Supplemental Materials

Supplemental methods and materials

Lentivirus production

Hek293T cells were transfected with CAR lentiviral vector and the packaging plasmids pRSV.REV, pMDLg/p.RRE, and pVSV-G in OptiMem and Lipofectamine 2000. Supernatant was harvested at 24h and 48h, filtered to remove cell debris, and virus was concentrated by ultracentrifugation for 2h at 25,000rpm and 4C. Virus was resuspended in RPMI, aliquoted to 100μL/vial, and stored at -80C. Virus titer was determined using viral transduction of Hek293T cells and subsequent measurement of GFP or CAR expression.

ELISpot experiment

Healthy donor T cells were stained for TCRvβ5.1, TCRvβ9, or TCRvβ12 and the negative fraction was sorted by FACS. An unsorted population was also maintained. $0.2x10^6$ T cells were cultured with HLA-matched antigen presenting cells (APCs) and peptides for cytomegalovirus (CMV), Epstein-Barr virus (EBV), or influenza (flu) in pre-coated IFNγ ELISpot plates (R&D Systems). K562 cells engineered to express HLA-A2 were used as the APCs.[2] These cells and the pooled peptides[1] were obtained from the Carreno lab (University of Pennsylvania). No peptide or CD3/CD28 beads (Invitrogen) were respectively used as negative and positive controls. Each condition was run in triplicate. After 24 hours, the plate was stained, and the number of spots corresponding to cytokine release was analyzed using the Immunospot plate reader (Cellular Technology Ltd.).

In vitro killing of SupT1 cell lines

Lytic function of CARTs was tested against SupT1 cell lines engineered to express either TCRvβ12 or TCRvβ9. CAR+ T cells were incubated with 50,000 target cells at 3:1, 1:1, and 0.1: E:T ratios in opaque white, flat-bottom 96-well plates. Total cell number was equilibrated across CARTs to account for differences in transduction efficiencies. Each condition was run in duplicate or triplicate. Cells were cultured in phenol-free RPMI and incubated overnight at 37C and 5% CO_2. After 16-20 hours, plates were centrifuged at 1300rpm for 5 minutes. 100μL of media was removed from each well and luciferase buffer from the Luc-Screen Extended-Glow Luciferase Reporter Gene Assay system was added according to the manufacturer's protocol (Invitrogen). Bioluminescence readings were obtained using a microplate reader. Cytotoxicity was calculated using the following equation: 100*(1-experimental condition/average control condition (target cells alone)).

In vitro cytotoxicity of patient cells

Prior to co-culture, patient PBMCs with a TCRvβ12 dominant clone were stained with CellTrace Violet according to the manufacturer's protocol (Invitrogen). $2.0x10^6$ patient cells were incubated with CAR+ or NTD T cells at a 1:1 E:T ratio in a 12-well plate. Total cell number was equilibrated across CARTs to account for differences in transduction efficiencies. Cells were cultured in RPMI and incubated at 37C and 5% CO_2. After 48 hours, cells were prepared for flow cytometry as described in the main text, using the Beckman Coulter IOTest Beta Mark kit, TCRαβ-APC, and CD3-BV605. LIVE/DEAD Fixable Aqua was used to determine live cell populations. Using the HTS plate reader, 75μL of the 200μL total was recorded and run to completion for each well. Gating on single live target cells was performed according to forward and side scatter characteristics, positivity for CellTrace Violet, and exclusion of the viability dye. Gates were then set for CD3/TCRαβ expression, and then in PE/GFP for each of the stains in the IOTest Beta Mark Kit (Supplemental Figure 5B).

Prior to co-culture, TCRvβ5.1+ patient PBMCs were stained with Invitrogen eBioscience Cell Proliferation Dye eFluor 670 according to the manufacturer's protocol. $0.5x10^6$ patient cells were incubated with CAR+ or NTD T cells at 3:1, 1:1, and 0.1:1 E:T ratios in a 96-well plate. Total cell number was equilibrated across CARTs to account for differences in transduction efficiencies. Each condition was run in triplicate. Cells were cultured in RPMI and incubated at 37C and 5% CO_2. After 16-20 hours, the cells were prepared for flow cytometry analysis using CD3-BV605 and TCRvβ5.1-PE antibodies. LIVE/DEAD Fixable Aqua was used to determine live cell populations. Using the HTS plate reader, 75μL was recorded and run to completion for each well. Gating on single live target cells was performed according to forward and side scatter characteristics, positivity for the cell proliferation dye, and exclusion of the fixable viability dye. Gates were then set on the CD3+/TCRvβ5.1+ population (Supplemental Figure 5D). Cytotoxicity was calculated using the following equation: 100*(1-Count(Live cells in experimental condition)/average count(Live cells in target + NTD condition)).

