Supplemental Methods

Flow cytometry

T cell immunophenotypes were investigated with flow cytometry using combinations of the following antibodies: anti-human FcR, anti-CD3 (HIT3a), anti-CD19 (H1B19), anti-CD4 (Sk.3), anti-CD8 (HIT8a), anti-CD57 (HNK-1), anti-Lag3 (3DS223H), anti-Tim3 (F38-2E2), anti-PD1 (EH12.2H7), anti-CD39 (A1), anti-CD127 (A019D5), anti-CD45RA (HI100), anti-CD45RO (UCHL1), anti-CD62L (DREG-56), anti-CCR7 (G043H7), anti-CD25 (BC96), anti-CD43 (CD43-10G7), anti-CD95 (DX2), anti-CD27 (LG.7F9), anti-CD40 (5C3), and anti-CD154 (CD40L; clone 24-31). All antibodies were purchased from Biolegend, eBioscience, or Jackson ImmunoResearch. Data were acquired using an LSRFortessa flow cytometer (BD Bioscience) and analyzed with FlowJo Software (Treestar).

Cytotoxicity assay

To assess cytotoxic function of CAR T cells, Raji-ffLuc target cells were labeled overnight with ^{51}Cr (PerkinElmer, Waltham, MA), washed, and dispensed at 2×10^3 cells per well in 96 well round bottom plates. FACS-sorted CD8$^+$ tCD19$^+$ or CD4$^+$ tCD19$^+$ T cells from mixed or separate cultures were added at various effector to target (E:T) ratios. After 4 hours, supernatants were harvested into 96 well Lumaplates, air-dried overnight, and counts assayed with a TopCount (PerkinElmer, Waltham, MA). Specific lysis was calculated as previously described (1).

RNA-seq of CD8$^+$ CAR T cells

Total RNA was isolated from FACS-sorted tCD19$^+$ CD8$^+$ CAR T cells at either day 8 (without restimulation) or day 14 (after restimulation with CD20$^+$ LCL cells at day 7) using the RNeasy kit (Qiagen). Total RNA integrity was assessed using an Agilent 4200 TapeStation (Agilent) and quantified using a Trinean DropSense96 spectrophotometer (Caliper Life Sciences). Day 8 RNA-seq libraries were prepared from total RNA using the TruSeq RNA Exome library prep kits (Illumina), and day 14 libraries

were prepared from total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech) and the Nextera XT DNA Library Preparation Kit (Illumina). Library size distribution was validated using an Agilent 4200 TapeStation. Additional library QC, blending of pooled indexed libraries, and cluster optimization was performed using a Qubit 2.0 Fluorometer (Life Technologies-Invitrogen). RNA-seq libraries were pooled (12-plex for day 8 samples and 17-plex for day 14 samples) and clustered onto 1 (day 8) or 3 (day 14) flow cell lanes. Sequencing was performed using an Illumina HiSeq 2500 in rapid mode employing a paired-end, 50 base read length (PE50) sequencing strategy. Image analysis and base calling were performed using Illumina's Real Time Analysis v1.18 software, followed by demultiplexing of indexed reads and generation of FASTQ files, using Illumina's bcl2fastq Conversion Software v1.8.4.

RNA-seq analysis

FASTQ files were filtered to exclude reads that did not pass Illumina's base call quality threshold. For the day 14 samples, STAR v2.5.2a (2) with 2-pass mapping was used to align paired-end reads to the human genome GRCh38.p12 assembly, employing UCSC hg38 annotations. FastQC 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and RSeQC 3.0.0 (3) were used for quality control, including insert fragment size, read quality, read duplication rates, gene body coverage, and read distribution in different genomic regions. FeatureCounts (4) from the Subread 1.6.0 package was used to quantify gene-level expression values, which were used in the Bioconductor package edgeR 3.20.9 (5) to detect differential gene expression between conditions. Genes with low expression were excluded by requiring at least one count per million in at least N samples (N is equal to one less than the number of samples in the smallest group). The filtered expression matrix was normalized by TMM method (6) and subjected to differential expression testing by edgeR using the GLM LRT method and paired donor as a blocking factor. Genes were deemed differentially expressed if absolute fold changes were 1.5 or higher and FDRs were less than 0.05. Bioconductor package GOseq v1.30.0 (7) was used to perform enrichment analysis on differentially expressed genes against REACTOME pathways (8). Strong co-expression of

multiple genes within a pathway may bias GOseq analysis because GOseq considers all genes in a pathway as independent. This is particularly problematic for smaller pathways where co-expressed genes may constitute a large proportion of pathway genes. We filtered out pathways with ≤ 50 genes in which a small number of principal components explain the majority of gene expression variation of the pathway. Day 8 samples were processed at a later time, where more recent versions of the same tools were utilized, with notable differences being that GENOCODE V31 gene annotations were used, and the samples were prepared using a strand specific library preparation kit. While technical differences from using different library preparation methods may introduce a batch effect, samples from both days were not directly compared, mitigating this potential issue. Genes were ranked by logFC in descending order for GSEAPreranked (9) Gene Set Enrichment Analysis against selected gene sets from the Molecular Signatures DataBase (MSigDB [https://www.gsea-msigdb.org/gsea/msigdb/index.jsp]).

Evaluation the effect of CD40L-CD40 and CD70-CD27 interactions

Resting $CD4^+$ or $CD8^+$ T cells were stimulated with anti-CD3/CD28 antibody-coated beads and immediately transferred either to tissue culture plate wells coated with 1 µg/ml agonistic anti-CD40 antibody (clone 82111, R&D Systems) or 1 µg/ml of agonistic anti-CD27 (BPS Bioscience), or transferred to uncoated wells, where 1 µg/ml of antagonistic anti-CD40L (clone 24-31, Biolegend) or 1 µg/ml of antagonistic anti-CD70 (clone 113-16, Biolegend) were added to the culture medium. For experiments with agonistic antibodies, $CD8^+$ cells were cultured without $CD4^+$ cells, and for experiments with antagonistic antibodies, $CD8^+$ cells were co-cultured with $CD4^+$ cells at a 60:40 CD4:CD8 ratio. Cells were transduced with 1.5.3-NQ-28-BB-z lentivirus on day 1 after bead stimulation, and beads were removed at day 4. At day 8, cells were counted, and phenotypic exhaustion and memory markers were assessed by flow cytometry as described above. For the proliferation assay, T cells were harvested and resuspended in pre-warmed PBS (0.1% BSA) at a final concentration of $1x10^6$ cells/ml. The same volume of diluted CTV solution (5 µM) was added and incubated at 37 °C for 10 min. Staining was quenched by

the addition of 5 volumes of ice-cold culture medium to the cells and 5 minutes of incubation on ice. T cells were centrifuged, resuspended in culture medium (without IL-2), and mixed in a 1:1 ratio with irradiated Raji-ffLuc cells for restimulation. After 4 days of incubation, cells were evaluated by flow cytometry for CTV dilution. In some experiments supernatants of CTV-stained cells were harvested 24 hours after restimulation and cytokines measured by Luminex assay.

In vivo tumor model experiments

Seven- to 9-week-old randomized female NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NOD/SCID/$\gamma^{-/-}$ [NSG]) mice were bred in-house. Mice were injected with 5 x 10^5 Raji-ffLuc tumor cells via tail vein. At 7 days after tumor injection, mice were injected by tail vein with a suboptimal dose of either 1 x 10^6 or 2 x 10^6 tCD19$^+$ CAR-modified T cells, derived from healthy donors, that had been expanded either in separate CD4$^+$ and CD8$^+$ cell cultures (using cells restimulated with LCL at day 7 and harvested at day 14-15) and then mixed at a 1:1 ratio of tCD19$^+$ T cells prior to infusion, or in mixed cultures initiated at either a 70:30 or 60:40 CD4:CD8 ratio. Mixed CD4:CD8 T cells that were untransduced or transduced with a vector containing the tCD19 marker but no CAR ("empty vector") were used as negative controls. Mice were euthanized per institutional guidelines for symptoms of progressive tumor growth, including hind-limb paralysis or > 20% weight loss. The primary endpoint was tumor response (total tumor burden over time) as measured using bioluminescence imaging. Luciferase activity was analyzed in mice anesthetized with isoflurane 10 minutes after intraperitoneal injection of D-luciferin potassium salt (BioVision) at 150 mg/kg. Sample sizes were based on expected effect sizes based on prior experiments using this model. No exclusion criteria were set other than the age at study initiation. No mice were excluded from the analysis. Confounders such as cage location were not controlled. Investigators analyzing data were blinded but investigators collecting data were not blinded. Mice were imaged in a Xenogen IVIS Spectrum Imaging System (Caliper Life Sciences). Binning and exposure were adjusted to achieve maximum sensitivity

without leading to image saturation. Living Image software was used to analyze the luminescent image data. Total bioluminescent signal was obtained as photons/s/cm^2/sr.

Study Approval

Human subjects provided written informed consent under Institutional Review Board-approved research protocols at the Fred Hutchinson Cancer Center (FHCC) in accordance with the Declaration of Helsinki. Mouse experiments were conducted under a protocol approved by the FHCC Institutional Animal Care and Use Committee.

Data Sharing Statement

All data associated with this study are present in the paper or the supplemental materials available with the online version of this article. The RNA-seq data are accessible on a public depository (accession number GSE245427), which can be found on the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE245427. For other original data, please contact tillb@fredhutch.org.

Supplemental References

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8. Jassal B, Matthews L, Viteri G, Gong C, Lorente P, Fabregat A*, et al.* The reactome pathway knowledgebase. Nucleic Acids Res **2020**;48(D1):D498-D503 doi 10.1093/nar/gkz1031.
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Supplemental Data

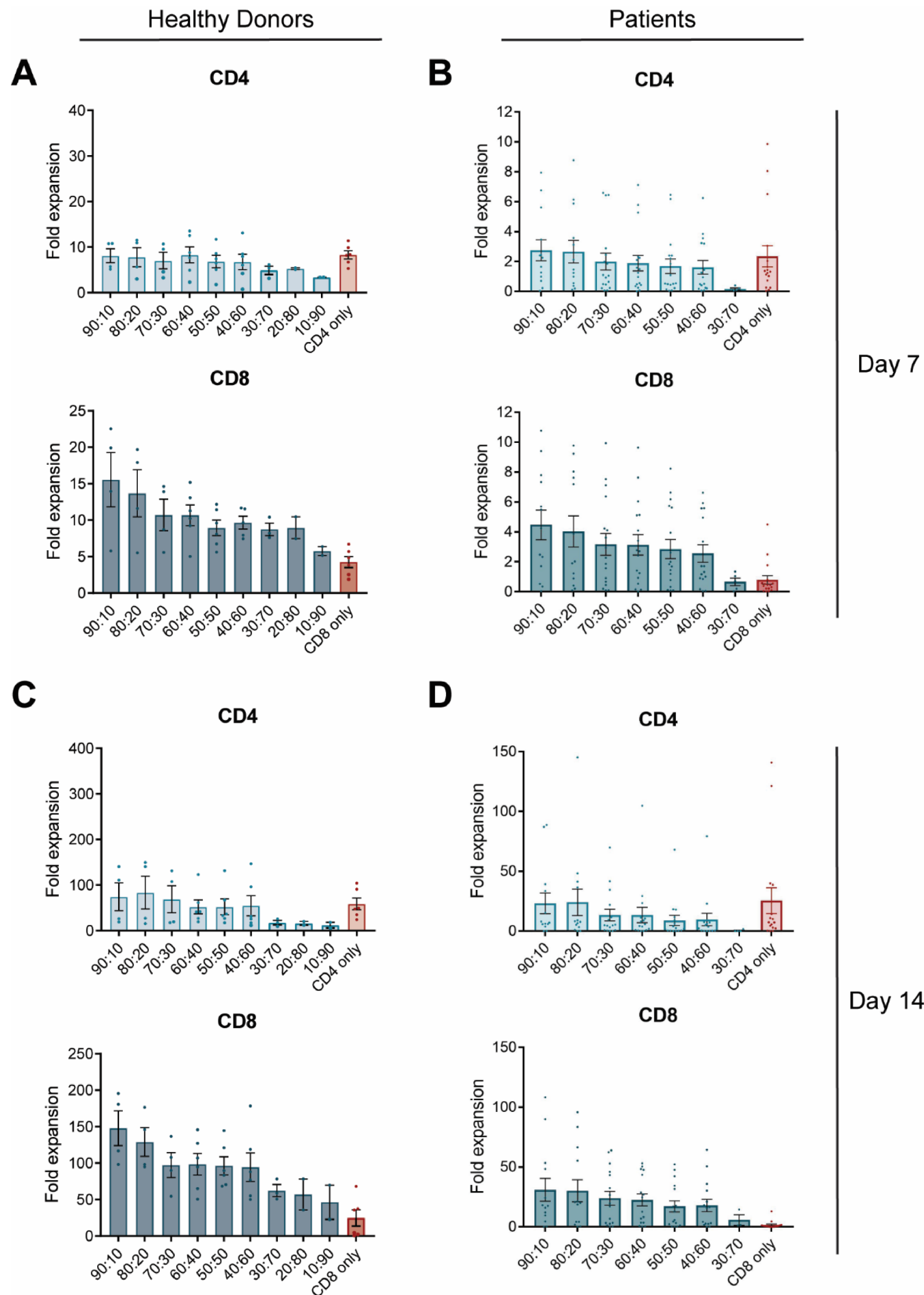


Figure S1. Fold expansion of CD4⁺ and CD8⁺ CD20-targeted CAR T cells manufactured in isolation or in CD4⁺/CD8⁺ co-cultures. The fold expansion of CAR T cells correlating with Figure 1 experiments are shown for cells derived from healthy donors or lymphoma patients, either at day 7 (before restimulation) or day 14 (after restimulation with CD20⁺ target cells). CD4:CD8 ratios at culture initiation are shown on the x-axis. Please see Figure 1 legend for experimental details. Individual data points are shown, along with mean ± SEM.

