (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), CD19-PE (Millipore, Burlington, Massachusetts, USA).

Flow cytometric measurements were performed on a MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) or BD LSRFortessa (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA).

Intracellular cytokine stain assay

To determine the secretion of IFN-γ and TNF-α upon target-cell contact, T cells were co-cultured with $CD19^+$ Raji cells at an E:T ratio of 1:1. Intracellular cytokine stain was performed after 24 hours.

Cytotoxicity assay

$CD19^+$ NALM6 cells were labeled according to CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and co-cultured with untransduced T cells, CAR-T cells, $_{RV}19BB_{CR}TCR^+$ CAR-T cells and $_{RV}19BB_{CR}TRBC^-$ CAR-T cells for 48 hours at various E:T ratios. Absolute count of remaining CellTrace Violet-positive cells was calculated using the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and set into relation to the count of CellTrace Violet-positive cells of control wells (target cells only).

Proliferation assay

T cells were labeled according to CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and co-cultured with Raji cells (E:T ratio 1:1). Cell proliferation was determined before and after 72 hours by flow cytometry according to the supplier's information.

Cell lines

NALM6 ($CD19^+$ B-precusor ALL) and Raji ($CD19^+$ Burkitt lymphoma) were cultured in RPMI 1640 (Biochrom, Berlin, Germany) with 10% fetal bovine serum (Sigma-Aldrich CHEMIE, Steinheim, Germany), 1% Penicillin-Streptomycin (Gibco, Life Technologies, Darmstadt, Germany) and 1% L-

glutamine 200mM (Biochrom, Berlin, Germany). Cell lines were routinely tested for identity by short-tandem repeat analyses (DSMZ, Braunschweig, Germany) and regularly checked for mycoplasma contamination using the PlasmoTest™ – Mycoplasma Detection Kit (InvivoGen, Toulouse, France).

CAR enrichment and depletion of CD3⁺ T cells

Eleven days after electroporation, magnetic CAR enrichment and depletion of $CD3^+$ (TCR^+) T cells were performed. Therefore, T cells were labeled with CD3 MicroBeads, human (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the supplier's protocol. $CD3^+$ T cells were depleted using LD Columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's information. CD3-depleted cells were stained with anti-c-myc-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10min, washed once and then labeled with anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the supplier's information. Magnetic enrichment of CAR-T cells was performed using a LS Column (Miltenyi Biotec, Bergisch Gladbach, Germany). Successful CAR enrichment and CD3 depletion was analyzed by flow cytometry. After depletion/ enrichment, TCR^-CAR^+ T cells were cultured for one day and were then used for further analyses or frozen. Non-electroporated conventional CAR-T cells are termed $_{RV}$19BB_TCR^+, CAR-T cells electroporated with Cas9 and a non-binding gRNA are termed $_{RV}19BB_{CR}TCR^+$ and untransduced, non-electroporated T cells (UTs) served as comparator for CAR-T cells with knockout of the TCRβ chain.

