Figure S1

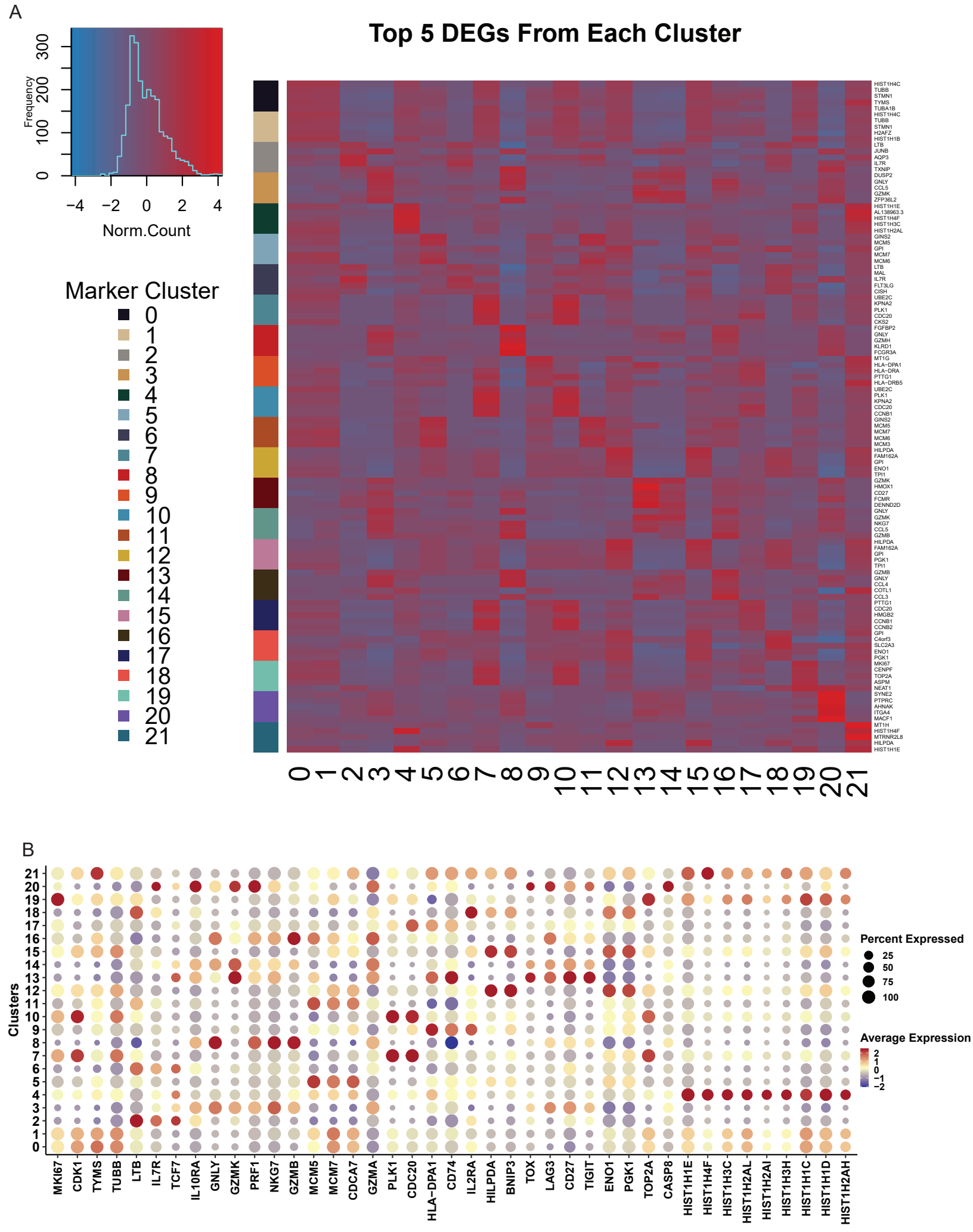

A
Top 5 DEGs From Each Cluster
Frequency
0
100
200
300
−4
−2
0
2
4
Norm.Count
Marker Cluster
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
HIST1H4C
TUBB
STMN1
TYMS
TUBA1B
HIST1H4C
TUBB
STMN1
H2AFZ
HIST1H1B
LTB
JUNB
AQP3
IL7R
TXNIP
DUSP2
GNLY
CCL5
GZMK
ZFP36L2
HIST1H1E
AL138963.3
HIST1H4F
HIST1H3C
HIST1H2AL
GINS2
MCM5
GPI
MCM7
MCM6
LTB
MAL
IL7R
FLT3LG
CISH
UBE2C
KPNA2
PLK1
CDC20
CKS2
FGFBP2
GNLY
GZMH
KLRD1
FCGR3A
MT1G
HLA-DPA1
HLA-DRA
PTTG1
HLA-DRB5
UBE2C
PLK1
KPNA2
CDC20
CCNB1
GINS2
MCM5
MCM7
MCM6
MCM3
HILPDA
FAM162A
GPI
ENO1
TPI1
GZMK
HMOX1
CD27
FCMR
DENND2D
GNLY
GZMK
NKG7
CCL5
GZMB
HILPDA
FAM162A
GPI
PGK1
TPI1
GZMB
GNLY
CCL4
COTL1
CCL3
PTTG1
CDC20
HMGB2
CCNB1
CCNB2
GPI
C4orf3
SLC2A3
ENO1
PGK1
MKI67
CENPF
TOP2A
ASPM
NEAT1
SYNE2
PTPRC
AHNAK
ITGA4
MACF1
MT1H
HIST1H4F
MTRNR2L8
HILPDA
HIST1H1E
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
B
Clusters
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
MKI67
CDK1
TYMS
TUBB
LTB
IL7R
TCF7
IL10RA
GNLY
GZMK
PRF1
NKG7
GZMB
MCM5
MCM7
CDCA7
GZMA
PLK1
CDC20
HLA-DPA1
CD74
IL2RA
HILPDA
BNIP3
TOX
LAG3
CD27
TIGIT
ENO1
PGK1
TOP2A
CASP8
HIST1H1E
HIST1H4F
HIST1H3C
HIST1H2AL
HIST1H2AI
HIST1H3H
HIST1H1C
HIST1H1D
HIST1H2AH
Percent Expressed
25
50
75
100
Average Expression
2
1
0
−1
−2