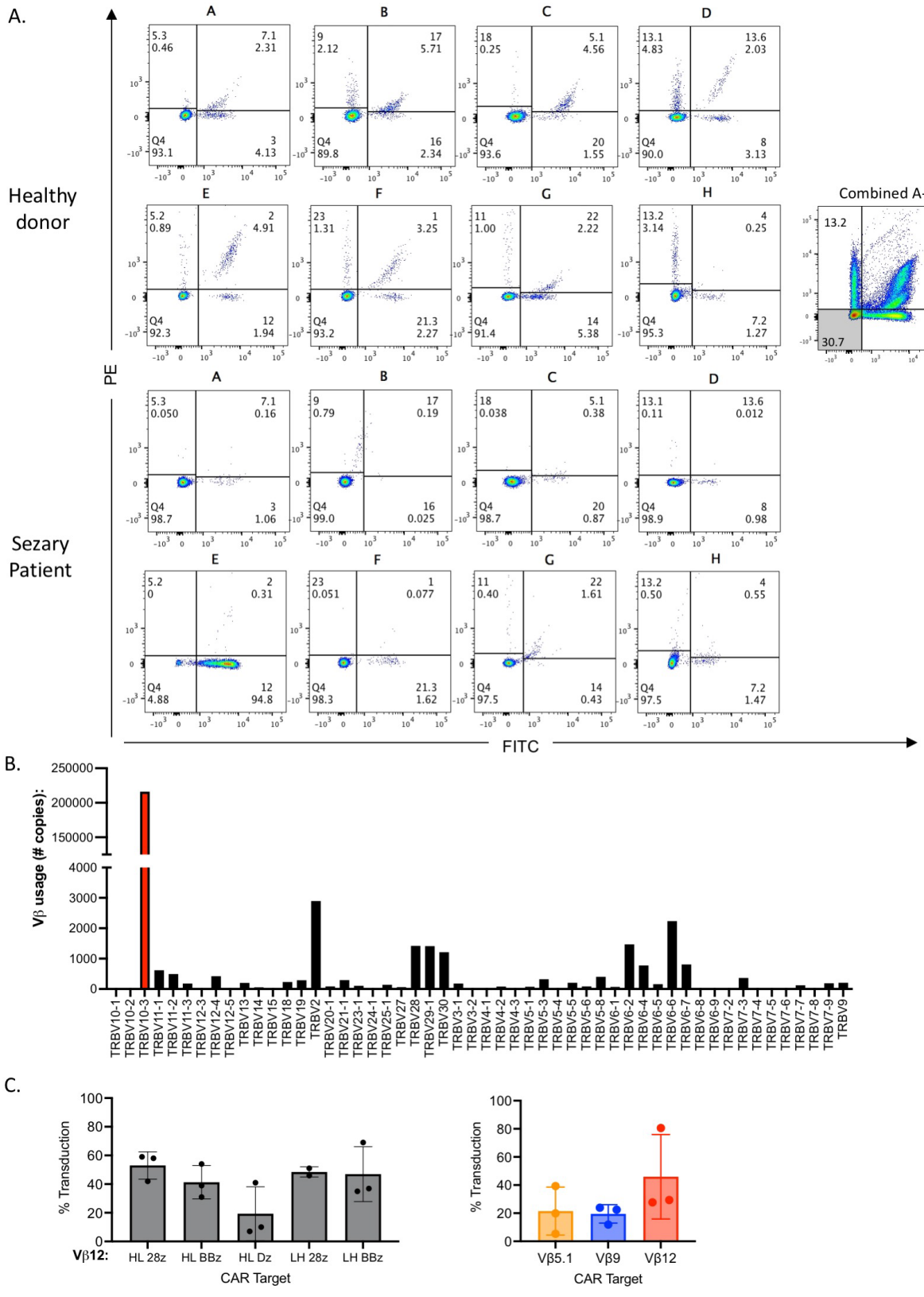
Experimental setup for activation of target patient cells

The vβ5.1+ patient sample was identified to be CD4+, so CD8+ CARTs were developed for this experiment to differentiate the targets from the effectors. A co-culture was set up as above at a 1:1 E:T ratio. After 5 days, cells were collected and prepared for flow cytometry analysis. In addition to the cell surface staining, the cells were stained for intracellular Ki67 using a Ki67-BV605 antibody and the Foxp3/Transcription factor staining buffer set

(eBiosciences) according to the manufacturer's protocol. A similar experiment was run using the vβ12+ patient sample. In this case, standard CD4/CD8 CARTs were utilized.