Histology and immunohistology

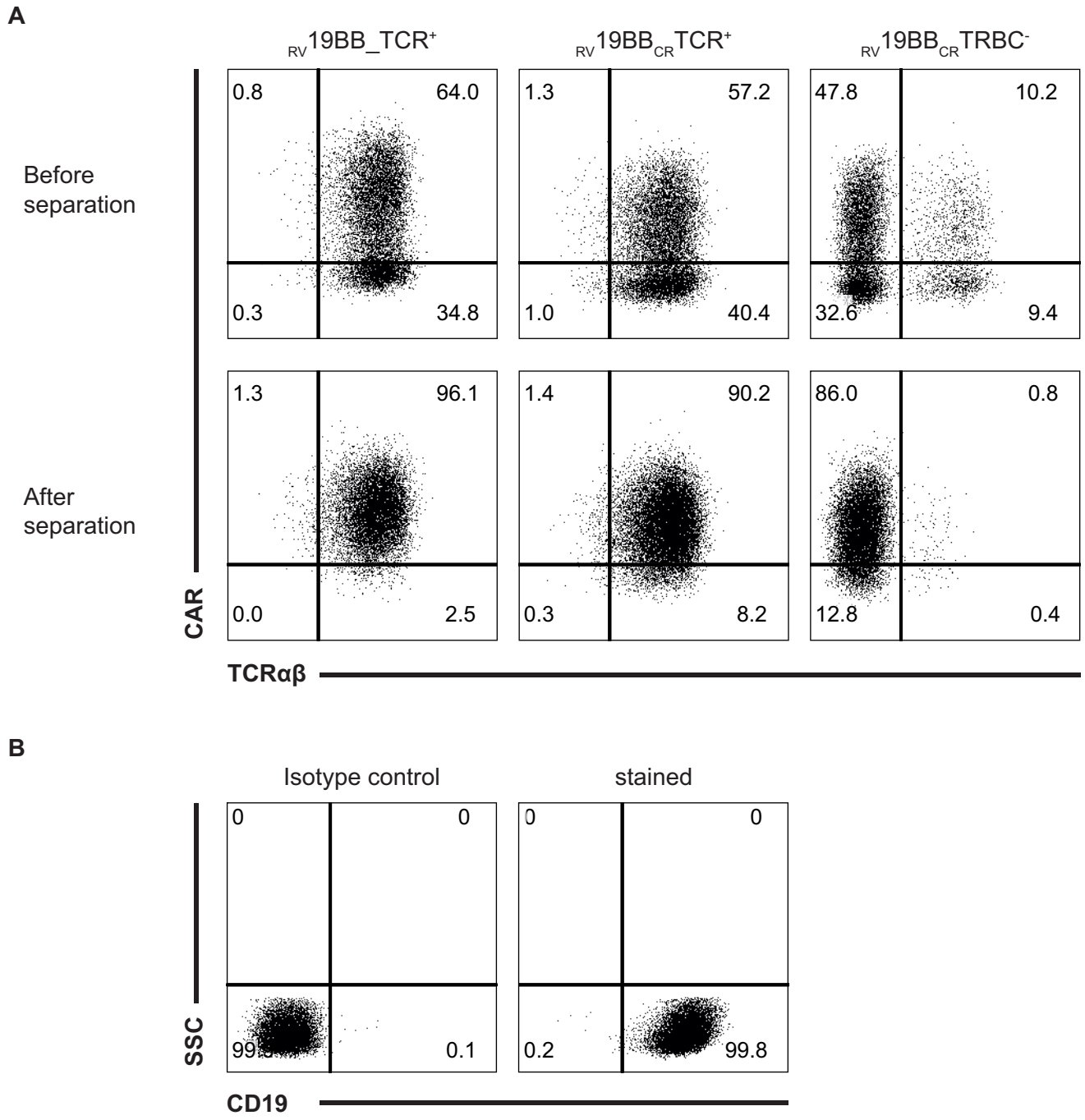
Parts of skin, intestines, spleen, liver, lung and brain were formalin-fixed for further analysis. Hematoxyli-Eosin staining was performed and histological scoring of GvHD applied. For immunohistochemistry of cleaved caspase 3 (CC3), 4 µm sections were cut and antigen retrieval was performed with microwave treatment using the Target Retrieval Solution (Agilent Technologies). Blockage of endogenous peroxidase was performed using 7.5% aqueous H_2O_2 solution at room temperature and blocking serum from the corresponding kits for 20 minutes. Slides were then incubated for 60 minutes with a primary antibody against CC3 (1:100 dilution, Cell signaling, #9661), and then incubated with a secondary anti-rabbit IgG antibody (MP-7401, ImmPress Reagent Kit, Peroxidase-conjugated) followed by target detection using DAB+ chromogen (Agilent Technologies). Slides were counterstained with hematoxylin Gill's Formula (H-3401, Vector).

Supplemental Figures

Supplemental Fig. 1 Surface expression profiles of CAR-T cells and ALL PDX cells. (A) Exemplary FACS plots of CAR and TCRαβ surface expression in conventional CAR, $_{RV}$19BB$_{CR}$TCR^{+}CARs and $_{RV}$19BB$_{CR}$TRBC^{-}CAR-T cells at day 12 after transduction. Upper row represents distribution before separation. Lower row shows expression pattern after CAR enrichment ± CD3 depletion using MACS beads. (B) CD19 surface expression of ALL PDX cells. $_{RV}$19BB$_{CR}$TCR^{+}=CAR-T cells electroporated with nonsense gRNA, $_{RV}$19BB$_{CR}$TRBC^{-}=CAR-T cells electroporated with TRBC-targeting gRNA

Supplemental Fig. 2 NALM6 leukemia bearing mice treated with CAR-T cells. NSG mice were injected i.v. with 1x10^{5} NALM6 cells, followed by i.v. T-cell injection (2x10^{7} cells) of untransduced T cells, $_{RV}$19BB$_{CR}$TCR^{+}CARs T cells and $_{RV}$19BB$_{CR}$TRBC^{-}CAR-T cells three days after. (A) At indicated time points after T-cell injection NALM6 leukemia burden was monitored by bioluminescence in vivo imaging. (B) CD4/CD8 ratio was determined in samples of peripheral blood. $_{RV}$19BB$_{CR}$TCR^{+}=CAR-T cells electroporated with nonsense gRNA, $_{RV}$19BB$_{CR}$TRBC^{-}=CAR-T cells electroporated with TRBC-targeting gRNA,

Supplementary Figure 1



Supplementary Figure 2