Supplemental Methods

Cell lines

B16-F10 tumor cells lines were obtained from the ATCC (Catalog # CRL-6475**)**. These were further modified to express a tandem minigene encoding epitopes from gp100 (EGSRNQDWL), H60 (LTFNYRNL), TRP1 (TAPDNLGYM), TRP2 (SVYDFFVWL) and OVA (SIINFEKL) as described in in Supplemental Figure 6 (B16-MG cells). Platinum-E (PLAT-E; Catalog # RV-101) and Platinum-GP (PLAT-GP; #RV-103) retrovirus packaging lines were purchased from Cell Biolabs (San Diego, CA). MC38 tumor cells(1) were a gift from Lary Kane (University of Pittsburgh). We modified 4G4 thymoma cells (2) to create cells that report TCR signaling (4G4R). TCRα/β-knockout human CD8α^+ Jurkat reporter cells were obtained from BlueSphere Bio. B6WT3 mouse embryonic fibroblast cells(3) were obtained from Robert Hendrick (University of Pittsburgh).

Culture media

Complete RPMI (cRPMI; used for all cultures that included T cells and MC38 cells) is RPMI (Gibco) supplemented with 10% FBS (Hyclone), NaPyruvate 1mM, non-essential amino acids, L-glutamine 2mM, Hepes 10mM and 2-beta-mercaptoethanol 50mM. PLAT-E media (used for all retrovirus generation) is DMEM with L-glutamine (Gibco), 10% FBS, NaPyruvate 1mM and 1x nonessential amino acids.

4G4 cell modifications

Briefly, 4G4 cells which do not express TCRα or TCRβ chains were transduced with two retroviruses, one encoding murine CD3α, CD3ε, CD3γ and CD3ζ chains linked to ametrine (poly-CD3; gift of Dario Vignali (4)) and the second expressing mouse CD8α. Ametrine$^+$ CD8α^+

cells were purified by FACS, and subcultures were selected based on rapid growth and high levels of surface CD8 expression.

Calculations of tumor volumes

Tumor volume was determined using the formula: ½*(Length *Width^2)

$$\text{Volume Elipsoid: } \frac{Length\ x\ Width^2}{2}$$

(Where L is length and W is width of the tumor). Percent change in tumor volume was calculated with the following formula:

% Change in tumor volume= (Tumor volume at the end of treatment-tumor volume on the start of treatment/ tumor volume on the start of treatment) *100

$$\%\ \text{Change in Tumor Volume} = \frac{Tumor\ Volume\ at\ "day\ 20" - Tumor\ volume\ at\ "day - 1"}{Tumor\ volume\ at\ "day\ -1"}\ x\ 100$$

Mice were euthanized if the tumor diameter exceeded 20mm, if they had lost >20% of their starting body weight on 2 consecutive measurements, or if they were moribund.

Creating TCR-expressing retrovirus.

VSV-G pseudotyped virus for Jurkat transduction. MSCV-based plasmids containing TCRα and TCRβ chains (1 µg/well) were individually co-transfected into PLAT-GP cells (1.5x10^5 cells/well) with VSV-G-expressing vector (1 µg/well) using 2 µL/well LipoJet™ (SignaGen #SL100468) in 24-well plates. 48 hours later, supernatants were collected.

Ecotropic retrovirus production for primary T cell transduction. 5x10^6 PLAT-E cells were seeded in T75 flasks. At 80% confluency, the media was replaced with 10mls complete RPMI (cRPMI) and the cells were transfected with 20µg TCR-expressing plasmid using the LipoJet™ In Vitro Transfection Kit (Ver. II). 18 hours later, the media was replaced with 10mL cRPMI and the cells

were incubated for an additional 30 hours. At 48 hours post-transfection, media was harvested, and debris was cleared through centrifugation. RetroX viral concentration reagent (Takara) was added to the supernatant and virus was concentrated according to the manufacturer's instructions.

Virus titering

4G4R cells were infected with dilutions of virus stock in 3μg/ml polybrene and centrifuged at 1000g for 90 minutes at 37° C. Cells were returned to the incubator and media was changed 18 18 hours later. Titers were determined based on the expression of mCherry or TCRβ 48-72 hours later (see Supplemental Figure 7).

Jurkat cell transduction

VSV-G-pseudotyped retroviral supernatants were added to Jurkat cells ($1x10^5$ cells/well) with 4 μg/mL polybrene (Sigma-Aldrich #107689) and centrifuged at 37°C for 90 min without braking after which the cells were returned to the incubator. Media was changed in next day. To puromycin-select transduced cells, puromycin was added to the media (2μg/ml) for 48 hours and then changed with fresh cRPMI media for cell expansion.

Neoantigen prediction

Whole genome sequencing (WGS) and sequence alignments of MC38 cells were carried out by the University of Pittsburgh Genomics Core. This initial alignment was then refined by GATK base recalibration of insertions and deletions (5), followed by indel realignment and duplicate removal, all following GATK best practices recommendations (5,6). Variant calling was performed on WGS data using three variant callers: Mutect, Mutect2, and Strelka (7,8). This multi-tool approach aimed to minimize false positives inherent in variant-calling algorithms.

Variants identified by at least two out of the three tools were annotated using Ensembl Variant Effect Predictor(9). Variants resulting in an amino acid change were selected, yielding 807 neoantigen candidates. NetMHCPan 4.0 was employed to estimate the EC50s of these predicted peptides to H-2K^{b} or H-2D^{b} (10). Using a publicly available bulk RNA-Seq dataset for MC38 cells (11), the transcripts per million (TPM) for each gene encoding potential neoantigens were calculated using STAR aligner (12) and geneCount (Illumina), so as to be able to incorporate gene expression into choosing putative neoantigens to pursue. TPM, predicted epitope binding scores, and variant allele fractions were each converted to percentile ranks across the candidate neoantigen dataset to place all features on a comparable scale. These normalized values were then integrated using a weighted linear scoring framework to generate a composite score reflecting the predicted likelihood that a candidate neoantigen will elicit a CD8^{+} T cell response.

Molecular deconvolution of TCR-encoding plasmids.

After a TCR expressed in Jurkat cell line was found to be desirable, the sequences and specificity of the TCR were confirmed by the “molecular deconvolution” of the vectors. Briefly, plasmid generated from the bulk E. coli cultures from the original Gibson assembly product was transformed into bacteria, followed by plating on agarose plates. Six to eight colonies were selected, expanded, and plasmid DNA was isolated. The plasmids were sequenced and used to create individual lentivirus supernatants. Jurkat reporter cells were transformed, reselected, and screened against B6WT3 expressing the appropriate antigen.

Sanger sequencing of TCRβ PCR products and TCR retroviral vector plasmids

Bead-purified second round TCRβ chain amplification products were Sanger sequenced directly with a single primer (GAAGAAAACCCCGGTCCC) without subcloning (Azenta). The TCRα and

TCRβ regions of single colony-derived plasmids were sequenced with the same primer used to sequence purified TCRβ chains plus three other primers (TTGGCTTTTGACCCCC, AGGTTCTGGGTTCTGGATGT, GGAGTCACATTTCTCAGATCCT) so as to cover all Gibson insertions.

Transduction of primary T cells

T cells were purified from C57BL6 mice splenocytes using Easy Sep Mouse T cell negative selection kits (Stem Cell, cat No. 19851). For TCX 1.0, on day 0, purified T cells were cultured at a 1:1 ratio with anti-CD3/CD28 Dynabeads (Thermo-Fisher/Gibco, cat. No. 11453D) in cRPMI with 50U/ml human IL-2 (Peprotech) for 48 hours at 37°C. For TCX2.0, T cells were activated in a 12 well plate with 3µg/well plate-bound anti-CD3 (clone 2C11, Biolegend, cat no. 100302) with soluble anti-CD28 (2µg/ml, clone 37.51, Biolegend, cat No. 102116), and anti-CD137 (10µg/ml, clone 3H3, InvivoMAb Bio-X Cell, cat. No BE0239) in cRPMI with 50U/ml IL-2 for 48 hours at 37°C. After 48 hours, activated T cells were harvested and subjected to endogenous TCR α/β knockout using the Stem Technologies CRISPR-Cas9 ARCITect system (Stem Cell Technologies, Vancouver, British Columbia, CA). In brief, crRNA containing sequences targeting the TCRα constant region (GAGACCGAGGATCTTTTAAC) and the TCRβ constant region (GCCCCTGGCCAAGCACACGA) and Tracr RNA complexes were prepared as per the manufacturer's protocol. gRNA-Cas9 complexes were prepared by combining Cas-9 (Stem Cell, cat no. 76004) with crRNA-Tracr RNA complexes followed by electroporation of activated T cells using with Lonza electroporation system and the P3 cell solution kit (Cat.no PB P3-U2250). Following electroporation, cRPMI with IL-2 (50U/ml) was added to the cells which were rested for 1hr at 37°C. These electroporated T cells were then transduced with matched titers (10 infectious units per cell) of retroviral supernatants encoding CRISPR-resistant TCRs of interest.

Transduced cells were washed the next day and expanded in cRPMI and IL-2 (50U/ml) for 4 additional days. Endogenous TCR knockout and TCR transduction was measured by flow cytometry by staining cells with antibodies against CD8, TCRβ and using MHC-multimers when appropriate. Functional assays and cell transfer for ACT were performed at day 8 post-activation.

T cell killing assays

Target specific killing by engineered T cells was analyzed using the xCELLigence system (Agilent). Published protocols were adapted for use with B6WT3, MC38 and B16-derived cell lines. Prior to being plated all target cell lines were pretreated with IFN-γ. In brief, xCELLigence gold E-plates were blocked for 1 hour at 37°C with 0.1% BSA in PBS. Plates were washed twice with PBS, media was added and allowed to equilibrate at 37°C, followed by the addition of cells (in triplicate for each condition). Adhesion and growth were recorded on the xCELLigence reader for 24 hrs. For MC38 killing assays, 80,000 MC38 cells were added the day prior to adding 10^5 live cells for each TCR-modified product. For killing of B6WT3 cells, 25,000 B6WT3 cells were seeded the day prior to adding 10^5 live cells for each TCR-modified product. For peptide-specific killing assays, the cell layers were pulsed with peptide and returned to the xCELLigence for 1-2 hours prior to the addition of T cells and incubation for 24 hours at 37°C. Relative cell indices were calculated based on dividing the conductance at each time-point by the maximum conduction during the experiment. Areas under curve were calculated for each type of treatment. Statistical analysis was done by one-way ANOVA.

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