Figure S1. Single cell transcriptomics of pre-and post-infusion CAR T cells. A, Heatmap of the top 5 differentially expressed genes from each transcriptional cluster. **B**, Dot plot of relevant proliferation, T cell functional state, and histone-related genes.

Figure S2

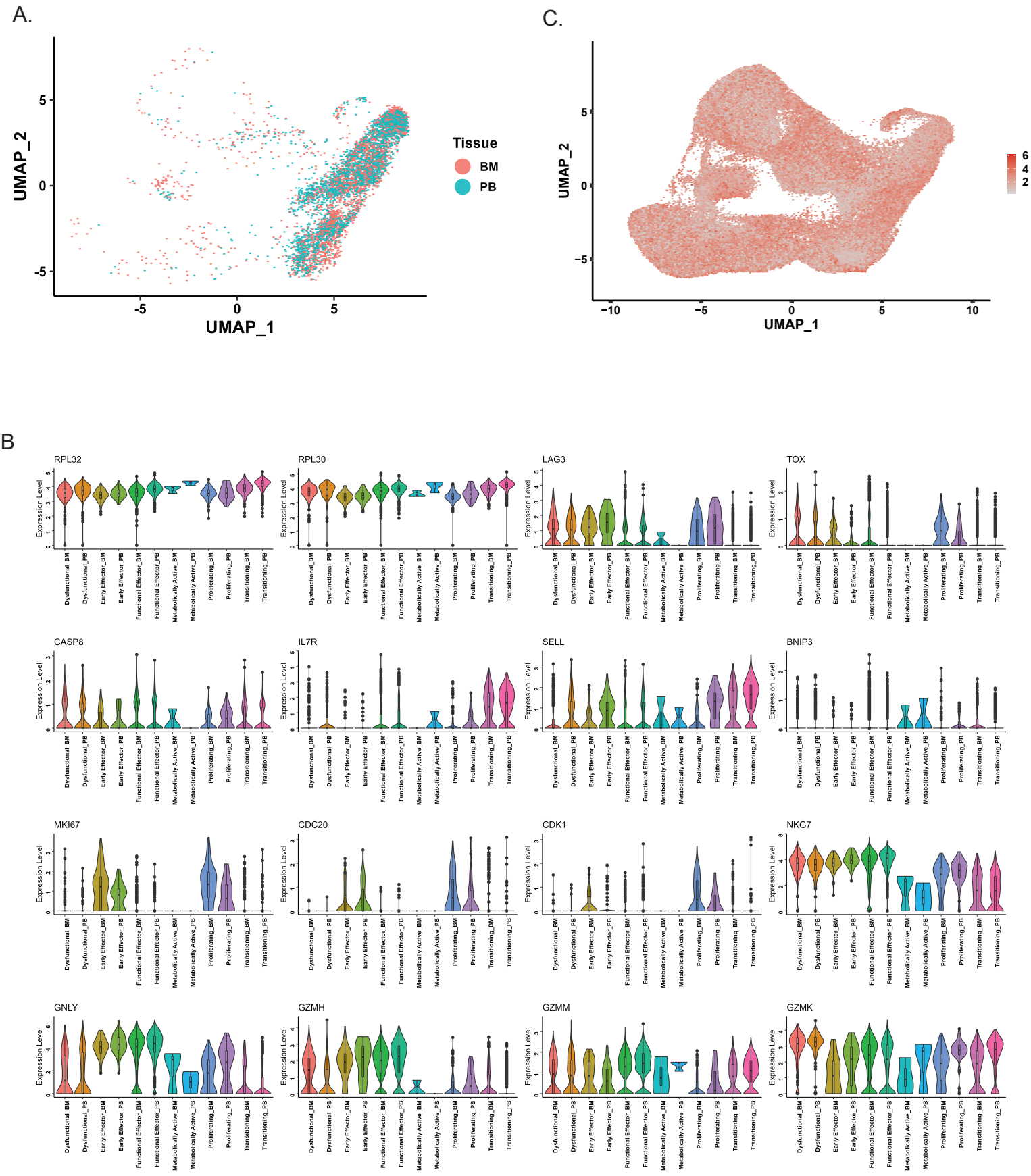

A.
UMAP_2
UMAP_1
Tissue
BM
PB
C.
UMAP_2
UMAP_1
6
4
2
B
RPL32
RPL30
LAG3
TOX
CASP8
IL7R
SELL
BNIP3
MKI67
CDC20
CDK1
NKG7
GNLY
GZMH
GZMM
GZMK
Expression Level
Dysfunctional_BM
Dysfunctional_PB
Early Effector_BM
Early Effector_PB
Functional Effector_BM
Functional Effector_PB
Metabolically Active_BM
Metabolically Active_PB
Proliferating_BM
Proliferating_PB
Transitioning_BM
Transitioning_PB

Figure S2. Post-infusion peripheral blood and bone marrow CAR T cells have comparable transcriptional signatures. A, UMAP of week 4 and month 4 post-infusion CAR T cells colored by sample type; bone marrow is colored in pink and peripheral blood is colored in blue. **B**, Violin plots comparing gene expression across peripheral blood (PB) and bone marrow (BM) CAR T cells from each functional group. **C**, UMAP of pre- and post-infusion CAR T cells colored by detected expression of CD19-CAR transcript.