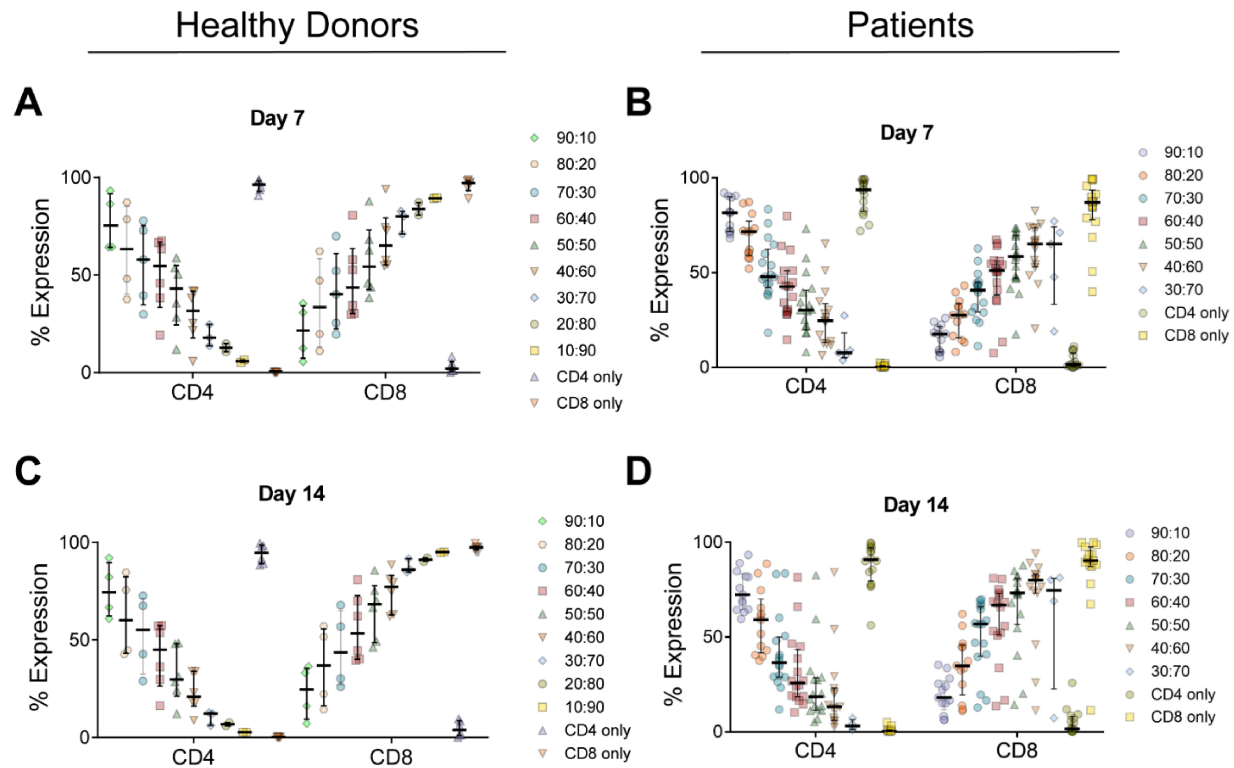


Figure S2. Percentages of CD4⁺ and CD8⁺ cells at cell harvest based on various CD4:CD8 ratios at culture initiation. See Figure 1 legend for experimental setup. The percentages used to calculate the ratios in Figure 1 (C-F) are shown for healthy donors (A, C) and patients (B, D) at day 7 (A, B) or day 14-15 (C, D). Non-zero values for CD8 in "CD4-only" and for CD4 in "CD8-only" reflect limitations in purity achieved in the selection process.

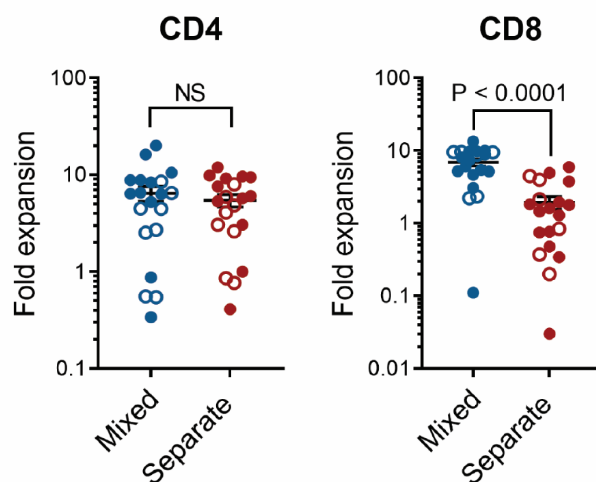


Figure S3. Expansion of 3rd-generation CD20 CAR T cells manufactured in separate or mixed cultures without restimulation. The fold expansion of CD4$^+$ and CD8$^+$ T cells transduced with 1.5.3-NQ-28-BB-z and expanded in separate (red) or in a mixed 60:40 CD4:CD8 ratio at culture initiation (blue) is shown for cells harvested at day 9 without restimulation (n = 20: 14 healthy donors [filled circles] and 6 patients [open circles]). The mean ± SEM is shown along with individual values. Statistical comparisons were made using paired t-tests for samples meeting criteria for normality based on D'Agostino & Pearson normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.

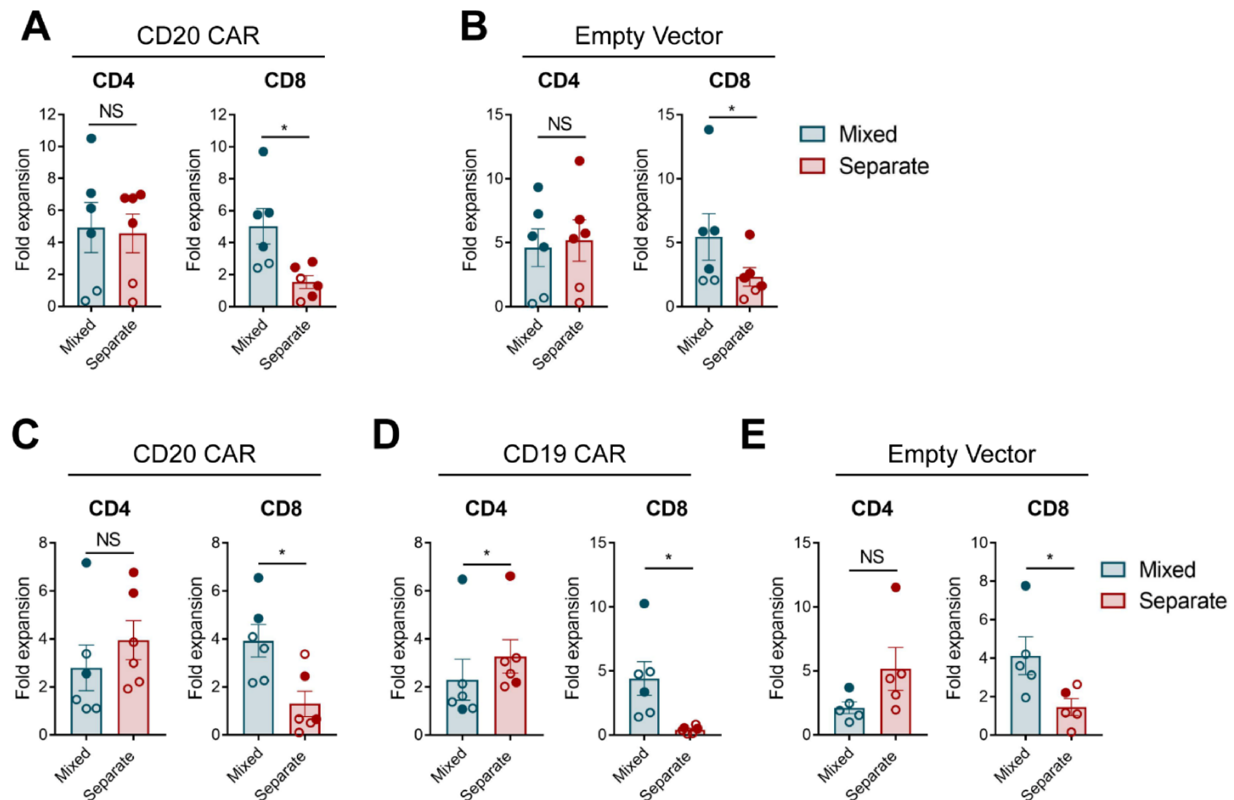


Figure S4. Impact of mixed cultures on CD8⁺ cell growth is not limited to CD20 CAR T cells. (A-B) CD4⁺ and CD8⁺ cells (n = 6: 4 healthy donors [filled circles] and 2 patients [open circles]) were stimulated, then either mixed at a 60:40 CD4:CD8 ratio or maintained in separate cultures, then transduced with lentiviral vector encoding the 1.5.3-NQ-28-BB-z CD20 CAR plus tCD19 (A) or a vector encoding the tCD19 without the CAR (B, "empty vector"), and expanded in culture for 7 days. (C-E) In separate experiments, cells were manufactured as in (A-B) but in addition to the CD20 CAR (C) and empty vector (E), cells were also transduced with a lentiviral vector encoding the hCD19-BB-z CAR (D) (n = 6: 2 healthy donors and 4 patients; the experiment with one of the healthy donors did not include an empty vector control). The mean fold expansion of CD4⁺ and CD8⁺ cells relative to their starting number at day 7 is shown (± SEM). Statistical comparisons were made using paired t-tests for samples meeting criteria for normality based on D'Agostino & Pearson or Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria (*: $P < 0.05$).

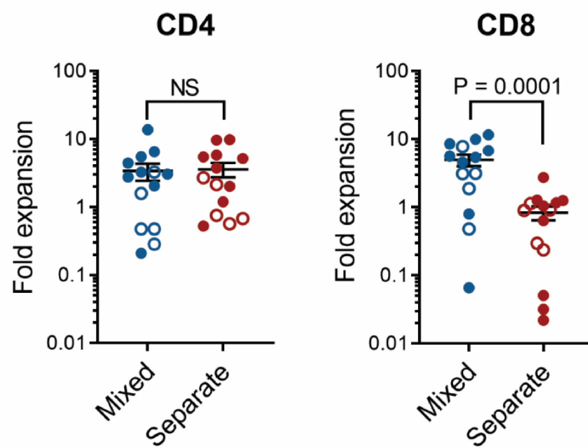


Figure S5. Expansion of 2nd generation CD20 CAR T cells manufactured in separate or mixed cultures without restimulation. The fold expansion of $CD4^+$ and $CD8^+$ T cells transduced with 1.5.3-NQ-28-z and expanded in separate (red) or in a mixed 60:40 CD4:CD8 ratio at culture initiation (blue) is shown for cells harvested at day 9 without restimulation (n = 14: 9 healthy donors [filled circles] and 5 patients [open circles]). The mean ± SEM is shown along with individual values. P-values were determined using Wilcoxon matched-pairs signed rank test.