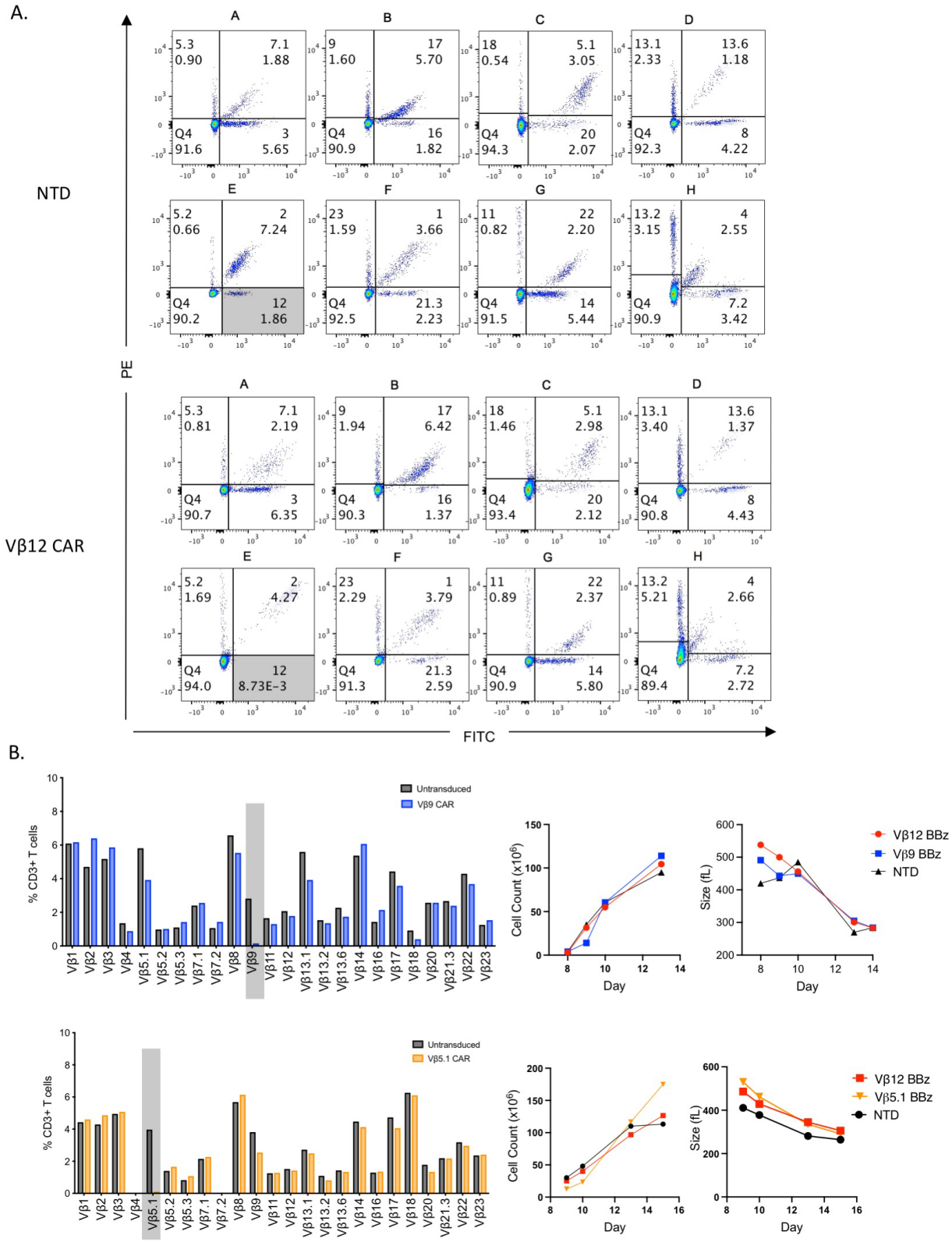
To examine potential reciprocal lysis of effector cells by healthy target cells at low E:T ratios, healthy donor T cells were sorted by FACS for vβ12- and vβ12+ populations. The vβ12- fraction was transduced with TCRvβ12-CARs and the vβ12+ fraction was expanded in parallel. A co-culture was set up as above at a 1:10 E:T ratio where target cells were stained with a cell tracking dye. After 48 hours, cells were collected and analyzed by flow cytometry.

Supplemental Figures



Supplemental figure 1. (A) Dot plots of healthy donor and Sézary patient T cells that were phenotyped using 8 stains (A-H) containing a total of 24 antibodies specific for a TCRvβ family. A healthy donor sample was also stained with all

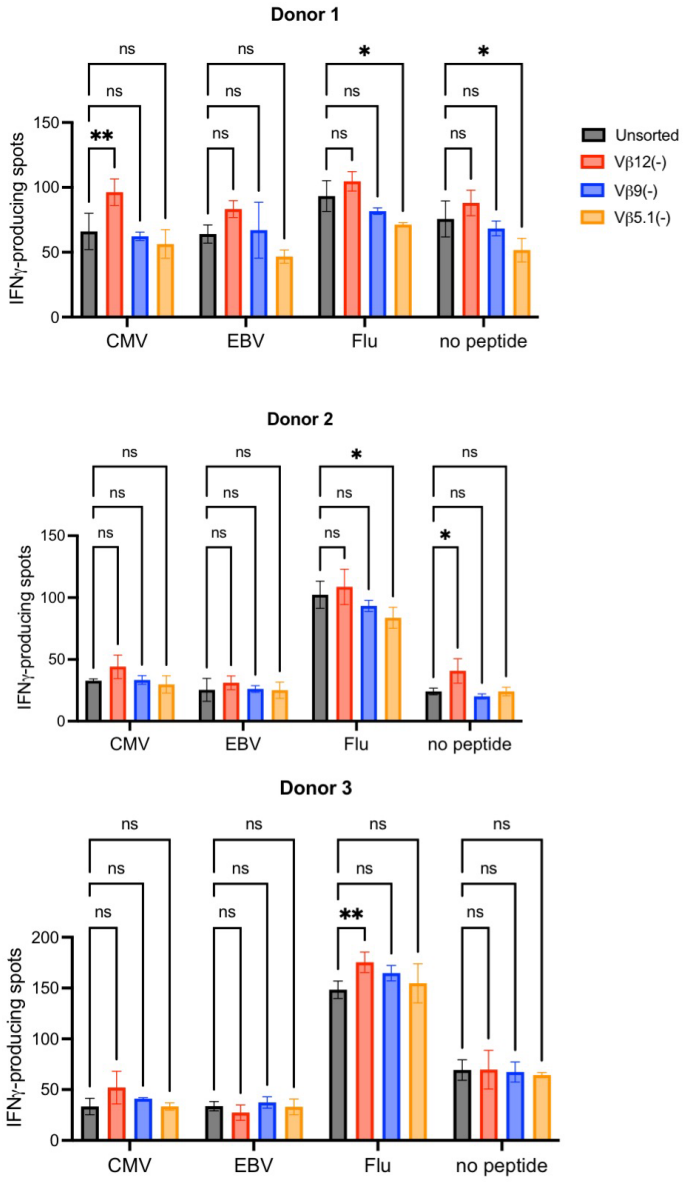
antibodies simultaneously to determine the percent of the T cell population not covered by the antibodies (gray box). This is in agreement with the information provided with the Beckman Coulter Beta Mark IO Test. (B) TRBV sequencing data confirming that the Sézary patient sample contains one dominant malignant clone in TCRvβ12 (TRBV10-3) amongst the healthy population in the other TCRvβ families. (C) Transduction of healthy donor (n=3) T cells engineered to express various CAR constructs that target TCRvβ families as determined by protein L staining and flow cytometry analysis.