Figure S3

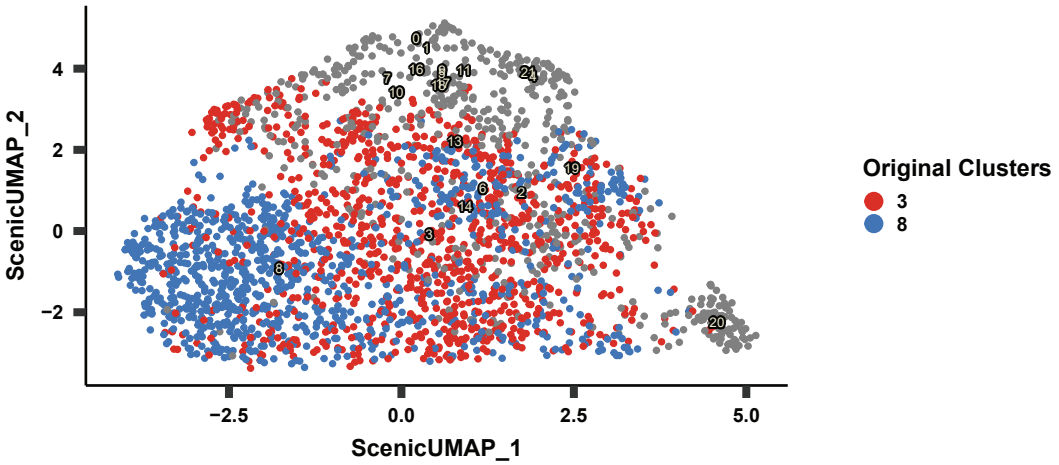


Figure S3. Clusters 3 and 8 exhibit distinct transcription factor regulatory networks. UMAP of downsampled CAR T cells clustered by differences in transcription factor regulons. Cluster 3 cells are colored red, cluster 8 cells are colored blue, and all other clusters are colored gray.

Figure S4

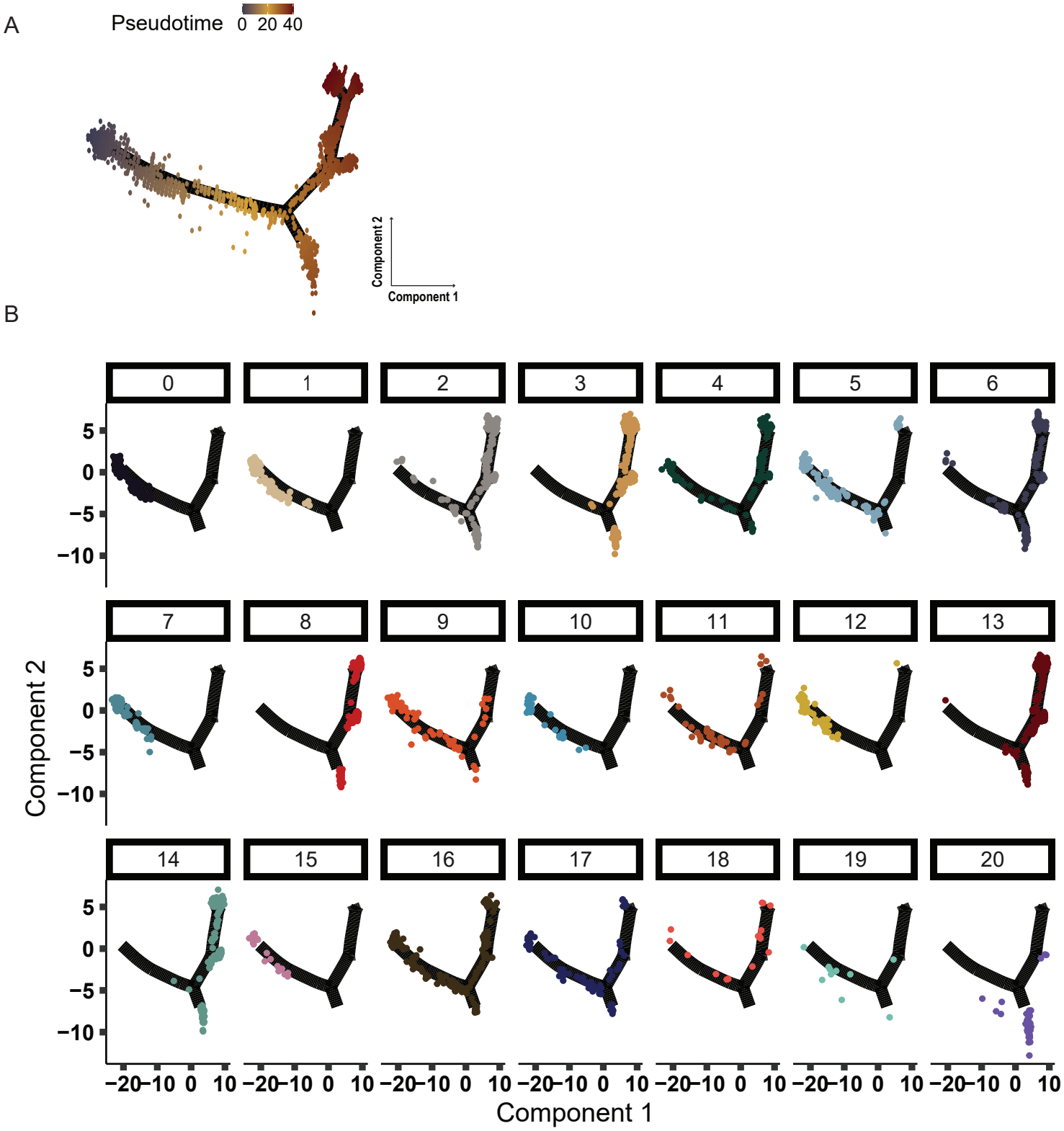


Figure S4. Characterization of CAR T cells in pseudotime. A, Monocle pseudotime map of CAR T cell transcriptional trajectory, colored by pseudotime gradient. **B**, Monocle pseudotime maps depicting each transcriptional cluster.