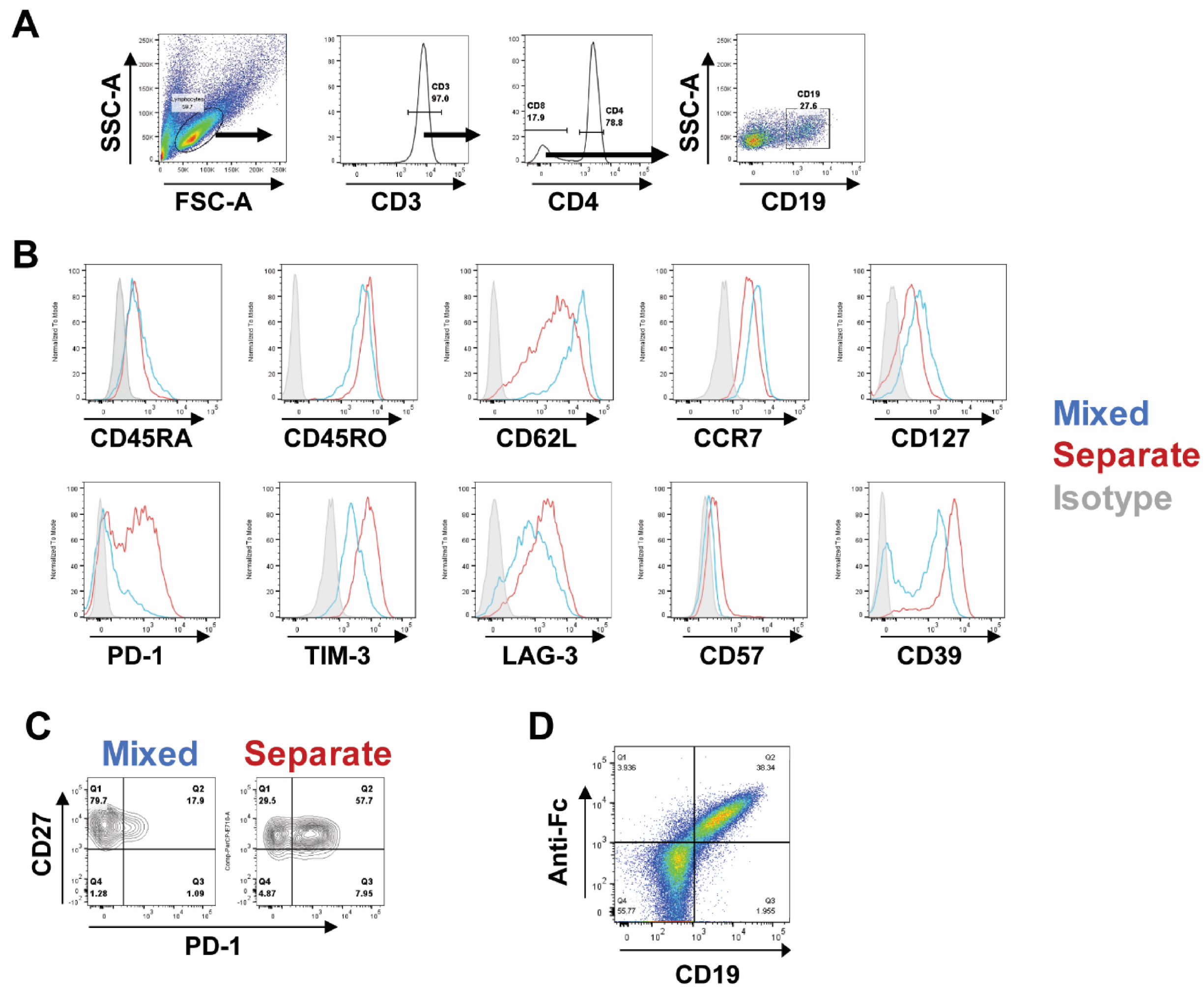


Figure S6. Gating strategy and representative histograms of CD8⁺ immunophenotypic analysis. (A) Gating strategy used for immunophenotyping in Figures 2, S7, S8, and S9. (B) Representative histograms for day 14 CD8⁺ patient-derived 1.5.3-NQ-28-BB-z CAR T cells grown in CD4⁺ co-cultures or separate CD8-only cultures. (C) Representative contour plots for CD27/PD-1 analysis to assess CD27⁺/PD-1⁻ cells, gated on CD8⁺ tCD19⁺ cells from day 14. (D) Dot plot showing co-expression of the CAR (anti-human IgG Fc staining the CAR spacer domain) and tCD19 in transduced T cells.

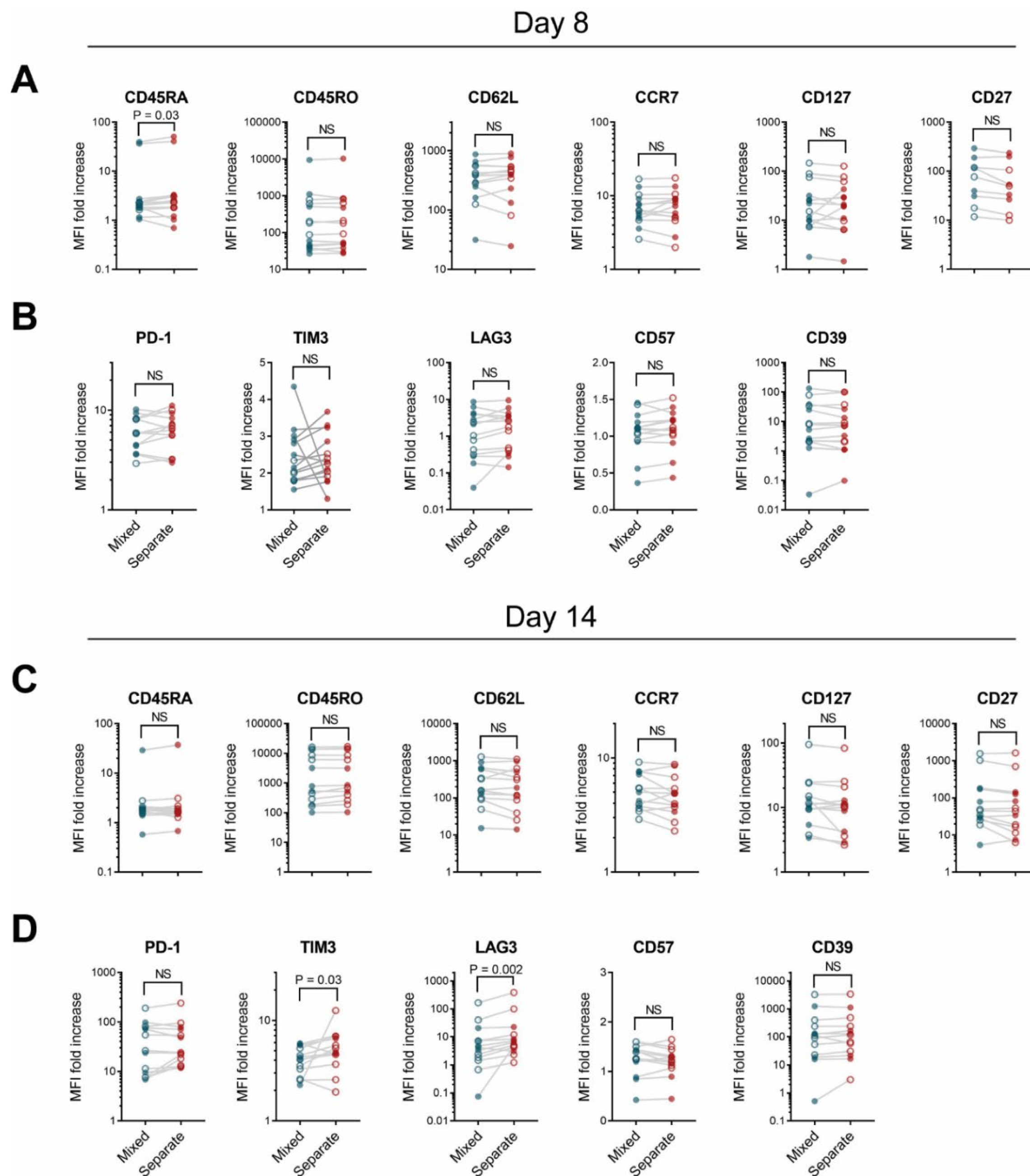


Figure S7. Phenotypes of CD4⁺ 3rd generation CD20 CAR T cells cultured alone or with CD8⁺ T cells. CD4⁺ or CD8⁺ enriched PBMC from healthy donors (filled circles) or patients (open circles) were stimulated, then either mixed at a 60:40 CD4:CD8 ratio or maintained in separate cultures, then transduced with 1.5.3-NQ-28-BB-z CD20 CAR lentiviral vector. (A-B) Cells were harvested on day 8 of cell culture without restimulation (n = 14: 9 healthy donors and 5 patients), or (C-D) restimulated with irradiated CD20⁺ LCL cells on day 7 and harvested on day 14 (n = 13: 6 healthy donors and 7 patients). Cells were evaluated by multiparameter flow cytometry for markers of memory and differentiation (A, C) or exhaustion (B, D). Data represent the fold increase in geometric mean fluorescence intensity (MFI) over isotype control gated on viable CD4⁺ tCD19⁺ CAR T cells. P-values were determined using paired two-tailed t-tests for markers meeting criteria for normality based on D'Agostino & Pearson or Shapiro-Wilk tests, or Wilcoxon matched-pairs signed rank test for markers not meeting normality criteria.

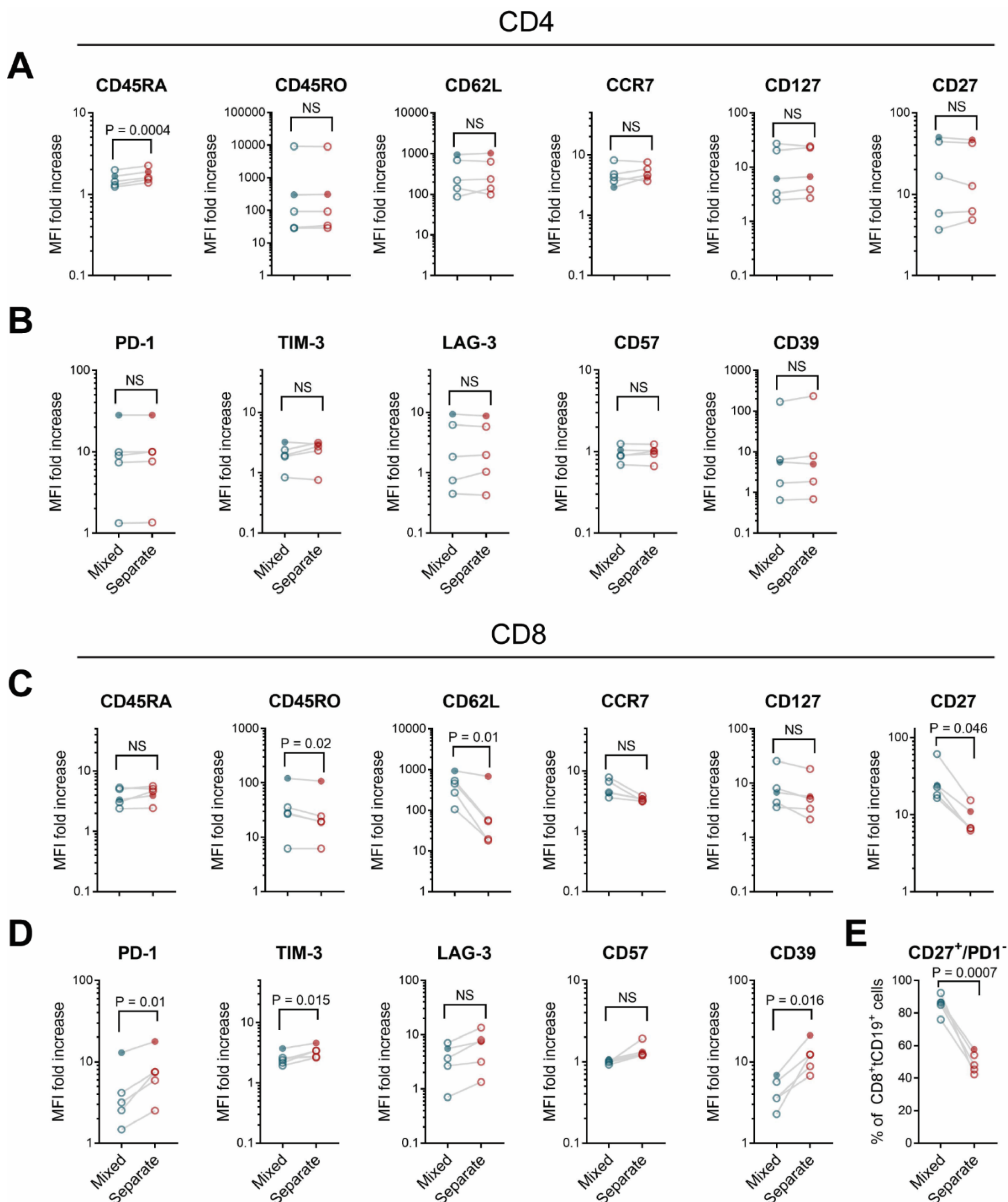


Figure S8. Phenotypes of CD19-targeted CAR T cells cultured in separate or mixed CD4⁺ and CD8⁺ cultures. CD4⁺ or CD8⁺ enriched PBMC were stimulated, then either mixed at a 60:40 CD4:CD8 ratio or maintained in separate cultures, then transduced with the hCD19-BB-z CAR lentiviral vector. Cells were harvested on day 8 of cell culture without restimulation. CD4⁺ CAR T cells (A-B), and CD8⁺ CAR T cells (C-E) were evaluated by multiparameter flow cytometry for markers of memory and differentiation (A, C), exhaustion (B, D), or for the percentage of CD27⁺ PD1⁻ cells among CD8⁺ CAR T cells (E). Data represent the fold increase in geometric mean fluorescence intensity (MFI) over isotype control gated on viable CD4⁺ or CD8⁺ CAR⁺ T cells for T cells derived

from healthy donors (n = 1, filled circle) or patients (n = 4, open circles). P-values were determined using paired two-tailed t-tests for markers meeting criteria for normality based on Shapiro-Wilk tests, or Wilcoxon matched-pairs signed rank test for markers not meeting normality criteria.

