PNAS
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Supplementary Information for:

Immunological ignorance is an enabling feature of the oligo-clonal T cell response to melanoma neoantigens

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Datasets S1 to S10

www.pnas.org/cgi/doi/10.1073/pnas.1906026116

Methods and Materials

Tumor samples

Next Generation Sequencing and Neoantigen Prediction

DNA samples were subjected to exome sequencing using Roche NimbleGen SeqCap EZ Human Exome Library v3.0. The resulting captured DNA was sequenced by Illumina HiSeq2000 platform with 100 bp paired-end reads. The raw exome sequencing reads were aligned against the GRCh37 version of the human reference genome and were merged and subsequently de-duplicated with Picard (v1.46). Detection of somatic mutations was performed using the union of three variant callers: SAMtools (intersected with Somatic Sniper), VarScan and Strelka. Variants were filtered as previously described and manually reviewed using the Integrated Genomics Viewer in order to obtained high-confidence variant calls (1). For cDNA-capture sequencing, tumor DNase-treated RNA samples were processed with MicroPoly(A)Purist™ Kit (Ambion) to enrich for poly(A) RNA and TruSeq Stranded Total RNA Sample Prep kit (Illumina, 20ng ribosomal RNA-depleted total RNA) used for cDNA synthesis. All NuGen cDNA sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® with minor modifications and processed as described previously(2). cDNA-capture data was aligned with Tophat v2.0.8 (params: --library-type fr-firststrand --bowtie-version=2.1.0). Expression levels were calculated with Cufflinks v2.0.2 (params--max-bundle-length=10000000--num-threads 4) and the resulting FPKM values were used to filter expressed gene isoforms with FPKM > 1. Identification and prioritization of candidate neoantigen resulting from amino acid substitutions (AAS) corresponding to coding / expressed missense mutations (MM) were obtained using pVACseq pipeline(3). Briefly, AAS encoding MM were translated into a 21-mer amino acid FASTA sequence, with ideally 10 amino acids flanking the substituted amino acid on each side. Each 21-mer amino acid sequence was then evaluated through a suite of HLA class I peptide binding algorithm to predict high affinity HLA class I 9-mer and 10-mer peptides for the AAS- as well as the WT sequence. Any AAS-peptide with binding affinity IC50 value< 500nm were considered for further analysis.

Peptides: Peptides were obtained lyophilized from American Peptide Company (>95% purity), dissolved in 10% DMSO in sterile water and tested for sterility, purity, endotoxin and residual organics. Peptide binding to HLA class I molecules was determined using the fluorescence polarization assay (Pure Protein, L.L.C.)(4).

DC manufacturing: Monocyte-derived immature DCs were generated in 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, Berlex) and 20ng/mL IL-4 (Miltenyi Biotec) as described(2). Six days after culture initiation, immature DCs were cultured with irradiated (10,000 rad) GMP-grade CD40L-expressing K562 cells, 100u/mL IFN-g (Actimmune, InterMune Inc.), poly I:C (Invivogen, Inc) and R848 (Invivogen, Inc.) for 16h to generate mDC. Two hours prior to infusion, mDC were pulsed (50ug/10^6 cells/mL) separately with each peptide (10 AAS-peptides and 2 gp100 peptides, G209-2M and G280-9V) and, for dose 1 only, influenza virus vaccine (Sanofi Pasteur) was added to provide a source of recall antigen for CD4+ T cells. IL-12p70 production by vaccine DC was measured by ELISA (eBioscience) according to the manufacturer's instructions. All mDC vaccine doses were prepared at time of immunization from either freshly isolated (dose1) or cryopreserved (doses 2-3) PBMC. The initial priming dose was $1.5x10^7$ DC per peptide ($1.35x10^8$ DC total), in remaining doses, patients received $5x10^6$ DC per peptide ($4.5x10^7$ DC total). Cyclophosphamide (300 mg/m^2) was given 96h prior to the first DC dose. Patients underwent clinical evaluation prior to each mDC infusion.