Figure S5

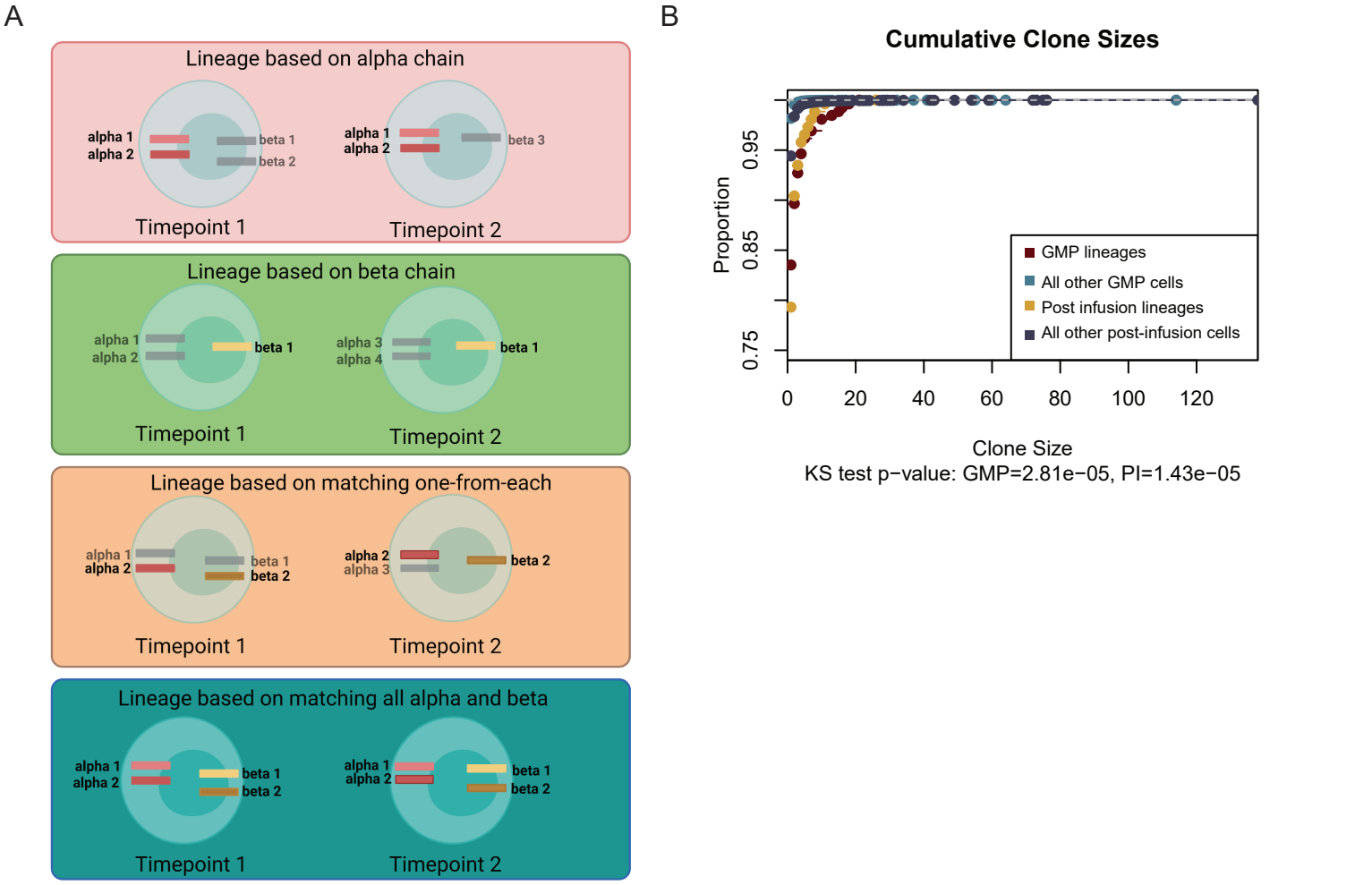


Figure S5. TCR lineage tracking. A, Different TCR α and β matching schemes. The complementarity determining region 3 (CDR3) of each α and β chain is sequenced to determine expressed alleles of each chain. Scheme 1 (red) describes classifying two CAR cells as in the same lineage when all observed α alleles match exactly while disregarding the β chain. Scheme 2 (green) assigns cells to lineages based on complete matching in only the β chain. Neither scheme 1 nor 2 fully represents the TCR by neglecting the contribution of the other chain. Since cells can differentially express distinct alleles of both the α and β chains of the TCR, the strictest definition of a lineage would be cases where all alphas and all betas must match between two or more cells (scheme 4; bottom). We detected the fewest number of lineages when requiring exact matches of all alleles (Supplementary Table 5). Thus, we defined lineages using a "one-from-each" approach (scheme 3; orange), where cells that match their most highly expressed (as a stringency filter) α and β chains are designated as lineages. **B**, Cumulative clone sizes of: GMP (dark red) and post-infusion (yellow) CAR T cell lineages, and GMP (light blue) and post-infusion (dark blue) CAR T cells without observed lineages (all other GMP/post-infusion cells). Graph represents the proportion of cells (y-axis) cumulatively encompassed by increasing clone sizes.