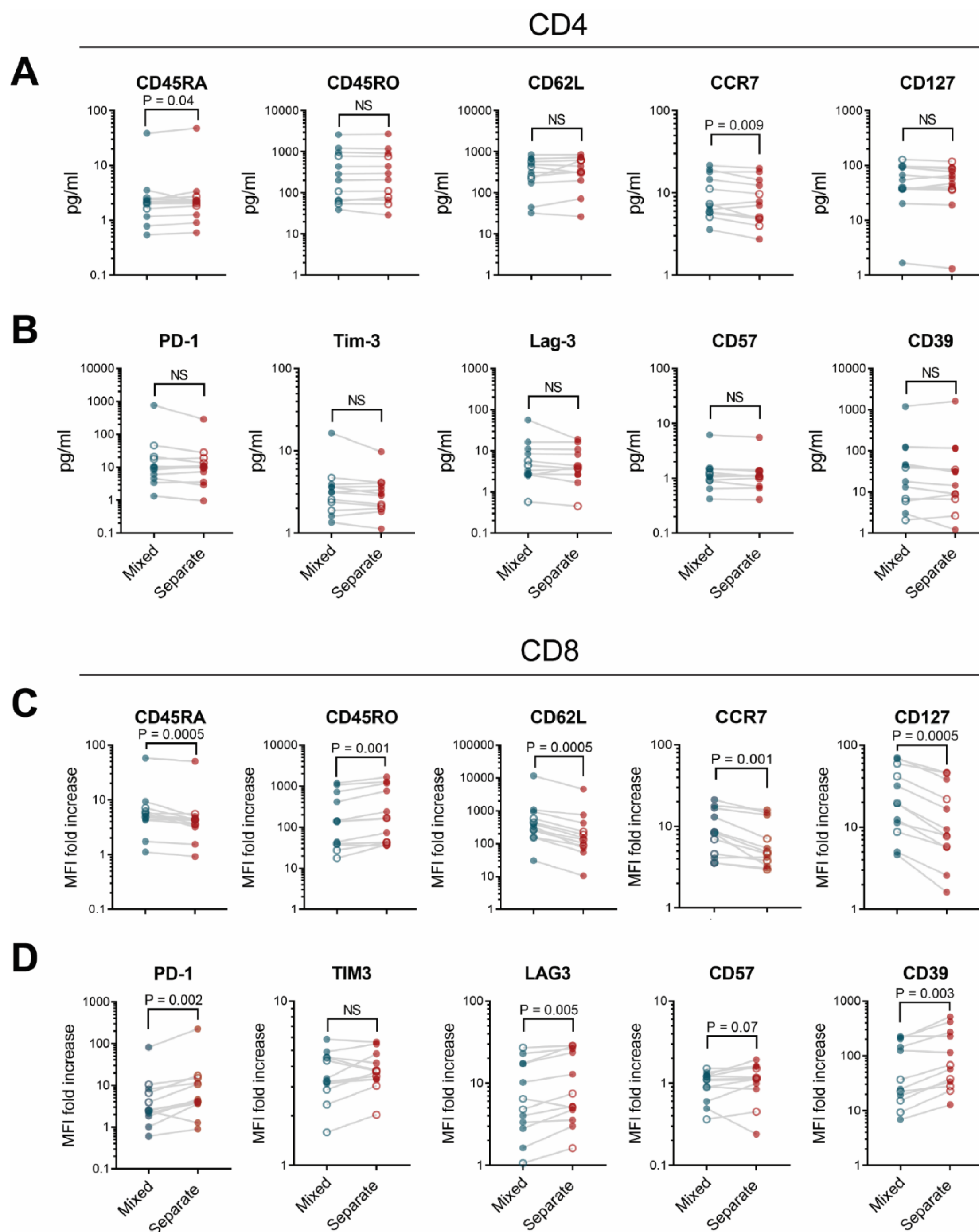
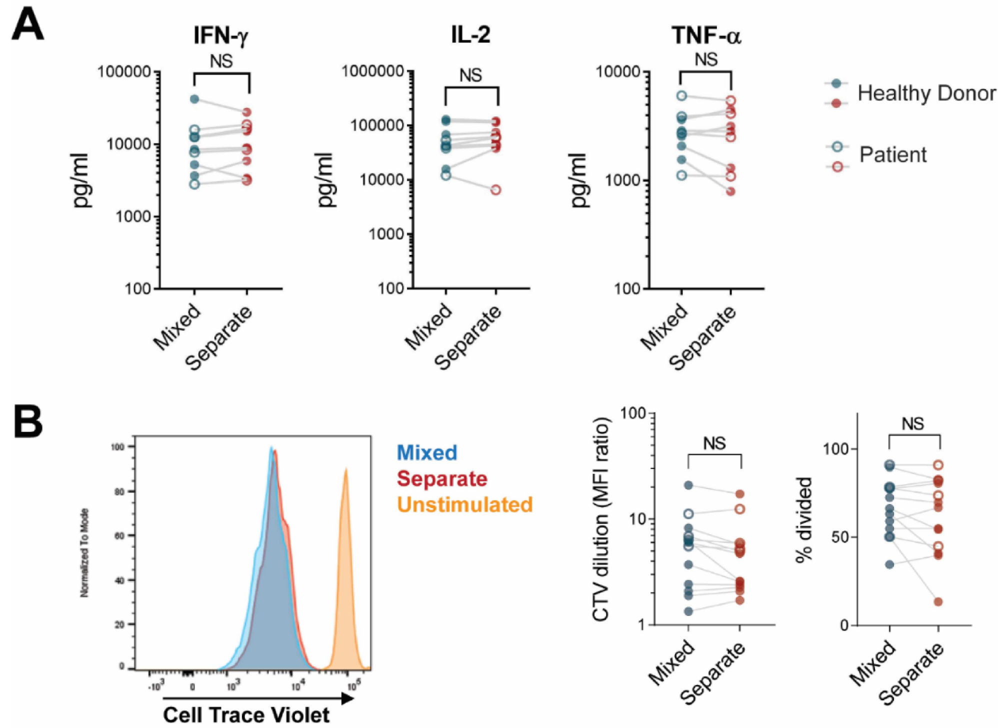


Figure S9. Phenotypes of 2nd generation CD20-targeted CAR T cells with CD28 costimulatory domain cultured in separate or mixed CD4⁺ and CD8⁺ cultures. CD4⁺ or CD8⁺ enriched PBMC were stimulated, then either mixed at a 60:40 CD4:CD8 ratio or maintained in separate cultures, then transduced with the 1.5.3-NQ-28-z CAR lentiviral vector. Cells were harvested on day 8 of cell culture without restimulation. CD4⁺ CAR T cells (A-B), and CD8⁺ CAR T cells (C-D) were evaluated by multiparameter flow cytometry for markers of memory and differentiation (A, C) or exhaustion (B, D). Data represent the fold increase in geometric mean fluorescence intensity (MFI) over isotype control gated on viable CD4⁺ or CD8⁺ CAR⁺ T cells for T cells derived from a healthy donors (n = 8, filled circles) or patients (n = 4, open circles). P-values were determined using paired two-tailed t-

tests for markers meeting criteria for normality based on D’Agostino & Pearson or Shapiro-Wilk normality tests, or Wilcoxon matched-pairs signed rank test for markers not meeting normality criteria.


Day 8
A
IFN-γ
IL-2
TNF-α
NS
pg/ml
Mixed
Separate
Healthy Donor
Patient
B
Mixed
Separate
Unstimulated
Normalized To Mode
Cell Trace Violet
CTV dilution (MFI ratio)
% divided

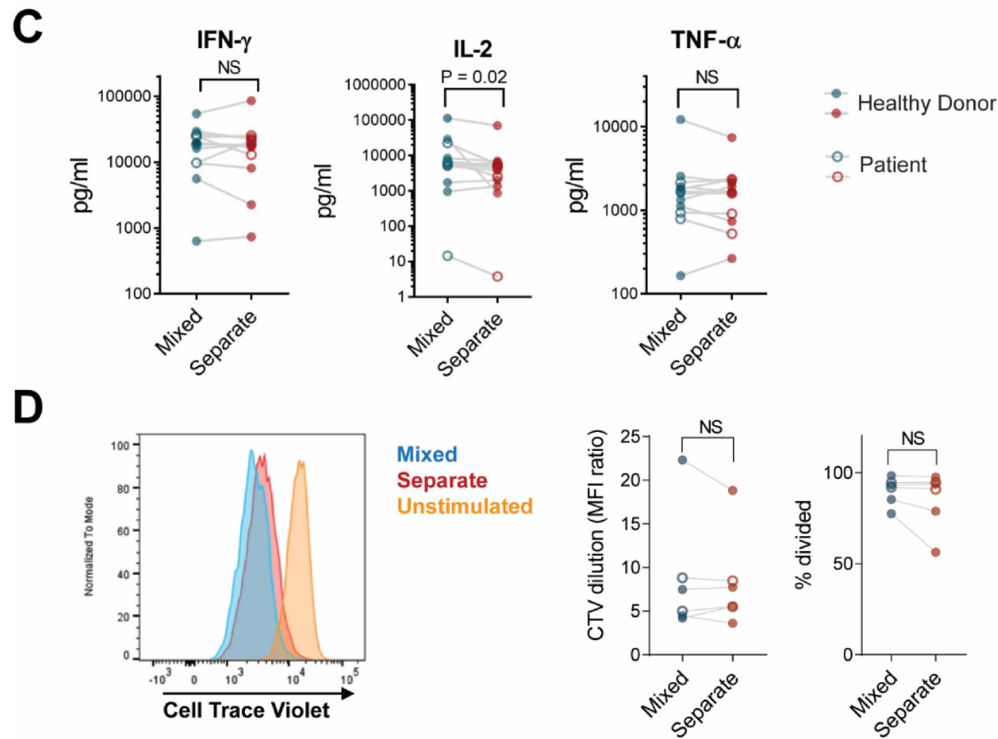
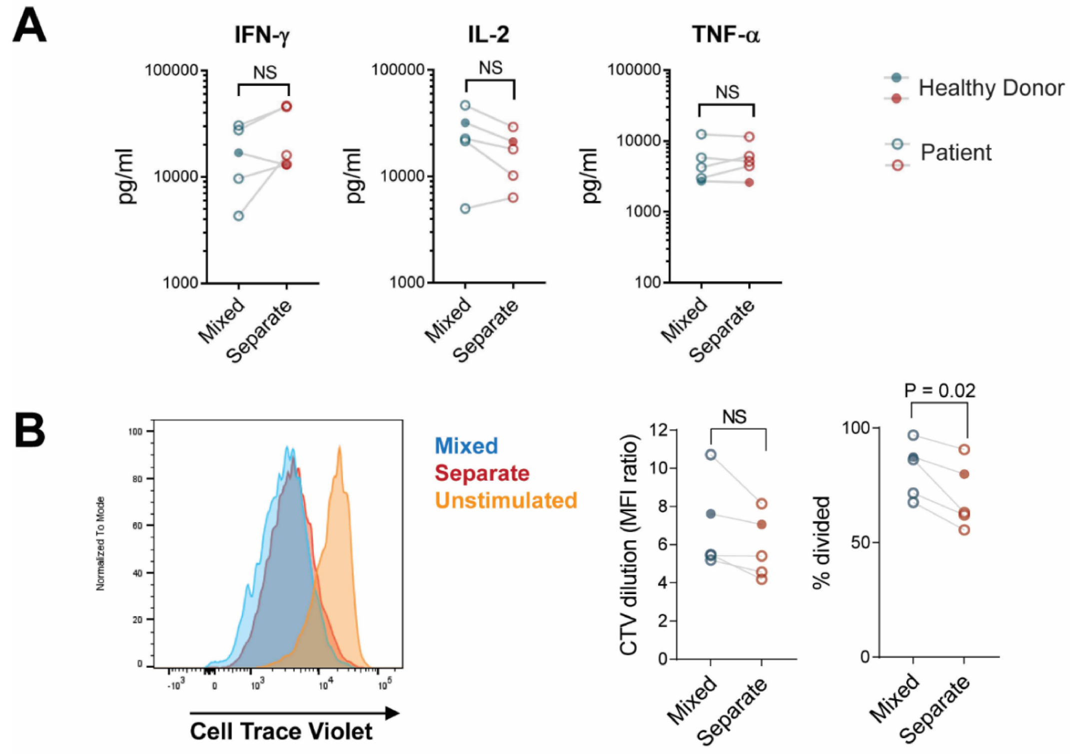

Day 14
C
IFN-γ
IL-2
TNF-α
NS
P = 0.02
pg/ml
Mixed
Separate
Healthy Donor
Patient
D
Mixed
Separate
Unstimulated
Normalized To Mode
Cell Trace Violet
CTV dilution (MFI ratio)
% divided

Figure S10. In vitro function of 3rd generation CD4+ CAR T cells manufactured in co-cultures with CD8+ cells is similar to that of CD4+ CAR T cells cultured separately. CD4+ and CD8+ enriched PBMC from patients (open circles) or healthy donors (filled circles) were stimulated with beads, mixed at a 60:40 ratio of CD4:CD8 cells or kept in separate cultures, transduced with 1.5.3-NQ-28-BB-z CD20 CAR, and expanded. Cells were harvested on day 8 of cell culture without restimulation (A, B), or restimulated with irradiated CD20+ LCL cells on day 7 and harvested on day 14 (C, D). FACS-sorted CD4+ tCD19+ T cells were labeled with Cell Trace Violet (CTV) and incubated with irradiated CD20+ Raji-ffLuc cells at a 1:1 ratio. (A, C) Supernatants were harvested at 24 hours and the indicated cytokines were measured by Luminex assay (n = 9: 4 patients and 5 healthy donors for day7; n = 12: 4 patients and 8 healthy donors for day 14). (B, D) Proliferation of the sorted cells after 4 days based on CTV dilution was assessed by flow cytometry. Representative histograms are shown in the left panel, and summary data of geometric MFI ratio of stimulated to unstimulated cells and % divided cells are shown in the right panels (n = 13: 4 patients and 9 healthy donors for day 7; n = 6: 2 patients and 4 healthy donors for day 14). P-values were determined using paired two-tailed t-tests for samples meeting criteria for normality based on D’Agostino & Pearson or Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.