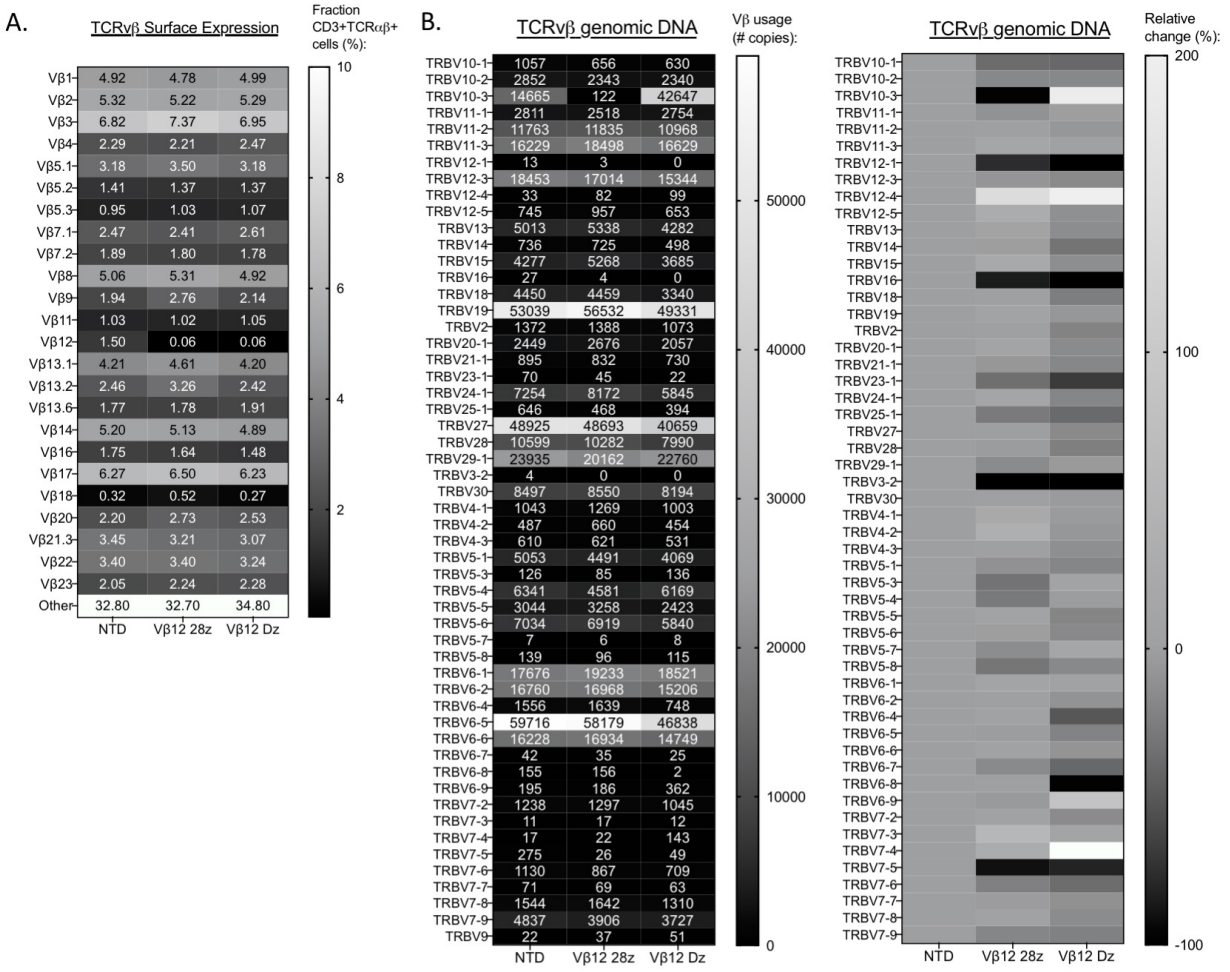
Supplemental figure 2. (A) Dot plots of stains for TCRvβ families in expanded T cells that were either NTD or engineered to express a CAR against TCRvβ12. The target population is eliminated (gray box) via fratricide when the CAR is expressed. Clonograms from expanded T cells that were either NTD or engineered to express a CAR against

TCRvβ9 (B) or TCRvβ5.1 (C) and their expansion kinetics. Expansion kinetics for CARTs against TCRvβ12 is also shown (red) for both expansions.