Figure S6

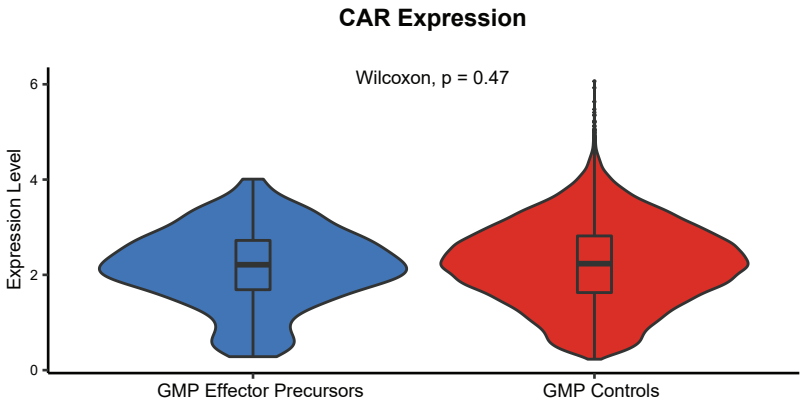


Figure S6. The distinct transcriptional signature between GMP effector precursors and non-precursors is not driven by differences in CAR expression. Violin plots comparing CAR transcript expression level between effector precursors (blue) and non-precursors (red) in the GMP. Adjusted p-value = 1.

Figure S7

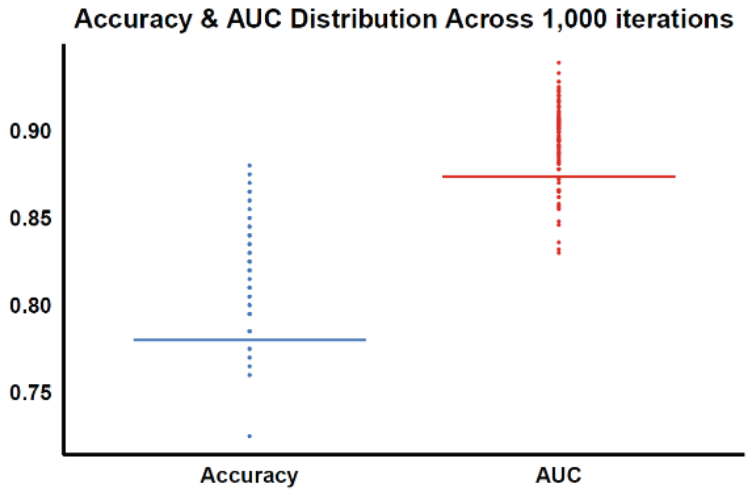


Figure S7. Summary of repetitive iterations of effector precursor classifier performance. Box plot summarizing the distributions of both the accuracy and the AUC across all 1,000 iterations. The box plot was graphed to show the median, upper and lower hinges (25th and 75th percentiles), two whiskers (1.5*IQR), and all outlier points (points > 1.5*IQR).