Neoantigen T cell responses: Kinetic and magnitude of T cell response to AAS-encoding and gp100-derived peptides was performed using PBMC collected weekly as described (5). Briefly, fresh PBMC obtained by Ficoll-Hypaque gradient centrifugation were cultured with 40ug/mL peptide and IL-2 (50U/mL). On day 10 (peak of response,(5)), neoantigen-specific T cell frequencies were determined by staining with HLA-A*02:01/peptide dextramers (Immudex), followed by addition of FITC-CD4, -CD14, -CD19 (Invitrogen) and -CD56 (BD Pharmigen), APC-CD8 (Invitrogen). Cells were washed, resuspended in FACS buffer containing 7AAD. Twenty-five thousand events in the CD8+ gate was collected using a hierarchical gating strategy that included FSC/SSC and excluded 7AAD-positive (dead cells) and CD4/14/19/56-positive cells. Data was acquired and analyzed using Flow-Jo software. For functional characterization, neoantigen-specific T cell lines were generated using autologous mDC and antigen loaded artificial antigen presenting cells at a ratio of 1:1 as previously described(2). Recognition of antigen was assessed in 4h ^{51}Cr-release assays using peptide-pulsed or tandem mini-gene construct (TCM)-expressing artificial antigen presenting cells as described(2). The latter allows for assessment of processing and presentation of neoantigens. TCR $\alpha\beta$ cloning was performed using the SMARTer profiling kit (Takara Bio) starting with mRNA and sequence verification of full-length cDNA (Illumina MiSeq) followed by cloning into pTRP vector plasmid. Purified LV particles are used for transduction of Jurkat (J76TPR) reporter cells kindly provided by Dr. Peter Steinberger (Institute for Immunology, Medical University of Vienna)(6) and analyzed on a Fortessa BD flow cytometer after antigen-stimulation using peptide-pulsed (1 ug/ml) K562/HLA-A*02:01 transfectants.

Proteomic Analysis. Production and Isolation of HLA-A*02:01/ Peptide Complexes**:** Cell lines (721.221 and A375) expressing sHLA-A*02:01 were transduced with Mel66 patient neoantigen expressing TMC (Tandem Minigene Construct). Cells were grown in roller bottles and sHLA/peptide complexes were purified from supernatants by affinity chromatography with the anti-VLDLr antibody. Eluate fractions containing sHLA/peptide complexes were brought to a final acetic acid concentration of 10%, pooled, and heated to denature HLA, and peptides were isolated from alpha chain and β_2m using an Ultracel 3 kDa molecular weight cutoff cellulose membrane (EMD Millipore) and lyophilized. The same process was performed with the TMC encoding the corresponding wildtype sequences. Mass Spectrometric Analysis: Synthetic peptides corresponding to the mutant and wild type sequences were separately resuspended in 10% acetic acid at 1µM and were fractionated by RP-HPLC with an acetonitrile gradient in 10 mM ammonium formate at pH 10. Peptide-containing fractions were dried, resuspended in 10% acetic acid and subjected to nanoscale RP-HPLC at pH 2.5 utilizing an Eksigent nanoLC coupled to a TripleTOF 5600 (AB Sciex) quadrupole time-of-flight mass spectrometer (LC/MS). Information dependent acquisition (IDA) was used to obtain MS and MS/MS fragment spectra for peptide ions. The sequence of each peptide was determined by observed mass and fragment ions, and the 1st dimension fraction number and LC/MS retention times were recorded. Next, peptides purified from TMC expressing cells were resuspended in 10% acetic acid and HPLC fractionated under the same conditions and gradient method. The HPLC fractions corresponding to those containing the synthetic peptides were then subjected to the same LC/MS conditions. Resulting spectra were found positive for the presence of the mutant peptides if the following criteria were met: 1. LC/MS elution time was within 2 minutes of the synthetic, and 2. Fragment ion masses matched those of the synthetic with an accuracy of ± 25 ppm. PeakView® Software version: 1.2.0.3 was used for exploring and interpreting of the LC/MS data. Instrument parameters and first and second dimension gradient methods details have been previously described (2).

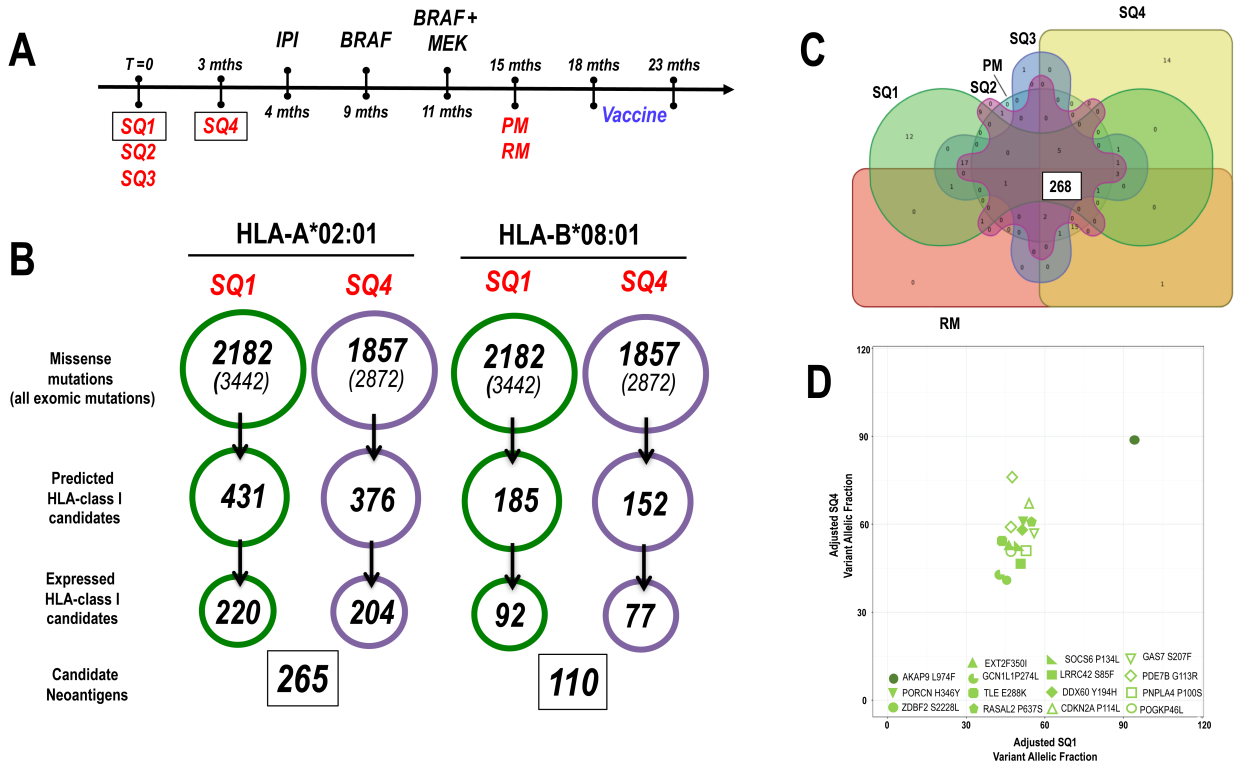

Fig. S1. Neoantigen Identification, distribution and recognition. (A) Schematic overview of MEL66 treatment and tissue collection. (B) Distribution of somatic (exomic and missense) mutations identified in metachronous tumors SQ1 and SQ4 of patient MEL66. HLA-A*02:01 and -B*08:01 binding candidates were identified in silico among AAS, expression of genes encoding mutated proteins determined by transcriptome sequencing. Numbers in square represents expressed candidate neoantigens in tumor samples. (C) Venn diagrams show expression among tumors of mutated genes encoding neoantigens. Shared neoantigens (268/350) among the 6 tumors is indicated in the white square. (D) Classification of neoantigens according to Sciclone analysis, solid shapes denote vaccine candidates, open shapes denote neoantigens identified in TILs. All neoantigens were encoded by SNV in cluster 1 (CNA or LOH) or 2 (clonal) as shown in Figure 1.

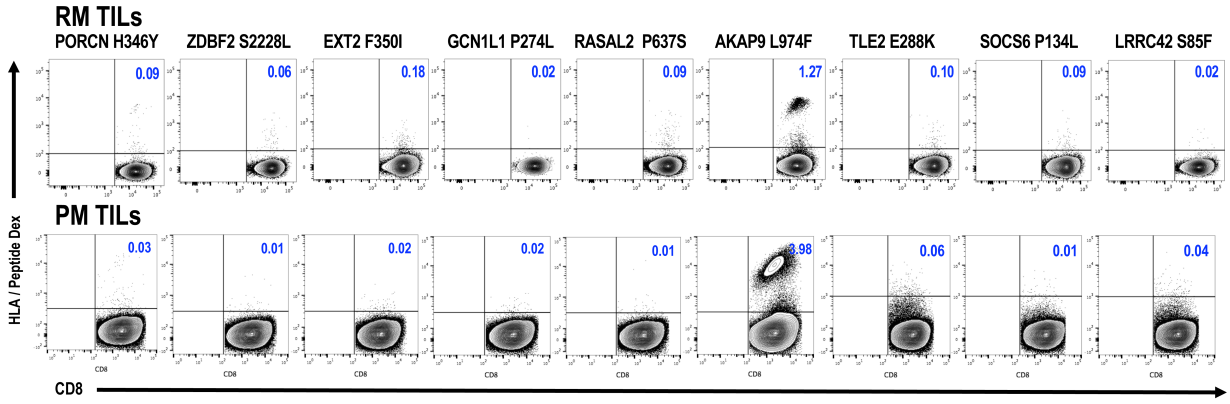

Fig. S2. MEL66 TIL reactivity to vaccine candidate neoantigens. Unmanipulated TILs isolated from tumors RM and PM were stained with p-HLA multimers and anti-CD8 and analyzed by flow cytometry. Twenty-five thousand events were collected on the CD8+ T cell gate.

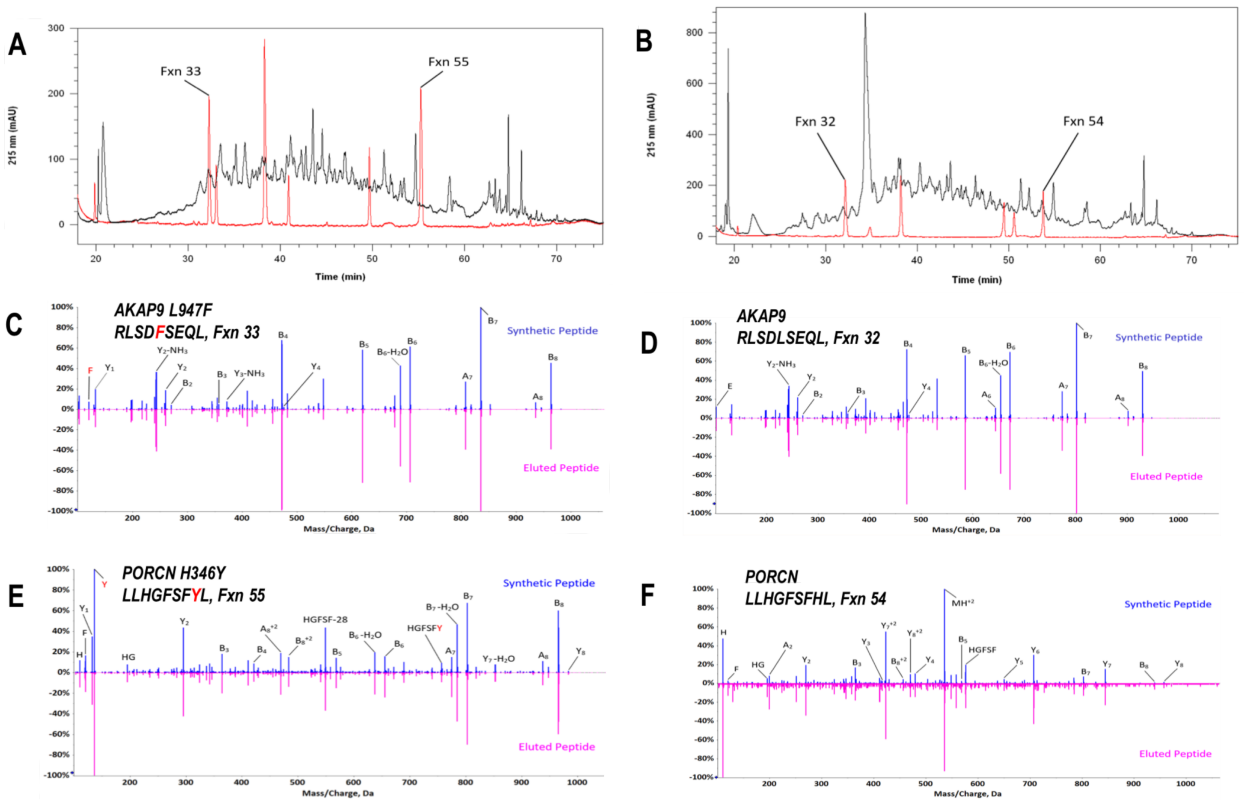

Fig. S3. Processing and presentation of AAS- and WT- AKAP9 and PORCN peptides. RP-HPLC fractionation of HLA-A*02:01 peptides eluted from (A) AAS- and (B) WT TMC expressing cell lines (black trace) and the synthetic peptide mixture (red trace) with fractions 32,33, 54 and 55 indicated. MS/MS fragmentation pattern of the eluted and corresponding synthetic peptides of (C) AKAP9 L947F, (D) AKAP9, (E) PORCN H346Y, and (F) PORCN. In each panel the pink trace (bottom) represents the eluted peptide and the blue trace (top) represents the synthetic peptide.

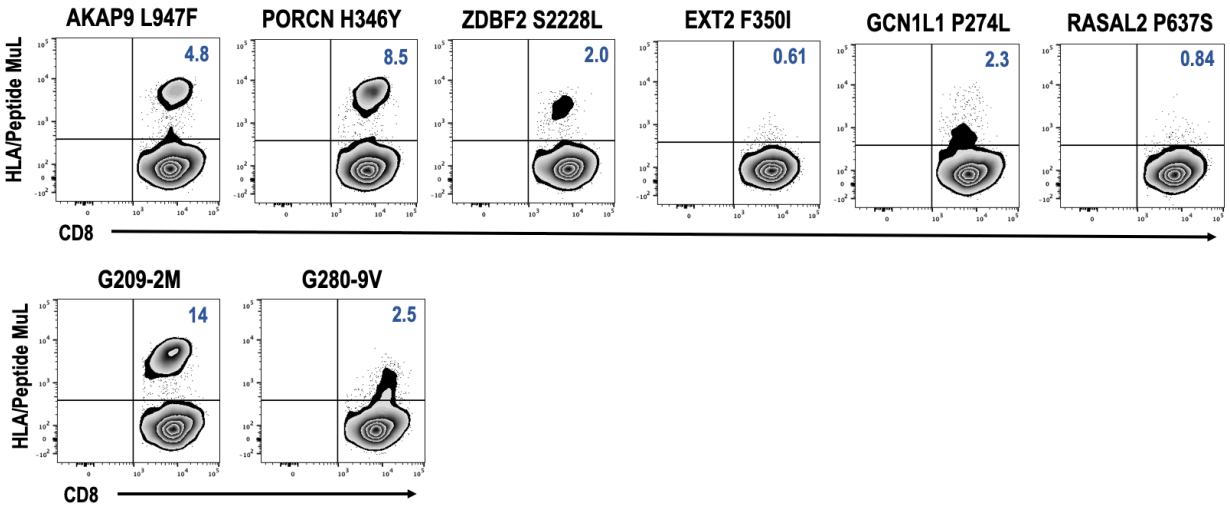

Fig. S4. Pattern of CD8+ T cell immunity to non-ignored and ignored neoantigens and shared antigens is maintained long-term. PBMC isolated from peripheral blood collected 60 weeks after last vaccination were cultured for 10 days in the presence of indicated peptide (40ug/mL) and IL-2 (100U/mL), stained with p-HLA multimer and analyzed by flow cytometry. Numbers in dot plot represent percentage of antigen-specific T cells in lymph/CD8+gated cells.

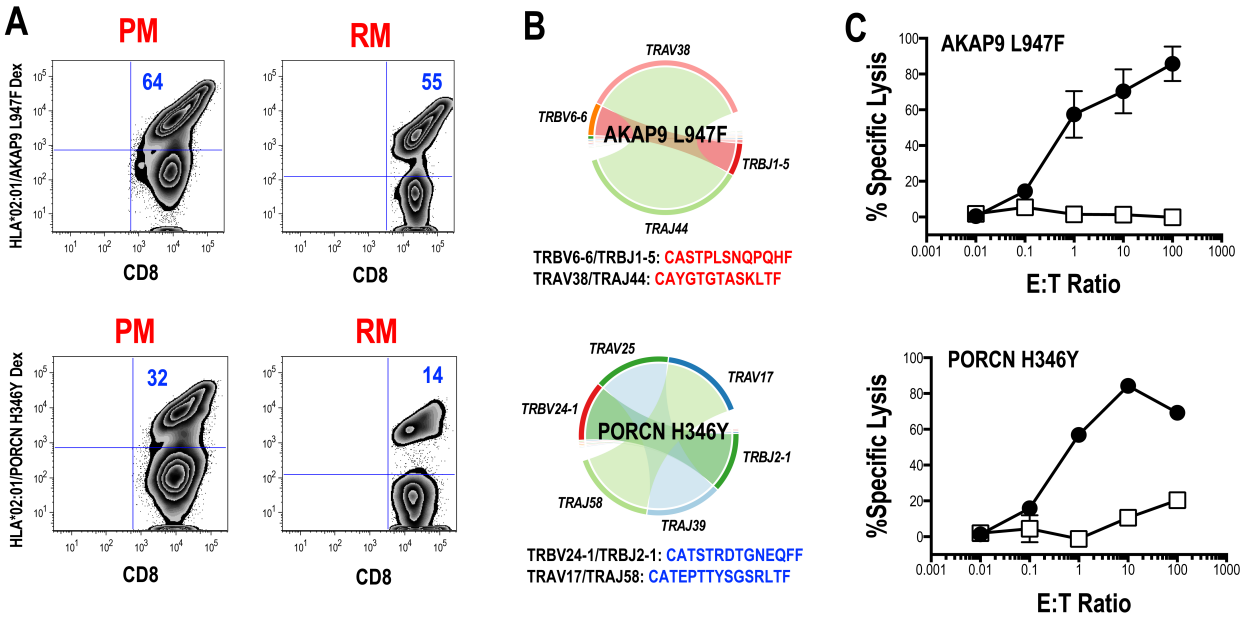

Fig. S5. Characterization of AKAP L947F- and PORCN H346Y- specific TILs isolated from RM/PM tumors. AKAP9 L947F and PORCN H346Y reactivities of TIL isolated from RM and PM tumors. (A) TILs were cultured in vitro in the presence of peptide and IL-2 for 10 days followed by p-HLA multimer staining. (B) Circos plots representing TCRVB and TCRVA transcript frequencies identified in AKAP L947F- and PORCN H346Y- specific TILs. CDR3VB and VA amino acid sequences identified for each TCR are shown. For PORCN H346Y only TRBV24/TRAV17 yield a productive TCR. (B) AKAP9 L947F- and PORCN H346Y- specific TILs recognition of AAS (closed circles) and WT (open squares) peptides as determined in a standard 4h ^{51}CR-release assay using peptide titration on T2 target cells. Percent specific lysis of triplicates is shown for each peptide concentration; spontaneous release is <15%. A representative experiment of 2 independent evaluations is shown.

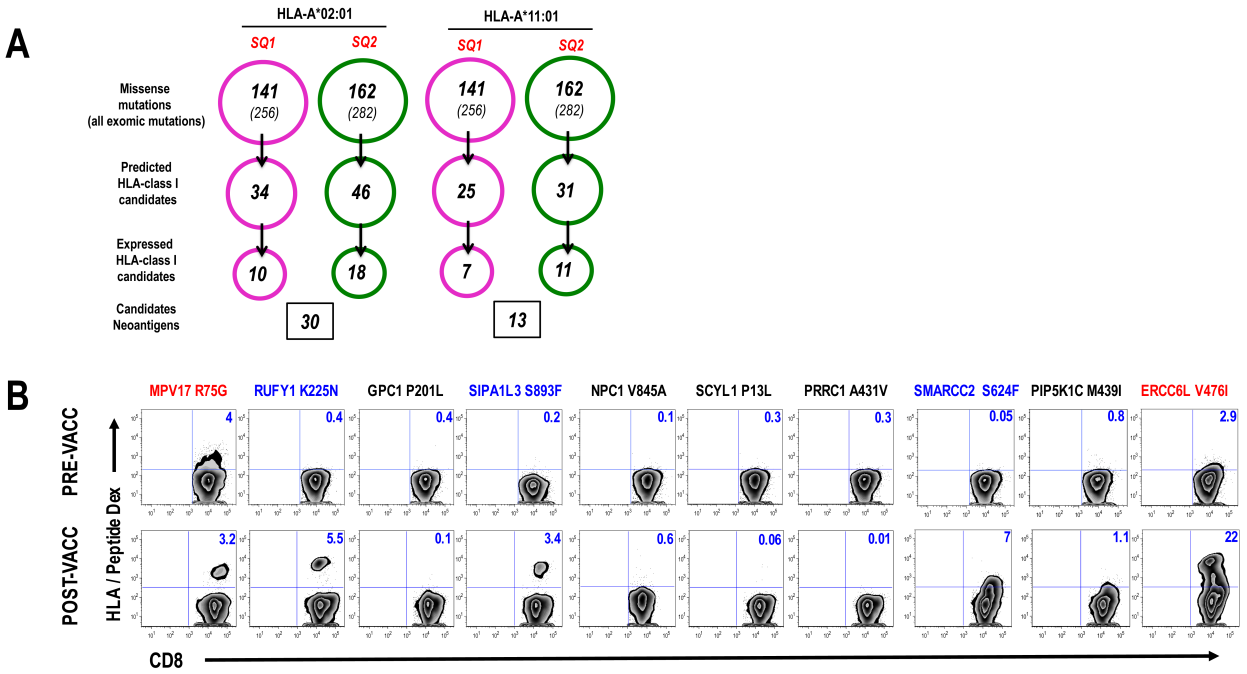

Fig. S6. MEL69 Neoantigen Identification and recognition. (A) Distribution of somatic (exomic and missense) mutations identified in metachronous tumors SQ1 and SQ2 of patient MEL69. HLA-A*02:01 and -A*11:01 binding candidates were identified in silico among AAS, expression of genes encoding mutated proteins determined by transcriptome sequencing. Numbers in square represents expressed candidate neoantigens among tumor samples. (B) Pre- and post-vaccine PBMC were cultured in vitro in the presence of peptide and IL-2 for 10 days followed by p-HLA multimer staining. Color coding identified pre-existing (red) and vaccine-induced (blue) neoantigens. Numbers in dot plot represent percentage of neoantigen-specific T cells in lymph/CD8+ gated cells.

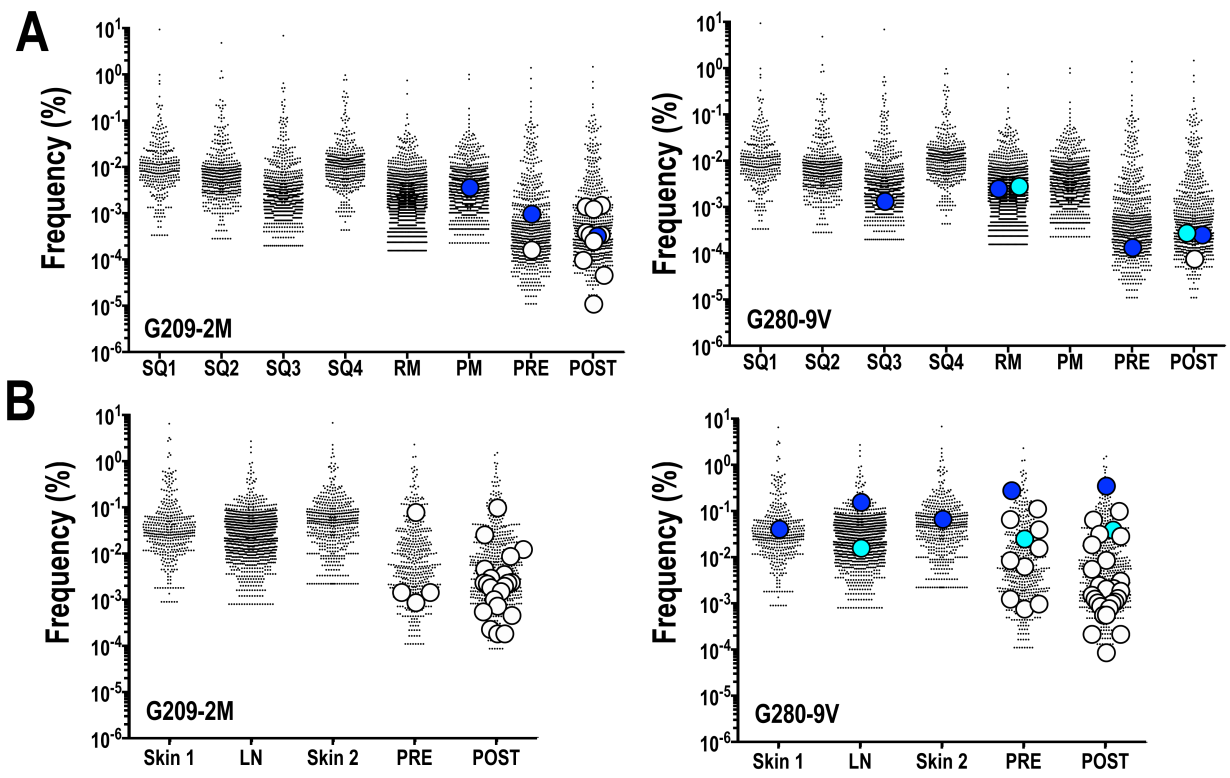

Fig. S7. GP100 TCR diversity and tumor distribution of T cell clonotypes. (A) MEL66 and (B) MEL21 G209-2M- and G280-9V-specific TCR clonotypes distribution among corresponding tumors. Peripheral blood pre- and post-vaccine CD8+ T cells as well as tumors were characterized for gp 100-specific TCRVB repertoires using G209-2M or G280-9V-specific TCRVB_CDR3 reference libraries. Each colored dot represents a unique neoantigen TCR clonotypes. White dots represent unique neoantigen TCR clonotypes found only in blood-derived samples. Identities of neoantigen-specific TCR clonotypes are listed in Dataset S10. For clarity, only TCR clonotypes with tumor frequencies > 0.00001 are shown.

Legends to Datasets:

Dataset 1: Patient MEL66 list of HLA-A*02:01 and HLA-B*08:01 candidate peptide neoantigens identified in SQ1 and SQ4 tumors.

Dataset 2: Ion Torrent platform sequencing results of 350 MM encoding putative neoantigens in patient MEL66 tumors.

Dataset 3: Patient MEL66 list of HLA-A*02:01 candidate peptide neoantigens tested in peripheral blood and TILs for immunogenicity.

Dataset 4: Characteristics of candidate peptide neoantigens formulated in vaccine given to patient MEL66.

Dataset 5: Patient MEL66 neoantigen-specific TCRVβ CDR3 reference libraries.

Dataset 6: Neoantigen-specific TCRVβ clonotypes identified in patient MEL66 tumors.

Dataset 7: Patient MEL69 list of HLA-A*02:01 and HLA-A*11:01 candidate peptide neoantigens identified in SQ1 and SQ2 tumors.

Dataset 8: Characteristics of candidate peptide neoantigens formulated in vaccine given to patient MEL69.

Dataset 9: Neoantigen-specific TCRVβ clonotypes identified in patients MEL21, MEL38 and MEL69 tumors.

Dataset 10: GP100-specific TCRVβ clonotypes identified in patients MEL66 and MEL21 tumors.

SI References:

1. Hundal J*, et al.* (2019) Accounting for proximal variants improves neoantigen prediction. *Nat Genet* 51(1):175-179.
2. Carreno BM*, et al.* (2015) Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science* 348(6236):803-808.
3. Hundal J*, et al.* (2016) pVAC-Seq: A genome-guided in silico approach to identifying tumor neoantigens. *Genome Med* 8(1):11.
4. Buchli R*, et al.* (2005) Development and validation of a fluorescence polarization-based competitive peptide-binding assay for HLA-A*0201--a new tool for epitope discovery. *Biochemistry* 44(37):12491-12507.
5. Carreno BM*, et al.* (2013) IL-12p70-producing patient DC vaccine elicits Tc1-polarized immunity. *J Clin Invest* 123(8):3383-3394.
6. Rosskopf S*, et al.* (2018) A Jurkat 76 based triple parameter reporter system to evaluate TCR functions and adoptive T cell strategies. *Oncotarget* 9(25):17608-17619.