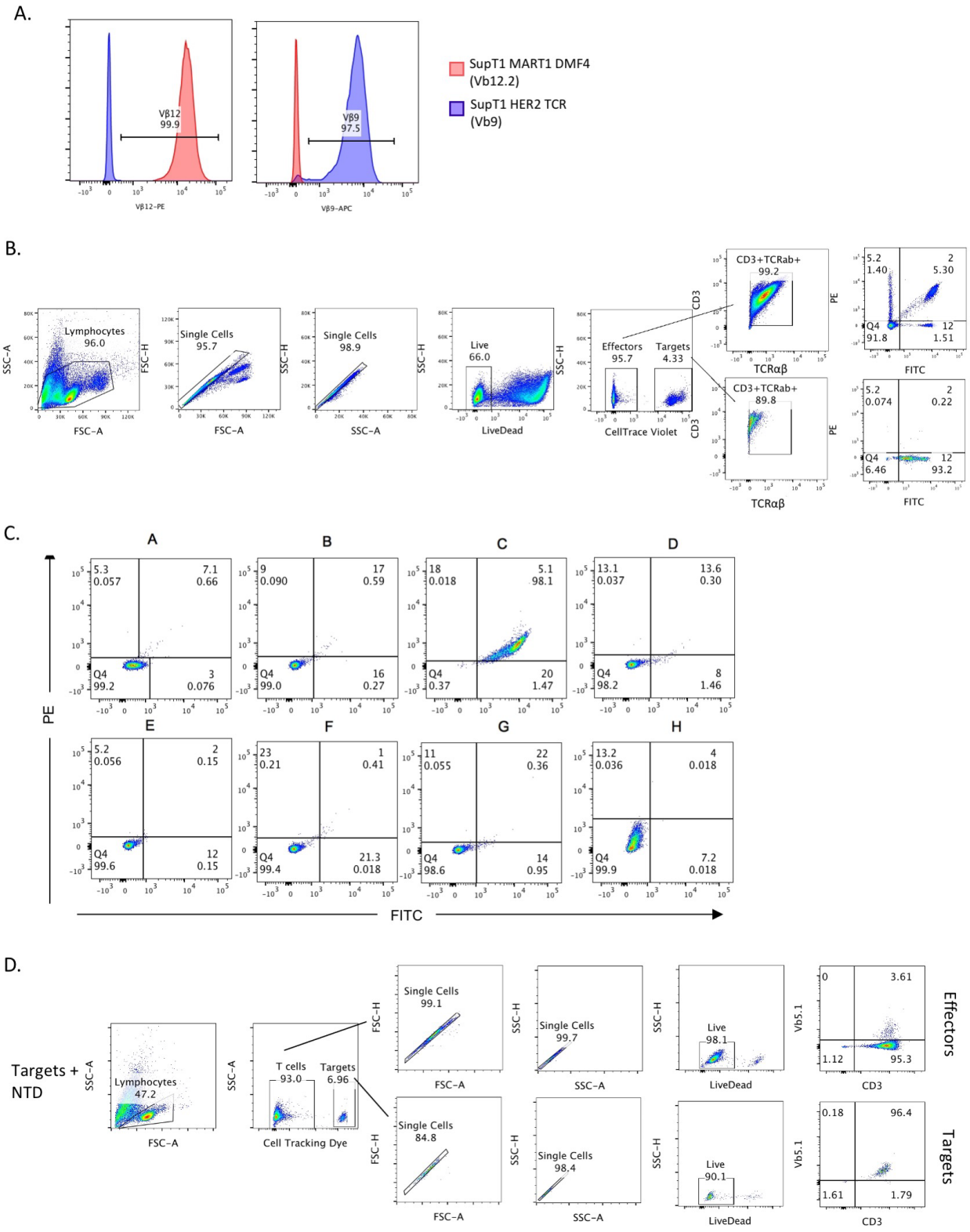
A.



Supplemental figure 3. (A) Healthy T cells from 3 donors were collected and sorted by FACS to remove populations expressing either TCRvβ12, 9, or 5.1. Unsorted cells were used as a control. All T cells were stimulated with peptides against CMV, EBV, and influenza in the presence of antigen-presenting cells. Each graph represents an individual donor. IFNγ-producing spots identified in each condition are shown. Analysis was done to compare the number of cells responding to that of unsorted cells. P value style: 0.1234 (n.s), 0.0332 (*), 0.0021 (**) by two-way ANOVA with Sídák multiple comparison test.