CD4
A
IFN-γ
IL-2
TNF-α
NS
pg/ml
Mixed
Separate
Healthy Donor
Patient
B
Mixed
Separate
Unstimulated
Normalized To Mode
Cell Trace Violet
CTV dilution (MFI ratio)
% divided
P = 0.02

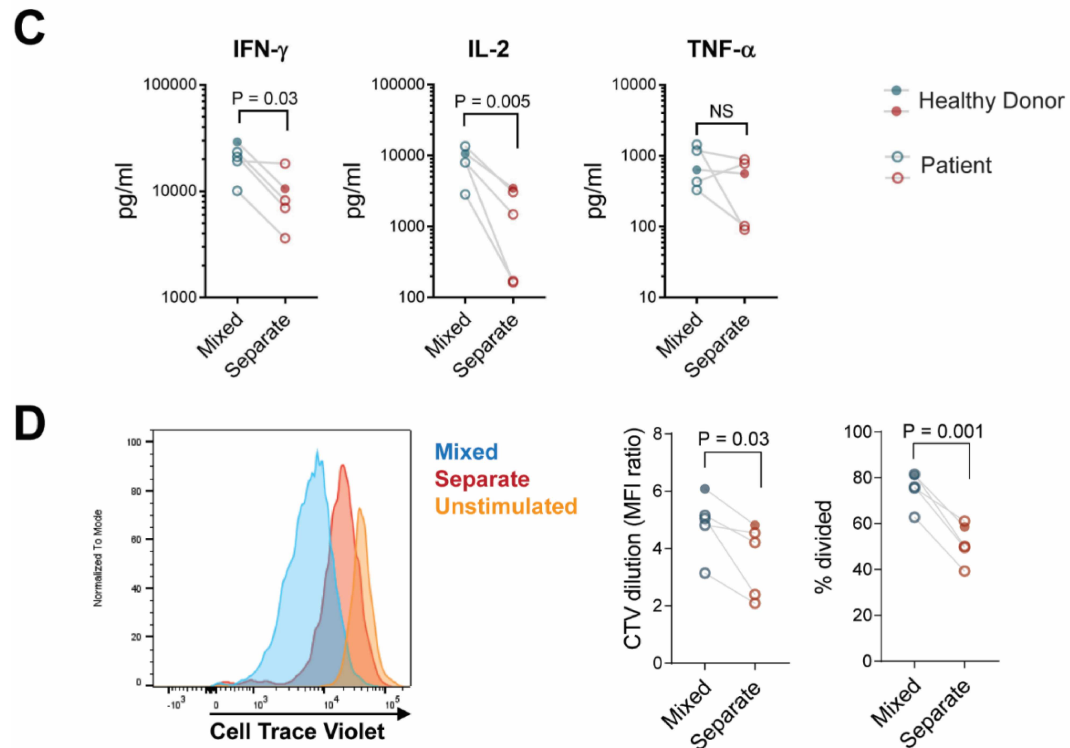
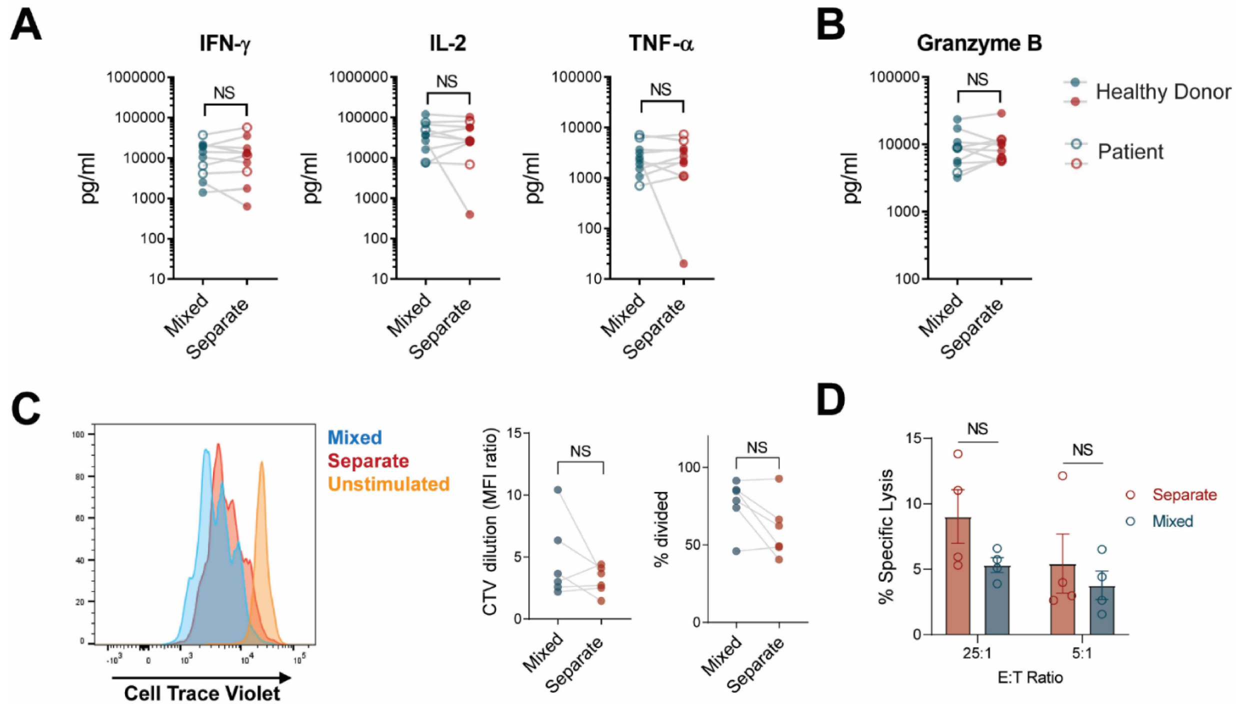

CD8
C
IFN-γ
IL-2
TNF-α
P = 0.03
P = 0.005
NS
pg/ml
Mixed
Separate
Healthy Donor
Patient
D
Mixed
Separate
Unstimulated
Normalized To Mode
Cell Trace Violet
CTV dilution (MFI ratio)
% divided
P = 0.03
P = 0.001

Figure S11. Impact on CD4$^+$ and CD8$^+$ cell function of mixed vs. separate cell production of CD19-targeted CAR T cells. CD4$^+$ and CD8$^+$ enriched PBMC from patients (open circles; n = 4) or healthy donors (filled circles; n = 1) were stimulated, then either mixed at a 60:40 CD4:CD8 ratio or maintained in separate cultures, transduced with lentiviral vector encoding the hCD19-BB-z CAR, and expanded in culture for 8 days. FACS-sorted CD8$^+$ CAR$^+$ T cells were labeled with Cell Trace Violet (CTV) and incubated with irradiated CD19$^+$ Raji-ffLuc cells at a 1:1 ratio. (A, C) Supernatants were harvested at 24 hours and the indicated cytokines were measured by Luminex assay. (B, D) After 4 days, proliferation of the sorted cells was assessed by measuring dilution of CTV by flow cytometry. Representative histograms are shown in the left panel, and summary data of geometric MFI ratio of stimulated to unstimulated cells and % divided cells and are shown in the right panels. P-values were determined using paired two-tailed t-tests for samples meeting criteria for normality based on Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.


CD4
A
IFN-γ
IL-2
TNF-α
B
Granzyme B
NS
pg/ml
Mixed
Separate
Healthy Donor
Patient
C
Mixed
Separate
Unstimulated
Cell Trace Violet
CTV dilution (MFI ratio)
% divided
D
% Specific Lysis
Separate
Mixed
25:1
5:1
E:T Ratio

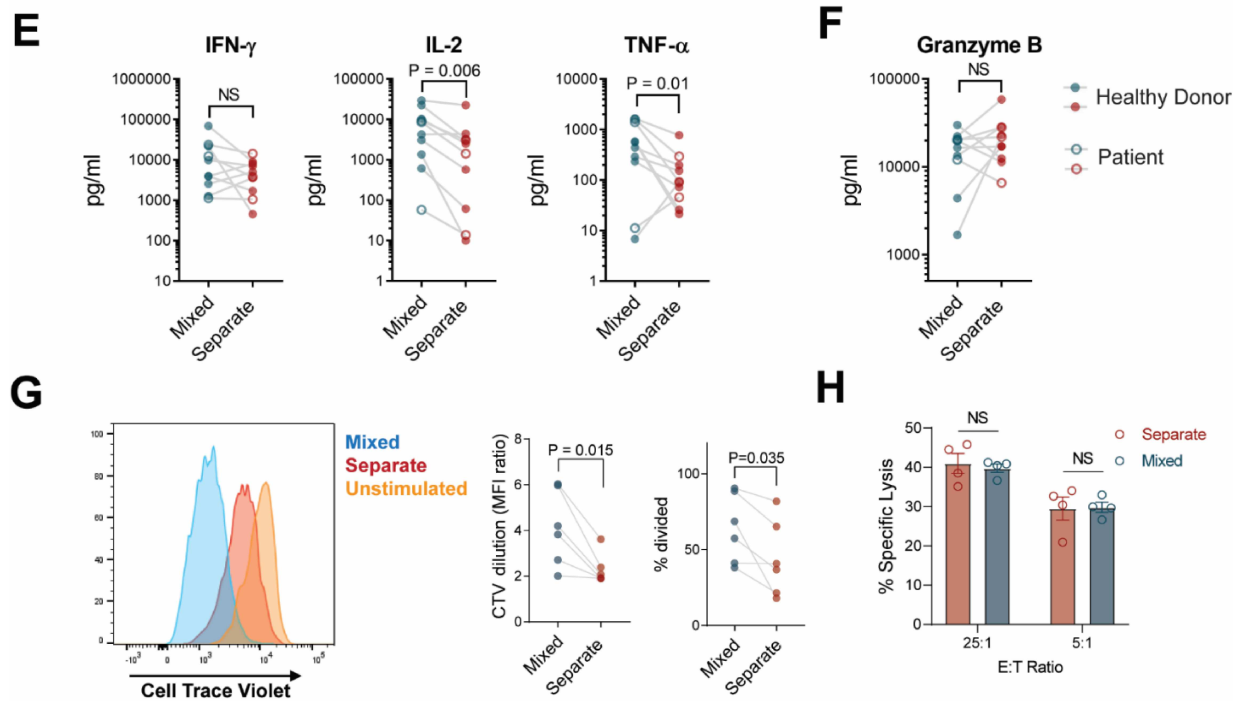

CD8
E
IFN-γ
IL-2
TNF-α
F
Granzyme B
NS
P = 0.006
P = 0.01
pg/ml
Mixed
Separate
Healthy Donor
Patient
G
Mixed
Separate
Unstimulated
Cell Trace Violet
CTV dilution (MFI ratio)
P = 0.015
% divided
P=0.035
H
% Specific Lysis
Separate
Mixed
25:1
5:1
E:T Ratio

Figure S12. Impact on CD4$^+$ and CD8$^+$ cell function of mixed vs. separate cell production of 2nd generation CD20-targeted CAR T cells with CD28 costimulatory domain. CD4$^+$ and CD8$^+$ enriched PBMC from patients (open circles) or healthy donors (filled circles) were stimulated with beads, mixed at a 60:40 ratio of CD4:CD8 cells or kept in separate cultures, transduced with 1.5.3-NQ-28-z CD20 CAR, and expanded. FACS-sorted CD4$^+$ tCD19$^+$ cells (A-C) or CD8$^+$ tCD19$^+$ T cells (E-G) were incubated with irradiated CD20$^+$ Raji-ffLuc cells at a 1:1 ratio. In some experiments, T cells were labeled with Cell Trace Violet (CTV) prior to co-incubation. (A-B, E-F) Supernatants were harvested at 24 hours and the indicated analytes were measured by Luminex assay (n = 10: 3 patients and 7 healthy donors). (C, G) Proliferation of the sorted cells after 4 days based on CTV dilution was assessed by flow cytometry. Representative histograms are shown in the left panel, and summary data of geometric MFI ratio of stimulated to unstimulated cells and % divided cells are shown in the right panels (n = 6 healthy donors). (D, H) Specific lysis of Raji-ffLuc target cells in ^{51}Cr-release cytotoxicity assays using FACS-sorted CD4$^+$ tCD19$^+$ (D) or CD8$^+$ tCD19$^+$ (H) cells. Mean values ± SEM at the indicated E:T ratios are shown (n = 4 patients). P-values were determined using paired two-tailed t-tests for samples meeting criteria for normality based on D'Agostino & Pearson or Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.

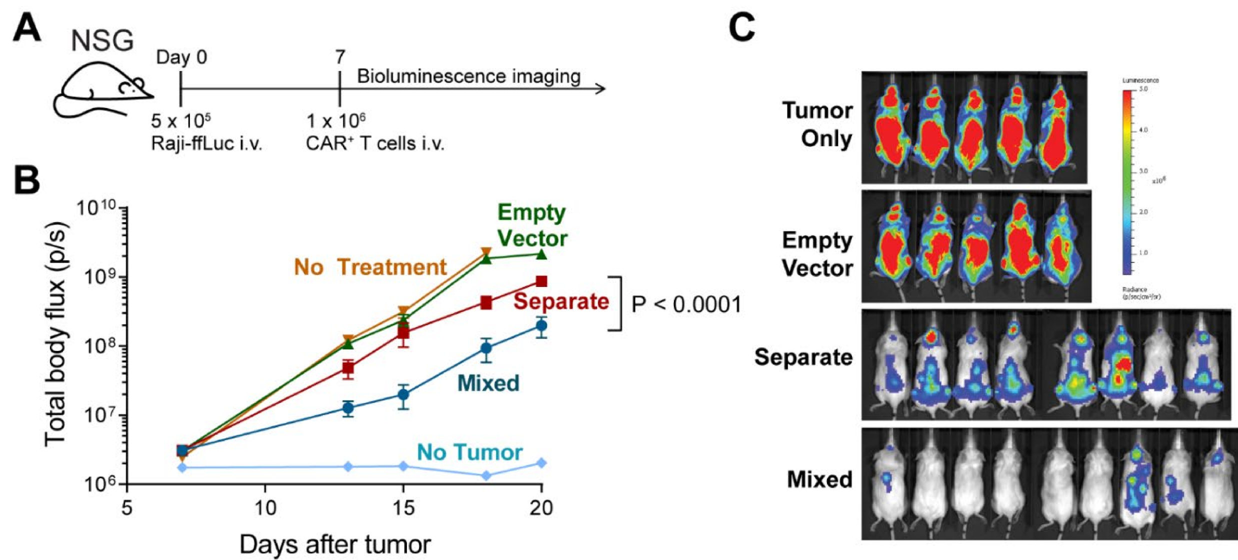


Figure S13. Impact of mixed vs. separate CD4⁺ and CD8⁺ cell cultures on in vivo anti-tumor activity. NSG mice (n = 9 for mixed CAR group, n = 8 for separate CAR group, n = 5 for empty vector and no treatment groups, n = 1 for no tumor group) were injected i.v. with 5 x 10^5 Raji-ffLuc tumor cells, followed 7 days later by a suboptimal dose of 1 x 10^6 $tCD19^+$ CD20 CAR T cells (1.5.3-NQ-28-BB-z), that had been either cultured at a 70:30 CD4:CD8 ratio or expanded in separate parallel $CD4^+$ and $CD8^+$ cultures and formulated at a 1:1 ratio prior to injection. The mixed cells were at a 1:1.8 CD4:CD8 ratio at the time of infusion. Empty vector T cells expanded as mixed cultures were included as a control. Mice were imaged twice weekly by bioluminescence imaging. (A) Experimental schema. (B) Average tumor burden per group over time as measured by total body bioluminescence. The mean luminescence values ± SEM are shown. The tumor burden over time was greater in the separate group compared with the mixed group based on a two-way repeated measures ANOVA, time x treatment group, $F_{(4, 60)} = 18.51$, $P < 0.0001$; time factor, $F_{(1.355, 20.32)} = 47.11$, $P < 0.0001$; treatment group factor, $F_{(1, 15)} = 16.87$, $P = 0.0009$. (The overall model including no treatment, empty vector, separate, and mixed groups showed: time x treatment group, $F_{(9, 69)} = 51.47$, $P < 0.0001$; time factor, $F_{(1.178, 27.09)} = 238.1$, $P < 0.0001$; treatment group factor, $F_{(3, 23)} = 47.6$, $P < 0.0001$; day 20 values were excluded for all groups in the overall model due to several mice in the control groups that had died prior to that timepoint). (C) Dorsal images of each mouse at day 18 is shown.

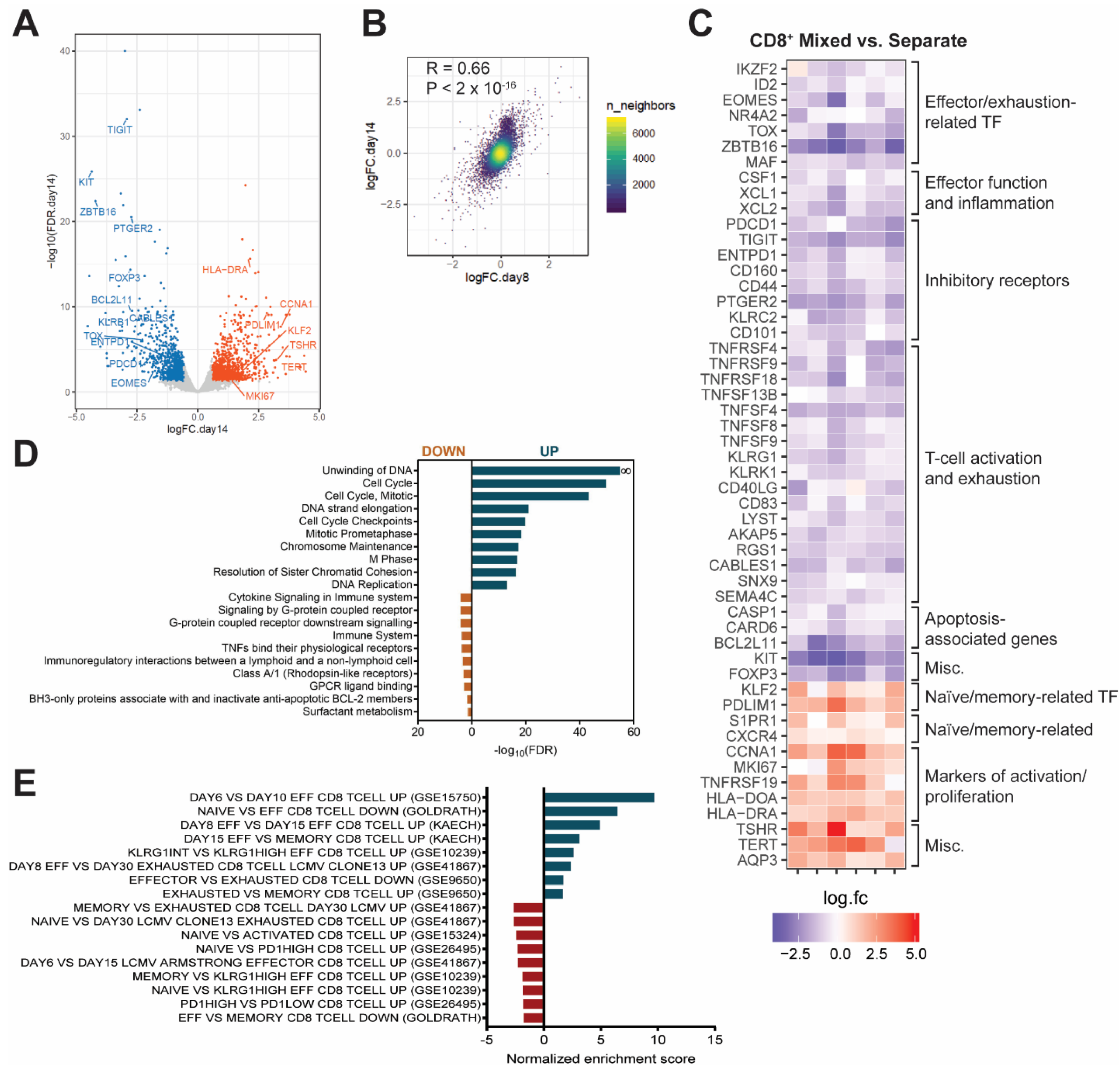


Figure S14. Distinct transcriptional signatures after restimulation at day 14 in CD8⁺ CAR T cells cultured in presence or absence of CD4⁺ T cells. Gene expression profiles of CD8⁺ tCD19⁺ CD20-targeted CAR T cells (1.5.3-NQ-28-BB-z) cultured either with CD4⁺ cells ("mixed") or alone ("separate") and sorted by flow cytometry 7 days after restimulation with CD20⁺ target cells (day 14 of cell culture) were evaluated by RNA-seq (n = 6: 1 patient and 5 healthy donors). (A) Volcano plot of false discovery rate (FDR) ($-\log_{10}$) versus fold change ($\log_2$) showing differentially expressed genes between mixed vs. separate CD8⁺ CAR T cells (FDR < 0.05 and $|\log_2 FC| \geq 0.585$ [≥ 1.5 fold change]), with upregulated and downregulated mRNAs shown in orange and blue, respectively. (B) Scatter plot of log fold change between mixed vs. separate CD8⁺ CAR T cells at day 14 (following restimulation) vs. at day 8 (without restimulation). (C) Heat map of fold change ($\log_2$) of selected differentially expressed genes (all with FDR < 0.05) between mixed vs separate CD8⁺ CAR T cells in the 6 individual subjects. (D) Pathway enrichment analysis of differentially expressed genes using REACTOME pathways was performed using R package GOseq, and the top 10 upregulated and downregulated pathways for mixed vs. separate CD8⁺ CAR T cells are shown. FDR was < 0.05 for all pathways. (E) Normalized enrichment scores from GSEA using selected gene sets related to CD8⁺ naïve, memory, effector, and exhausted cells from the MSigDB C7 database. Positive/negative scores indicate enrichment of the gene sets in up- or downregulated genes when comparing mixed vs. separate CD8⁺ CAR T cells. The FDR was < 0.05 for all the gene sets shown here. GEO datasets are indicated in parentheses.

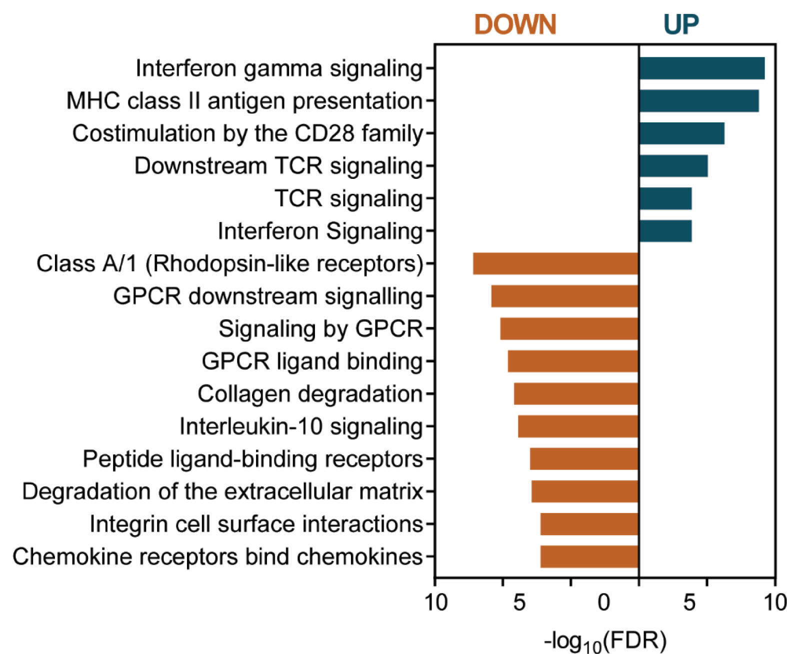
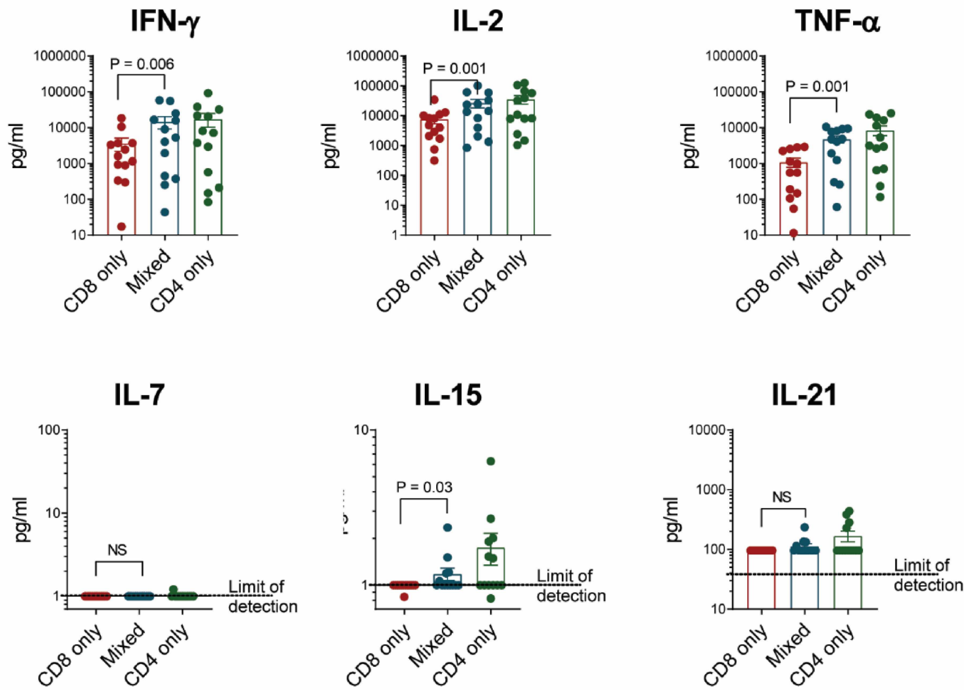


Figure S15. Pathway enrichment analysis of day 8 CD8⁺ CD20 CAR T cells. Gene expression profiles of CD8⁺ tCD19⁺ CD20-targeted CAR T cells (1.5.3-NQ-28-BB-z) cultured either in co-culture with CD4⁺ cells ("mixed") or alone ("separate") were sorted by flow cytometry at day 8 of cell culture and evaluated by RNA-seq (n = 6: 2 patients and 4 healthy donors). Enrichment analysis of differentially expressed genes using REACTOME pathways was performed by R package GOseq, and all significant upregulated and the top 10 downregulated pathways for mixed vs. separate CD8⁺ CAR T cells are shown. FDR was < 0.03 for all pathways.


