Combined analysis of antigen presentation and T-cell recognition reveals restricted immune responses in melanoma

Shelly Kalaora¹, Yochai Wolf¹, Tali Feferman², Eilon Barnea³, Erez Greenstein², Dan Reshef², Itay Tirosh¹, Alexandre Reuben⁴, Sushant Patkar⁶, Ronen Levy¹, Juliane Quinkhardt⁶, Tana Omokoko⁶, Nouar Qutob¹, Ofra Golani⁷, Jianhua Zhang⁴, Xizeng Mao⁴, Xingzhi Song⁴, Chantale Bernatchez⁸, Cara Haymaker⁸, Marie-Andrée Forget⁶, Caitlin Creasy⁸, Polina Greenberg¹, Brett W. Carter⁹, Zachary A. Cooper⁴, Steven A. Rosenberg¹⁰, Michal Lotem¹¹, Ugur Sahin¹², Guy Shakhar², Eytan Ruppin⁵, Jennifer A. Wargo¹²*, Nir Friedman²*, Arie Admon³*, Yardena Samuels¹**

¹ Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel
² Department of Immunology, Weizmann Institute of Science, Rehovot, Israel
³ Department of Biology, Technion, Haifa, Israel
⁴ Departments of Surgical Oncology and Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
⁵ Cancer Data Science Lab, National Cancer Institute, NIH, MD, USA
⁶ BioNTech Cell & Gene Therapies GmbH, Mainz, Germany
⁷ Department of Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel
⁸ Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
⁹ Department of Diagnostic Radiology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
¹⁰ National Cancer Institute, NIH, MD, USA
¹¹ Sharett Institute of Oncology, Hadassah Medical School, Jerusalem, Israel
¹² TRON – Translational Oncology at the University Medical Center of Johannes Gutenberg University GmbH, Mainz, Germany

* Contributed equally to this work
** Corresponding author: yardena.samuels@weizmann.ac.il

Running title: Antigen recognition is immunologically-restricted

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Corresponding author: Yardena Samuels, Weizmann Institute of Science, Rehovot, 76100, Israel. Phone: +972- 8-934-3631; Fax: +972-8-934-4373; Email: yardena.samuels@weizmann.ac.il

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Abstract
The quest for tumor-associated-antigens (TAAs) and neo-antigens is a major focus of cancer immunotherapy. Here we combine a neo-antigen prediction-pipeline and human-leukocyte-antigen (HLA)-peptidomics to identify TAAs and neo-antigens in 16 tumors derived from seven melanoma patients, and characterize their interactions with their TILs. Our investigation of the antigenic and T-cell landscapes encompassing the TAA and neo-antigen signatures, their immune reactivity, and their corresponding T-cell identities provides the first comprehensive analysis of cancer cell T-cell co-signatures, allowing us to discover remarkable antigenic and TIL similarities between metastases from the same patient. Furthermore, we reveal that two neo-antigen-specific clonotypes killed 90% of autologous melanoma cells, both in vitro and in vivo, showing that a limited set of neo-antigen-specific T-cells may play a central role in melanoma tumor rejection. Our findings indicate that combining HLA-peptidomics with neo-antigen predictions allows robust identification of targetable neo-antigens, which could successfully guide personalized cancer-immunotherapies.

Significance
As neo-antigen targeting is becoming more established as a powerful therapeutic approach, investigating these molecules has taken center stage. Here, we show that a limited set of neo-antigen-specific T-cells mediates tumor rejection, suggesting that identifying just a few antigens and their corresponding T-cell clones could guide personalized immunotherapy.

Introduction
Immunotherapy has become a leading cancer treatment, with therapies such as checkpoint blockade now commonly used against many tumor types, and has proven particularly successful in cutaneous melanoma (1). Melanoma cells present on their human leukocyte antigen class I (HLA-I) complex tumor associated antigens (TAAs), which are tissue-specific antigens that are
overexpressed in cancer cells, as well as unique mutated antigens, termed neo-antigens. Unlike TAAs, which are only differentially expressed, neo-antigens are truly unique to the cancerous tissue, thus increasing the likelihood of their recognition by host immune cells, predominantly tumor infiltrating CD8\(^+\) T-lymphocytes (2,3).

Neo-antigens have been identified in various tumors and have been shown to be promising immunotherapy targets (4,5). Mounting evidence suggests that HLA-restricted recognition of neo-antigens by T-cells contributes to the efficacy of most cancer immunotherapies and provides clues to the extension of immunotherapy to additional cancer types. Furthermore, efforts are underway to develop personalized cancer vaccines based on neo-antigen profiles (6-8). Thus, in-depth characterization of the T-cell antigenic targets is of great importance. To this end, we combine in this study HLA-peptidomics and a novel neo-antigen prediction pipeline with T-cell receptor (TCR) sequencing to comprehensively analyze cancer cell T-cell co-signatures. While HLA-peptidomics directly analyses the peptides bound to the cells’ HLA by liquid chromatography and tandem mass spectrometry (LC–MS/MS) (6,9-13), neo-antigen prediction based solely on computational tools and subsequent functional screening may skew toward an antigenic profile no longer presented by the cancer cells.

Our research pipeline (Figure 1) entailed whole-exome and RNA sequencing of 15 melanoma tumor samples derived from six patients and one melanoma cell line (Supplementary Table 1) in parallel to HLA-peptidome analysis of the HLA-I and HLA-II repertoires on the same tumor cells. Integrating the two datasets revealed the neo-antigens and TAAs present in each patient’s tumor cells. We found our neo-antigen identification approach to be highly complementary to current neo-antigen prediction approaches where peptide-HLA-I binding is derived using artificial neural networks (14,15). In parallel, we isolated tumor-infiltrating lymphocytes (TILs) from each tumor and characterized the T-cell repertoire by TCR sequencing. Neo-antigens and TAAs were then tested for their
ability to activate TILs to specifically kill melanoma cells using in vitro and in vivo imaging. The output was a detailed account of the repertoire of neo-antigens and TAAs together with TCRβ sequences of their specific TILs.

**Results**

**Identifying melanoma germ-line and neo-antigenic peptides**

Our HLA-peptidomics (9,11) profiling of the melanoma HLA-I and HLA-II antigens from 16 tumor samples (tumor clinical information is provided in Supplementary Table 2 (16)) identified 30,496 and 19,932 unique HLA-I-associated and HLA-II-associated peptides, respectively, which were derived from 10,852 and 4,327 different proteins, respectively (Supplementary Tables 3 and 4). Clustering of 8-13 amino acid HLA-I peptides identified from each patient showed, as expected, reduced amino acid complexity at the peptides’ second and ninth anchor residues (Supplementary Figure 1A). The length distribution of the identified peptides was consistent with those expected for class I and II HLA peptides (Supplementary Figure 1B).

Of all the HLA-bound peptides identified using HLA peptidomics, five were neo-antigens and 511 and 641 were unique HLA-I and HLA-II TAAs, respectively. Both HLA-I and HLA-II TAAs were derived from 117 different genes (Supplementary Table 5). Neo-antigen identification accuracy was validated by comparing the endogenous peptide spectra with synthetic peptide spectra (Supplementary Figures 2-4).

**High similarity between HLA-peptidome and TCR sequence data of melanoma lesions derived from the same patient**

While it is well established that RNA expression profiles of metastases derived from the same patient are highly comparable (Supplementary Figure 5), similar studies of the HLA peptide repertoires were not previously conducted. We observed substantial overlap among all peptides presented on the cells of different metastases derived from the same patient, with 25%-80% of peptides
detected in at least two metastases from the same patient (Figure 2A-B and Supplementary Table 6). When comparing TAAs and the total pool of HLA-I (Figure 2C) and HLA-II (Figure 2D) peptides derived from two metastases of patient 92, we discovered that the metastases not only presented many of the same peptides but also that their intensities were comparable (r=0.738 and 0.751 for HLA-I and HLA-II, respectively). This pattern was maintained in all patients and was more significant in HLA-I compared to HLA-II peptides (Supplementary Figure 6).

Homology of HLA peptide signatures was reflected at the TIL levels, as gathered from our TCRβ chain sequencing analysis (Supplementary Tables 7 and 8). TCR repertoires of the bulk TILs isolated from two metastases of the same patient were similar in clonotype composition and frequency (p<0.0001), while TIL repertoires were largely non-overlapping between patients (p<0.01) (Figure 2E). Further, the most abundant TCR clonotypes in a given patient were found at comparable frequencies in both metastases (Figure 2F and Supplementary Figure 7A). Interestingly, the TIL populations differed greatly in their clonality across metastases. The frequencies of the top-1, -10, -50 and -100 most abundant TCRs are plotted in Supplementary Figure 7B and the Gini index for TIL clonality is plotted in Supplementary Figure 7C.

Some TCRs were found to be identical by amino acid sequence in their complementarity-determining region 3 (CDR3) but divergent at the nucleotide level. The two most convergent TCRs in each metastasis were derived from between 2-8 different nucleotide sequences (Figure 2G-H and Supplementary Figure 8), and these sequences in many cases were generated by different V chains.

**TILs exhibit high specificity toward autologous melanoma cells**

We chose to focus on the 12T sample as it presented three neo-antigens, two of which induced T-cell reactivity. An *in vitro* imaging analysis of CMTMR-labeled
12TIL killing of GFP-tagged 12T melanoma cells revealed that autologous TILs, but not non-autologous counterparts (108TILs), could kill corresponding melanoma cells, with a staggering 50% of all T-cell killing occurring within the first hour of co-incubation (Supplementary Figure 9A-B and Supplementary Movies 1-6).

12TILs induced significant and specific tumor cell death in vivo, a finding obtained both with a tumor rejection assay (Supplementary Figure 10A) and with intravital two-photon microscopy in a mouse model (Supplementary Figure 10B and Supplementary Movie 7). Interestingly, 12TILs were enriched near the tumor vasculature (Supplementary Figure 10C and Supplementary Movie 8), had a slower velocity in vitro than non-autologous 108TILs (Supplementary Figure 10D-E and Supplementary Movie 9), and exhibited increased clustering relative to 108TILs (Supplementary Figure 10F), demonstrating their specificity to 12T melanoma cells.

Combining HLA-peptidomic and antigen prediction to identify immunogenic neo-antigens and TAAs

We further tested the reactivity of TILs to peptides identified by HLA-peptidomics by pulsing synthetic peptides onto EBV-transformed B-cells expressing matched HLA alleles and co-culturing these cells with TILs from the same patient. TIL reactivity was detected for three of the five neo-antigens identified by HLA-peptidomics: 55DTIL was reactive against a neo-antigen derived from oxysterol binding protein-like 8 (OSBPL8_{D>N}) (Supplementary Figure 11A). Notably, this neo-antigen, which was identified by HLA-peptidomics, was predicted to be a weak binder according to NetMHCpan (Supplementary Table 9). 12TIL was reactive against neo-antigens derived from mediator complex subunit 15 (MED15_{P>S}) (9) and Condensin-2 complex subunit H2 (NCAPH2_{S>Y}) (Figure 3A and Supplementary Figure 11B-C). Importantly, only the mutated peptides stimulated TIL release of interferon-γ (IFNγ), whereas the wild-type peptides
elicited no response. Two neo-antigens (TPD52L2_{S>L} from 12T and TAS2R43_{S>F} from 86B) did not induce a detectable IFNγ response in the respective TILs.

To identify neo-antigens that may have been missed as they are no longer presented by the tumor or may have been missed by HLA-peptidomics, but which had prior immunogenicity by the autologous TILs, we used NetMHCpan to predict the potential binding affinity of all neo-antigens to the patient-restricted HLA alleles (14,15). We then ranked the peptides based on their RNA expression as previously done (8). We then assessed the reactivity of the 50 most highly expressed predicted mutated peptides, using 25-mer synthetic peptides that included the mutation flanked by 12 amino acids on either side to allow for flexibility in the position of the mutated residue within the final processed and presented peptide. We further tested the reactivity of all possible minimal epitopes (derived from the 25-mers) that could be derived from the mutation to identify the exact neo-antigen sequence that elicited a TIL response (Figure 3B and Supplementary Table 10).

Four different 25-mer predicted peptides were reactive for patient 12TTILs, and each of these contained one-to-three different reactive minimal epitopes (Figure 3B). Minimal epitopes from the same gene that evoked reactivity were found to contain overlapping sequences, suggesting that these may be the core TCR recognition motifs. As seen in Supplementary Figure 12, all the predicted neo-antigen genes were expressed, suggesting that their lack of surface presentation was due to another escape mechanism. Interestingly, minimal epitopes predicted to bind with higher affinity according to NetMHCpan did not elicit T-cell responses (Supplementary Table 11). A similar analysis for patient 51 identified 11 neo-antigens: nine derived from point mutations, one from a frame shift mutation (in HLA-DRA), and one from a deletion (in MRPL44) (Supplementary Figure 13). As we observed that some predicted 25-mer peptides were processed and bound the HLA allotypes expressed by the 12T and 51T tumors, the prediction
algorithms allowed further identification of reactive peptides, emphasizing the complementarity of both systems.

Strikingly, in vitro killing assays involving 12T GFP-tagged melanoma cells revealed that the neo-antigen-enriched TIL population had an enhanced killing ability of their autologous melanoma cells compared to the bulk TIL population or the neo-antigen negative population (Figure 3C). TIL reactivity was also observed against many of the TAAs identified by HLA-peptidomics (Figure 3A and Supplementary Figure 14): 51ATILs, 92BTILs and 55DTILs demonstrated reactivity against 27 of 73, 24 of 33, and 4 of 64 of the TAAs identified from these patient samples, respectively (Supplementary Figure 14). Reactive TAAs were found in several metastases of the same patient, though the extent of peptide presentation (seen as intensity) was not an indicator of reactivity (Supplementary Figure 14). 42BTILs were not reactive against the identified peptides (tested peptides are listed in Supplementary Table 5).

**Neo-antigen-specific T-cell clone reactivity and frequency affects neo-antigen-specific TIL reactivity**

Using tetramers specific to the NCAPH2$_{S>Y}$ and MED15$_{P>S}$ neo-antigens and two reactive TAAs (ALTSTLISV derived from GPNMB and ALTPVVVTL derived from CDK4), we found that the percentage of tetramer-positive cells in 12TILs was 2.5% for MED15$_{P>S}$, 65.5% for NCAPH2$_{S>Y}$, 1% for GPNMB, and 0.3% for CDK4 (Figure 3D). To directly estimate the reactivity of neo-antigen-specific T-cells, we incubated bulk 12TILs with the autologous melanoma cells and stained them with an anti-4-1BB antibody (a marker for T-cell activation) and with the neo-antigen tetramers, and analyzed activated T-cell populations by flow cytometry. As seen in Figure 3E, 4-1BB expression on NCAPH2$_{S>Y}$-reactive T-cells (MFI=9,782, red) and MED15$_{P>S}$-reactive T-cells (MFI=8,824, blue) was higher than the intensity measured for cells reactive to the remainder of antigens presented by the melanoma cells (tetramer-negative, MFI=5,086, green). Thus, the high TIL response toward the mutant NCAPH2$_{S>Y}$ antigen (Figure 3A) was due to both a
high frequency of NCAPH2<sub>S</sub>-Y-specific T-cells and a high level of activation of each neo-antigen-specific T-cell. The TIL fraction reactive against the melanoma cells was 81% (Figure 3E).

Our assessment of the abundance of each clone targeting the two identified neo-antigens and two TAAs, their IFN<sub>γ</sub> secretion values, and the finding that neo-antigen-reactive clones are more reactive than those targeting TAAs allowed us to roughly estimate that reactive T-cells against all neo-antigens together accounted for approximately 64.6% of the TILs, those reactive to TAAs accounted for approximately 16.4% of reactive TILs, and the non-reactive T-cells accounted for approximately 6.4% of the TILs. Together, our complementary analyses characterized approximately 87.4% of the TIL composition (Supplementary Table 12). The remainder of the TIL population are likely T-cells targeting identified non-reactive antigens or other antigens no longer presented by the tumor.

We next evaluated the reactivity of autologous TILs in vivo. Mice were inoculated with 12T melanoma cells and an equimolar mixture of 12TILs and 108TILs, where 12TILs were stained with cell track violet to track their proliferation in vivo. Twenty four hours after TIL injection, mouse tumors were processed and analyzed by flow cytometry. TIL proliferation was assessed by cell track violet dilution, and their antigen specificity was assessed using tetramers of the two neo-antigens identified in sample 12T. The NCAPH2<sub>S</sub>-Y-specific and MED15<sub>P</sub>-S-specific populations proliferated extensively compared to the tetramer-negative population (Figure 3F), demonstrating that the neo-antigen-specific TILs were the most reactive TIL populations. Our in vitro data show a consistent increase in the reactivity of TILs specific to NCAPH2<sub>S</sub>-Y and MED15<sub>P</sub>-S compared to TILs reactive to other antigens. Our in vivo studies thus demonstrate that neo-antigen-specific TILs exhibit a higher level of proliferation compared to both non-autologous and non-neo-antigen-specific TILs.
TCR sequencing signatures recapitulate the neo-antigenic signatures in TILs and identify neo-antigen-specific TCR sequences

To identify the TCR sequences involved in neo-antigen-specific TIL reactivity, we used tetramers to isolate MED15$_{P>S}$- and NCAPH2$_{S>Y}$-binding T-cells and T-cells unstained by the tetramers. All three populations were sequenced to identify their TCR$\beta$-chains. We also sequenced TCRs of reactive and non-reactive TILs by coculturing bulk TILs with autologous melanoma cells and staining for the activation marker 4-1BB. Eleven TCRs (defined by the amino acid sequences of the CDR3 region of TCR$\beta$) dominated across the different samples: ten were found in the bulk TILs (Supplementary Table 13) and one in sample NCAPH2 (Figure 4A-B). These 11 TCRs comprised 90.75-99.94% of the productive sequences in all sorted samples, and their frequencies ranged from 0.01% to 58.4% in the bulk sample (Figure 4B). We identified two immunodominant NCAPH2$_{S>Y}$-specific T-cell clonotypes, which accounted for 86.6% (clone 1) and 12% (clone 11) of TILs isolated with the NCAPH2$_{S>Y}$ tetramer. The same TCR$\beta$ sequences were enriched in the 4-1BB-positive population compared to the 4-1BB negative population (Supplementary Figure 15). We further identified two T-cell clones for the MED15$_{P>S}$ antigen (clones 3 and 4, Figure 4B). As in the case of NCAPH2$_{S>Y}$, MED15$_{P>S}$ clones were enriched in the 4-1BB-positive population (Supplementary Figure 15).

Similar to the case of 12T, we identified a reactive TIL clone against the OSBPL8$_{D>N}$ neo-antigen identified in tumor 55C, which accounted for 76.11% of 55D TILs, and was the most abundant clone in the 4-1BB positive sample. The TCR sequence of the OSBPL8$_{D>N}$ clone was the third most frequent clone in the 55A tumor (1.48% of TIL population) and the thirty-fourth most frequent in the 55B tumor (0.2% of TIL population), suggesting that this T-cell clone may have played a role in targeting each of the three metastases of this patient to different extents (Supplementary Figure 16B and Supplementary Table 8).

Discussion
To the best of our knowledge, this is the first report that establishes the antigenic and T-cell landscapes encompassing both the TAAs and neo-antigen signatures of metastases derived from the same patient. The significant similarity of the HLA-peptidomes between metastases is reflected in the respective TCR signatures. This has strong implications for the process of choosing peptides and TCRs for patient treatment, as it points out that it is clearly essential not only to identify the presented immunogenic peptides, but those of them that are actually common among the patient metastases in order to mediate systemic therapeutic responses in patients with multiple synchronous metastases.

Our study demonstrates that HLA-peptidomics, which directly analyzes the peptides bound to the cells’ HLA, addresses the need to query neo-antigens and TAAs at high levels of accuracy and efficiency. