Supplemental Methods:

Cells lines and primary cells

The NALM6 cell line was purchased from ATCC, Manassas, VA, USA, and the MOLM13 cell line was a gift from the Jelinek Laboratory at the Mayo Clinic (purchased from DSMZ, Braunschweig, Germany). These cell lines were transduced with a luciferase-ZsGreen lentivirus (Addgene, Cambridge, MA, USA) and sorted to 100% purity. Cell lines were cultured in R10 (made with RPMI 1640 (Gibco, Gaithersburg, MD, US), 10% Fetal Bovine Serum (FBS, Millipore Sigma, Ontaria, Canada), and 1% Penicillin-Streptomycin-Glutamine (Gibco, Gaithersburg, MD, US). Primary cells were obtained from the Mayo Clinic Biobank for patients with acute leukemia under a Mayo Clinic Institutional Review Board (IRB) approved protocol. The use of recombinant DNA in the laboratory was approved by the Mayo Clinic Institutional Biosafety Committee (IBC).

Primary cells and CAR-T cells

Peripheral blood mononuclear cells (PBMC) were isolated from de-identified normal donor blood apheresis cones[32] obtained under a Mayo Clinic IRB approved protocol, using SepMate tubes (STEMCELL Technologies, Vancouver, Canada). T cells were separated with negative selection magnetic beads using EasySep™ Human T Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada). Monocytes were isolated using a Human Monocyte Isolation Kit from Miltenyi Biotec, Bergisch Gladbach, Germany, which isolates CD14+ monocytes. Primary cells were cultured in T Cell Medium made with X-Vivo 15 (Lonza, Walkersville, MD, USA) supplemented with 10% human serum albumin (Corning, NY, USA) and 1% Penicillin-Streptomycin-Glutamine

(Gibco, Gaithersburg, MD, USA). CART19 cells were generated through the lentiviral transduction of normal donor T cells as described below. Second generation CART19 constructs were *de novo* synthesized (IDT) and cloned into a third generation lentivirus under the control of the EF-1α promotor. The CD19 directed single chain variable region fragment was derived from the clone FMC63. A second generation 4-1BB co-stimulated (FMC63-41BBz) CAR construct was synthesized and used for these experiments. Lentiviral particles were generated through the transient transfection of plasmid into 293T virus producing cells (gift from the Ikeda lab, Mayo Clinic), in the presence of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), VSV-G and packaging plasmids (Addgene, Cambridge, MA, USA). T cells isolated from normal donors were stimulated using Cell Therapy Systems Dynabeads CD3/CD28 (Life Technologies, Oslo, Norway) at a 1:3 ratio and then transduced with lentivirus particles 24 hours after stimulation at a multiplicity of infection (MOI) of 3.0. To determine titers and subsequently MOI, after lentivirus particles were concentrated, titers were determined by transducing $1x10^5$ primary T cells in 100 ul of T cell medium with 50 ul of lentivirus. First, T cells were stimulated with CD3/CD28 beads and then transduced with lentivirus particles 24 hours later. Transduction was performed in triplicates and at serial dilutions. Fresh T cell medium was added one day later. Two days later, cells were harvested, washed twice with PBS, and CAR expression on T cells was determined by flow cytometry. Titers were determined based on the percentage of CAR positive cells (percentage of CAR+ cells x T cell count at transduction x the specific dilution / volume) and expressed as transducing units/mL (TU/mL). Magnetic bead removal was performed on Day 6 and CAR-T cells were harvested and cryopreserved on Day 8 for

future experiments. CAR-T cells were thawed and rested in T cell medium 12 hours prior to their use in experiments.

GM-CSF neutralizing antibody and isotype controls

Lenzilumab (Humanigen, Burlingame, CA) is a novel, first in class Humaneered® monoclonal antibody that neutralizes human GM-CSF. For *in vitro* experiments, lenzilumab or *InVivo*MAb human IgG1 isotype control (BioXCell, West Lebanon, NH, USA), 10 ug/mL was used. For *in vivo* experiments, 10 mg/kg of lenzilumab or isotype control were intraperitoneally injected daily for 10 days beginning on the day of CART19 injection. In some experiments, anti-mouse GM-CSF neutralizing antibody (*InVivo*MAb anti-mouse GM-CSF, BioXCel, West Lebanon, NH, USA) or the corresponding isotype control (*InVivo*MAb rat IgG2a isotype control BioXCel, West Lebanon, NH, USA) was also used, as indicated in the experimental schema.

Generation of GM-CSF$^{k/o}$ CART19:

A guide RNA (gRNA) targeting exon 3 of human GM-CSF was selected via screening gRNAs previously reported to have high efficiency for human GM-CSF.[23] This gRNA was ordered in a CAS9 third generation lentivirus construct (lentiCRISPRv2), controlled under a U6 promotor (GenScript, Township, NJ, USA). Lentiviral particles encoding this construct were produced as described above. T cells were dual transduced with CAR19 and GM-CSFgRNA-lentiCRISPRv2 lentiviruses, 24 hours after stimulation with CD3/CD28 beads. CAR-T cell expansion was then continued as described above. To analyze efficiency of targeting GM-CSF, genomic DNA was extracted from the GM-CSF$^{k/o}$ CART19 cells using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The DNA of interest was PCR amplified using

Choice Taq Blue Mastermix (Thomas Scientific, Minneapolis, MN, USA) and gel extracted using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA) to determine editing. PCR amplicons were sent for Eurofins sequencing (Louisville, KY, USA) and allele modification frequency was calculated using TIDE (Tracking of Indels by Decomposition) software available at https://tide.nki.nl.[33] Fig S1 describes the gRNA sequence, primer sequences, and the schema for generation of GM-CSF$^{k/o}$ CART19 schema.

T cell functional experiments

Cytokine assays were performed 24 or 72 hours after a co-culture of CAR-T cells with their targets at a 1:1 ratio as indicated. Human High Sensitivity T Cell Magnetic Bead Panel (Millipore Sigma, Ontario, Canada), Milliplex Human Cytokine/Chemokine MAGNETIC BEAD Premixed 38 Plex Kit (Millipore Sigma, Ontario, Canada), or Milliplex Mouse Cytokine/Chemokine MAGNETIC BEAD Premixed 32 Plex Kit (Millipore Sigma, Ontario, Canada) were performed on supernatants collected from these experiments or serum, as indicated. This was analyzed using Luminex (Millipore Sigma, Ontario, Canada). Intracellular cytokine analysis and T cell degranulation assays were performed following incubation of CAR-T cells with targets at a 1:5 ratio for 4 hours, in the presence of monensin (BioLegend, San Diego, CA, USA), hCD49d (BD Biosciences, San Diego, CA, USA), and hCD28 (BD Biosciences, San Diego, CA, USA). After 4 hours, cells were harvested and intracellular staining was performed after surface staining, followed by fixation and permeabilization with fixation medium A and B (Life Technologies, Oslo, Norway). For proliferation assays, CFSE (Life Technologies, Oslo, Norway) labeled effector cells (CART19), and irradiated target cells were co

cultured at a 1:1 ratio. In some experiments, CD14+ monocytes were added to the co-culture at a 1:1:1 ratio as indicated. Cells were co-cultured for 3-5 days, as indicated in the specific experiment and then cells were harvested and surface staining with anti-hCD3 (eBioscience, San Diego, CA, USA) and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA) was performed. PMA/ionomycin (Millipore Sigma, Ontario, Canada) was used as a positive non-specific stimulant of T cells, at different concentrations as indicated in the specific experiments. For killing assays, the $CD19^{+}Luciferase^{+}$ ALL cell line NALM6 or the $CD19^{-}Luciferase^{+}$ control MOLM13 cells were incubated at the indicated ratios with effector T cells for 24, 48, or 72 hours as listed in the specific experiment. Killing was calculated by bioluminescence imaging on a Xenogen IVIS-200 Spectrum camera (PerkinElmer, Hopkinton, MA, USA) as a measure of residual live cells. Samples were treated with 1ul D-luciferin (30ug/mL) per 100ul sample volume (Gold Biotechnology, St. Louis, MO, USA), for 10 minutes prior to imaging.