Day 1
IFN-γ
P = 0.006
IL-2
P = 0.001
TNF-α
P = 0.001
pg/ml
CD8 only
Mixed
CD4 only
IL-7
NS
Limit of detection
IL-15
P = 0.03
IL-21
NS

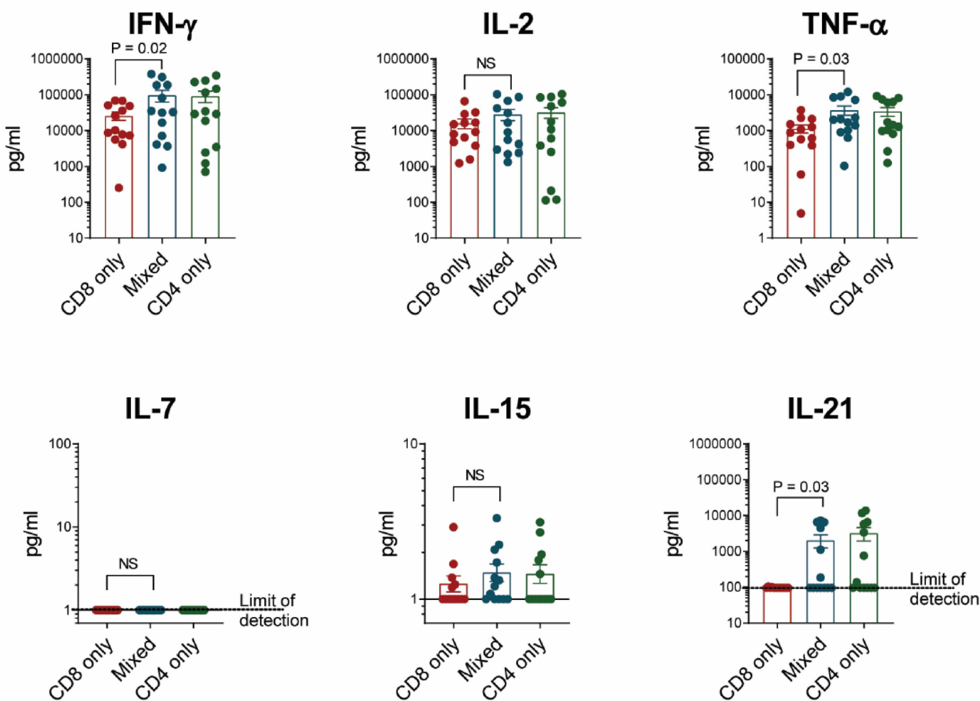

Day 4
IFN-γ
P = 0.02
IL-2
NS
TNF-α
P = 0.03
pg/ml
CD8 only
Mixed
CD4 only
IL-7
NS
Limit of detection
IL-15
NS
IL-21
P = 0.03

Figure S16. Levels of cytokines in mixed vs separate CD4⁺ and CD8⁺ cell cultures. Supernatants from CD4-only, CD8-only, or 70:30 mixed CD4:CD8 cultures of αCD3/28-bead-stimulated 1.5.3-NQ-28-BB-z CAR T cells were harvested at 1 and 4 days after culture initiation, and levels of the indicated cytokines were measured by Luminex. Mean values (± SEM) are shown. (n = 13: 2 patients and 11 healthy donors). P-values were determined using paired two-tailed t-tests for samples meeting criteria for normality based on D'Agostino & Pearson or Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.

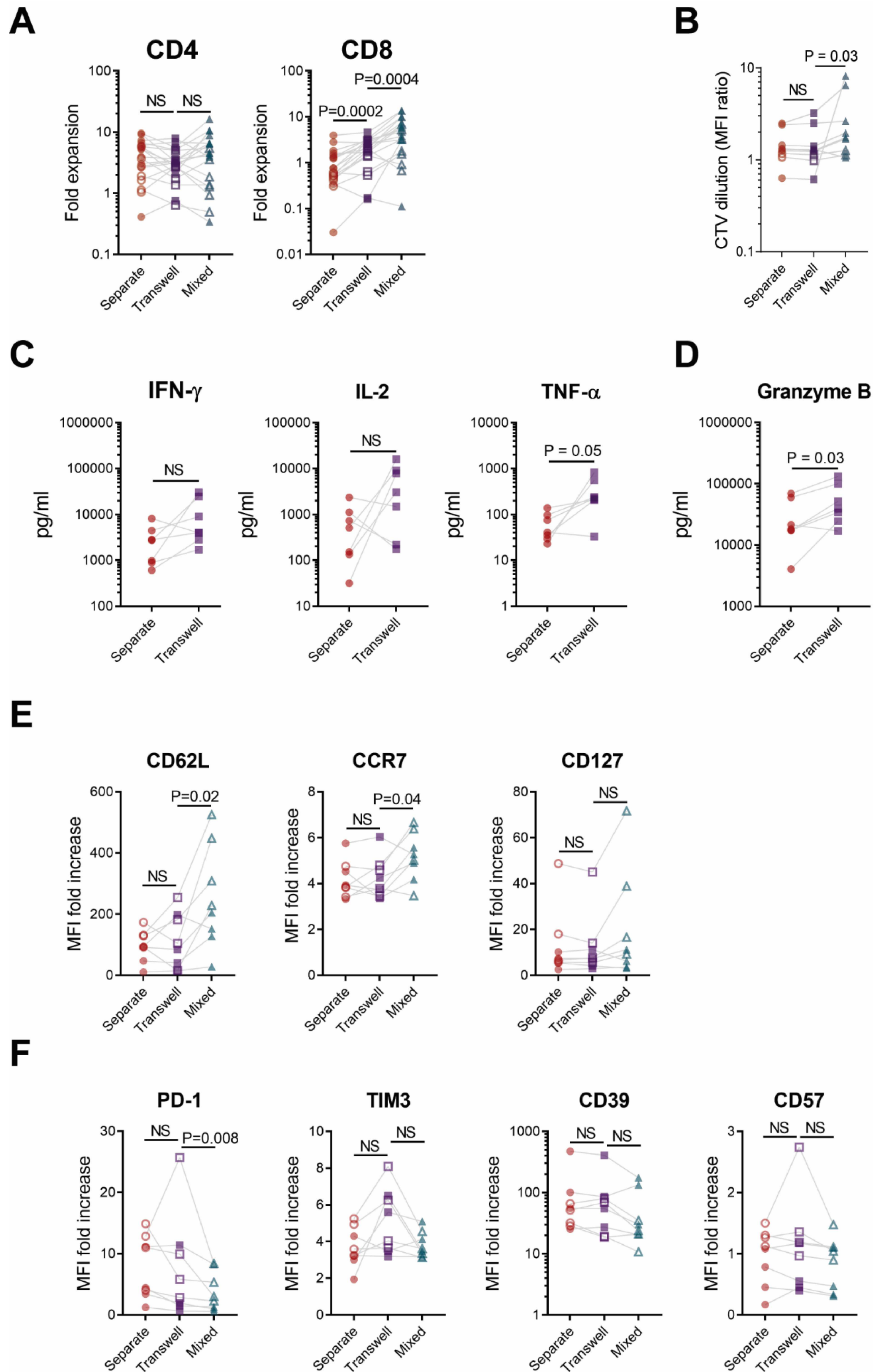

A
CD4
CD8
Fold expansion
NS
P=0.0002
P=0.0004
Separate
Transwell
Mixed
B
P = 0.03
CTV dilution (MFI ratio)
C
IFN-γ
IL-2
TNF-α
pg/ml
P = 0.05
D
Granzyme B
E
CD62L
CCR7
CD127
MFI fold increase
P=0.02
P=0.04
F
PD-1
TIM3
CD39
CD57
P=0.008

Figure S17. Impact of soluble factors vs. cell-cell contact between CD4$^+$ cells and CD8$^+$ cells on CD8$^+$ 3rd generation CAR T cell expansion, function, and phenotype. (A) CD4$^+$ and CD8$^+$ cells from healthy donors (filled markers, n = 12) or patients (open markers, n = 7) were transduced with 1.5.3-NQ-28-BB-z and cultured separately, or in transwells separated by a permeable membrane, or in mixed cultures. Fold expansion of CD4$^+$ and CD8$^+$ cells at day 7 is shown. (B) CD8$^+$ CAR T cells in some experiments from (A) were harvested at day 8, stained with Cell Trace Violet (CTV), restimulated with irradiated Raji-ffLuc cells, and assessed by flow cytometry for CTV dilution 4 days later. The ratio of geometric mean fluorescence intensity (MFI) of unstimulated to stimulated CAR T cells is shown (n = 9: 2 patients and 7 healthy donors). (C-D) CD8$^+$ CAR T cells from separate or transwell cultures were restimulated as in (B), supernatants were harvested at 24 hours after restimulation, and the analytes shown were quantified by Luminex assay (n = 7 healthy donors). (E-F) CD8$^+$ CAR T cells were harvested at day 8 of cell culture and selected memory (E) and exhaustion (F) markers were evaluated by flow cytometry. Values represent the fold increase over isotype control (n = 8: 4 healthy donors and 4 patients). P-values were determined using paired two-tailed t-tests for samples meeting criteria for normality based on D'Agostino & Pearson or Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.

