**Supplementary Methods**

**Patients and cells**

Whole exome sequencing (WES) and TCR sequencing of the tumor samples was performed as described in ([19](#_ENREF_19)), normal tissue was used to filter out germline variants. WES quality control metrics are found in **Supplementary Table 14**. WES data are available in the NCBI Sequence Read Archive public repository (BioProject ID PRJNA316754). RNA was extracted using the AllPrep DNA/RNA/miRNA Universal kit from QIAGEN as suggested by the manufacturer. Extracted RNA was then prepared for sequencing using the TruSeq RNA Access Library Prep reagents as recommended by the manufacturer and sequenced on a HiSeq Sequencer (Illumina). HLA typing was determined from the WES data by POLYSOLVER ([34](#_ENREF_34)).

WES and RNA sequencing of 12T was performed as described previously ([12](#_ENREF_12)). WES of 12T is available in dbSNP under accession 1062266. To increase the amount of HLA expressed by the 12T cell line, the cells were grown in the presence of 250 IU/ml IFNγ for 48 hours. Cell pellets with and without IFNγ treatment were used for HLA peptidomics.

To increase the number of identified TAAs from patient 55, the 55D cell line was also used for HLA peptidomics.

EBV transformed B-cells were purchased from the IHWG Cell and DNA Bank (cells were purchased on July 2016, March 2017 and July 2017). Hybridoma cells HB95 and HB145 were purchased from the ATCC (on August 2015) and were used to purify pan-HLA-I and pan-HLA-II antibodies for the preparation of the HLA affinity columns. All cell lines were tested regularly and were found negative for mycoplasma contamination January 2018 (EZ-PCR Mycoplasma Kit, Biological Industries). Cells were authenticated by Finger printing with STR profiling (Panel: PowerPlex\_16\_5Nov142UAGC, Size: GS500 x35 x50 x250, Analysis Type: Fragment (Animal), Software Package: SoftGenetics GeneMarker 1.85).

**Production and purification of membrane HLA molecules**

Tumor samples and cell pellets were homogenized through a cell strainer on ice with lysis buffer containing 0.25% sodium deoxycholate, 0.2mM iodoacetamide, 1mM EDTA, 1:200 Protease Inhibitors Cocktail (Sigma-Aldrich, P8340), 1mM PMSF and 1% octyl-b-D glucopyranoside in PBS. Samples were then incubated at 4˚C for 1 hour. The lysates were cleared by centrifugation at 48,000g for 60 minutes at 4˚C, and then were passed through a pre-clearing column containing Protein-A Sepharose beads.

HLA-I molecules were immunoaffinity purified from cleared lysate with the pan-HLA-I antibody (W6/32 antibody purified from HB95 hybridoma cells) covalently bound to Protein-A Sepharose beads or to Amino-Link beads (Thermo-Fisher Scientific, as reported previously ([12](#_ENREF_12),[35](#_ENREF_35)). HLA-II molecules were then purified by transferring the flow through onto similar affinity columns containing pan-HLA-II antibody (purified from HB-145 hybridoma cells). Affinity columns were washed first with 10 column volumes of 400mM NaCl, 20mM Tris–HCl and then with 10 volumes of 20mM Tris–HCl, pH 8.0. The HLA peptides and HLA molecules were then eluted with 1% trifluoracetic acid followed by separation of the peptides from the proteins by binding the eluted fraction to disposable reversed-phase C18 columns (Harvard Apparatus). Elution of the peptides was done with 30% acetonitrile in 0.1% trifluoracetic acid ([35](#_ENREF_35)). The eluted peptides were then cleaned using C18 stage tips as described previously ([36](#_ENREF_36)).