Multi-parametric flow cytometry

Anti-human and anti-mouse antibodies were purchased from Biolegend, eBioscience, or BD Biosciences (San Diego, CA, USA). Cells were isolated from *in vitro* culture or from peripheral blood of animals. After BD FACS lyse (BD Biosciences, San Diego, CA, USA), they were washed twice in phosphate-buffered saline supplemented with 2% FBS (Millipore Sigma, Ontario, Canada) and 1% sodium azide (Ricca Chemical, Arlington, TX, USA) and stained at 4 °C. For cell number quantitation, Countbright beads (Invitrogen, Carlsbad, CA, USA) were used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). In all analyses, the

population of interest was gated based on forward vs side scatter characteristics, followed by singlet gating, and live cells were gated following staining with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA). Surface expression of CAR was detected by staining with a goat anti-mouse F(ab')2 antibody (Invitrogen, Carlsbad, CA, USA). Flow cytometry was performed on three-laser cytometers, Canto II (BD Biosciences, San Diego, CA, USA) and CytoFLEX (Beckman Coulter, Chaska, MN, USA). Analyses were performed using FlowJo X10.0.7r2 software (Ashland, OR, USA) and Kaluza 2.0 software (Beckman Coulter, Chaska, MN, USA).

Xenograft mouse models

Male and female 8-12 week old NOD-SCID-IL2rγ$^{-/-}$ (NSG) mice were bred and cared for within the Department of Comparative Medicine at the Mayo Clinic under a breeding protocol approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). Mice were maintained in an animal barrier space that is approved by the IBC for BSL2+ level experiments.

NALM6 cell line xenografts

The CD19^{+}, luciferase^{+} ALL NALM6 cell line was use to establish ALL xenografts, under an IACUC approved protocol. Here, $1x10^{6}$ cells were injected intravenously (IV) via a tail vein injection. 4-6 days after injection, mice underwent bioluminescent imaging using a Xenogen IVIS-200 Spectrum camera (PerkinElmer, Hopkinton, MA, USA), to confirm engraftment. Imaging was performed 10 minutes after the intraperitoneal (IP) injection of 10ul/g D-luciferin (15mg/mL, Gold Biotechnology, St. Louis, MO, USA). Mice were then randomized based on their bioluminescent imaging to receive different treatments as outlined in the specific experiments. Typically, $1\text{-}1.5x10^{6}$

CAR-T cells (and an equivalent of total T cell number of untransduced (UTD) T cells) were injected IV per mouse. Transduction efficiency of CAR-T cells was typically approximately 50%. For example, with a 50% transduction efficiency of CAR-T cells, mice that received 1.5×10^6 CAR-T cells received 3 million total T cells, and the corresponding UTD mice received 3×10^6 UTD. Weekly imaging was performed to assess and follow disease burden. Bioluminescent images were acquired using a Xenogen IVIS-200 Spectrum camera (PerkinElmer, Hopkinton, MA, USA) and analyzed using Living Image version 4.4 (Caliper LifeSciences, PerkinElmer). Tail vein bleeding was done 7-8 days after injection of CAR-T cells to assess T cell expansion and cytokines and chemokines, and subsequently as needed. Mouse peripheral blood was subjected to red blood cell lysis using BD FACS Lyse (BD Biosciences, San Diego, CA, USA) and then used for flow cytometric studies. Antibody treated mice commenced daily antibody therapy (10mg/kg lenzilumab or isotype control) IP on the same day of CART cell therapy for a total of 10 days.

Primary patient-derived ALL xenografts

To establish primary ALL xenografts, NSG mice first received 30mg/kg busulfan IP (Selleckchem, Houston, TX, USA). The following day, mice were injected with 2.5×10^6 primary blasts derived from the peripheral blood of patients with relapsed or refractory ALL. Mice were monitored for engraftment for ~10-13 weeks. When $CD19^+$ cells were consistently observed in the blood (approximately 1 cell/uL), they were randomized to receive different treatments of CART19 (2.5×10^6 cells IV) and PBMCs derived from the same donor (1×10^5 cells IV) with or without antibody therapy (10mg/kg lenzilumab or isotype control IP for a total of 10 days, starting on the day they received

CAR-T cell therapy). Mice were periodically monitored for leukemic burden via tail vein bleeding.

Primary patient-derived ALL xenografts for CRS/NI

Similar to the experiments above, mice were IP injected with 30mg/kg busulfan (Selleckchem, Houston, TX, USA). The following day, mice received 1-3x10^6 primary blasts derived from the peripheral blood of patients with relapsed ALL. Mice were monitored for engraftment for ~10-13 weeks via tail vein bleeding. When serum $CD19^+$ cells were ≥10 cells/ul, the mice received CART19 (2-5x10^6 cells IV) and commenced antibody therapy for a total of 10 days, as indicated. Mice were weighed on a daily basis as a measure of their well-being. Mouse brain MRIs were performed 5-6 days post CART19 injection and tail vein bleeding for cytokine/chemokine and T cell analysis was performed 4-11 days post CART19 injection.