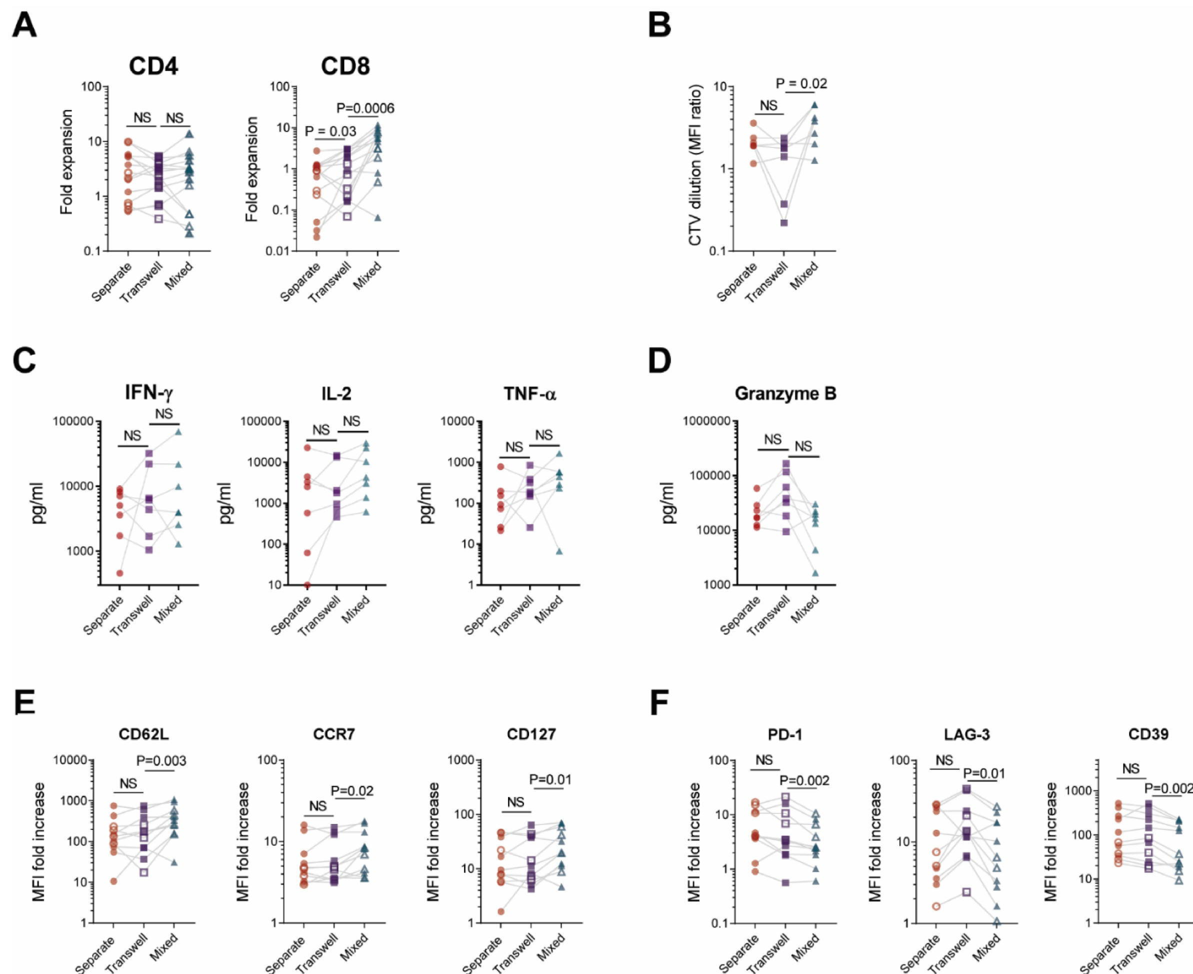


Figure S18. Impact of soluble factors vs. cell-cell contact between CD4⁺ cells and CD8⁺ cells on CD8⁺ 2nd generation CAR T cell expansion, function, and phenotype. (A) $CD4^+$ and $CD8^+$ cells from healthy donors (filled markers, n = 9) or patients (open markers, n = 5) were transduced with 1.5.3-NQ-28-z and cultured separately, or in transwells separated by a permeable membrane, or in mixed cultures. Fold expansion of $CD4^+$ and $CD8^+$ cells at day 7 is shown. (B) $CD8^+$ CAR T cells in some experiments from (A) were harvested at day 8, stained with Cell Trace Violet (CTV), restimulated with irradiated Raji-ffLuc cells, and assessed by flow cytometry for CTV dilution 4 days later. The ratio of geometric mean fluorescence intensity (MFI) of unstimulated to stimulated CAR T cells is shown (n = 9: 2 patients and 7 healthy donors). (C-D) FACS-sorted $CD8^+$ CAR T cells were restimulated as in (B), supernatants were harvested at 24 hours after restimulation, and the analytes shown were quantified by Luminex assay (n = 7 healthy donors). (E-F) $CD8^+$ CAR T cells were harvested at day 8 of cell culture and selected memory (E) and exhaustion (F) markers were evaluated by flow cytometry. Values represent the fold increase over isotype control (n = 11: 7 healthy donors and 4 patients). P-values were determined using paired two-tailed t-tests for samples meeting criteria for normality based on D'Agostino & Pearson or Shapiro-Wilk normality test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.

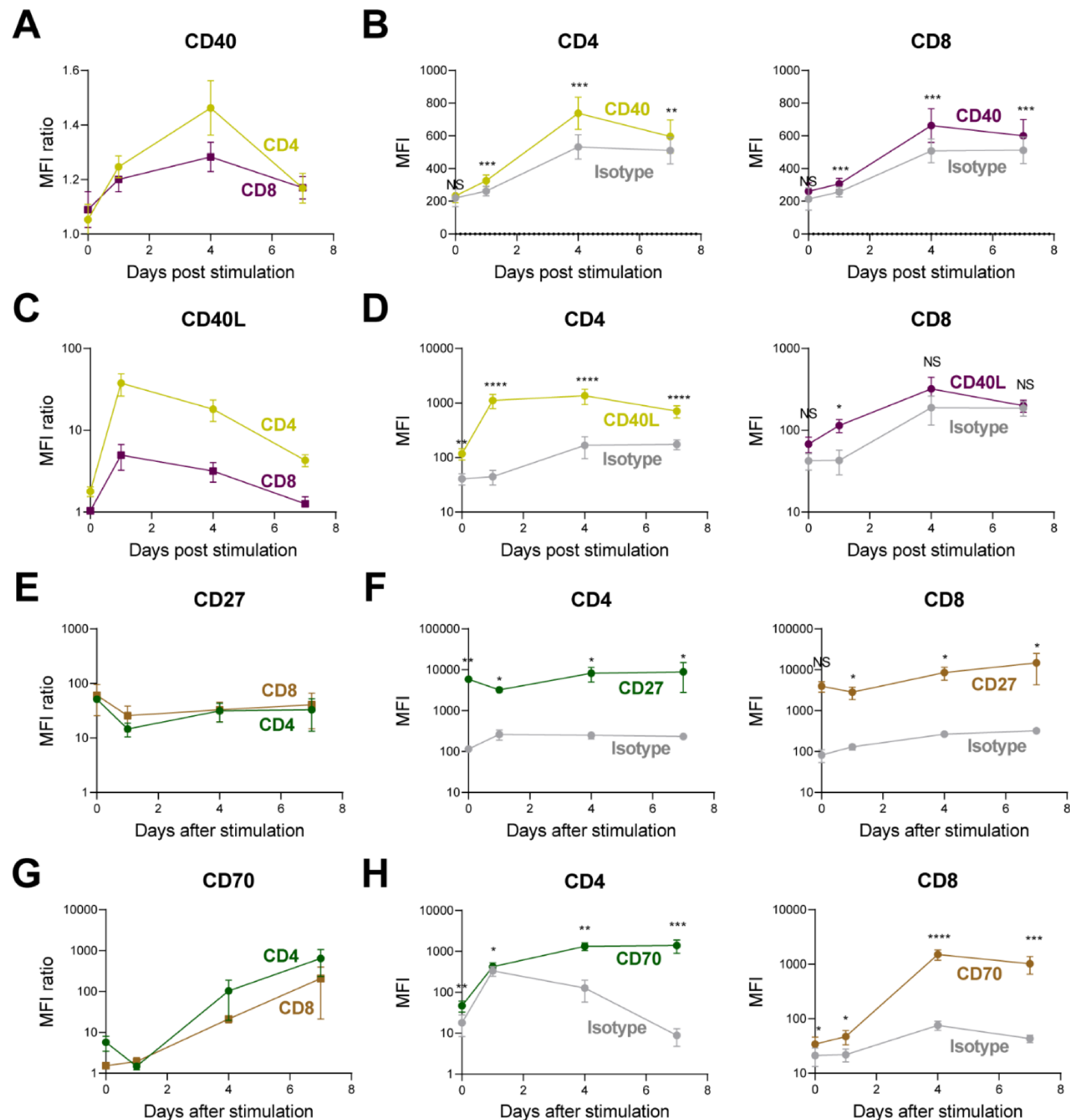


Figure S19. Expression of CD40, CD40L, CD27, and CD70 expression on CD4⁺ and CD8⁺ T cells. Expression of CD40 (A-B), CD40L (C-D), CD27 (E-F), and CD70 (G-H) was measured by flow cytometry at baseline, and at 1, 4, and 7 days after bead stimulation in 1.5.3-NQ-28-BB-z transduced T cells from mixed (70:30 ratio of CD4:CD8) CD4⁺/CD8⁺ cultures (n = 9: 5 healthy donors and 4 patients for A-D; n = 7: 3 healthy donors and 4 patients for E-F; and n = 3 healthy donors for G-H). (A, C, E, G) The mean (± SEM) ratios of geometric mean fluorescence intensity (MFI) vs. isotype are shown for CD4⁺ and CD8⁺ CAR T cells, (B, D, F, H). The mean (± SEM) geometric MFI of each marker and isotype control, gated on CD4⁺ or CD8⁺ cells, are shown separately. Statistical comparisons between CD40/CD40L/CD27/CD70 and isotype MFIs were made using multiple paired ratio t-tests (or multiple Wilcoxon tests for data sets not meeting normality by Shapiro-Wilk test), with false discovery rate (Q) calculated using two-stage step-up method of Benjamini, Krieger, and Yekutieli (*: Q ≤ 0.05, **: Q ≤ 0.01, ***: Q ≤ 0.001, ****: Q ≤ 0.0001).

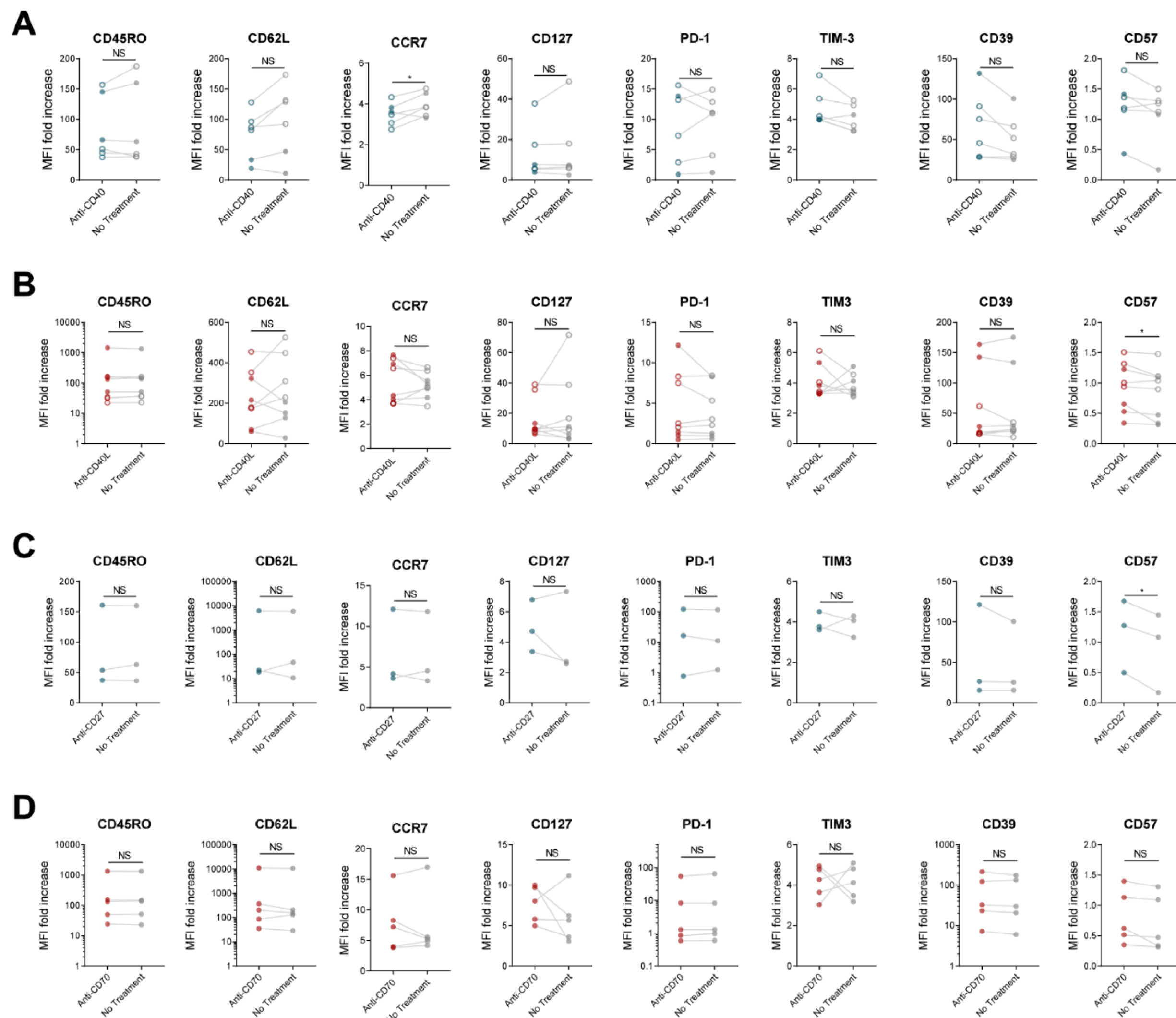


Figure S20. Effect of CD40 or CD27 signaling on CD8⁺ CAR T cell phenotype. (A, C) CD8⁺ T cells were isolated, stimulated with anti-CD3/CD28 beads in tissue culture wells with or without plate-bound agonistic anti-CD40 (A; n = 6: 4 patients [open circles] and 2 healthy donors [closed circles]) or anti-CD27 (C; n = 3 healthy donors) antibodies, and transduced with 1.5.3-NQ-28-BB-z lentiviral vector. (B, D) CD4⁺ and CD8⁺ T cells were mixed at a 60:40 CD4:CD8 ratio and stimulated with anti-CD3/CD28 beads in the presence or absence of antagonistic anti-CD40L (B; n = 8: 4 patients [open circles] and 4 healthy donors [closed circles]) or anti-CD70 (D; n = 5 healthy donors) antibodies. For all cultures, cells were harvested on day 8 of cell culture, and markers of memory, differentiation, and exhaustion were measured by flow cytometry. Data represent the fold increase in geometric mean fluorescence intensity (MFI) over isotype control, gated on viable CD8⁺ tCD19⁺ CAR T cells. Statistical comparisons were made using paired two-tailed t-tests for samples meeting criteria for normality based on Shapiro-Wilk test, or Wilcoxon matched-pairs signed rank test for samples not meeting normality criteria.

Dataset S1. (separate file)

Differentially expressed genes in CD8⁺ CAR T cells expanded in mixed vs. separate cultures at day 8 following initial stimulation (first tab).

Differentially expressed genes in CD8⁺ CAR T cells expanded in mixed vs. separate cultures at day 14-15 of cell culture, 7 days after restimulation with CD20⁺ target cells (second tab).