Supplementary Materials and Methods:

Supplementary Methods

Clinical Protocol. The trial was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board, the U.S. Food and Drug Administration, and the National Institutes of Health Recombinant DNA Advisory Committee. It is registered at clinicaltrials.org as NCT1640301. Individuals with features of 'high-risk' acute myeloid leukemia (AML) and their respective fully human leukocyte antigen (HLA)-matched (10/10) related or unrelated donors expressing HLA-A*02:01 (HLA-A2) were eligible for treatment.

Patient Selection. HLA-A2 genotype was confirmed by high-resolution typing (*49*) prior to pre-hematopoietic cell transplant (HCT) formal enrollment. High-risk features included: AML with adverse genetic abnormalities (*50*), AML beyond 1st remission, primary refractory AML, therapy-related AML, AML arising in patients with antecedent hematologic disorders, or AML with evidence of minimal residual disease (MRD) or overt disease at time of HCT (*50-52*). Exclusion criteria included: active central nervous system (CNS) disease, HIV seropositivity, grade ≥3 graft-versus-host disease (GVHD), and no available Epstein-Barr Virus (EBV)-seropositive matched donor. On this two-arm clinical trial, patients with no detectable disease post-HCT received treatment with T cell receptor (TCR) transgenic $T_{TCR\text{-}C4}$ on the Prophylactic Arm, and patients with evidence of disease post-HCT received $T_{TCR\text{-}C4}$ on the Treatment Arm (*10*).

Detailed case report of Patient 1. The first individual described here was a 27-year-old man who, at the age of 15 was diagnosed with AML trisomy 8, translocation (12;14). His AML proved resistant to daunorubicin, AraC, etoposide and dexamethosone, followed by high-dose cytarabine, and mitoxantrone induction. The patient finally achieved a complete remission after

single agent gemtuzumab ozogamicin (Mylotarg). He underwent a first 10/10 matched related mobilized allogeneic hematopoietic stem cell transplant (HCT) with a preparative regimen of Samarium 153-ethylene diamine tetramethylene phosphonate (^{153}Sm-EDTAP) and Mephalan. HCT was successful with full engraftment and no evaluable disease (NED) detected post-HCT. His AML relapsed seven years later, with a new deletion 5q (23q33) and was now located in the marrow (61% blasts), in the central nervous system (67% blasts) and within a S2-S3 nerve root myelosarcoma. He received systemic re-induction with azacytidine and Mylotarg, serial intrathecal methotrexate (IT MTX) and 24 Gy of radiation in 12 fractions to the sacrum which again achieved a complete response. He received a second reduced-intensity HCT from a 10/10 matched unrelated donor after a preparative regimen of Treosulfan, Fludarabine and 200cGy of total body irradiation. This second HCT achieved 100% donor engraftment and NED post-HCT. He continued to receive IT MTX for 7 months post-HCT and his AML relapsed 7 months after treatment discontinuation with predominantly cervical C5 and C7 myelosarcomas and minimal marrow involvement (0.002% blasts). He received a cycle of systemic azacitine (75mg/m^2 x 7 days) and Mylotarg (days 8, 11, 14) as well as 24 Gy irradiation in 24 fractions to the cervical spine. Two months after the start of this last treatment and immediately prior to a first infusion of 10^{10} $T_{TCR\text{-}C4}$/m^2 on the Treatment Arm, he had no evaluable bone marrow AML. The patient was monitored and showed no evidence of GVHD or toxicity to cells expressing low Wilms' tumor antigen 1 (WT1) including the hematopoietic system, kidneys, lung, heart, liver, and skin were observed. He remained with NED and no additional treatments for 368 days before relapsing with a bladder myelosarcoma. His concurrent bone marrow aspirate was positive for AML blasts. He received a second infusion of 10^{10} $T_{TCR\text{-}C4}$/m^2, radiation to the bladder, and then additional azacitidine and Mylotarg, filgrastim, cladribine, cytarabine, and mitoxantrone (G-

CLAM) chemotherapy and hydroxyurea which did not control his AML. In accordance with the patient and his family, no additional treatments were administered, and he succumbed to progressive AML a year after his 2nd T_{TCR-C4} infusion. Although the patient was followed closely after the last relapse, the study team respected his wishes for minimal intervention and sample collection, which limited the amount of material obtained.

Detailed case report of Patient 2. The second patient described was a 47-year-old man who at the age of 46 was diagnosed with a myelodysplastic syndrome at the stage of refractory anemia with excess blasts (blasts constituted 15.6% of marrow cells). He had multiple cytogenetic abnormalities including translocation of chromosome 11 and 12, trisomy 8 and 21 and deletion of the long arm of chromosome 17. A month later he presented with circulating blasts and received chemotherapy with daunorubicin and cytarabine on a scheduled 7 +3, 2 cycles of decitabine, daunorubicin and cytarabine, and decitabine followed by mitoxantrone, etoposide and cytarabine. Chemotherapy did not avert progressive disease, and no remission was achieved. His circulating blast count was then maintained with hydroxyurea. He was enrolled in a transplant protocol specifically designed for patients with residual leukemia and received a myeloablative induction of clofarabine and busulfan prior to the infusion of peripheral blood stem cells from a male matched unrelated donor. Post-transplant immunosuppression consisted of methotrexate on days 1, 3, 6 and 11, as well as tacrolimus that was tapered 56 days later. Twenty-eight days after transplant, he presented with minimal residual disease (0.28% abnormal marrow blasts). He received two 7-day cycles of azacytidine separated by a month, but his disease continued to progress. A bone marrow biopsy performed 94 days after HCT showed 12.9% blasts and cytogenetics now showed evidence of clonal evolution with additional inversion of chromosome 7. Chromosome gene array testing (CGAT) performed on the latter marrow showed deletions of

chromosomes 9q13q31, 9q32q33 and 17q11.2 in >80% host cells (host cells constituted 80% of the bone marrow cells). Of note, no WT1 copy number aberrations, no single nucleotide polymorphisms or copy neutral loss of heterozygosity were detected. Twelve days prior to the infusion of 10^{10} $T_{TCR\text{-}C4}/m^2$ on the protocol Treatment Arm (150 days after HCT), his marrow showed 69% blasts; immediately prior to cell infusion his complete blood count included a total of 8030 white blood cells/mcl with 66% blasts (5310 cells/mcl), 9% neutrophils (720 cells/mcl) and 13% lymphocytes (1040 cells/mcl). Although his absolute blast count moderately decreased immediately after infusion, his AML subsequently progressed despite persistence of $T_{TCR\text{-}C4}$ at greater than 3% of $CD8^+$ T cells in the peripheral lymphocyte compartment. He succumbed to progressive disease 15 days after $T_{TCR\text{-}C4}$ transfer.

Assessment of disease status. Morphology, multiparameter flow cytometry (*53*), standard cytogenetics (*54*), or genomic technologies (*55*) were routinely performed on bone marrow aspirates that were obtained from the patients. Any detectable residual disease was considered to indicate positivity for MRD.

Isolation of TCR_{C4} and lentiviral vector construction. TCR_{C4} was selected from a screen of more than 1000 T cell clones from more than 50 HLA-$A2^+$ healthy donors based on its comparatively higher functional avidity for the $WT1_{126\text{-}134}$ peptide (RMFPNAPYL) as previously described (*10*). TCR_{C4} was inserted into the pRRLSIN.cPPT.MSCV/GFP.wPRE vector obtained from the lab of Richard Morgan after excising the green fluorescent protein (GFP) gene (*56*).

Generation of T_{TCR-C4}. All ex vivo processing of infusion products was performed in the FHCRC cGMP Cell Processing Facility (CPF) at as previously described (*10*). Briefly, substrate $CD8^+$ T cells for generating the TCR_{C4}-transduced T cells (T_{TCR-C4}) were obtained from an aliquot of the granulocyte colony stimulating factor (G-CSF)-stimulated peripheral blood mononuclear cells (PBMC) donation (mobilized leukapheresis) of the patients' corresponding allogeneic stem cell donors. Cells were stimulated with the HLA-A2-restricted EBV ($EBV_{280-288}$ BMLF1 [GLCTLVAML]) peptide, transduced using the pRRLSIN.cPPT.MSCV/$TCR_{C4}\beta$-P2A-$TCR_{C4}\alpha$/wPRE lentiviral vector and sorted by flow cytometry; cells were labeled with peptide:HLA tetramers that selectively bound the WT1-specific TCR labeled with allophycocyanin (APC) and the EBV-specific TCR labeled with phycoerythrin (PE); double positive cells were selected followed by a secondary, non-specific expansion and infusion into the patient. Total production time was 4 to 6 weeks. Quality control of the infused products included assessment of inserted lentiviral vector copy number per cell (≤5 copies per cell), envelope vesicular stomatitis virus (VSV)-G DNA by qPCR as a surrogate marker for replication-competent lentivirus (RCL) (<10copies/50ng DNA) and binding to the $WT1_{126-134}$ HLA-A2-restricted tetramer (≥30% of live cells). HLA-A2/$WT1_{126-134}$ specific tetramers (produced by the FHCRC Immune Monitoring Core facility) were used to detect T_{TCR-C4} in PBMCs collected after infusions, with a staining sensitivity of 0.01% of total $CD8^+$ T cells, as previously described (*57*).

Clonotype identification in the pre-infusion product and tracking by high throughput TCRβ sequencing. To identify clonotypes composing infused T_{TCR-C4}, high-throughput TCRβ sequencing (HTTCS) analysis was performed on HLA-A2/$WT1_{126-134}$ $tetramer^+$ cells from the

pre-infusion product to obtain >99% purity (*58*). As the TCR_{C4} sequence was codon-optimized, HTTCS could not identify the introduced WT1-specific TCR. Instead, HTTCS identified clonotypes based on the endogenous EBV-specific TCR sequence. To track the identified clonotypes, HTTCS was performed on whole PBMC obtained after transfer (*58*). Briefly, DNA was extracted from T cell products and sorted PBMC using Qiagen Maxi DNA isolation kits (QIAGEN Inc.). TCRβ complementary determining region (CDR)3 regions were amplified and sequenced from 750ng of extracted DNA by Adaptive Biotechnologies Corp using the ImmunoSEQ assay with "deep" resolution, as previously described (*59*). Raw sequence data was filtered using the Adaptive bioinformatic website based on the TCRβ V, D and J gene definitions provided by the International ImMunoGeneTics collaboration (IMGT) (*60*) using the IMGT database (www.imgt.org). Productive nucleotide sequences were used for all tracking experiments. The limit of detection of HTTCS was set at 0.001% of all TCR reads, below which frequency could not be reliably determined (*60*). Only clonotypes present in the infusion products were tracked in PBMC obtained after infusions. Infusion product 'bystander' clonotypes were defined as those not detected in vivo post-infusion and not expanded during the ex vivo culture process, and were eliminated for further analysis (*58*). The frequency of each HTTCS-detected clonotype is based on all TCR Vβ reads which include $CD4^+$ and $CD8^+$ T cells.

Flow cytometry on T cells. $T_{TCR\text{-}C4}$ in infusion products and post-transfer PBMCs were identified by binding to the APC dye-labeled HLA-A2:$WT1_{126\text{-}134}$ tetramer (FHCRC in-house production), and analyzed by flow cytometry after staining for 30 minutes with fluorochrome-conjugated monoclonal antibodies to CD16 (1:150 dilution, PE-Cy5, clone 3G8, Becton Dickinson. 555408), CD19 (1:80 dilution, PE-Cy5, clone HIB19, Becton Dickinson, 555414; for

dump channel), CD4 (1:50 dilution, brilliant ultraviolet (BUV) 496, clone SK3, Beckton Dickinson, 612936), CD8 (1:80 dilution, Qdot 605, clone 3B5, Life Technologies, Q22157), CD28 (1:200 dilution, brilliant violet (BV) 421, clone CD28.05, BioLegend, 302930), CD27 (1:200 dilution, Fluorescein isothiocyanate (FITC), clone M-T271, BioLegend, 356404), CD62L (1:50 dilution, PE, clone SK11 Becton Dickinson, 341012) and CD45RO (1:100 dilution, Alexa Fluor 700, clone UCHL1, BioLegend, 304218). A representative example of the initial gating algorithm is shown in **fig. S2A**.

Intracellular cytokine staining. Cryopreserved PBMCs were thawed and rested overnight in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) (R10). Intracellular cytokine staining and stimulations were performed using a 15-color staining panel, as previously described (*61, 62*). The stimulation cocktail contained either $WT1_{126\text{-}134}$ peptide in R10 at a final peptide concentration of 1 μg/mL or R10 with an equivalent amount of dimethyl sulfoxide (DMSO) used to preserve the peptides (negative control) or staphylococcal endotoxin B (Signma, 11100-45-1, positive control). Modifications to the panel included excluding interleukin (IL)-21, CD56 and CXCR5, changing IL-2 to PE-dazzle 594 (1:100 dilution, clone MQ1-17H12, BioLegend, 500344). Cells were analyzed on an LSRII flow cytometer (Becton Dickinson), using FACS-Diva software. The resulting flow cytometry data were analyzed with FlowJo v9.9.6 (Treestar). Percent cytokine expression (% of tetramer$^+$ cells that are both cytokine$^+$ and tetramer$^+$ in the DMSO only sample [negative control]) was calculated for each sample. Percent tetramer$^+$cytokine$^+$ cells in the presence of DMSO without peptide varied among samples from 0-2.27% with a median of 0.745%. A representative example of the initial gating algorithm is shown in **fig. S2B**.

Serum EBV and CMV quantitation. EBV PCR was developed in-house using primers specific for the EBER gene (*63*); the limit of quantitative detection (the minimum virus concentration that gives a positive result in 95% of replicates) was 250 IU/mL (2.40 log IU/mL). Quantitative results less than 250 IU/mL are described as very low positive. CMV PCR was developed in-house using primers specific for the UL123 and gB genes (*64*); the limit of quantitative detection (the minimum virus concentration that gives a positive result in 95% of replicates) was 20 IU/mL (1.3 log IU/mL). Quantitative results less than 20 IU/mL are described as very low positive.