MRI acquisition

A Bruker Avance II 7 Tesla vertical bore small animal MRI system (Bruker Biospin) was used for image acquisition to evaluate central nervous system (CNS) vascular permeability. Inhalation anesthesia was induced and maintained via 3 to 4% isoflurane. Respiratory rate was monitored during the acquisition sessions using an MRI compatible vital sign monitoring system (Model 1030; SA Instruments, Stony Brook, NY). Mice were given an IP injection of gadolinium using weight-based dosing of 100 mg/kg, and after a standard delay of 15 min, a volume acquisition T1-weighted spin echo sequence was used (repetition time = 150 ms, echo time = 8 ms, field of view: 32 mm × 19.2 mm × 19.2 mm, matrix: 160 × 96 × 96; number of averages = 1) to obtain T1-weighted images. Gadolinium-enhanced MRI changes were indicative of blood-

brain-barrier disruption.[24] Volumetric analysis was performed using Analyze Software package developed by the Biomedical Imaging Resource at Mayo Clinic.

RNA-Seq on mouse brain tissue

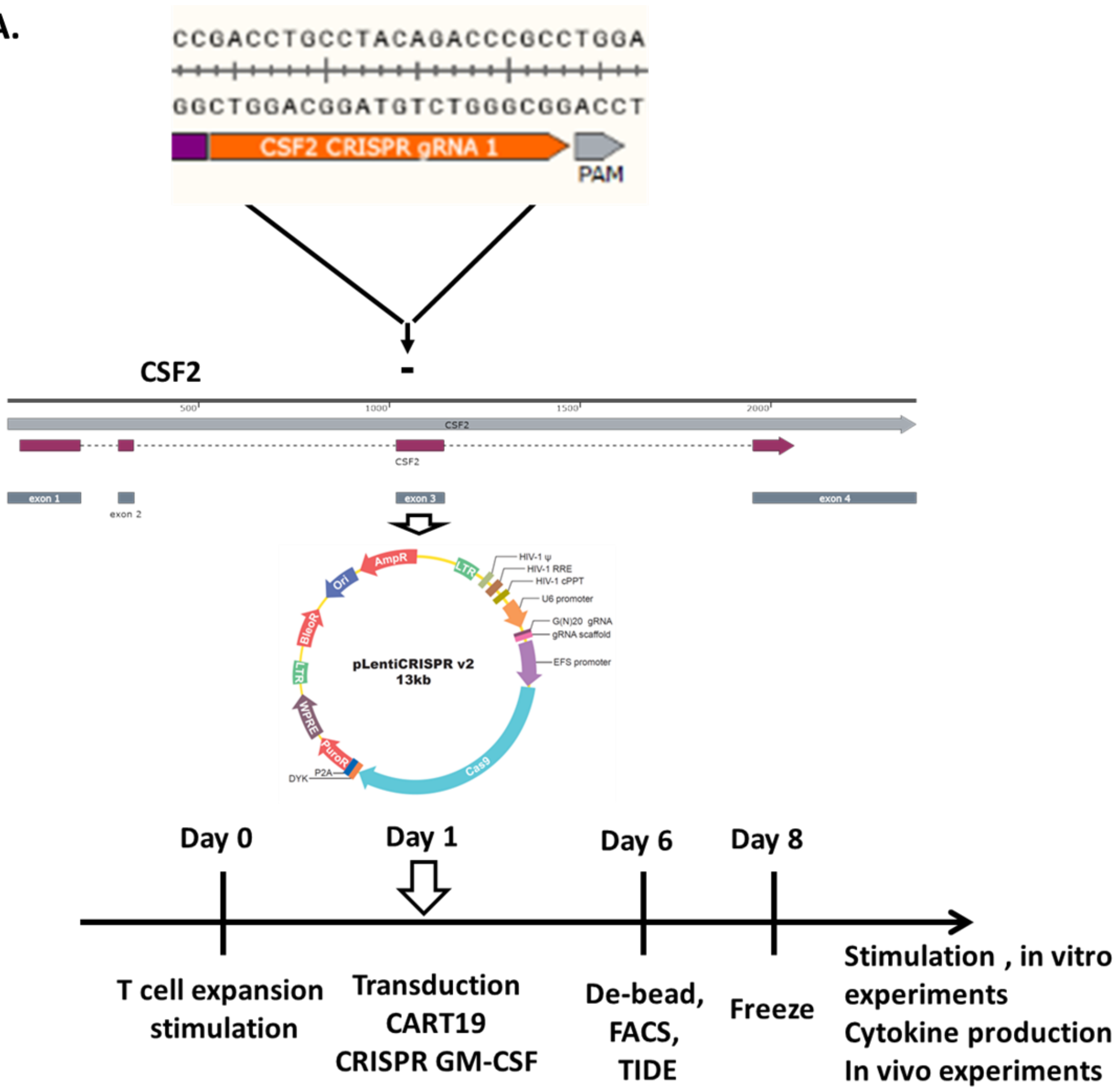
RNA was isolated using miRNeasy Micro kit (Qiagen, Gaithersburg, MD, USA) and treated with RNase-Free DNase Set (Qiagen, Gaithersburg, MD, USA). RNA-seq was performed on an Illumina HTSeq 4000 (Illumina, San Diego, CA, USA) by the Genome Analysis Core at Mayo Clinic. The binary base call data was converted to fastq using Illumina bcl2fastq software. The adapter sequences were removed using Trimmomatic,[34] and FastQC[35] was used to check for quality. The latest human (GRCh38) and mouse (GRCm38) reference genomes were downloaded from NCBI. Genome index files were generated using STAR[36], and the paired end reads were mapped to the genome for each condition. HTSeq[37] was used to generate expression counts for each gene, and DeSeq2[38] was used to calculate differential expression. Gene ontology was assessed using Enrichr.[39] Figure S2 summarize the steps detailed above. RNA sequencing data are available at the Gene Expression Omnibus under accession number GSE121591.