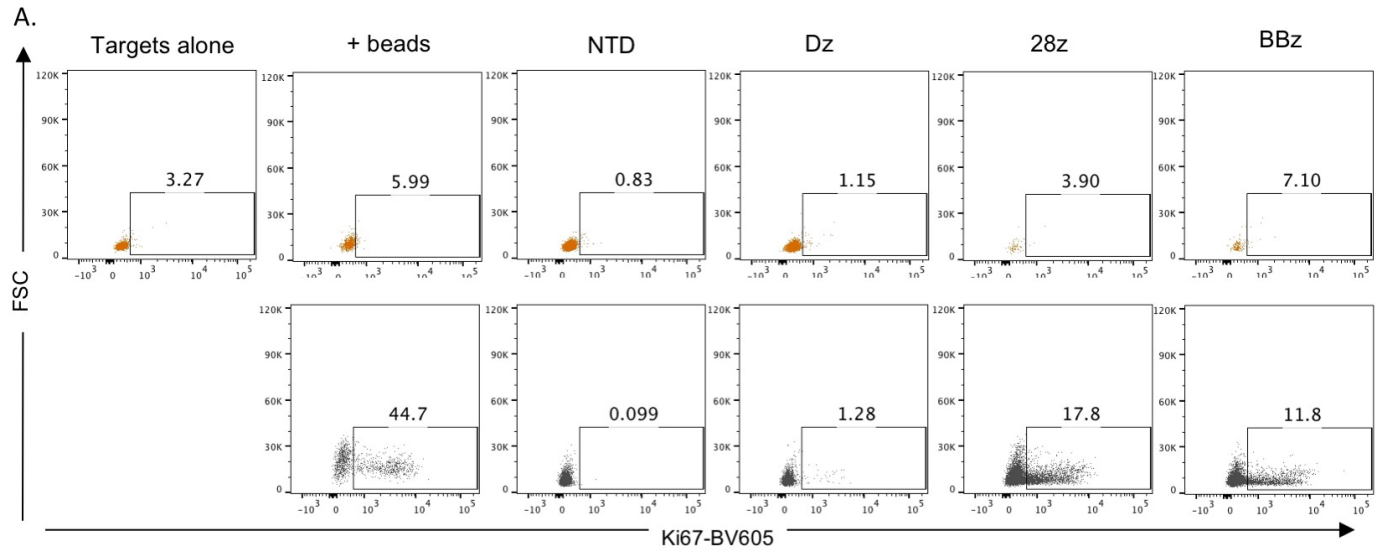


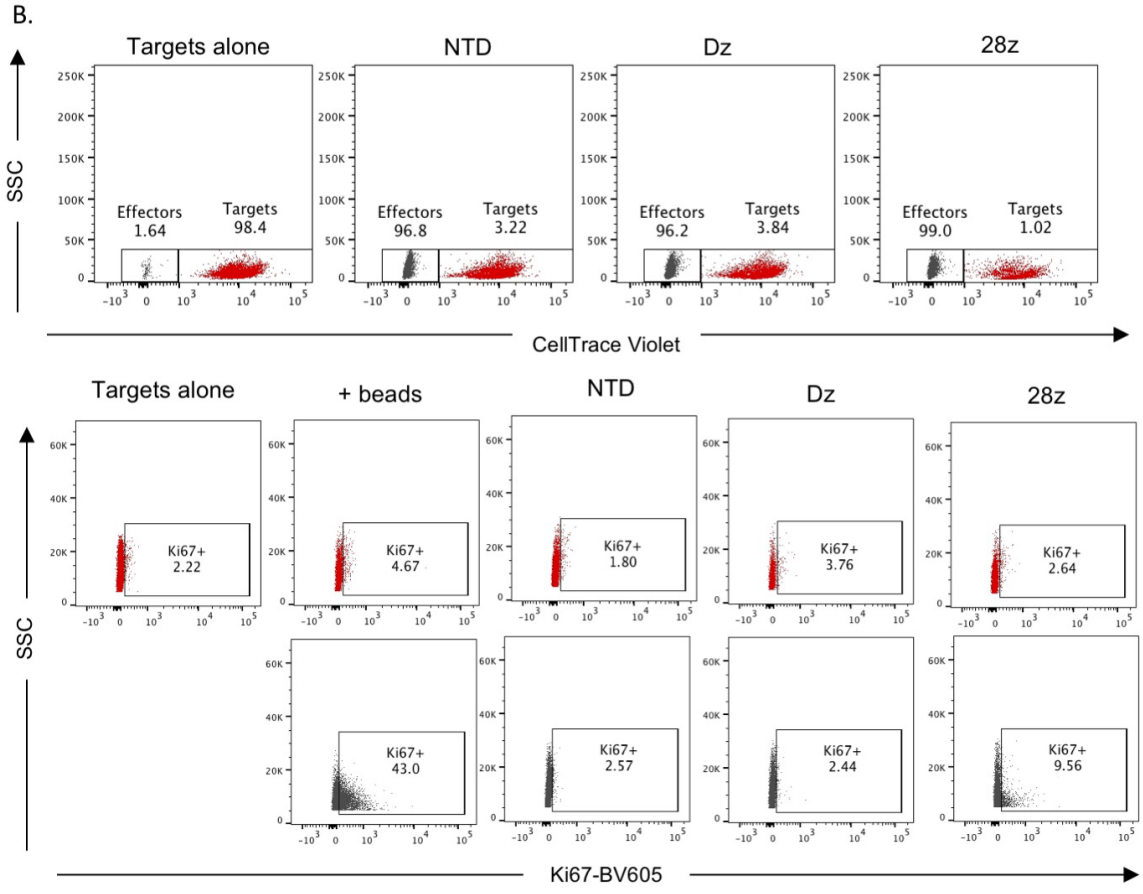
Supplemental figure 4. (A) Percentages of the 24 tested TCRvβ families in expanded CD3+TCRαβ+ T cells either NTD or engineered to express a truncated CAR against TCRvβ12 or a functional CAR against TCRvβ12. Cells in the "other" group were determined as cells that were CD3+TCRαβ+ but negative for all 24 TCRvβ antibodies. (B) Genomic analysis of TCRvβ families in the same CARTs, either represented as number of copies (left) or relative change compared to NTD cells (right).

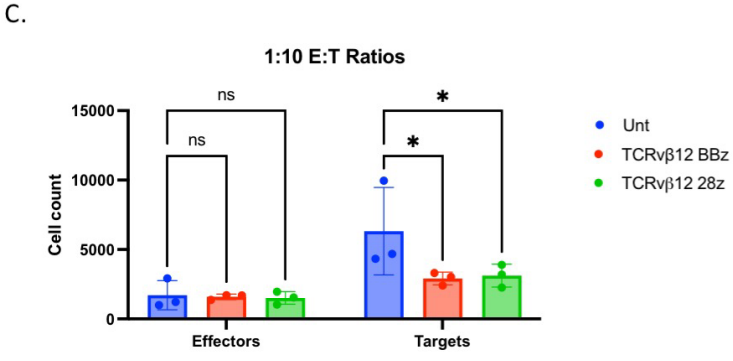


Supplemental figure 5. (A) Histogram demonstrating TCRvβ expression of SupT1 cells engineered to express TCRs that had TCRvβ components belonging to either TCRvβ12 or TCRvβ9. (B) Representative gating strategy for the co-culture experiment with the TCRvβ12+ Sézary patient sample, here shown with NTD effector cells. Target cells were

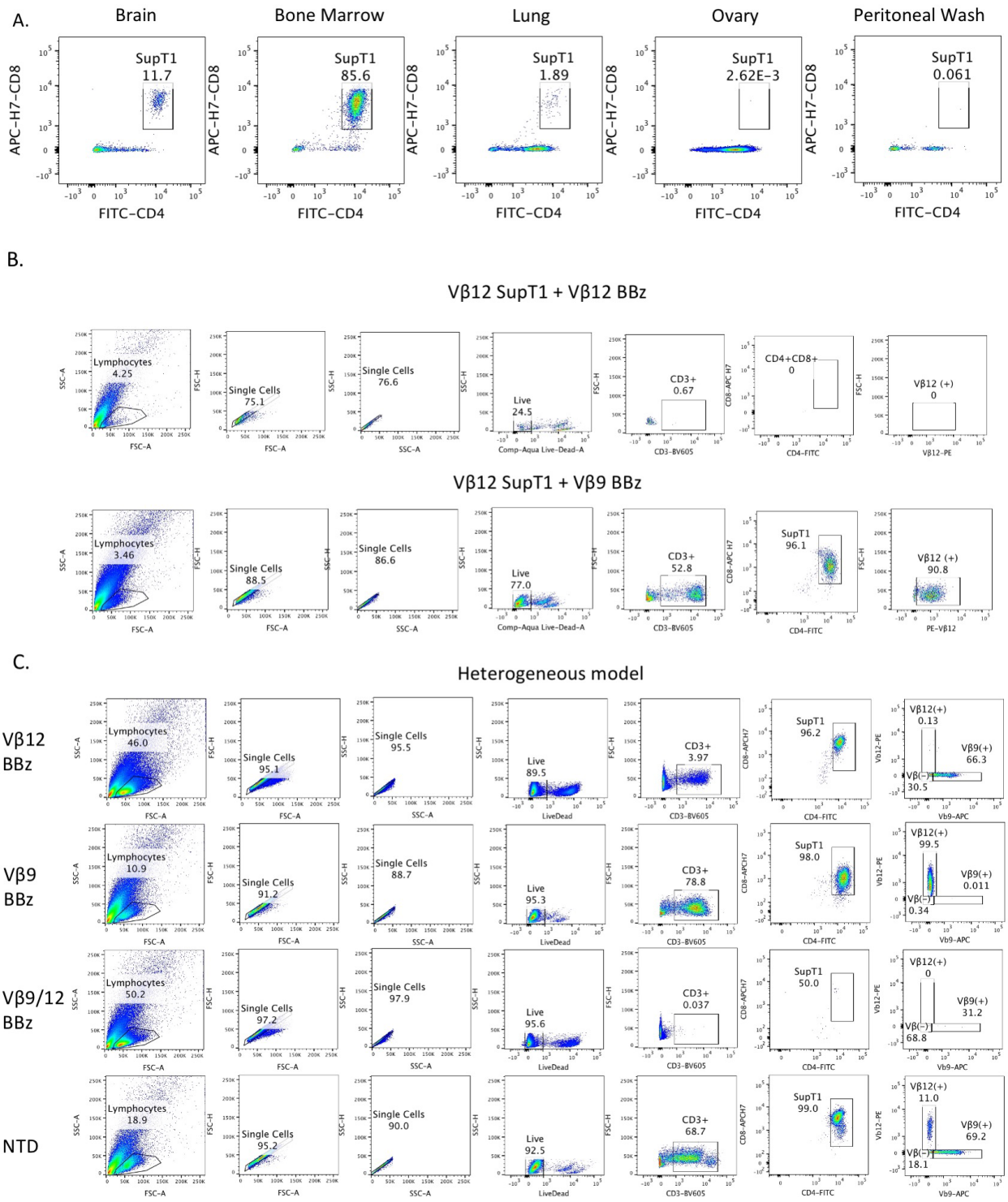
stained with CellTrace Violet prior to co-culture to distinguish them from effector cells. CD3+TCRαβ+ cells in each group were then analyzed for the 24 TCRvβ families. This representative plot used stain “E,” and shows TCRvβ5.2, 2, and 12 (clockwise from top left). (C) Dot plots of staining for 24 TCRvβ families in the Sézary patient sample determined to contain a dominant clone in TCRvβ5.1, representing 98.1% of the total T cell population. (D) Gating strategy for the co-culture experiment with the TCRvβ5.1+ Sézary patient sample. Target cells were stained with a cell tracking dye prior to co-culture to distinguish them from effector cells. The live, single cells in the target population were used to determine lysis by comparing their number to those with NTD T cells. Effectors and targets were further phenotyped for their CD3 and TCRvβ5.1 expression.


A.
Targets alone
+ beads
NTD
Dz
28z
BBz
FSC
3.27
5.99
0.83
1.15
3.90
7.10
44.7
0.099
1.28
17.8
11.8
Ki67-BV605


B.
Targets alone
NTD
Dz
28z
SSC
Effectors 1.64
Targets 98.4
Effectors 96.8
Targets 3.22
Effectors 96.2
Targets 3.84
Effectors 99.0
Targets 1.02
CellTrace Violet
Targets alone
+ beads
NTD
Dz
28z
SSC
Ki67+ 2.22
Ki67+ 4.67
Ki67+ 1.80
Ki67+ 3.76
Ki67+ 2.64
Ki67+ 43.0
Ki67+ 2.57
Ki67+ 2.44
Ki67+ 9.56
Ki67-BV605