Single Cell RNA Sequencing (scRNAseq). ScRNAseq (*65*) was performed on samples from two patients enrolled in NCT1640301. Patient 1 'remission' and 'relapse' samples were selected to be approximately 100 days after the last infusion to minimize changes associated with infusion of large numbers of ex vivo-manipulated cells. Patient 2 samples were selected before $T_{TCR\text{-}C4}$ infusion and five days after. PBMCs for both patients were thawed, washed, and labelled using the 10x Genomics 3' Chromium v2.0 (Patient 1) or 5' Chromium v2.0 (Patient 2) platform per manufacturer's instructions. Library preparation was performed as per manufacturer's protocol with no modifications. Library quality was confirmed by Illumina TapeStation high sensitivity (evaluates library size), qubit (evaluates dsDNA quantity), and KAPA qPCR analysis (KAPA Biosystems, evaluates quantity of amplifiable transcript). Samples were mixed in equimolar fashion and sequenced on an Illumina HiSeq 2500 *rapid run* mode according to standard 10x genomics protocol.

Computational analysis for scRNAseq on Patient 1. The Cell Ranger Single-Cell Software Suite (v2.0.0) was used to perform sample demultiplexing, barcode processing and single-cell 3' gene counting (http://10xgenomics.com/). First, raw base BCL files were demultiplexed using the Cell Ranger *mkfastq* pipeline into sample-specific FASTQ files. Second, these FASTQ files were individually processed using the Cell Ranger *count* pipeline. Reads, which contain cDNA inserts, were aligned to the hg38 human reference genome (Ensembl) and the known transgene codon-optimized sequence using Spliced Transcripts Alignment to a Reference (STAR) (*66*). Aligned reads were then filtered for valid cell barcodes and unique molecular identifiers (UMIs). Cell barcodes with 1-Hamming-distance from a list of known barcodes were examined. UMIs with sequencing quality score > 10% and not homopolymers were retained as valid UMIs. A UMI with 1-Hamming-distance from another UMI with more reads, for a same cell and a same gene was corrected to this UMI with more reads. The default estimated number of cells used were 10,000 cells for both 'remission' and 'relapse' timepoints. Samples from both time points were aggregated using the Cell Ranger *aggr* pipeline resulting in one gene-barcode count matrix to be used for downstream analyses. A correction for sequencing depth was performed during the aggregation.

Statistical analysis for scRNA-seq on patient 1 PBMC. Standard analyses were performed using the Seurat R package (*67, 68*). Following sequence alignment and filtering, cells with a percentage of mitochondrial genes > 20%, a unique gene counts < 200 or > 15,000 UMIs were removed. Based on these thresholds, 68 cells were discarded, resulting in a data set of 7,688 cells for downstream analyses. Only genes with at least one UMI count detected in at least three cells were kept (16,590 genes). A library-size normalization was performed for each cell. UMI counts

were divided by the total number of UMI in each cell and multiplied by 10,000. Data were then log transformed and corrected for unwanted sources of variation such as the number of detected UMIs and percentage of mitochondrial content using the *ScaleData* R function as described in the Seurat manual. As high mitochondrial gene expression might be suggestive of low-quality libraries (*69*), the corrected-normalized gene-barcode matrix was used as input to perform Principal component analysis (PCA), clustering, and Uniform Manifold Approximation and Projections for Dimension Reduction (UMAP) analyses, whereas the normalized gene-cell barcode matrix was used as input for the differential expression analysis as described below. PCA was performed using the top 2,000 most variable genes. The top 20 principal components (PCs) were then down-selected for UMAP visualization and clustering. UMAP with these parameters (n.epochs = 400, min.dist = .2, local.connectivity = 20) was performed using the *RunUMAP* R function. Cell clustering was performed using a graph-based clustering method implemented in Seurat (*FindNeighbors* and *FindClusters* R function) using a resolution of 0.25.

Differential expression analysis was performed using the R package Model-based Analysis of Single-cell Transcriptomics (MAST) (*70*) implemented within the *FindMarkers* R function from the Seurat R package. MAST provides functionality for significance testing of differential expression using a Hurdle model, gene set enrichment and facilities for visualizing patterns in residuals indicative of differential expression. It is a two-part generalized linear model that simultaneously models the rate of expression over the background of various transcripts (logistic regression), and the positive expression mean (Gaussian). These tests are two sided and adjust for the bimodal nature of scRNAseq data. Differential expression was performed between clusters, and between conditions (such as transgenic versus endogenous $CD8^+$ T cells). In each case, the

normalized gene-cell barcode matrix was used as input. The model included the cellular detection rate (CDR), defined as the total of UMIs in a given cells, as a covariate to correct for biological and technical nuisance factors that can affect the number of genes detected in a cell (such as cell size and amplification bias). Genes were declared significantly differentially expressed at a false discovery rate (FDR) of 5% and a fold-change > 1.5.

Computational and statistical analysis for scRNA-seq on patient 2. A relaxed quality control (QC) strategy was used, and we chose to only exclude cells with large mitochondrial proportions as proxy for cell damage. A stringent cutoffs (2 Median Absolute Deviation; MAD) was used for percent of mitochondrial genes, resulting in a data set of 10,700 cells for downstream analyses (2,477 cells were filtered out). Only genes with at least one UMI count detected in at least three cells were retained (17,475 genes).

Data integration and standard analyses were performed using the Seurat R package (*68*). Gene expression data integration was performed using the time point as factor, resulting in two different datasets. To integrate the gene expression values, we first separately normalized each of our partitions using the *SCTransform* R function (*71*) with *vars.to.regress = "percent.mito"*. Data integration was then performed using the two R functions *FindIntegrationAnchors* and *IntegrateData* (with *dims = 1:50*). PCA was performed on the integrated dataset was performed using the *RunPCA* R function. The top 50 PCs were then used for UMAP visualization and clustering. UMAP with this parameter (*min.dist = .01*) was performed using the *RunUMAP* R function. Cell clustering was performed using a graph-based clustering method implemented in Seurat (*FindNeighbors* and *FindClusters* R function) with different resolutions. Differential

expression analysis was performed using the R package, MAST (*70*), implemented within the *FindMarkers* R function from the Seurat R package as described in the *Statistical analysis for scRNA-seq on patient 1 PBMC* section

Immunohistochemistry (IHC). WT1 IHC was performed by a College of American Pathologists Clinical Laboratory Improvement Amendments (CLIA) certified laboratory with a standard clinical protocol. Paraffin-embedded core bone marrow biopsies or particle preparations were sectioned (4μM) and antigen retrieval performed by heat treatment in 10mM sodium citrate buffer (pH 6) before staining with DAKO mouse anti-WT1 monoclonal antibody (mAb) clone 6F-H2, at a 1:800 dilution. IHC for HLA Class I (HLA-A, B, C) was performed in a CLIA certified laboratory using Paraffin-embedded core bone marrow biopsies or particle preparations were sectioned (4μM). Antigen retrieval was performed at heat treatment at 95°C for 20 minutes in DIVA citrate buffer (pH 6.0), before staining with mouse monoclonal major histocompatibility complex (MHC) Class I antibody clone EMR8-5 (Abcam) at a 1:1000 dilution.

AML HLA-A2 flow cytometry. Patient PBMCs were assessed at relapse for HLA-A2 expression using Flow Cytometry. Briefly, PBMCs containing ≥89% AML were stained for 30 minutes CD34 (1:50 dilution, BV421, clone 561, BioLegend) to identify AML blasts and for HLA-A2 (1:600 dilution, APC, clone BB7.2, Beckton Dickinson). Samples were evaluated on an FACSymphony flow cytometer and analyzed as described above.

Gene expression analysis from archived formalin fixed paraffin embedded (FFPE) samples and peripheral blood. Tumor tissues was obtained post-second HCT and pre-$T_{TCR\text{-}C4}$ from a malignant chloroma (**Fig. 3A**) and at the time of relapse from PBMCs (day 581 after the first $T_{TCR\text{-}C4}$ infusion). Both samples contained ≥89% AML. For FFPE tissues, total RNA was extracted using the AllPrep DNA/RNA FFPE Kit (Qiagen); for PBMCs, RNA was extracted using Blood RNEasy miniprep kit (Qiagen). RNA gene expression quantification was performed using the nCounter Immunology Panel (Human V2, code set NS_Immunology_v2_c2328) that contains 579 immunology related human genes plus 15 internal housekeeping control genes per manufacturer's protocol (NanoString Technologies). The raw counts were normalized using the geometric mean of the internal controls using nSolver software (Version 4.0) and all samples passed QC parameters (**fig. S6**) (*72*). For the blood sample collected after $T_{TCR\text{-}C4}$ infusion, the normalized counts for the panel-represented genes encoding the cap proteins ([*PSMC2*] and [*PSMD7*]) and the catalytic sites of the immunoproteasome (IP) and (β5i [*PSMB8*], β1i [*PSMB9*], β2i [*PSMB10*], β5 [*PSBM5*] and β2 [*PSMB7*]) were expressed as a fold-change compared to expression in the cervical chloroma sample obtained before $T_{TCR\text{-}C4}$ infusions. Of note, although the purity of both samples was ≥89% AML, the pre-$T_{TCR\text{-}C4}$ sample was a chloroma and considered to be nearly 100% AML, whereas the relapsed PBMC sample on which RNA gene expression was performed contained only 89% AML. Thus, the calculated reduction of β1i [*PSMB9*] is likely an underestimate due to immune and hematopoietic cells (expressing elevated immunoproteasome genes) in the PBMC sample.

WT1 sequencing. Clinical sequencing for oncogenic mutations including for WT1 was performed on a post-relapse bone marrow biopsy specimen from day 58 after 2nd infusion using

the University of Washington OncoPlex platform. Performance details and characteristics of this technology have been previously described (*73*). Tumor percentage in the specimen was 35%. The study quality was "high," full length WT1 was sequenced with good coverage of all exons, and average WT1-specific coverage for this sample was 452X.

Whole Exome sequencing. Isolated AML cells were sent to University of Washington Center for Precision Diagnostics for whole exome sequencing. >95% of genes had at least 20X coverage. A single nucleotide polymorphism was detected in [*PSMB9*], which was a G to A transition at position 179 (c.179G>A) yielding a change from an arginine to histidine (p.Arg60His). This polymorphism is reported to occur in 26.8% of the population (NCBI ALFA allele frequency, global population) (*74*).

T cell isolation, activation, and transduction for cytotoxicity assays, live tumor-cell killing assays, and in vivo studies. $CD8^+$ and $CD4^+$ T cells were isolated from healthy HLA-A*02:01 donor PBMCs using the EasySep Human positive selection kits (STEMCELL Technologies) following the manufacturer's protocol. T cells were seeded at 10^6 T cells/ml with 50 IU/ml IL-2 and were activated with either CTS Dynabeads CD3/CD28 (Life Technologies), T Cell TransAct, human (Miltenyi Biotec), or 30ng/ml OKT3 antibody according to manufacturer's instructions. After 24 to 48 hours of stimulation, the stimulant (beads, TransAct, or OKT3) was removed and T cells were transduced with lentiviral particles encoding TCR_{C4}, $TCR_{37\text{-}45}$ or an irrelevant virus-specific TCR ($TCR_{Irr.}$). In some cases, transduction efficiency was enhanced by spinfecting T cells at 30°C and 1055 x g for 90 minutes with 5 µg/mL polybrene (Sigma-Aldrich) or 10mg/ml protamine sulfate. Because $TCR_{37\text{-}45}$ is dependent on CD8 expression in

assays involving use of $CD4^+$ cells, the CD8α and CD8β chains were added to the existing lentiviral constructs to generate double positive $CD4^+$ ($dpCD4^+$) and 'enhanced' $CD8^+$ ($eCD8^+$) T cells. Cells were maintained every 2 to 3 days with IL-2 and media supplemented with 5 to 10% human serum, L-glutamine (Thermo Fisher Scientific) or glutamax (Thermo Fisher Scientific) and antibiotics (penicillin (200U/ml) and streptomycin (0.1mg/ml) (Thermo Fisher Scientific). After seven days, T cells were stained for 30 min and then selected for $CD8^+$ (1:100, BV421, clone RPA-T8, Becton Dickinson, 562428) and tetramer-binding (APC, FHCRC) using flow cytometry and subsequently placed in non-specific rapidly expanding protocol (REP) (*75*) for 9 to 12 days before use. Cells used for ^{51}Cr release assays, live-tumor assays, recognition assays, and in vivo studies were used after sort purification. Cells used in the AML cytotoxicity assays underwent Cas9 electroporation before WT1-specific TCR transduction to knockout both their TCRα and TCRβ chains of the endogenous TCR (see CRISPR methods below).

Assessment of $WT1_{126\text{-}134}$ dependence on specific immunoproteasome subunits. Four HEK-293EBNA cell lines, each expressing one proteasome subtype (*29, 76*), were used to study processing of the peptide $WT1_{126\text{-}135}$ by the four proteasome isoforms. The day before transfection, 30,000 HEK-293 cells were seeded in collagen-treated 96-well plates (Greiner) in 100µl Iscove's Modified Dulbecco Medium (IMDM) 10% FBS supplemented with essential amino acids, penicillin (200U/ml) and streptomycin (0.1mg/ml). The next day, 293 cells were transfected in duplicate using LT-transit (Mirus Bio), 50 ng HLA-A*02:01 and the indicated concentration of the pCMV6-XL4-WT1 variant D cDNA (Origene). As control, 1µM peptide $WT1_{126\text{-}135}$ was pulsed on HLA-A2 transfected cells for 30 minutes. Peptide was removed before addition of $T_{TCR\text{-}C4}$. After 24 hours, transfected cells were incubated with 10,000 $T_{TCR\text{-}C4}$ in 200µl IMDM medium supplemented with 10% FBS, essential amino acids, penicillin (200U/ml),

streptomycin (0.1mg/ml) and 25 U/ml recombinant IL-2. After 20 hours of co-culture, the culture supernatant was collected, and tumor necrosis factor (TNF)-α released by $T_{TCR\text{-}C4}$ was measured using WEHI -164 cl13 cells as previously described (*77*).