Figure S8

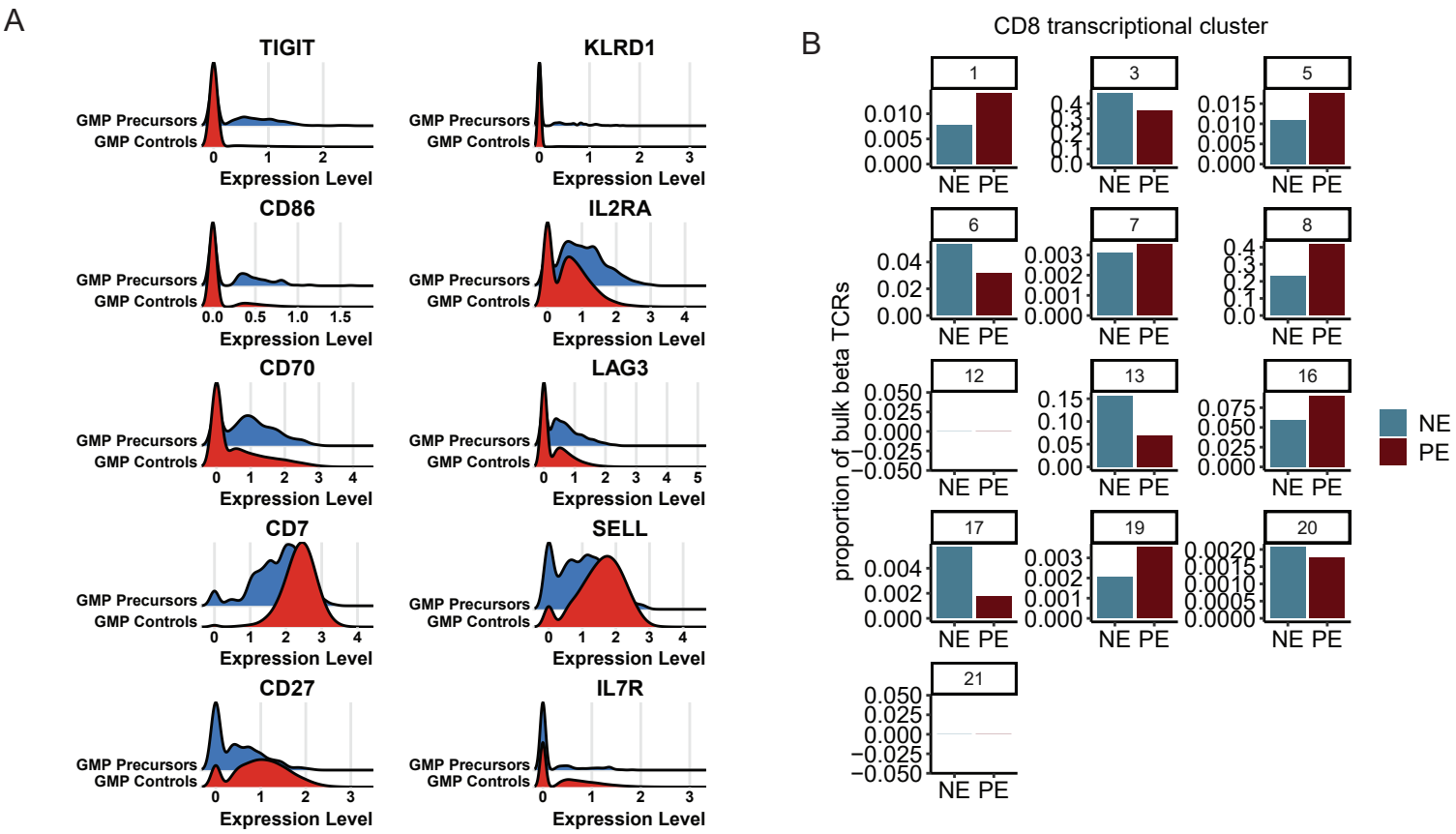
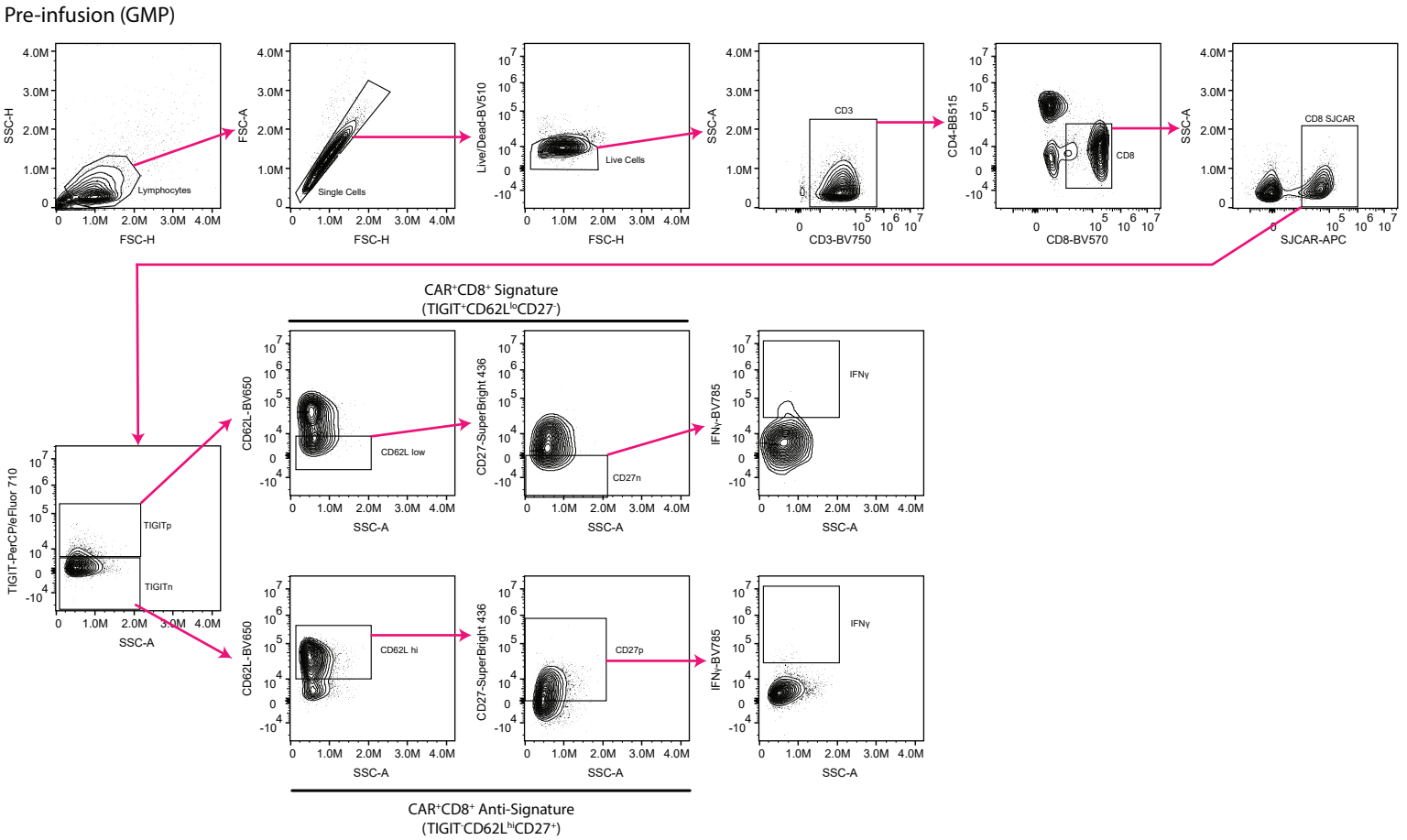


Figure S8. Selection of surface targets for pre-effector CAR T cell surface phenotype validation and results of enrichment. A, Histograms comparing expression of a subset of genes encoding surface proteins in effector precursors (blue) and non-effector precursors (red). **B**, Bar plots representing the inferred proportions of post-infusion CD8 transcriptional clusters linked to Patient 11 GMP CAR T cells sorted to approximate either the pre-effector signature (PE) or the opposing, non-effector signature (NE). TCRβ chains were sequenced in bulk from each sort, and cluster-annotated post-infusion cells from CD8 clusters that shared those β sequences were used to infer the post-infusion transcriptional cluster those lineages would track to.