Statistics

Prism Graph Pad (La Jolla, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA) were used to analyze data. The high cytokine concentrations in the heat map were normalized to “1” and low concentrations normalized to “0” via Prism. Statistical tests are described in the figure legends.

Figure S1. Generation of GM-CSF$^{k/o}$ CART19 cells.

A.



B.

gRNA/Primer	Sequence
CSF2 Primer For	TGACTACAGAGAGGCACAGA
CSF2 Primer Rev	TCACCTCTGACCTCATTAACC
CSF2 CRISPR gRNA 1	GACCTGCCTACAGACCCGCC

Figure S1. Generation of GM-CSF$^{k/o}$ CART19 cells. Experimental schema: depicts the schema (A), gRNA sequence (B), and primer sequences (B) for generation of GM-CSF$^{k/o}$ CART19. To generate GM-CSF$^{k/o}$ CART19 cells, gRNA was cloned into a Cas9 lentivirus vector under the control of a U6 promotor and used for lentivirus production. T cells derived from normal donors were stimulated with CD3/CD28 beads and dual transduced with CAR19 virus and CRISPR/Cas9 virus 24 hours later. CD3/CD28 magnetic bead removal was performed on Day +6, and GM-CSF$^{k/o}$ CART19 cells or control CART19 cells were cryopreserved on Day 8.

Figure S2. Flow chart for procedures used in RNA sequencing.

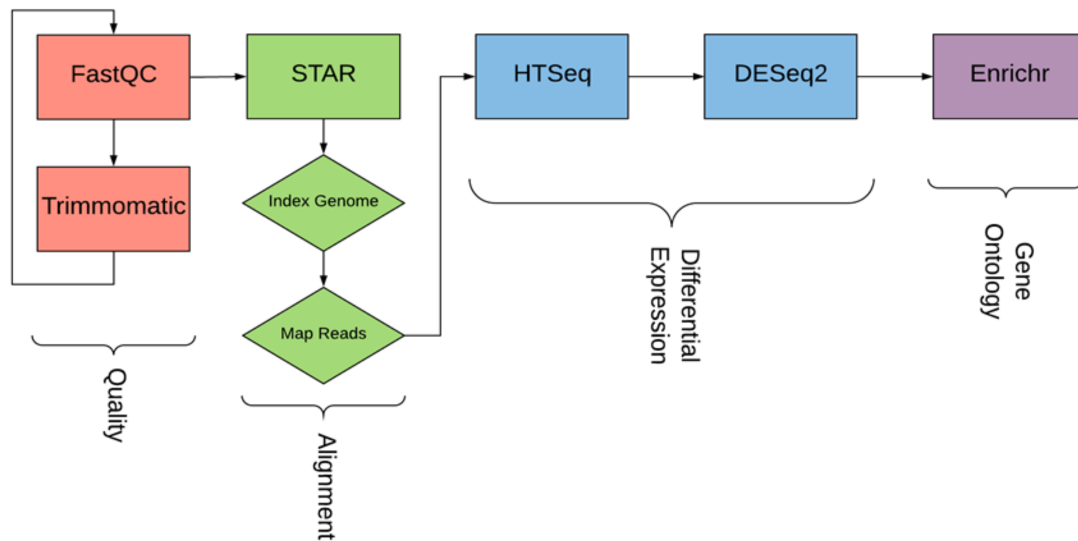


Figure S2. Flow chart for procedures used in RNA sequencing.

The binary base call data was converted to fastq using Illumina bcl2fastq software. The adapter sequences were removed using Trimmomatic,[28] and FastQC[29] was used to check for quality. The latest human (GRCh38) and mouse (GRCm38) reference genomes were downloaded from NCBI. Genome index files were generated using STAR,[30] and the paired end reads were mapped to the genome for each condition. HTSeq[31] was used to generate expression counts for each gene, and DeSeq2[32] was used to calculate differential expression. Gene ontology was assessed using Enrichr.[33]

Figure S3. Lenzilumab+CAR-T cell treated mice have comparable survival compared to isotype control+CAR-T cell treated mice in a high tumor burden relapse xenograft model of ALL.

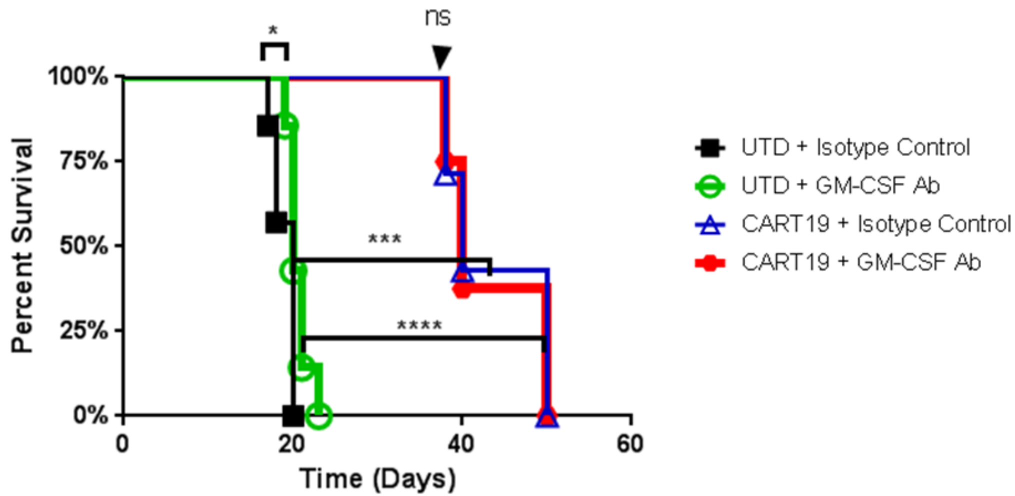


Figure S3. Lenzilumab+CAR-T cell treated mice have comparable survival compared to isotype control+CAR-T cell treated mice in a high tumor burden relapse xenograft model of ALL. n=2 experiments, 7-8 mice per group, representative experiment depicted, **** $p<0.0001$, *** $p<0.001$, * $p<0.05$, log-rank.

Figure S4. Representative TIDE sequence to verify genome alteration in the GM-CSF CRISPR Cas9 knockout CAR-T cells.

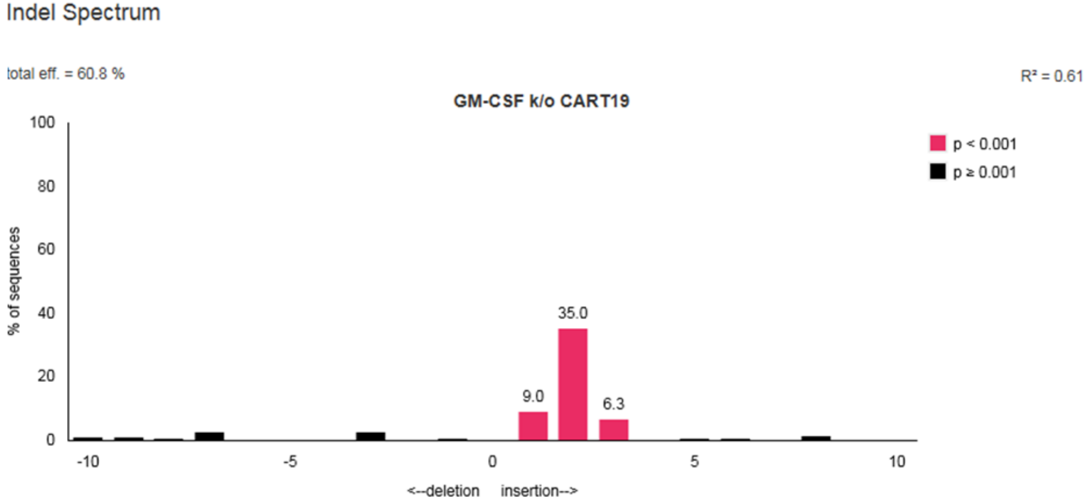


Figure S4. Representative TIDE sequence to verify genome alteration in the GM-CSF CRISPR Cas9 knockout CAR-T cells. n=2 experiments, representative experiment depicted.

Figure S5. GM-CSF knockout CAR-T cells *in vivo* show slightly enhanced control of tumor burden compared to wild type CAR-T cells in a high tumor burden relapse xenograft model of ALL.

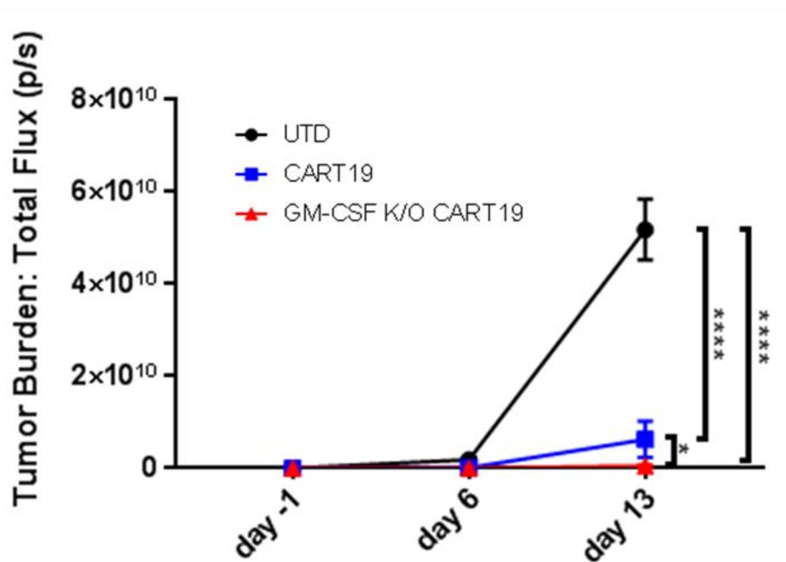


Figure S5. GM-CSF knockout CAR-T cells *in vivo* show slightly enhanced control of tumor burden compared to wild type CAR-T cells in a high tumor burden relapse xenograft model of ALL. Days post CAR-T cell injection listed on x-axis, 5-6 mice per group (2 remained in UTD group at day 13), representative experiment depicted, **** $p<0.0001$, * $p<0.05$, 2 way ANOVA, mean$\pm$SEM.

Figure S6. Patient derived xenograft model for neuro-inflammation and cytokine release syndrome with CART19+GM-CSF Ab treatment.

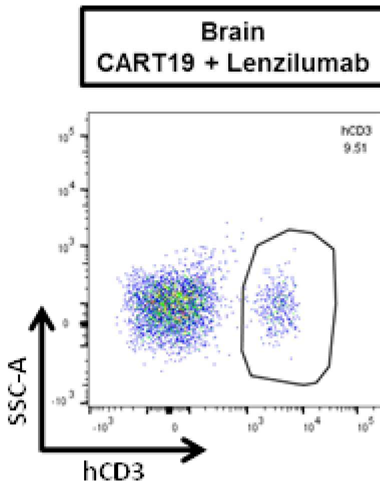


Figure S6. Patient derived xenograft model for neuro-inflammation and cytokine release syndrome with CART19+GM-CSF Ab treatment. High tumor burden primary ALL xenografts treated CART19+GM-CSF Ab treatment show human CD3 cell infiltration of the brain (Figure S6) compared to untreated PDX controls (Figure 4D). 3 mice per group, representative image.

Table S1. Table of canonical pathways altered in brains from patient derived xenografts after treatment with CART19 cells in tabular format.