**Identification of eluted HLA peptides**

The HLA peptides were dried by vacuum centrifugation, solubilized with 0.1% formic acid, and resolved on capillary reversed phase chromatography on 0.075x300 mm laser-pulled capillaries, self-packed with C18 reversed-phase 3.5μm beads (Reprosil-C18-Aqua, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany)([37](#_ENREF_37)). Chromatography was performed with the UltiMate 3000 RSLCnano-capillary UHPLC system (Thermo Fisher Scientific), which was coupled by electrospray to tandem mass spectrometry on Q-Exactive-Plus (Thermo Fisher Scientific). The HLA peptides were eluted with a linear gradient over 2 hours from 5 to 28% acetonitrile with 0.1% formic acid at a flow rate of 0.15µl/minute. Data were acquired using a data-dependent “top 10” method, fragmenting the peptides by higher-energy collisional dissociation. Full scan MS spectra were acquired at a resolution of 70,000 at 200 m/z with a target value of 3x106 ions. Ions were accumulated to an AGC target value of 105 with a maximum injection time of generally 100 milliseconds. The peptide match option was set to Preferred. Normalized collision energy was set to 25% and MS/MS resolution was 17,500 at 200 m/z. Fragmented m/z values were dynamically excluded from further selection for 20 seconds. The MS data were analyzed using MaxQuant ([38](#_ENREF_38)) version 1.5.3.8, with 5% false discovery rate (FDR). Peptides were searched against the UniProt human database (July 2015) and customized reference databases that contained the mutated sequences identified in the sample by WES. N-terminal acetylation (42.010565 Da) and methionine oxidation (15.994915 Da) were set as variable modifications. Enzyme specificity was set as unspecific and peptides FDR was set to 0.05. The match between runs option was enabled to allow matching of identifications across the samples belonging the same patient.

**Identification of tumor associated antigens (TAAs)**

We used the lists of known cancer and melanoma antigens described in the Cancer/Testis database ([39](#_ENREF_39)) (CTDatabase, <http://www.cta.lncc.br/>), peptide database ([40](#_ENREF_40)) (<https://www.cancerresearch.org/scientists/events-and-resources/peptide-database>) and a previously described human melanoma dataset ([41](#_ENREF_41)).

**Analysis of T-cell reactivity by IFN-γ release assay**

To evaluate the ability of the identified peptides to elicit an immune response, EBV-transformed B cells that express the correct HLA allele were first loaded with the candidate peptides at a concentration of 10 μM for 2 hours at 37 °C. All synthetic peptides were purchased from GeneScript and used as crude peptides for reactivity validation. Further analysis of the neo-antigens was done using >95% pure peptides. Following three washing steps, the loaded B cells were co-cultured with the TILs in which the antigen was identified at a ratio of 1:1 (105 cells) for an overnight incubation. The amount of soluble IFNγ secreted from the TILs were measured by ELISA assay (Biolegend).

**Flow cytometry analysis**

TILs were co-cultured with autologous melanoma cells for 24 hours and then stained with anti-4-1BB (309809, Biolegend), anti-CD3 (300307, Biolegend), and anti-neo-antigens or TAAs tetramers (NIH Tetramer Facility). For the *in vivo* proliferation analysis the injected 12TILs were stained pre-injection with Cell Track Violet. At 24 hours after TILs injection the tumors were collected and processed for flow cytometry. Tumors were dissected, macerated mechanically using a scalpel, and incubated in RPMI medium containing 2 mg/ml collangenase IV, 1 mg/ml DNase, 2 mg/ml hyaluronidase (all from Sigma) at room temperature for 2 hours. Following incubation the tissues were processed and filtered through 70-m mesh, and the pellet was used for subsequent staining. TILs were later stained with anti-CD3 and anti-neo-antigen tetramers. TILs were first gated for live cells, then for CD3+ cells, and then were subdivided according to their staining with the tetramers to evaluate the fraction of cells that had divided and partially lost their Cell Track Violet signal. A BD LSR II flow cytometer (BD Biosciences) was used, and data were analyzed using the FlowJo software. Neo-antigenic and reactive vs. non-reactive clones were isolated by staining with the relevant tetramer or anti-4-1BB antibody. Sorting was done using the BD FACSAria III Cell Sorter (BD Biosciences).

**TCR sequencing**

Genomic DNA from sorted and bulk TIL populations of 12TIL was produced by the QIAamp DNA blood mini kit (QIAGEN). CDR3 regions were sequenced by ImmunoSeq (Adaptive Biotechnologies), with primers annealing to V and J segments, resulting in amplification of rearranged VDJ segments from each cell. The ImmunoSeq Analyzer was used to identify rearrangements that were differentially abundant between the bulk and 4-1BB positive samples. TCR sequencing was done on genomic DNA that was purified from tumor samples of the different metastases described previously ([19](#_ENREF_19)). TCR sequencing of thesorted and bulk TIL populations of 55DTIL was performed using RNA that was isolated from the TILs using RNeasy mini kit (QIAGEN). Libraries were produced with the SMARTer Human TCR  Profiling Kit (Clontech) and were sequenced using the Illumina MiSeq system. Data were analyzed using VDJtools and MiXCR.

**Fluorescence-based *in vitro* killing assay**

Fluorescent target cells were plated (for 12T we used 2x105 cells) for 8 hours and then incubated for 3 hours with TILs at effector to target ratios ranging from 0.5:1 to 8:1. Three different TIL populations were used for the assay: bulk TILs, a neo-antigen-enriched TIL population that was isolated by sorting the cells according to tetramer staining, and the tetramer-negative-sorted TIL population. Loss of the fluorescent content was used to quantify target cell death ([42](#_ENREF_42)). After incubation, non-adherent TILs and dead target cells were washed away with PBS. The fluorescence of remaining live target cells was quantified using a Typhoon-9410 laser flatbed scanner (GE Healthcare, USA). Fluorescence reading was focused 3 mm above the plate surface. The percentage of specific lysis was calculated as 100×(C-X)/C, where C is fluorescence in the TIL-free condition and X is fluorescence in the presence of TILs.

***In vitro* live cell imaging**

Melanoma cells (2.5x105) were plated on a glass bottom MatTek plate in 2 ml of medium overnight. TILs were added to the plate immediately before imaging at a 1:1 ratio with the melanoma cells. Cellular motility was visualized. Images were taken at 20 seconds intervals for 4 hours using a Nikon Eclipse Ti inverted spinning-disc confocal microscope with a 20× air objective. Images were acquired using Andor iQ software.

***In vivo* rejection assay**

Approval for all the research in mice was granted from the Institutional Animal Care and Use Committee (IACUC) committee at the Weizmann Institute of Science (IACUC # 29350816-3). Eight-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Harlan ([43](#_ENREF_43))) were injected intradermally with 1x106 12T cells in Matrigel. After 7 days, 4x106 12TILs in PBS were injected intratumorally with 2000 U of IL-2; both TILs-injected and non-injected mice were given 2000 U of IL-2 daily for 5 days. Tumors were measured daily using calipers.

**Live two-photon microscopy of melanoma tumors**

Eight-week-old male NSG mice (Harlan) were injected intradermally with either a mix of 12T-GFP and non-GFP (ratio 1:2) or a mix of 12T-GFP and 108T-non GFP (ratio 1:10). A total of 107 cells were injected in 100 μl with 100% Matrigel. At day 6 post melanoma injection, either 12T TILs or 108T TILs were thawed and left to recover in full media supplemented with 6000 U/ml IL-2 for 24 hours. At day 7 post melanoma injection, TILs were labeled with either CMTMR or Cell Track Violet (Molecular Probes), and 5x106 TILs were injected intratumorally in PBS supplemented with 2000 U IL-2.

Mice were anesthetized with 100 mg/kg ketamine, 15 mg/kg xylazine and 2.5 mg/kg acepromazine, supplemented hourly with half this dose. Mice were placed on a warmed stage and kept at a core temperature of 37°C. A skin flap was separated from the abdominal muscle to expose the intradermal tumor which was then covered with a glass-bottom imaging chamber. For vascular imaging mice were retro-orbitally injected with 3µl of 2 µM Q-tracker 655 quantum dots (Life Science Technologies).

We used an Ultima™ Multiphoton Microscope (Prairie Technologies) incorporating a pulsed Mai Tai™ Ti-sapphire laser (Newport Corp.). The laser was tuned to 840 nm to simultaneously excite Cell Track Violet, EGFP, CMTMR, and quantum dots. A water-immersed 20× (NA 0.95) objective from Olympus was used. To create a time-lapse sequence, a 50-μm-thick volume of the tumor was typically scanned at 5-μm Z-steps every 30-50 seconds.

**Quantification of cell motility**

The movement of TILs was analyzed using Volocity software (PerkinElmer) to calculate two-dimensional velocity *in vitro*. Average velocities are reported throughout the manuscript. The movement of TILs *in vivo* was analyzed using Imaris software (BitPlane). TILs were segmented and tracked in three-dimensions as spot objects. Segmentation and tracking was manually corrected, and the average velocities of 12TIL and 108TIL were compared. The movies show some leakage from the green (melanoma) channel into the red channel; false-detection due to this leakage was ignored. Minimal distance from closest neighbor was calculated by the Imaris "spot-to-spot closest distance" extension.

**Calculation of neo-antigen and TAA frequencies in the TIL**

To estimate the percentages of reactive and non-reactive T cells against neo-antigens and reactive T cells against TAAs we used IFNγ secretion values and tetramer staining values. The percentages of reactive and non-reactive T cells against the MED15 and NCAPH2 were also calculated using their IFNγ secretion values and tetramer staining values. The percentages of reactive T cells against the predicted neo-antigens were calculated by their IFNγ secretion values compared to the MED15 IFNγ secretion values and MED15 tetramer staining. The percentages of reactive T cells against the TAAs was calculated using their IFNγ secretion values and then normalized to the GPNMB and CDK4 IFNγ secretion values to estimate the percentages of TILs against each antigen according to the percentages of GPNMB and CDK4 from the tetramer staining. Most of the TAAs appear to have a low activation state (per cell) as shown in **Figure 3E**. We divided the IFNγ secretion values to group TILs at the same level of magnitude and estimated the percentage of T cells.

**Sequencing of the mutations from cDNA**

RNA was purified from cultured cells using RNAeasy mini kit (Qiagen), and cDNA was prepared using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). The regions containing the mutation sites were amplified by PCR using KAPA HiFi HotStart ReadyMix PCR Kit (KAPAbiosystems). The PCR primer pairs used were: MED15 (5′-GGTGTCCCCTGAAGACCTTG-3’) and (5′-CTGGGCCCAGGTGTTGAG-3’); TPD52L2 (5′-GCCGGCCAAGATATCAACCT-3’) and (5′-GATCCGACAGGGGCTTGTC-3’); CULB4 (5′-ATCAGACCACCCAGAAGTCA-3’) and (5′-AATGCTTCTTTCATGGCATTG-3’); OLFML3 (5′-ATCCAGTGCTCCTTTGATGC-3’) and (5′-CCTCCTCCTCTTTCTTCCTCA-3’); SNRPA (5′-ACCCGCCCTAACCACACTAT-3’) and (5′-GTGCCTTTCATCTTGGCAAT-3’). PCR was performed using 100 ng cDNA and the following parameters: 1 cycle at 95 °C for 3 minutes; 30 cycles at 98 °C for 20 seconds, annealing temperature of 65 °C for 15 seconds, 72 °C for 60 seconds; and 1 cycle at 72 °C for 1 minute. PCR products were cleaned with QIAquick PCR Purification Kit (Qiagen) and sequenced using a 3730 DNA Analyzer (ABI). Sequencing primers were the same as the PCR primers.