Collection, sorting, and RNA extraction of normal donor peripheral blood stem cells and primary AMLs. For our study, we had access to historical data from six normal donor mobilized peripheral stem cells and peripheral blood or bone marrow AML samples from 38 patients. All donors and patients signed the informed consent for Fred Hutch protocol #1690: Fred Hutch/University of Washington Hematopoietic Diseases Repository. Cryopreserved samples were thawed, and RNA was extracted as previously described (*78*). Briefly, thawed cells were stained for 30 minutes with CD45 (APC-H7, clone 581, BioLegend, 560178) to identify lymphoid and myeloid populations, as well as CD34 (APC, Clone 581, BioLegend, 343510) and CD117 (PE, clone 104D2, BioLegend, 313204) to identify the immunophenotype of leukemic blasts. Viable AML blasts were sorted on a BD FACS Aria II instruments for viable AML blasts. Cells from the $CD34^+$ enriched normal donor mobilized peripheral stem cells and leukemic blast populations were then lysed in RLT-Plus buffer (Qiagen) supplemented with β-mercaptoethanol (Sigma-Aldrich), and RNA were extracted by using the AllPrep DNA/RNA Mini kit (Qiagen) and quantified by using the Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA integrity number (RIN) was determined on an Agilent 2200 TapeStation (Agilent Technologies).

RNA sequencing on viable leukemia blasts and normal mobilized peripheral blood stem cells. RiboErase (Roche) was utilized to deplete ribosomal RNA, and transcriptome libraries were generated using a KAPA Stranded RNA-Seq Library Preparation Kit. Paired-end 50 bp or 75 bp reads were sequenced on either an Illumina HiSeq 2500 instrument or NovaSeq

6000 instruments (Illumina). A pipeline of established bioinformatics tools, including FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), STAR2 (*66*), RSeQC (*79*), Subread/featureCounts (*80*), was used by the Fred Hutch bioinformatics shared resource to assess data quality, map reads to GRCh37/hg19 and compute gene-level expression based on GENCODE gene annotation V31 (*81*). The expression matrix was processed by edgeR (*82*) and normalized using the Trimmed Mean of M-values (TMM) method (*83*).

Identification of the HLA-A*0201-restricted WT1-specific TCR$_{37\text{-}45}$.

1. **Generation of WT1 epitope-specific T cell lines.** Antigen-specific T cell lines were generated from two donors that were specific for the following WT1 peptides: WT1$_{10\text{-}18}$ (ALLPAVPSL), WT1$_{22\text{-}31}$ (GGCALPVSGA), WT1$_{37\text{-}45}$ (VLDFAPPGA), WT1$_{38\text{-}46}$ (LDFAPPGAS), WT1$_{187\text{-}195}$ (SLGEQQYSV), WT1$_{235\text{-}243}$ (CMTWNQMNL), WT1$_{238\text{-}246}$ (WNQMNLGAT), and WT1$_{242\text{-}250}$ (NLGATLKGV). Ten lines were generated from each donor targeting each epitope (160 total) as previously described (*75*). Briefly, dendritic cells (DCs) were derived from the plastic adherent fraction of PBMCs after culture for two days (days -2 to 0) in media supplemented with 1000 U/ml IL-4 and 800 U/ml Granulocyte-Macrophage colony stimulating factor (GM-CSF). On day -1, a maturation cocktail containing 10ng/ml TNF-α, 10ng/ml IL-1β, 1000 U/ml IL-6 and 1000ng/ml prostaglandin E2 (PGE_2)was added. On day 0, DCs were harvested, washed, and pulsed with peptide. $CD8^+$ T cells were isolated from PBMCs using anti-CD8 microbeads and stimulated with peptide-pulsed DCs in the presence of 30ng/ml IL-21. Cultures were fed every 2 to 3 days by exchanging half of the medium and adding 12.5 U/ml IL-2, 5ng/ml

IL-7 and 5ng/ml IL-15. T cells were re-stimulated every 10 days by culturing at a 1:2 ratio with irradiated, peptide-pulsed, autologous PBMCs.

2. **Sort-purification of WT1-specific T cell lines.** T cell lines from the most responsive donor PBMCs were stained with peptide-HLA-A*02:01 tetramers specific for $WT1_{37\text{-}45}$ and $WT1_{235\text{-}243}$. The optimal tetramer concentration for staining was determined by titrating each tetramer on a cell population containing antigen-specific T cells and evaluating specific staining above background (non-specific) T cells. The tetramer-stained cells were sorted for high tetramer expression and expanded using a modified REP with IL-2 (50 U/mL), OKT3 (1 mg/mL), and 100-fold excess of irradiated feeder cells in 8 mL of RMPI-1640 supplemented with 10% heat inactivated human serum, 25 mM HEPES, 12 mM L-glutamine, and 55 μM β-mercaptoethanol (CTL media). Cultures were maintained with CTL media and 50 U/mL IL-2 every 2 to 3 days.
3. **Evaluation of antigen-specific cytokine production in response to HLA-$A2^+$ K562 cells.** Sorted antigen-specific T cells were co-cultured at a 1:1 ratio with HLA-A2-transduced K562 cells ($A2^+$ K562). After 6 hour incubations in the presence of golgi-inhibitors (BD GolgiPlug and GolgiStop), cells were surface-stained with anti-CD8 and then fixed (BD Cytofix/Cytoperm) before intracellular labelling with anti-interferon (IFN)-γ in BD Perm/Wash buffer. The cells were then analyzed by flow cytometry to determine the percentage of IFN-γ^+ cells for each sample.
4. **Isolation and expression of a $WT1_{37\text{-}45}$-specific TCR.** $WT1_{37\text{-}45}$-specific T cells that exhibited the most robust IFN-γ response when cultured with HLA-$A2^+$ K562s, were further sorted for the highest population of tetramer binding T cells (about 1% of the total population). The sorted $tetramer^{hi}$ cells were lysed, RNA was isolated, and full-length

TCRα- and TCRβ- encoding genes (*TRA* and *TRB*) were amplified by RACE PCR (TakaraBio). A dominant TCRA/TCRB clonotype was evident after sequencing, which was confirmed to be $WT1_{37\text{-}45}$ specific by expression in Jurkat cells followed by $WT1_{37\text{-}45}$ specific tetramer staining. This TCR ($TCR_{WT1\text{-}37\text{-}45}$ #1) was then synthesized as a codon-optimized P2A-linked lentiviral expression construct in the pRRLSIN.cPPT.MSCV/GFP.wPRE vector after excising the GFP gene (*56*).

5. **Lysis of $A2^+$ K562 cells by Primary T cells expressing WT1-specific TCRs**. Target HLA-$A2^+$ K562 cells or control (HLA-$A2^-$) K562 cells were labeled with ^{51}Cr (Perkin Elmer) overnight, washed and incubated in triplicate with effector T cells expressing either TCR_{C4} or $TCR_{WT1\text{-}37\text{-}45}$ #1 at various effector to target (E:T) ratios. Supernatants were harvested for γ-counting after a 4 hour incubation and percent specific lysis was calculated.

Immunoblot of proteasome catalytic subunits from patient sample or HEK-293 cell lines expressing specific proteasome isoforms. For the relapsed patient sample, PBMCs were depleted of $CD3^+$ cells using EasySep $CD3^+$ selection kit II (StemCell) following manufacturer's instructions. $CD3^+$ cells were discarded leaving an AML- enriched blast population. Blasts or cell lines were then counted and lysed at 40M cells/mL for 15 minutes on ice using Pierce IP lysis buffer (Thermo Fisher Scientific) with 1X proteasome inhibitor (Thermo Fisher Scientific). Lysates were centrifuged at 13,000G for 10 minutes to clear.

Assembled proteasome complexes were isolated using co-immunoprecipitation with and anti-α2 antibody, MCP21 (Enzo Lifesciences, BML-PW8105) and immunoblotted with different

immunoprecipitation beads, as previously described (*84*) (*29*). Briefly, Dynabeads M-270 epoxy beads (Invitrogen) were functionalized with MCP21 at 35 μg antibody per mg Dynabeads following manufacturer's instructions. Cleared lysate was quantified using a BCA assay Kit (Thermo Fisher Scientific) and 700 ng protein was incubated with 1mg functionalized beads to capture assembled proteasome complexes. Complexes were eluted in 0.1M Glycine buffer pH 2 to 2.5 and neutralized with equal volume Tris pH 7.5.

Proteasome composition was assessed by immunoblot. Elution fractions were separated on a denaturing 4-12% polyacrylamide gel and then transferred to a PVDF (Invitrogen) membrane, using the mini-blot module (Invitrogen). Membranes were blocked for 1 hour at room temperature in TBS containing 5% of dry milk and 0.1% Tween 20, and washed three times in TBS, 0.1% Tween and incubated with the relevant primary antibody. Standard proteasome (SP) and immunoproteasome beta subunits were probed over night in a solution of TBS, 5% milk powder, and 0.1% Tween-20 for β1[patient AML: (1:500 dilution, Santa Cruz Bio, sc-374405), cell lines: (1:500 dilution, Enzo Life sciences, BLM-PW8140)], β2 (1:250 dilution, Enzo Lifesciences, BML- PW8145), β5 (1:1000 dilution, Enzo Lifesciences, BML-PW8895), β1i (1:500 dilution, Enzo Lifesciences, BML- PW8840), β2i(1:250 dilution Novus, NBP1-8660-), β5i (1:1000 dilution, Enzo Lifesciences, BML- PW8355) and the shared beta-subunit β7 (1:1000 dilution, Enzo Lifesciences, BML- PW8135) was used as a loading control.

Transduction of HEK-293EBNA cells expressing the IP or SP with WT1 and HLA-A*02:01. HEK-239EBNA cell lines expressing either the IP or SP (*84*) were serially transduced with lentiviral constructs containing HLA-A*02:01 then WT1 (pCVL-A) (*85*). Cells were cultured on CELLCOAT Type I Collagen plates (Greiner bio-one) in IMDM medium

supplemented with L-Glutamine, 1%, non-essential ammino acids (NEAA), 1X, and 10% FBS. Hygromycin and puromycin were added to select for IP expressing cells. Cells were first transduced with HLA-A*02:01; positive cells (APC) were isolated using the Sony MA900. HLA-A2$^+$ cells were then transduced a second time with full-length WT1 (variant D) in the pCVL-A (*85*) co expressing eGFP. Cells were again stained for HLA-A2 and double positive HLA-A2$^+$GFP$^+$ cells were selected using the Sony MA900 WT-1 expression was further confirmed by IHC

Cytokine analysis of eCD8$^+$ and dpCD4$^+$ T$_{TCR37\text{-}45}$ cells. PBMCs were thawed from a healthy donor. CD8$^+$ and CD4$^+$ T cells were selected using CD8 and CD4 positive selection kits (Stem Cell Technologies). CD8$^+$ and CD4$^+$ T cells were transduced with a polycistronic construct containing TCR$_{37\text{-}45}$ and CD8αβ to yield eCD8$^+$ and dpCD4$^+$ T cells respectively. 5.0 x10^5 cells of either eCD8$^+$, dpCD4$^+$, or a combination eCD8$^+$/dpCD4$^+$(1:1 ratio; 2.5 x10^5 cells each) were co-cultured with T2 cells exogenously loaded with 10μg WT1$_{37\text{-}45}$ peptide at an E:T ratio of 5:1 for 4, 24, or 48 hours before supernatant was collected for analysis. Cytokine concentrations were determined using the R&D systems Luminex (*86*) platform according to manufacturer's instructions. Based on the inability of CD4$^+$ T cells to control tumor without co expression of the CD8αβ co-receptor (**Fig. 5K** and **fig. S15**), suggesting a lack of TCR activation through TCR signaling, we did not assess the cytokine profile of CD4$^+$ T cells alone. Similarly, CD8$^+$ cells expressing only TCR$_{37\text{-}45}$ showed identical tumor control as eCD8$^+$ T cells and were not assessed separately.

Cytotoxicity assays of HEK-293EBA IP and SP cell lines. Cytotoxic activity of mock-

transduced, $T_{TCR\text{-}C4}$ -transduced and $TCR_{37\text{-}45}$ -transduced $CD8^+$ T were examined by assessing the capacity of the T cells to lyse WT1- and HLA-A2-transduced HEK-293-EBNA cell lines expressing either the IP or SP in a 4 hour ^{51}Cr release assay at the indicated effector to target (E:T) ratios as previously described (*87*).

AML cell death assays. The endogenous TCR can non-specifically react with the patient sample (only HLA-A2 is matched) necessitating an additional CRISPR step to view specific TCR signaling. Because TCR_{C4}, $TCR_{37\text{-}45}$, and $TCR_{Irr.}$ are codon optimized, they are not susceptible to the crisprRNA (crRNA) used. To measure T cell-induced AML lysis, CRISPR edited T cells were transduced with $TCR_{37\text{-}45}$, TCR_{C4}, and TCR_{Irr} and stained with CFSE and cultured with primary AML cells at the indicated E:T ratios in triplicates for 18 hours as previously described (*88*). Briefly, after the culture period, surviving AML cells were identified by staining for CD45 (1:600 dilution, PE Texas Red, clone 2D1, BioLegend), CD34 (1:50 dilution, BV421, clone 561, BioLegend) and CD38 (1:160 dilution BUV395, clone HB7, Beckton Dickinson) for 30 minutes and quantified with Flow Count beads (Molecular Probes). Percent killing was calculated by the formula: (absolute number of AML targets in co-culture/absolute number of AML targets in target only control well) x 100.