Figure S9

A


Pre-infusion (GMP)
SSC-H
FSC-H
Lymphocytes
FSC-A
Single Cells
Live/Dead-BV510
Live Cells
SSC-A
CD3
CD3-BV750
CD4-BB515
CD8
CD8-BV570
CD8 SJCAR
SJCAR-APC
CAR+CD8+ Signature
(TIGIT+CD62LloCD27-)
TIGIT-PerCP/eFluor 710
TIGITp
TIGITn
CD62L-BV650
CD62L low
CD27-SuperBright 436
CD27n
IFNγ-BV785
IFNγ
CD62L hi
CD27p
CAR+CD8+ Anti-Signature
(TIGIT-CD62LhiCD27+)

B

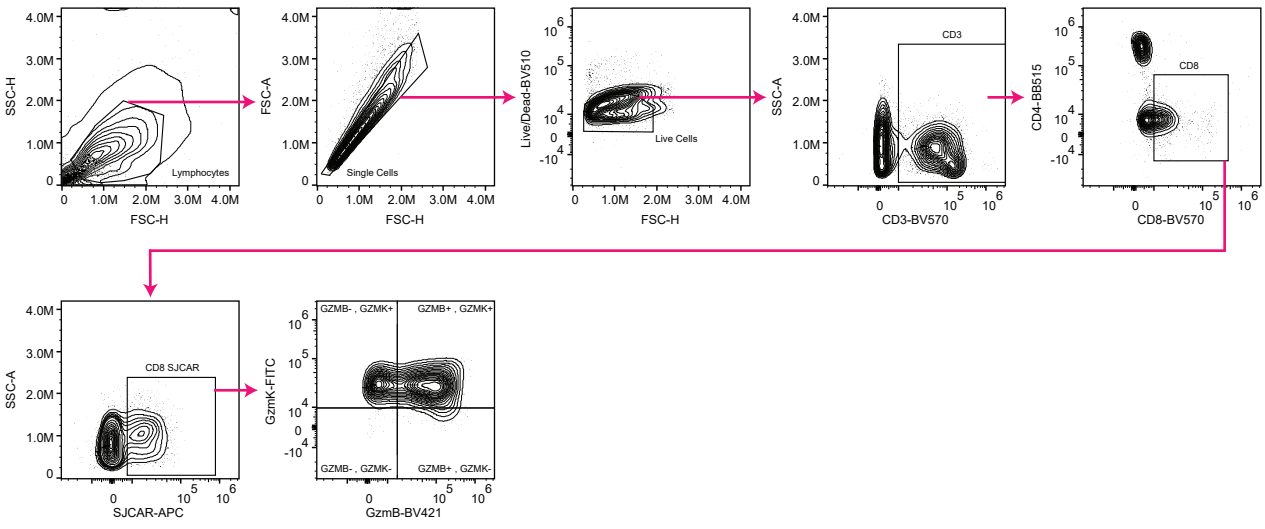

Post-infusion (Patient Collection)
SSC-H
FSC-H
Lymphocytes
FSC-A
Single Cells
Live/Dead-BV510
Live Cells
SSC-A
CD3
CD3-BV570
CD4-BB515
CD8
CD8-BV570
CD8 SJCAR
SJCAR-APC
GZMB- , GZMK+
GZMB+ , GZMK+
GZMB- , GZMK-
GZMB+ , GZMK-
GzmK-FITC
GzmB-BV421

Figure S9: Flow cytometry gating strategies for surface phenotyping and intracellular cytokine staining of SJCAR19 GMP and post-infusion samples. All samples were gated on lymphocyte-sized, single, live, CD3+, CD4–, CD8+, CAR+ cells. A, Gating strategy applied to GMP (pre-infusion) samples to evaluate IFNγ production from the CD8+CAR+ signature (TIGIT+, CD62Llo, CD27–) and CD8+CAR+ anti-signature (TIGIT–, CD62Llo, +CD27+) lymphocytes. **B,** Gating strategy applied to post-infusion samples to assess frequencies of TOX and IFNγ-producing cells across CD8+ CAR T cells with differential staining of GzmB and GzmK. IFNγ (interferon gamma); TIGIT (T Cell Immunoreceptor with Ig and ITIM Domains); GzmB (granzyme B); GzmK (granzyme K); TOX (Thymocyte Selection Associated High Mobility Group Box); CCR5 (C-C Motif Chemokine Receptor 5); CX3CR1 (C-X3-C Motif Chemokine Receptor 1). Gating strategy prepared using FlowJo v10.7.1 software (TreeStar).