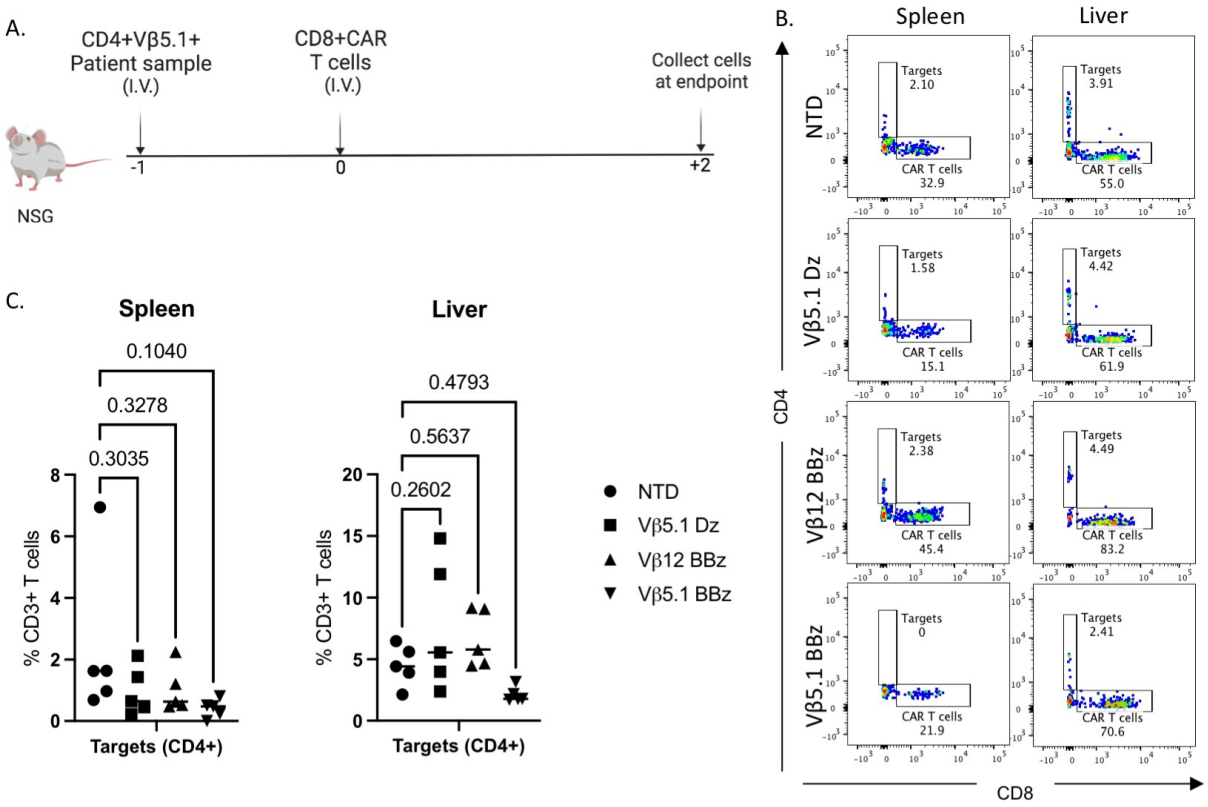

C.
1:10 E:T Ratios
Cell count
15000
10000
5000
0
ns
ns
*
*
Effectors
Targets
Unt
TCRvβ12 BBz
TCRvβ12 28z

Supplemental figure 6. (A) Representative plots of Ki67 expression by target (top, orange) or effector (bottom, gray) cells at the end of a 5-day co-culture. (B) Representative plots from a 5-day co-culture of TCRvβ12+ patient sample with TCRvβ CARTs. Dot plots of effector and target cells at the end of the culture identified by CellTrace Violet (top). Ki67 expression of target (red) or effector (gray) cells. (C) Quantification of absolute count of effector and target cells after 48-hour co-culture of CARTs and sorted, healthy, autologous TCRvβ12+ T cells, analyzed by fow cytometry. P value style: 0.1234 (n.s), 0.0332 (*), 0.0021 (**) by two-way ANOVA with Sídák multiple comparison test.



Supplemental figure 7. (A) Representative dot plots of SupT1 (CD4+CD8+) cells identified in various organs of a mouse at the endpoint of the study. The brain and bone marrow were selected for collection for all other mice, but further data is shown only from brain tissue for simplicity. (B) Gating strategy for identifying TCRvβ12+ cells in

brains collected from mice at the endpoint of the study. Data from a representative mouse from the treatment and control group are shown. (C) Gating strategy for identifying TCRvβ12+ and TCRvβ9+ cells in brains collected from mice at the endpoint of the study. Data from a representative mouse from the treatment and control groups are shown.



Supplemental figure 8. (A) Experimental setup of in vivo experiment using TCRvβ5.1-expressing patient cells. The endpoint was decided based on preliminary studies that indicated the cells could be detected 3 days after engraftment but, due to the lack of expansion, could not be detected beyond that time. N=5 mice per group. (B) Representative dot plots from mice whose spleens and livers were collected and analyzed for target (CD4+) and CART (CD8+) cells within CD3+ cells. (C) Quantification of patient cells identified in mice at the endpoint. Analysis by one-way ANOVA with Dunnett's multiple comparison test.

References

1. Wells DK, van Buuren MM, Dang KK, et al. Key Parameters of Tumor Epitope Immunogenicity Revealed Through a Consortium Approach Improve Neoantigen Prediction. *Cell*. 2020;183(3):818-834.e13.
2. Bear AS, Blanchard T, Cesare J, et al. Biochemical and functional characterization of mutant KRAS epitopes validates this oncoprotein for immunological targeting. *Nat. Commun.* 2021;12(1):4365.