Conical Pathway	Adj P-Value	Genes
regulation of immune response (GO:0050776)	9.45E-14	IFITM1, ITGB2, TRAC, ICAM3, CD3G, PTPN22, CD3E, ITGAL, SAMHD1, SLA2, CD3D, ITGB7, SLAMF6, B2M, NPDC1, CD96, BTN3A1, ITGA4, SH2D1A, HLA-B, HLA-C, BTN3A2, HLA-A, CD8B, SELL, CD8A, CD226, CD247, CLEC2D, HCST, BIRC3
cytokine-mediated signaling pathway (GO:0019221)	1.36E-12	IFITM1, SP100, TRADD, ITGB2, IL2RG, SAMHD1, IL27RA, OASL, CNN2, IL18RAP, RIPK1, CCR5, IL12RB1, B2M, GBP1, IL6R, JAK3, CCR2, IL32, ANXA1, IL4R, TGFB1, IL10RB, IL10RA, STAT2, PRKCD, HLA-B, HLA-C, IL16, HLA-A, TNFRSF1B, CD4, IRF3, OAS2, IL2RB, FAS, TNFRSF25, LCP1, P4HB, IL7R, MAP3K14, CD44, IL18R1, IRF9, MYD88, BIRC3
T cell receptor complex (GO:0042101)	1.30E-11	ZAP70, CD4, CD6, CD8B, CD8A, CD3G, CD247, CD3E, CD3D, CARD11
T cell activation (GO:0042110)	2.07E-11	ITK, RHOH, CD3G, NLRC3, PTPN22, CD3E, SLA2, CD3D, CD2, ZAP70, CD4, PTPRC, CD8B, CD8A, LCK, CD28, LCP1, LAT
regulation of T cell activation (GO:0050863)	2.46E-10	PTPN22, LAX1, CCDC88B, CD2, CD4, LCK, SIT1, TBX21, TIGIT, JAK3, LAT, PAG1, CCR2
T cell receptor signaling pathway (GO:0050852)	4.35E-08	ITK, BTN3A1, TRAC, WAS, CD3G, PTPN22, BTN3A2, CD3E, CD3D, ZAP70, CD4, PTPRC, LCK, GRAP2, LCP2, CD247, CARD11, LAT, PAG1
positive regulation of cytokine production (GO:0001819)	1.57502E-07	GBP5, ANXA1, TGFB1, CYBA, PTPN22, PARK7, TMEM173, CCDC88B, MAVS, CD6, IRF3, CD28, RIPK1, SLAMF6, CD46, IL12RB1, TIGIT, IL6R, CARD11, MYD88, CCR2
T cell differentiation (GO:0030217)	2.36E-07	ZAP70, CD4, ANXA1, PTPRC, CD8A, LCK, CD28, RHOH, PTPN22, CD3D
cytokine receptor activity (GO:0004896)	2.43E-07	IL4R, IL10RB, IL10RA, IL2RG, CD4, CXCR3, IL2RB, CCR5, IL12RB1, IL7R, IL6R, CD44, CCR2
type I interferon signaling pathway (GO:0060337)	3.27E-07	IFITM1, SP100, IRF3, OAS2, STAT2, HLA-B, HLA-C, HLA-A, SAMHD1, IRF9, MYD88, OASL
response to cytokine (GO:0034097)	0.0004679	SIGIRR, IFITM1, SP100, HCLS1, RIPK1, PTPN7, IKBKE, IL6R, JAK3, IL18R1, MYD88, AES
regulation of innate immune response (GO:0045088)	0.001452	GBP5, GFI1, STAT2, ADAM8, NLRC3, PTPN22, SAMHD1, BIRC3
regulation of tumor necrosis factor production (GO:0032680)	0.003843	CD2, MAVS, CYBA, NLRC3, PTPN22, RIPK1, SLAMF1
T cell receptor binding (GO:0042608)	0.0102397	LCK, CD3G, CD3E
regulation of tumor necrosis factor-mediated signaling pathway (GO:0010803)	0.0124059	SHARPIN, TRADD, CASP4, RIPK1, TRAF1, BIRC3
positive regulation of myeloid leukocyte differentiation (GO:0002763)	0.0376647	CD4, HCLS1, RIPK1, EVI2B

Table S1. Table of canonical pathways altered in brains from patient derived xenografts after treatment with CART19 cells in tabular format.