Figure S10

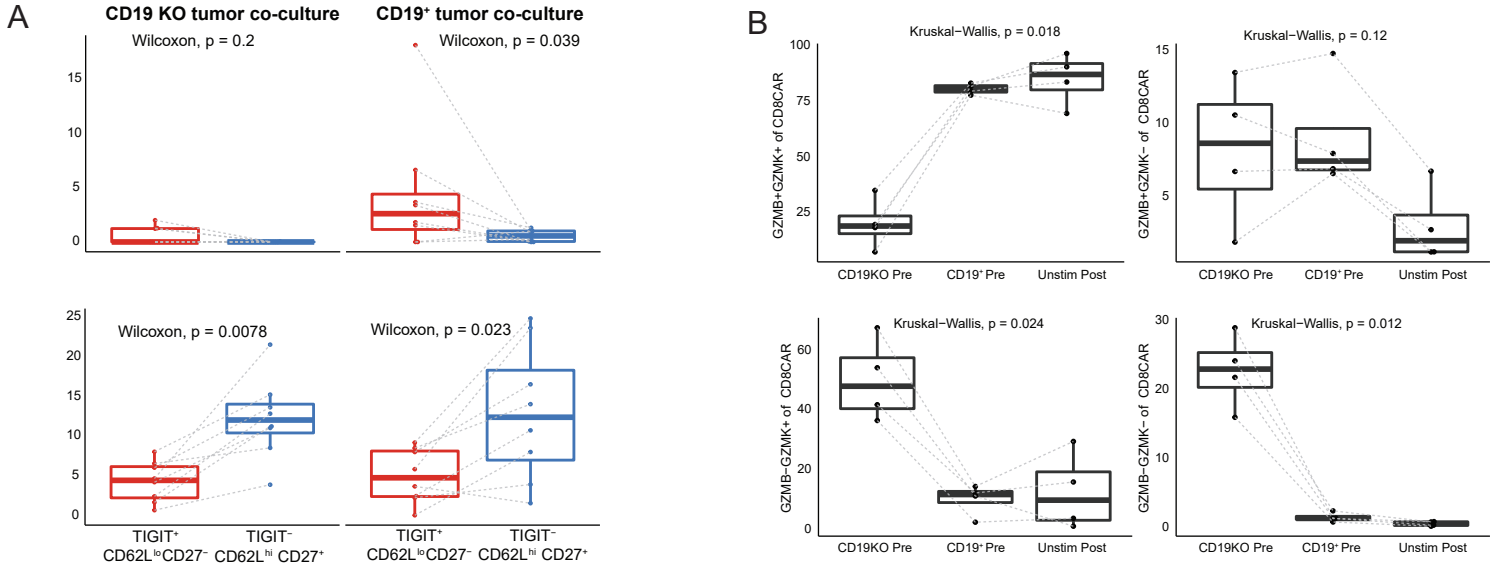


Figure S10. Flow cytometry data from validation patient cohort. A, Box plots comparing the percent of IFNγ-producing CD8+ CAR T cells (top) and TOX-producing CD8+ CAR T cells (bottom) between effector precursor pre-infusion cells (Tigit+, CD62Llo, CD27-; red) and pre-infusion cells with the opposing surface phenotype (Tigit-, CD62Lhi, CD27+; blue) co-cultured either with CD19 KO tumor (left) or CD19+ tumor (right). Dashed lines pair individual patients across samples. Data is from GMP samples of patients not included in the initial transcriptional profiling dataset (patients 16-23). **B**, Box plots comparing the percent of GZMB+ GZMK+ (top left), GZMB+ GZMK- (top right), GZMB- GZMK+ (bottom left), and GZMB-GZMK- (bottom right) CD8+ CAR T cells across pre-infusion CAR T cells co-cultured with either CD19KO or CD19+ tumor and unstimulated post-infusion CAR T cells. Patients included in analysis are 16, 17, 22, and 23 from the validation cohort.

Figure S11

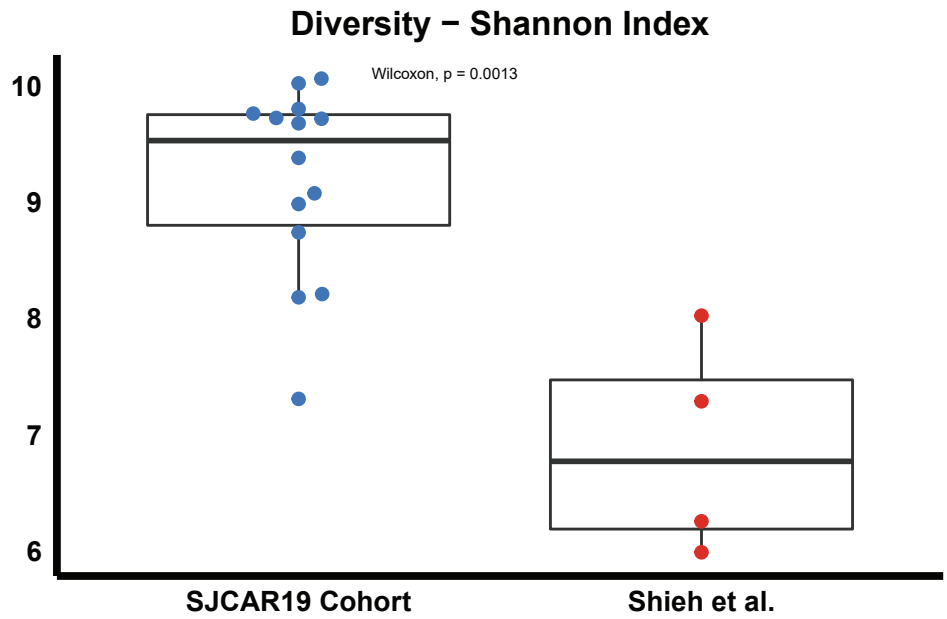


Figure S11. GMP products generated from pediatric patients exhibit extreme clonal diversity compared to those generated from adult patients. Box plots comparing the β chain diversity of CAR T cell TCRs from the GMP product in pediatric patients from the current study compared to those from adult patients in a previously published study (Sheih et al 2020, Nature Communications). The Shannon-Wiener index was calculated for each patient, and a Wilcoxon test was performed to evaluate statistical significance.