To measure T cell induced AML apoptosis, T cells with endogenous TCR α and β chains knocked out with CRISPR were transduced with $TCR_{37\text{-}45}$, TCR_{C4}, or TCR_{irr} and cultured with PBMC from patient 1's relapsed AML at a 3:1 E:T ratio (3.0×10^6 T cells, 1.0×10^6 AML) for 1 hour. The cells were harvested and stained with a viability dye (TONBO Biosciences #13-0870-T100) and cell surface markers for 30 minutes, CD34 (1:50 dilution, BV421, BioLegend, 343610), CD8 (1:100 dilution, APC-Cy7, BD Biosciences, 557760) and CD45 (1:300 dilution,

PE, BioLegend, 368510). The cells were fixed with 4% Formaldehyde (CellSignal, 47746) and permeabilized with 100% Methanol (CellSignal, 13604) according to manufacturer's instructions, followed by intracellular stain for 1 hour for cleaved caspase-3 (1:50 dilution, FITC, CellSignal, 96695) and flow cytometry analysis. Percent cleaved-caspase 3 expressing $CD8^-$ $CD34^+$ cells were quantified.

CRISPR-Cas9 Gene editing by electroporation. $CD8^+$ and $CD4^+$ T cells were selected from healthy PBMCs using the EasySep Human CD8 Positive Selection kit II and Human $CD4^+$ T Cell isolation kit (STEMCELL Technologies). T Cells were seeded at 10^6 T cells/mL with 50 IU/mL IL-2 and activated with T Cell TransAct, human (Miltenyi Biotec) according to manufacturer's instructions; TransAct was removed after 48 hours. Cells were then electroporated with Cas9 complexed with CRISPR-RNA (crRNA) targeting the TCRα and TCRβ to knock out the endogenous TCR, sequences for TRAC Exon 1 and TRBC Exon 1 were 5'-AGAGTCTCTCAGCTGGTACA-3' and 5'- GGAGAATGACGAGTGGACCC -3' respectively. To generate gRNAs, crRNAs and tracrRNAs were thawed, mixed at 1:1 v/v ratio (160μM stock) and incubated at 37°C for 30 minutes. To create the ribonucleoprotein (RNP) mixture, Cas9 protein (MacroLab, Berkeley, 61μM stock) and poly-L-glutamic acid (Sigma-Aldrich, 100 mg/mL in H_2O stock) were both added to the gRNA at a 1:1 v/v ratio (Poly-L-glutamic Acid:Cas9 protein). Poly-L-glutamic acid increases stability of the Cas9 (*89*). T cells were resuspended in Lonza P3 Buffer ($1x10^6$ per 20μL) and 4 μL of the RNP mixture was added to each sample. The Cell/RNP mixture was transferred to a 16 well nucleocuvette strip (Lonza) and electroporated using the pulse code EH115 (Lonza, Nucleofector Device). Following electroporation, 80μL of prewarmed medium was added and the samples recovered for 15

minutes at 37°C. Cells were then transferred to 1mL of prewarmed media and allowed to rest for 1 hour at 37°C before transduction with lentivirus encoding TCR_{C4}, $TCR_{37\text{-}45}$ or $TCR_{Irr.}$. An aliquot of untransduced cells was reserved to assess TCRα/TCRβ knockout efficiency by flow cytometry 3 days later, efficiency was determined as percent $CD3^-$. Cells were allowed to grow for 7 days after transduction, then FACS sorted for $CD8^+$tetramer$^+$ (BV421, APC), then placed into REP for 7 to 9 days.

Live tumor-cell killing assays. T cell killing capacity was monitored using the IncuCyte S3 Live-Cell Analysis System (Essen BioScience,). Two human solid tumor lines - pancreatic cancer cell line, Panc-1 (ATCC, CRL-1469), and breast cancer cell line, MDA-MB-468 (ATCC, HTB-26) tumors were transduced with the IncuCyte NucLight Red Lentivirus Reagent (Sartorius) and seeded (10,000 cells/well) in 48-well plates (Corning). When comparing WT1-specific TCRs, wells were cocultured in triplicate at an E:T ratio of 10:1 with either no T cells, or with TCR-transduced $CD8^+$ T cells. When investigating if $CD4^+$ T cells could help sustain an anti-tumor response, wells were cocultured in triplicate at an E:T ratio of 4:1 with no T cells, $CD8^+$ T cells, $dpCD4^+$ T cells or a $CD8^+$/$dpCD4^+$ T cell mixture. $CD8^+$ and $CD4^+$ T cells were transduced with a lentiviral construct containing CD8αβ along with $TCR_{37\text{-}45}$. For all assays, red fluorescence was recorded at 2 hour intervals for 96 hours or 156 hours. Tumor rechallenge (10,000 cells/well) was conducted at 48 hours and 96 hours. Images were analyzed using the IncuCyte S3 v. 2019b software (Essen BioScience) with total red object area (μm^2/well) corresponding to the degree of tumor cell death over time.

In vivo $T_{TCR37\text{-}45}$ therapy model. Experiments were performed following the approved IACUC protocol 50898. In accordance with best practices, the ARRIVE checklist was followed for in vivo studies.

The HLA-A2$^+$ WT1$^+$ pancreatic cancer cell line,Panc-1 (ATCC, CRL-1469), was transduced to express a firefly luciferase (PANC-1_{Luc}); individual clones were assessed for high luciferase expression. Clone#9 was selected for a high degree of transgene expression and used in subsequent in vivo studies.

Female NSG (NOD-*scid* IL2Rgamma^{null}, Jackson Laboratory 005557) mice (*90*) of similar age (six to eight weeks) were injected intraperitoneally (I.P.) with 1.25 x 10^5 PANC-1_{Luc} cells. As a 'prevention' model of adoptive T cell therapy, 3 days later mice either received no T cells at all (tumor only), or therapeutic $T_{TCR37\text{-}45}$ T cells consisting of 2x10^6 each eCD8$^+$ and dpCD4$^+$. As a 'treatment' model of adoptive T cell therapy 14 days post tumor injection, tumor burden was measured and mice with no detectable tumor were excluded from the study and remaining mice with detectable tumor wereenrolled in the study and randomly assigned to treatment groups. a one-way ANOVA with Tukey's multiple comparison test was used to confirm there was no significant difference in tumor burden between the groups ($p \geq 0.64$). Mice either received no T cells at all (tumor only), or therapeutic T cells ($T_{TCR37\text{-}45}$, T_{TCRC4} ,or $T_{TCRirr.}$) consisting of 10x10^6 each eCD8$^+$ and dpCD4$^+$. Mice in both studies were imaged and weighed weekly. The mice were administered 1μL of Luciferin per gram of body weight at 15 mg/mL of concentration I.P. under isoflurane anesthesia. After 15 minutes the mice were imaged while still under isoflurane anesthesia. At week 4, $T_{TCR37\text{-}45}$ treated mice received a second injection of $T_{TC37\text{-}45}$ T cells (2.0x10^6 each CD4$^+$ and CD8$^+$). Mice were euthanized on week 8.

Supplementary Figures

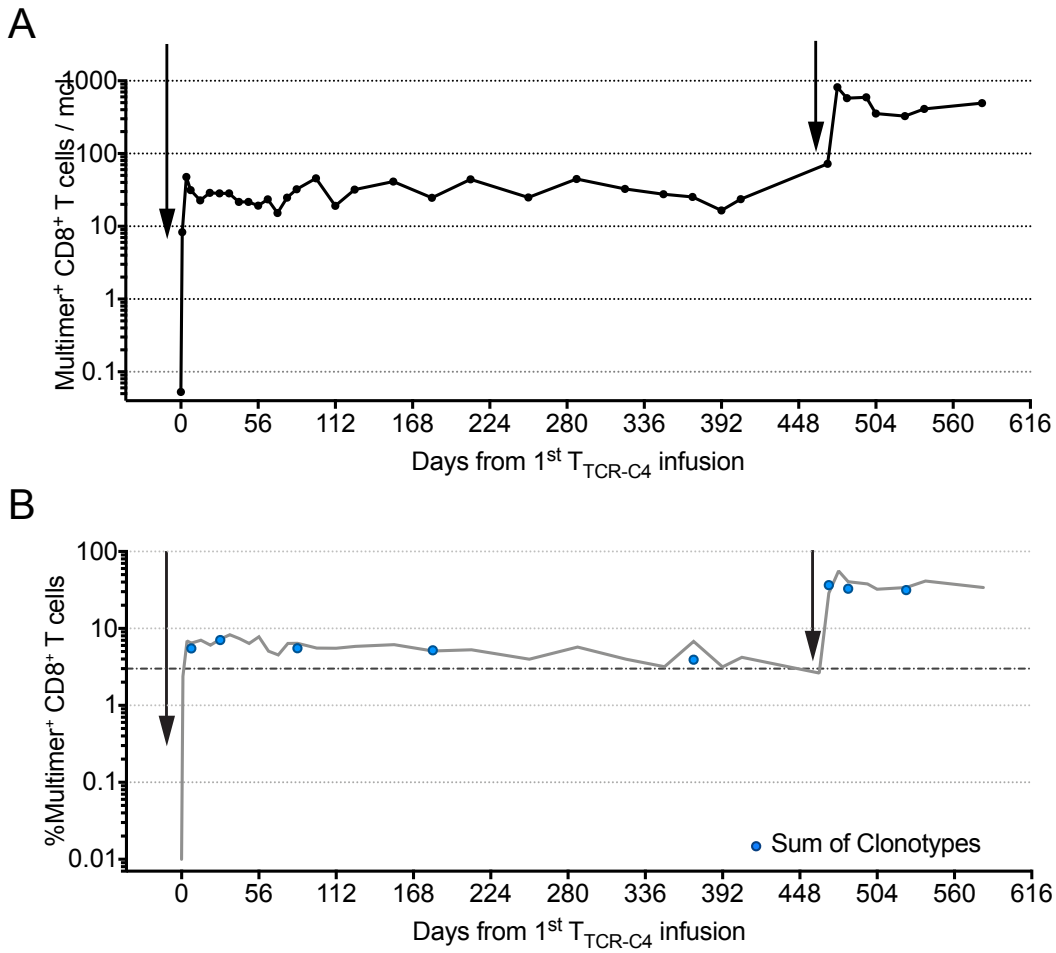

Fig. S1. Patient 1 in vivo persistence kinetics of transferred $T_{TCR\text{-}C4}$. (A) Multimer^{+}CD8^{+} T cells/µl (y-axis) were enumerated in PBMCs collected after infusion (x-axis, day relative to first infusion). Time of infusions are indicated with black arrows. **(B)** The relationship over time after infusion (x-axis) between the percent multimer^{+}CD8^{+}T cells (gray line) in PBMCs and the sum of detected frequencies of individual infusion product clonotypes (blue circles) is shown. The percentage of multimer^{+}CD8^{+} T cells (y-axis) were measured in PBMCs collected after infusions (x-axis, day relative to first infusion). Time of infusions indicated with black arrows. The thick dashed line indicates the persistence cutoff defined in this trial as 3% multimer^{+}CD8^{+} T cells.

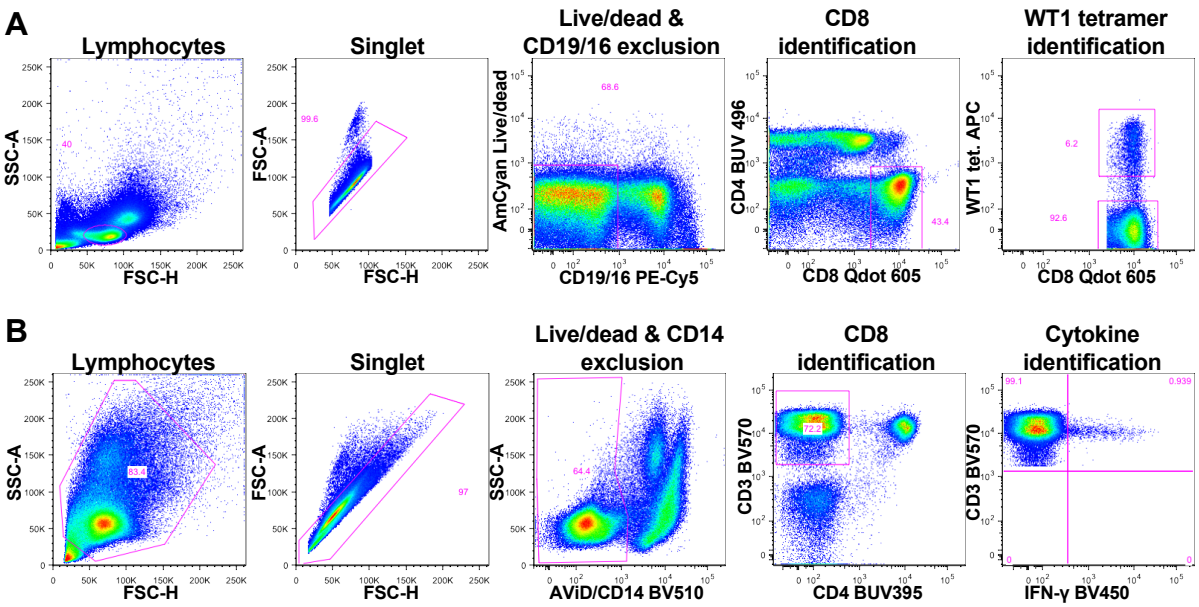

Fig. S2. Gating strategies for surface and intracellular stains. (A) The gating strategy for surface staining is shown. Panels from left to right: Initial gating was on lymphocytes (side scatter (SSC)-area (A) versus forward scatter (FSC)-height (H)), followed by singlet exclusion (FSC-A versus FSC-H), followed by exclusion of dead cells and CD19 and CD16-expressing cells (AmCyan Live/dead versus CD19/16 PE-Cy5), followed by CD8 identification (CD4 BUV 496 versus CD8 Qdot605), followed by $WT1_{126\text{-}135}$-HLA-A2-tetramer^{+} cells identifying $T_{TCR\text{-}C4}$ (WT1-tetramer APC versus CD8 Qdot605). A representative example is shown. **(B)** The gating strategy for intracellular staining is shown. Panels from left to right: Initial gating was on lymphocyte identification (SSC-A versus FSC-H), followed by singlet exclusion (FSC-A versus FSC-H), followed by exclusion of dead cells and CD14-expressing antigen presenting cells (SSC-A versus AViD/CD14 BV510), followed by CD8 identification (CD3 BV570 versus CD4 BUV395), followed by cytokine identification (CD3 BV570 versus IFN-γ BV450). A representative example for IFN-γ is shown.

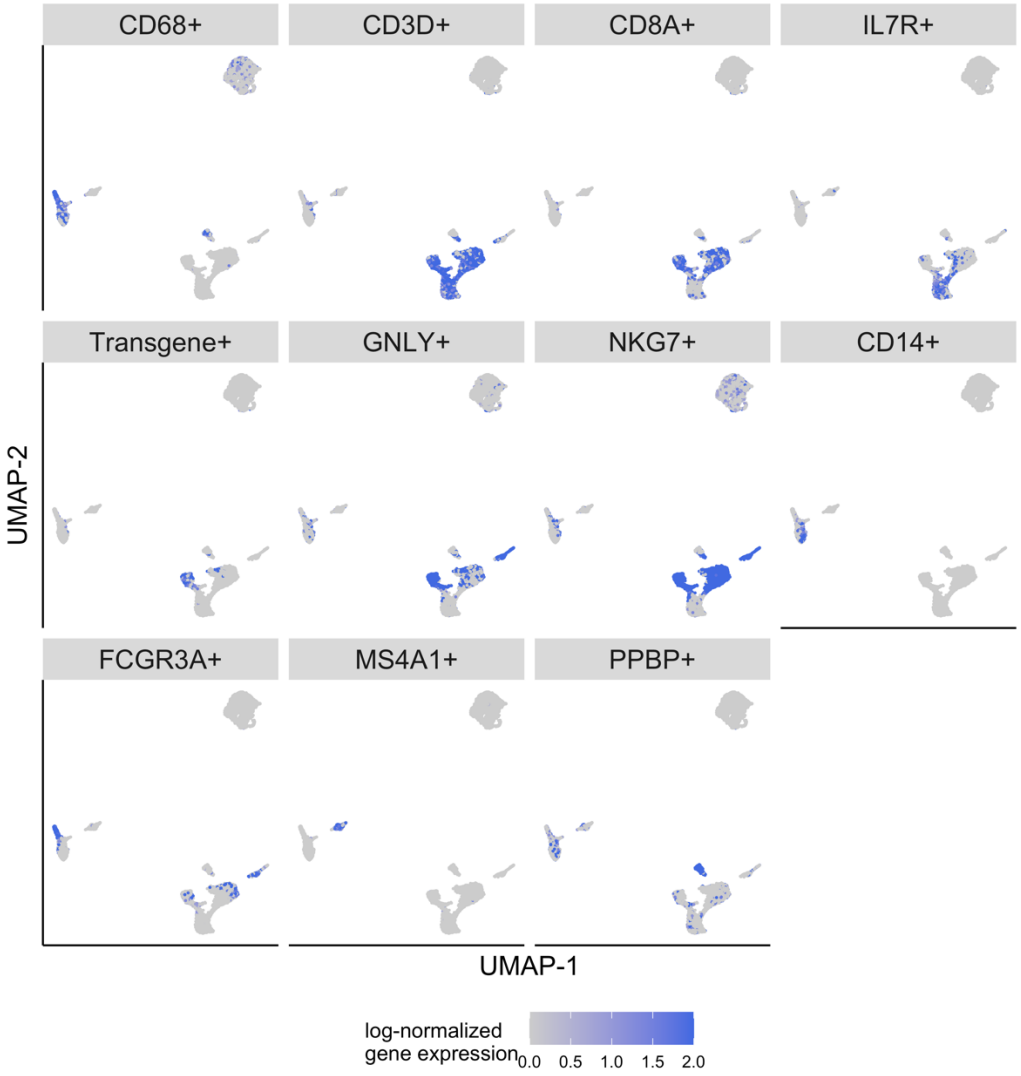

Fig. S3. Identification of cell populations in patient 1 peripheral blood. Selection of key markers that assisted in confirmation of clusters identified by principal component analysis and visualized with UMAP for both remission and relapse timepoints combined (n=7704, each represented with a dot). Gene names are shown. Blue color shows cells expressing indicated gene, with darker blue indicating greater expression.

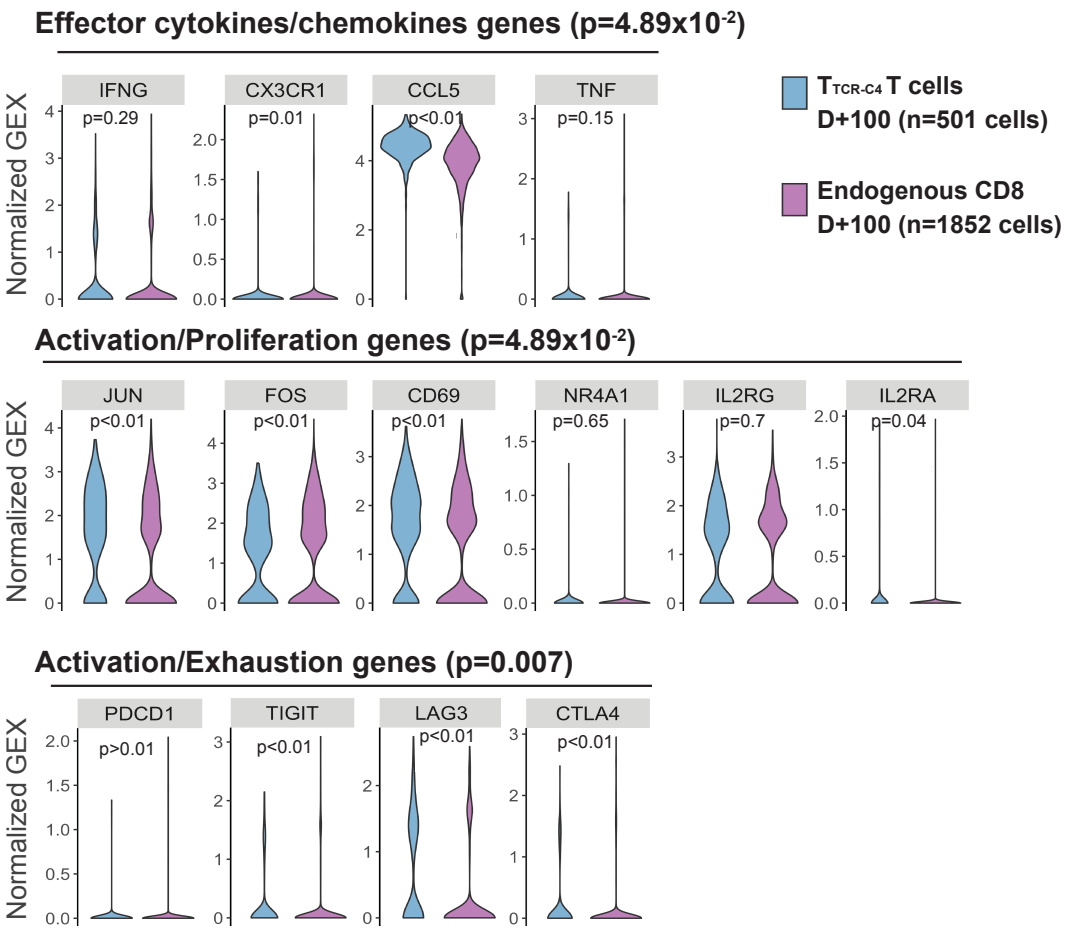

Fig. S4. Gene set differences between $T_{TCR\text{-}C4}$ and endogenous $CD8^+$ T cells at remission.

Expression of transcripts for gene-sets representing effector cytokine/chemokine production and T cell activation at remission for $T_{TCR\text{-}C4}$ (blue, n=501) and endogenous $CD8^+$ T cells (purple, n=1852). All graphs are shown as violin plots. The shape of the violin displays frequencies of values. Model-based Analysis of Single Cell Transcriptomics (MAST) was used to determine the significance shown on each plot. Significance thresholds were set a priori at a threshold of false discovery rate of 5% and positive or negative fold change > log2(1.5). Effector cytokine/chemokine genes: IFN-γ [*IFNG*], C-X-C motif chemokine receptor 1 [*CX3CR1*], C-C motif chemokine ligand 5 [*CCL5*] and TNF-α[*TNF*]. Activation/Proliferation genes: Jun proto-oncogene [*JUN*], Fos proto-oncogene [*FOS*], [*CD69*], nuclear receptor subfamily 4 group A

member 1 [*NR4A1*], IL-2 receptor subunit gamma [*IL2RG*], and IL-2 receptor subunit alpha [*IL2RA*]. Activation/Exhaustion genes: programmed cell death receptor-1 [*PDCD1*], T-cell immunoreceptor with Ig and ITIM domains [*TIGIT*], lymphocyte activating-3 [*LAG-3*] and cytotoxic T-lymphocyte associated protein 4 [*CTLA4*].

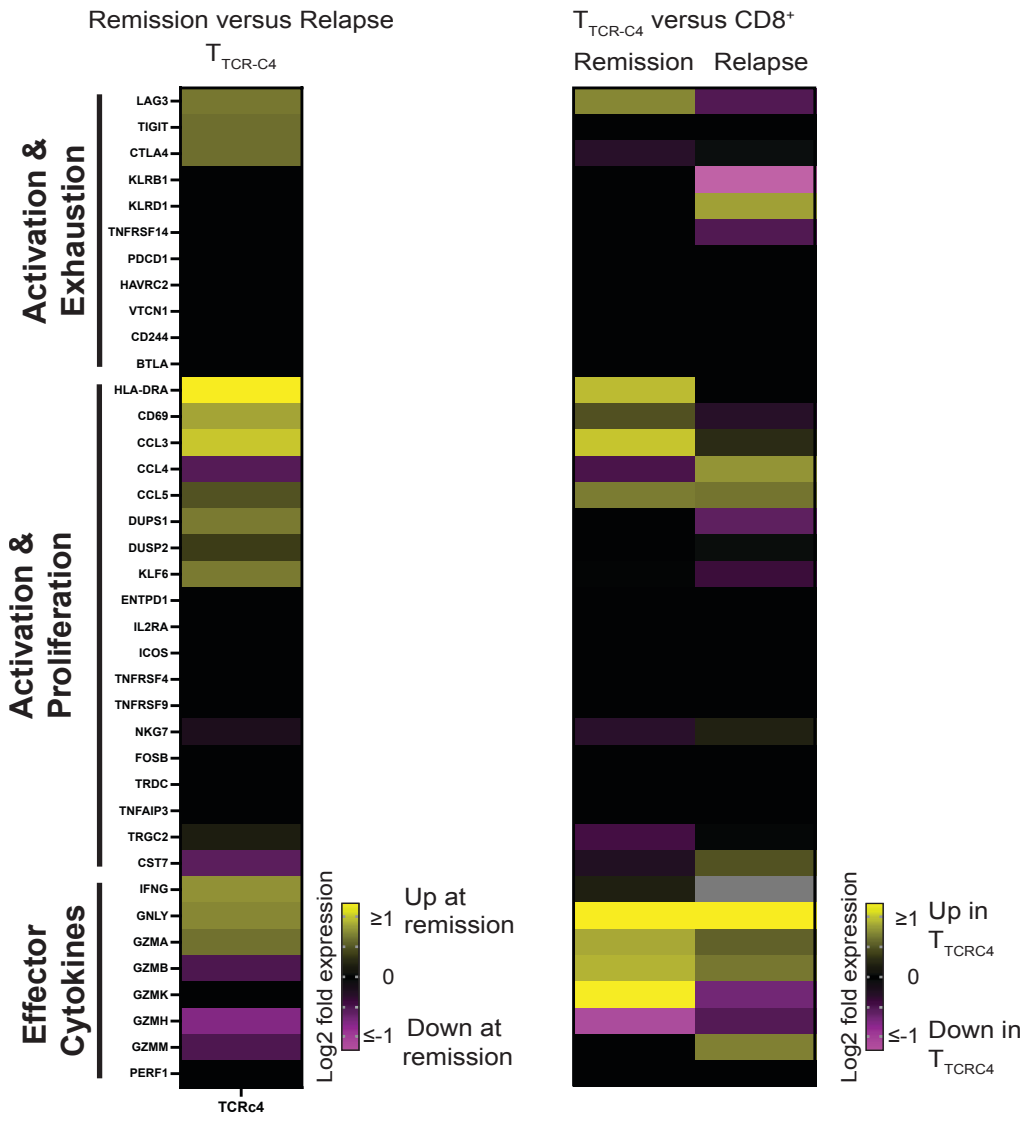

Fig. S5. Gene expression differences between $T_{TCR\text{-}C4}$ at remission and relapse and between $T_{TCR\text{-}C4}$ and endogenous $CD8^+$ T cells at both remission and relapse. Heat maps show gene expression differences (shown as log2 fold change) for a large set of activation & exhaustion genes, activation & proliferation genes, and effector cytokines. Comparisons: between $T_{TCR\text{-}C4}$ at remission and at relapse; $T_{TCR\text{-}C4}$ and endogenous $CD8^+$ T cells at remission; $T_{TCR\text{-}C4}$ and endogenous $CD8^+$ T cells at relapse. Gray indicates Log2 fold change could not be calculated.

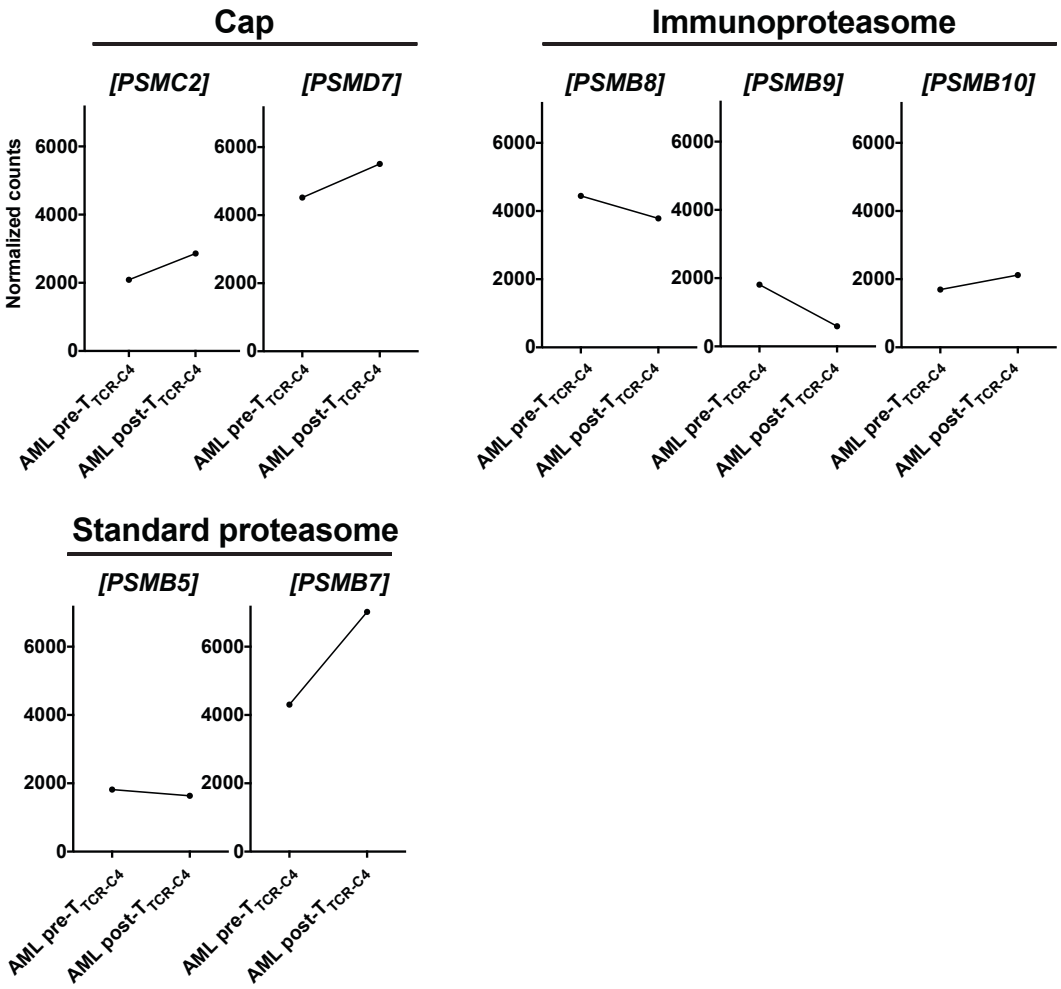

Fig. S6. Transcript expression of proteasome subunit in AML before and after $T_{TCR\text{-}C4}$ infusions. Normalized counts showing expression of proteasome beta subunits and two subunits in the regulatory cap in AML samples from Patient 1. No *PSMB6* (β1) probes were available for the panel used.

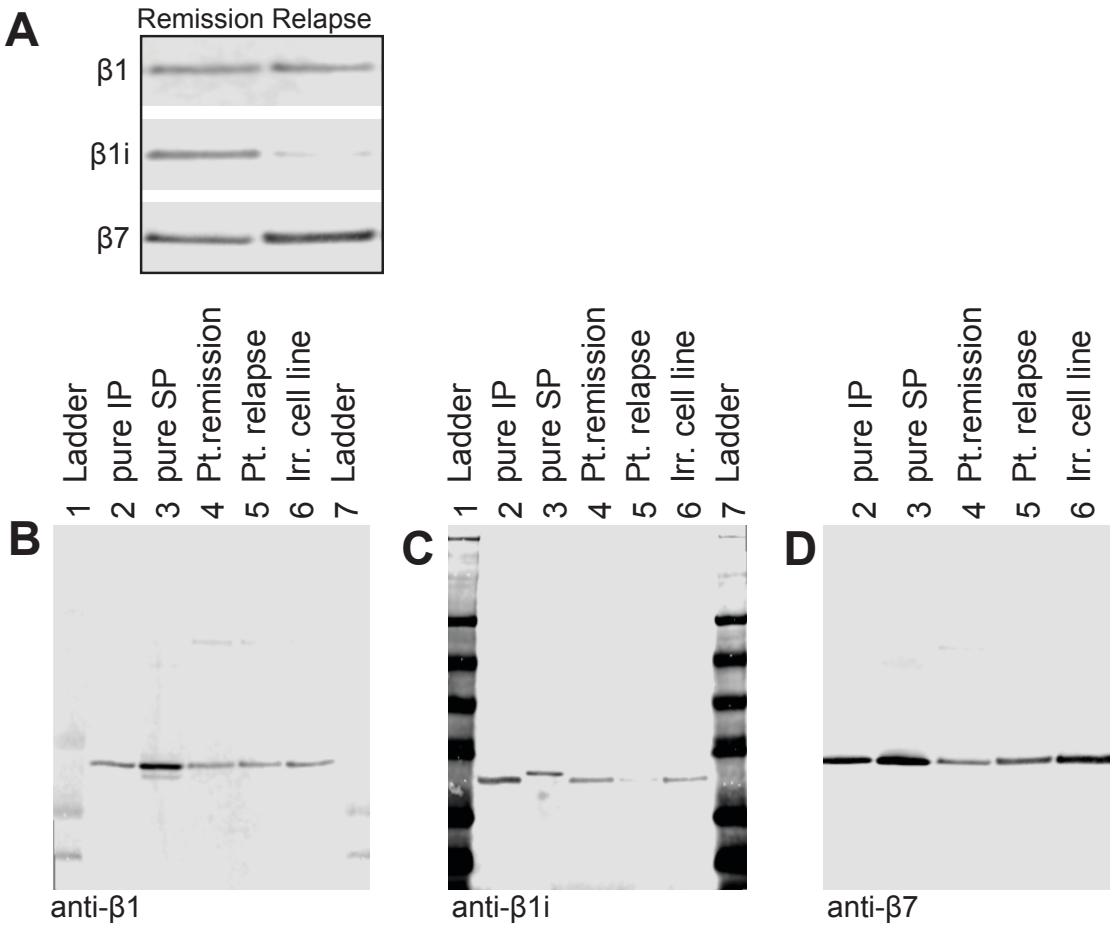

Fig. S7. Protein expression of proteasome subunits at remission and relapse. (**A**) Immunoblots show standard proteasome specific subunit β1, immunoproteasome subunit β1i, and shared proteasome subunit β7. Assembled proteasome complexes were immunoprecipitated from patient 1's PBMC samples during remission (complete sample) and from relapse (CD3 depleted AML). Proteasome subunit β7 is shared between proteasome complexes and serves as a loading control of immunoprecipitated fractions. (**B to D**) Full blots are shown for (**B)** anti-β1, (**C**) anti-β1i, and (**D**) anti-β7. Lanes are 1-Ladder, 2-purified Immunoproteasome (Thermo Fisher Scientific), 3-purified Standard proteasome (Novus Biologics), 4-Patient PMBCs at remission 5-Patient AML at relapse, 6-irrelevant cell line, 7-ladder.

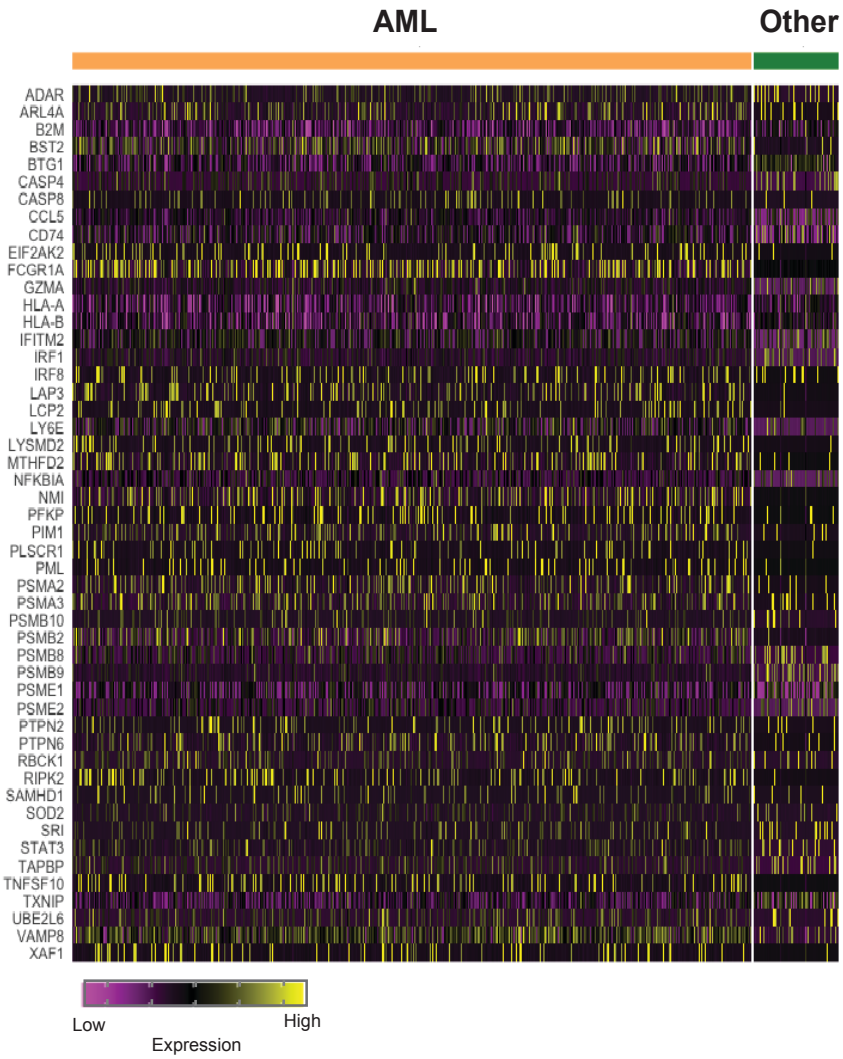

Fig. S8. IFN-γ response genes expressed in PBMCs isolated from Patient 1 after relapse.

Fifty of the most highly expressed IFN-γ response genes, in addition to IP subunits, are shown in AML and other immune cells in PBMCs isolated from patient 1.

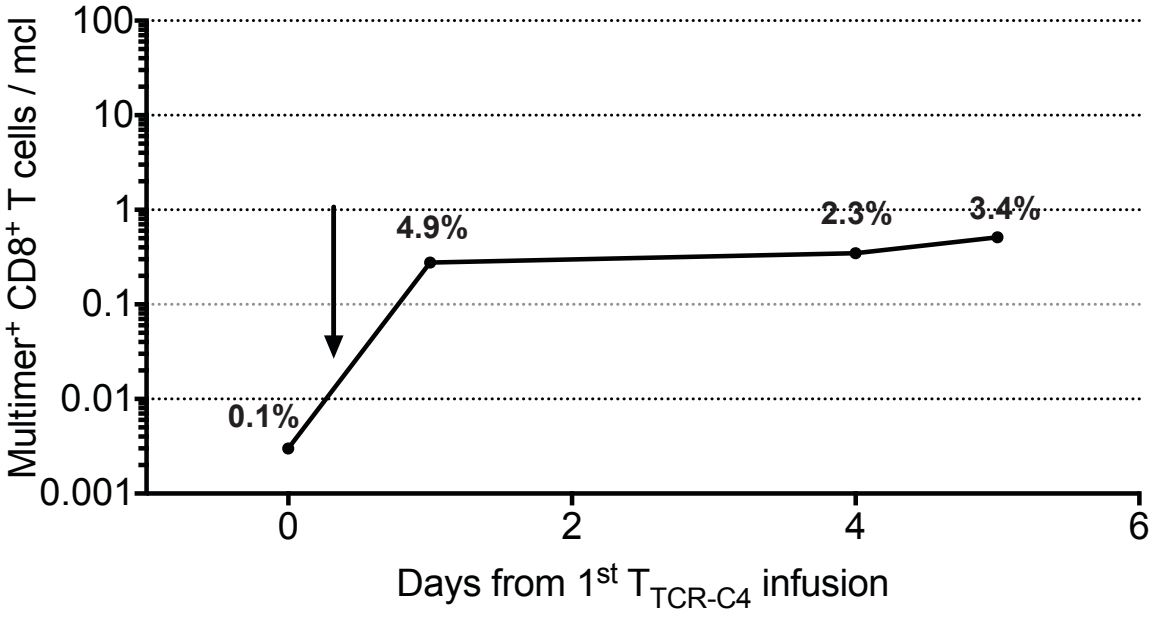

Fig. S9. In vivo persistence kinetics of transferred $T_{TCR\text{-}C4}$ were measured using samples from Patient 2: Multimer^{+}CD8^{+} T cells/μl (y-axis) were enumerated in PBMCs collected after infusion (x-axis, day relative to infusion). Time of infusion is indicated with black arrow. Percent multimer^{+} of CD8^{+} cells is shown above each timepoint.

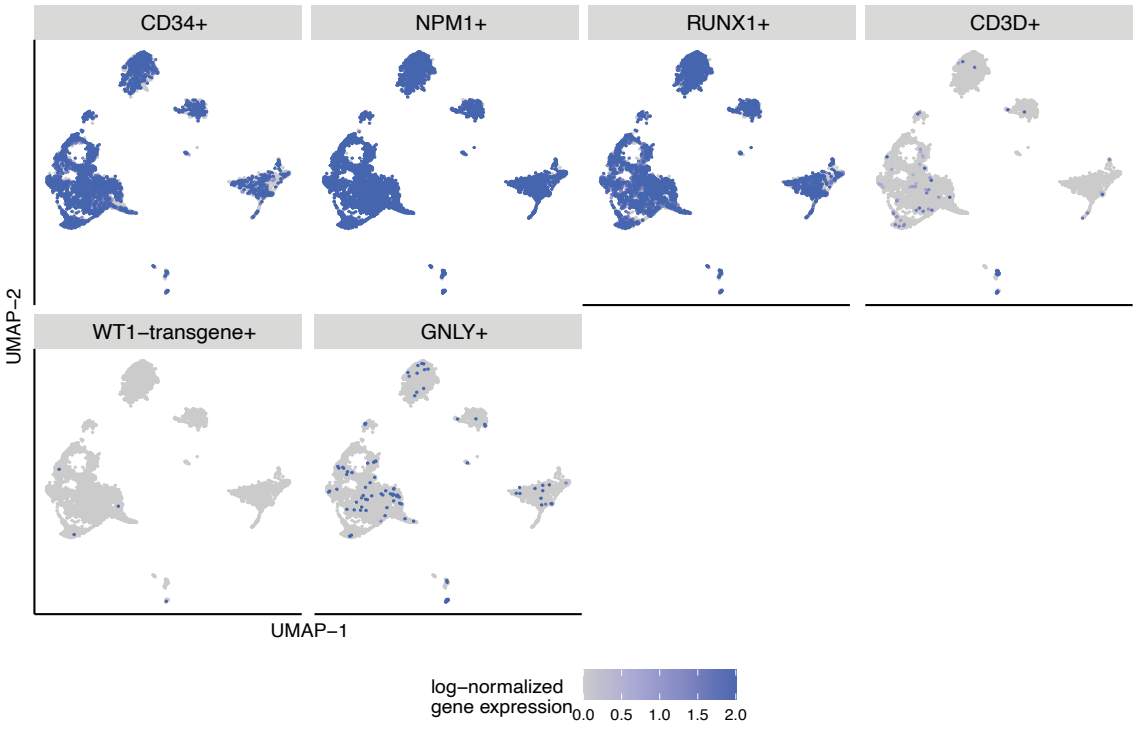

Fig. S10. Identification of cell populations in peripheral blood isolated from Patient 2. Selection of key markers that assisted in confirmation of clusters identified by principal component analysis and visualized with UMAP for both remission and relapse timepoints combined (n=10,700) are shown. Gene names are indicated. Blue color shows cells expressing indicated gene, with darker blue indicating greater expression.

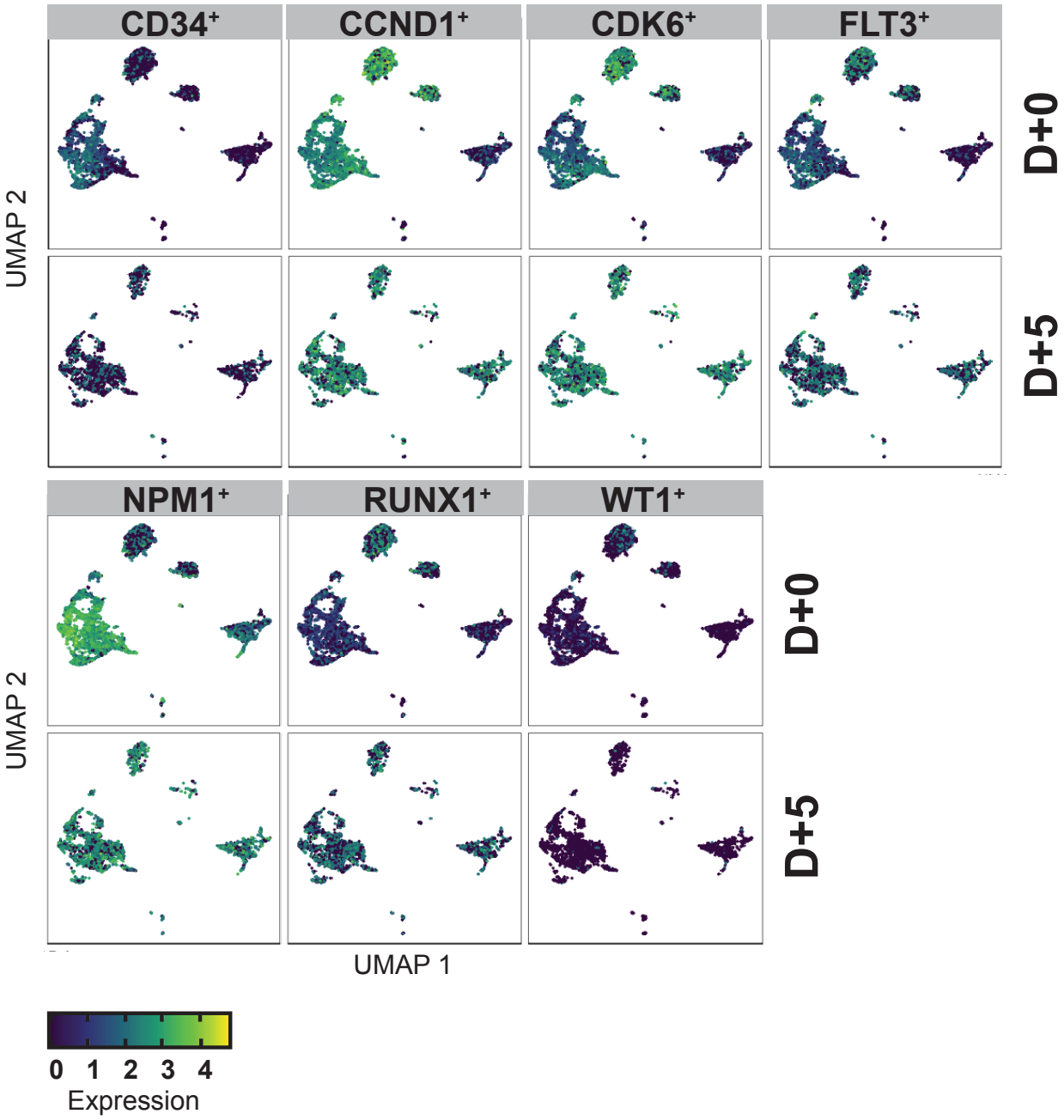

Fig. S11. Gene expression of AML-specific genes is shown for samples isolated from Patient 2 before and five days after $T_{TCR\text{-}C4}$ therapy. Key markers associated with AML that assisted in confirmation of AML visualized with UMAP for both remission (D+0, n=7,381) and relapse (D+5, n=3,319) timepoints are shown. Gene names are indicated. Purple through yellow coloring color shows degree of expression.

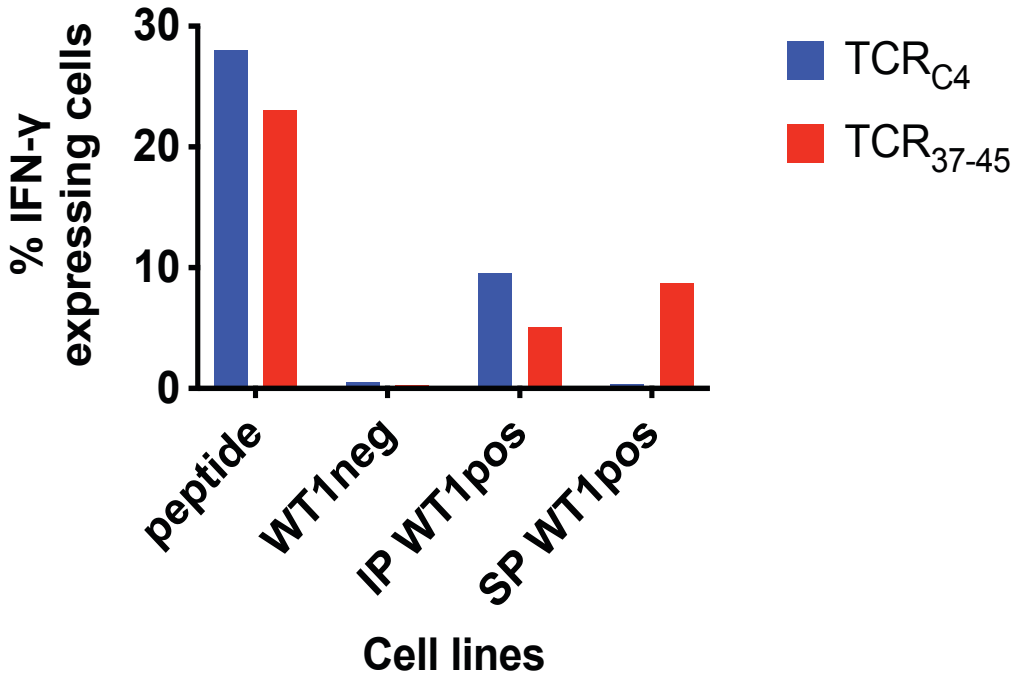

Fig. S12. T_{TCR-C4} and $T_{TCR37-45}$ differentially recognize HLA-A2- and WT1-transduced HEK293 cells expressing the IP or SP. $CD8^+$ T cells were selected from a healthy, HLA-A*02^+ donor and transduced with TCR_{C4} and $TCR_{WT1-37-45}$. T_{TCRC4} and $T_{TCR37-45}$ were co-cultured with target cell lines (indicated on the x-axis). Targets are HLA-$A2^+$ T2 cells loaded with 10ng/mL respective peptide (peptide), HLA-A2-transduced HEK 293 expressing SP (WT1neg), HLA-$A2^-$ and WT1-transduced HEK 293 cells engineered to express IP (IP WT1pos), and HLA-$A2^-$ and WT1-transduced HEK 293 cells expressing SP (SP WT1 pos). Target cells were co-cultured with T_{TCR-C4} or $T_{TCR37-45}$ for 20 hours at an E:T ratio of 1.5:1. Cells assessed for IFN-γ production with flow cytometry.

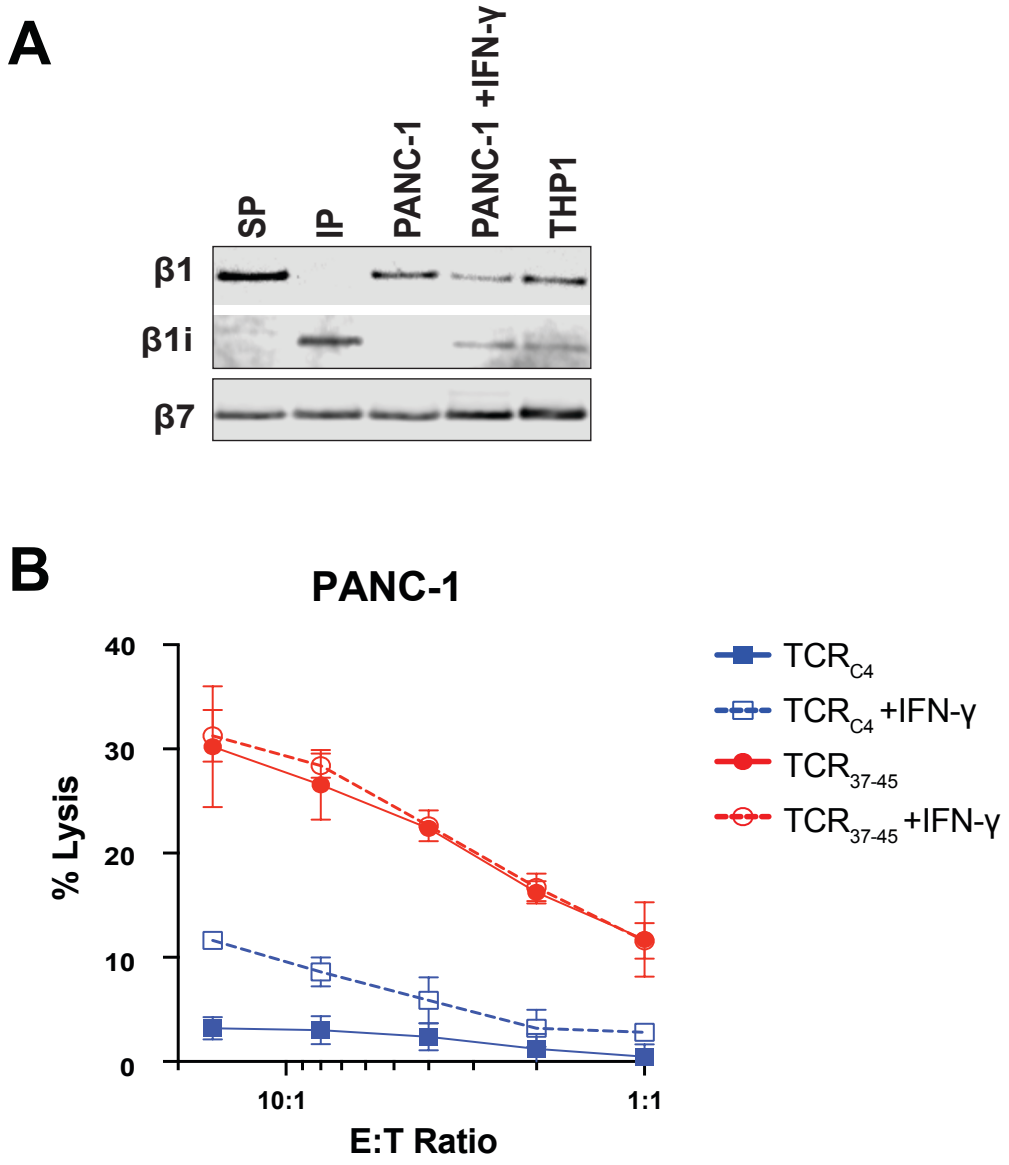

Fig. S13. 48 hour exposure to IFN-γ induces β1i expression and incorporation into proteasomes in PANC-1 cells, but does not completely rescue recognition by T_{TCR-C4} T cells. (**A**) Immunoblot of standard and immunoproteasome subunits β1 and β1i, respectively. PANC-1 cells were untreated or exposed to IFN-γ for 48 hours. All cells were lysed and assembled proteasome complexes were immunoprecipitated for immunoblot analysis. Proteasome subunit β7 is shared between both proteasome complexes and serves as a loading control of immunoprecipitated fractions. (**B**) Percent lysis is shown for PANC-1 cells that were either

exposed to IFN-γ for 48 hours (dotted lines) or untreated (solid lines) prior to culture with T cells expressing TCR_{C4} or $TCR_{37\text{-}45}$ at various E:T ratios. Data are presented as mean ± standard deviation.

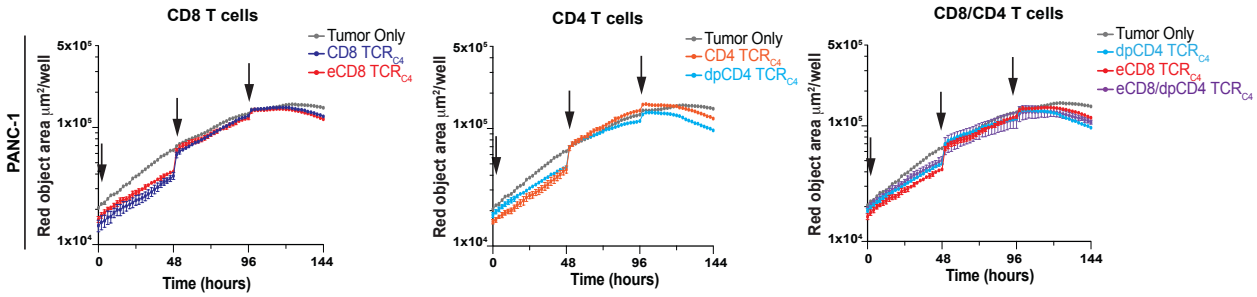

Fig. S14. In the absence of addition of exogeneous IFN-γ exposure, increased abundance of CD8αβ does not rescue TCR$_{C4}$ in a PANC-1 solid tumor model. Left: CD8$^+$ T cells were transduced with only TCR$_{C4}$ (light-blue line) or a polycistronic construct containing TCR$_{C4}$ and CD8αβ (red line, eCD8$^+$). Middle: CD4$^+$ T cells were transduced with only TCR$_{C4}$ (blue line) or a polycistronic construct containing TCR$_{C4}$ and CD8αβ (green line, dpCD4$^+$). Right: A comparison of eCD8$^+$ alone (red line), dpCD4$^+$ alone (green line) or eCD8$^+$/dpCD4$^+$ in combination (1:1 ratio, purple line) is shown. For all conditions, arrows indicate addition of tumor cells to culture. Standard error of triplicate wells are shown. Co-cultures were mixed at a 4:1 E:T ratio; total T cell numbers were kept consistent for all conditions.

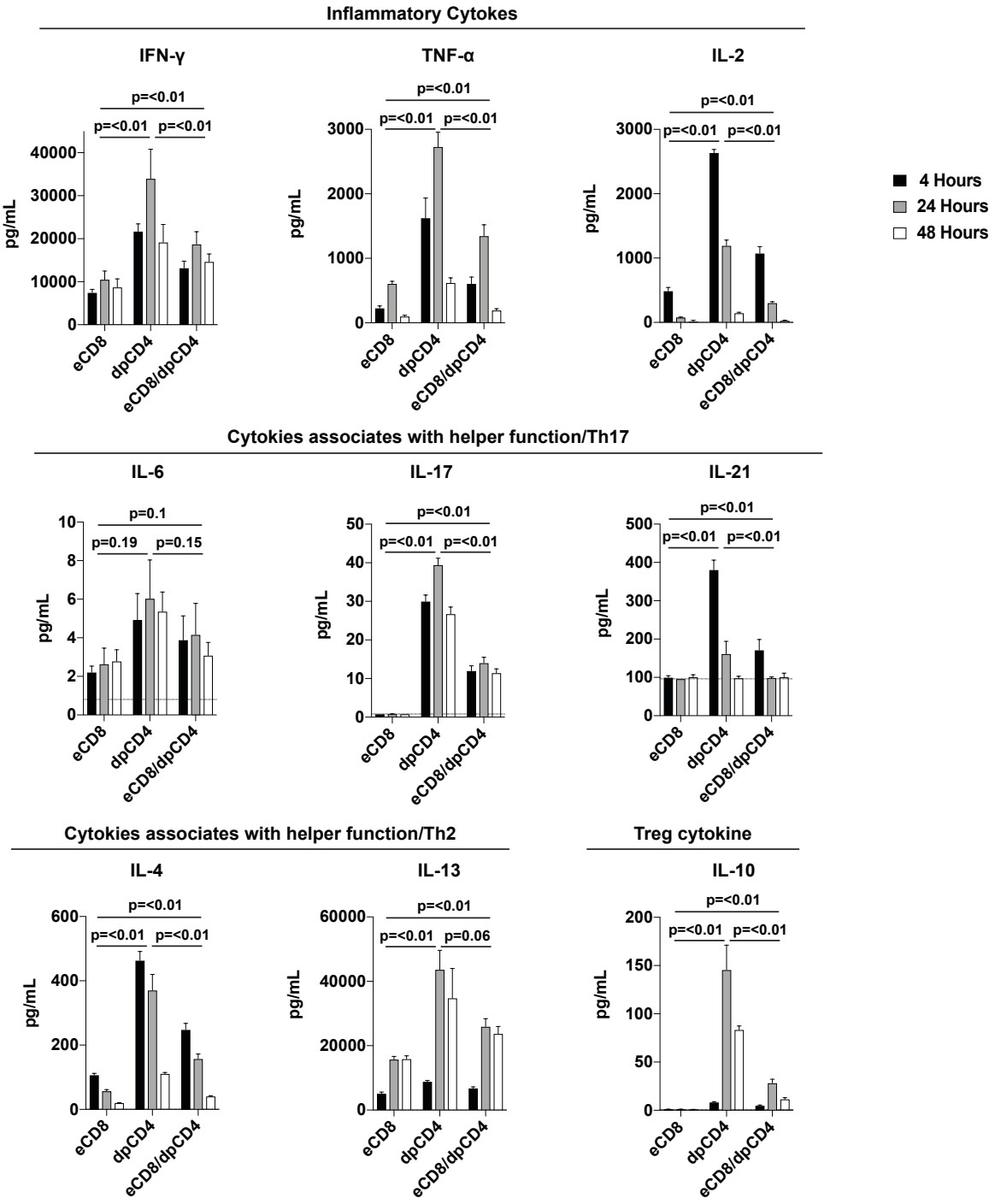

Fig. S15. Cytokine profiles of peptide stimulated eCD8$^+$, dpCD4$^+$ and eCD8$^+$/dpCD4$^+$ $T_{TCR37\text{-}45}$ T cells. Cytokine production by $T_{TCR37\text{-}45}$ T cells was measured after 4, 24, and 48 hours of stimulation with T2 cells exogenously loaded with 10µg peptide. Except for IL-6, all cytokines are found at significantly higher concentrations in cultures of dpCD4$^+$ cells compared

to $eCD8^+$ cells and the mixture of $eCD8^+/dpCD4^+$. The mixture of $eCD8^+/dpCD4^+$ produced significantly more cytokine than $eCD8^+$ cells alone. Data are presented as mean+standard deviation. Differences between groups were calculated using a student t test.

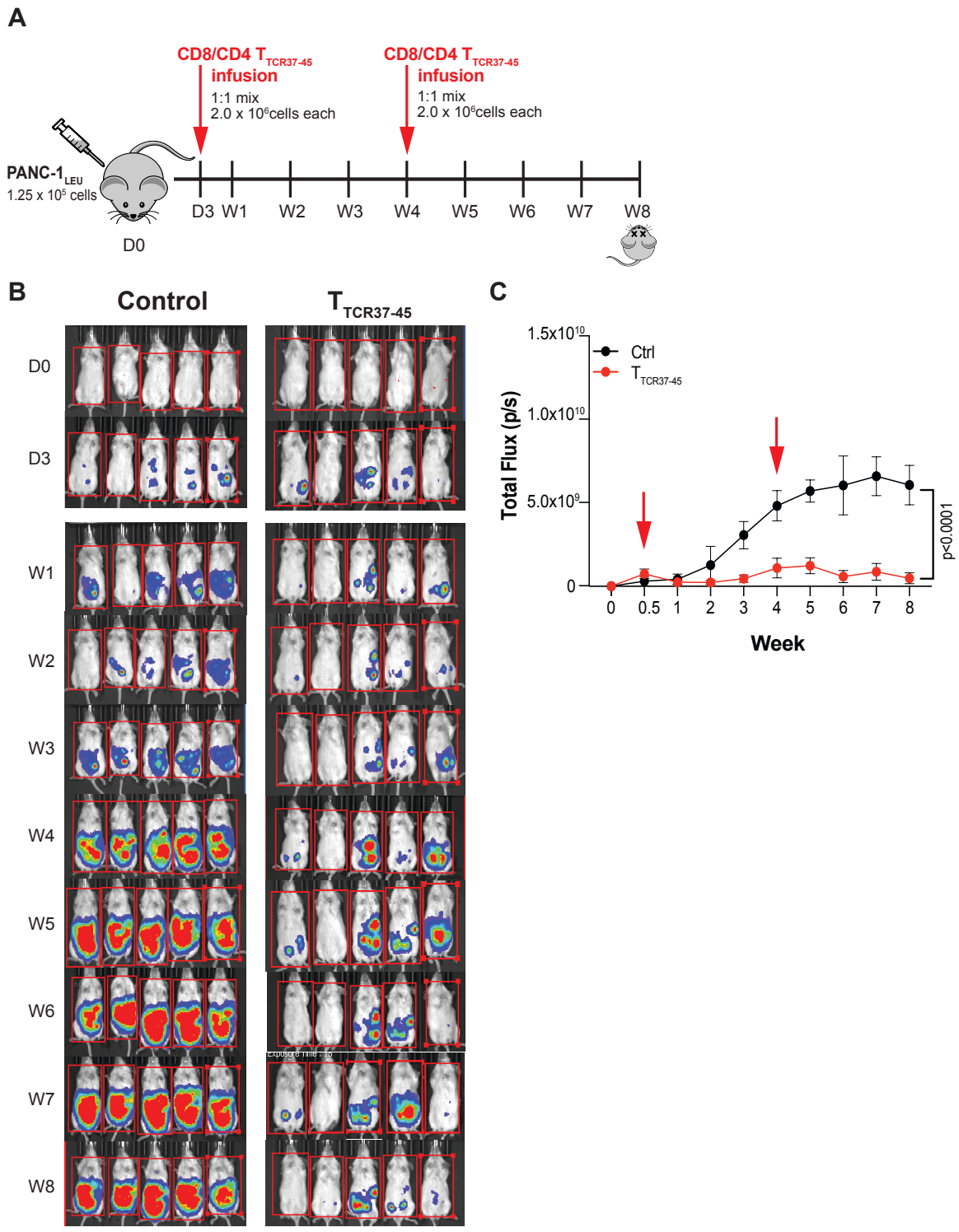

Fig. S16: $T_{TCR37-45}$ controls PANC-1 tumor growth in a prevention model with NSG mice.

(A) Schematic illustrating experimental time course. NSG (NOD-*scid* IL2Rgamma^{null}) mice were injected with 1.25 x 10^5 Panc-1-Luc cells intraperitoneally. On day 3 post-injection, tumor-

bearing mice were injected with a mix of $CD8^+$/dp$CD4^+$ $T_{TCR37\text{-}45}$ (2 x 10^6 of each, 1:1 ratio), and received a second injection of $CD8^+$/dp$CD4^+$ $T_{TCR37\text{-}45}$ at week 4. Control (Ctrl) mice did not receive therapeutic T cells. **(B)** Mice were imaged weekly as shown for a bioluminescence signal to gauge progression or regression of tumor in vivo. The mice were administered 1 μL of Luciferin per gram of body weight at 15 mg/mL of concentration intraperitoneally under isoflurane anesthesia. The incubation time was 15 minutes before the mice were imaged while still under isoflurane anesthesia. **(C)** Total flux (p/s) was measured to evaluate luciferase activity for the treated and untreated mice. Error bars indicate 95% confidence interval. The p-value was calculated by a two-way ANOVA. Mouse 3 and mouse 5 of the $T_{TCR37\text{-}45}$ treatment group were originally imaged in the incorrect order on week 7. For clarity, the larger image for this week only was cropped to individual mice and ordered correctly.

Supplementary Tables

Days after 1st $T_{TCR\text{-}C4}$ infusion	CMV (IU/mL)	EBV (IU/mL)
0	NDET	NDET
1	NDET	130
7	NDET	NDET
14	NDET	NDET
24	NDET	NDET
180	NDET	NDET
210	NDET	NDET
254	NDET	NDET

Table S1: Detection of CMV and EBV viremias after infusions. Patient serum was analyzed for presence of CMV and EBV virus (see **Supplementary Methods**) over the course of treatment. NDET: none detected.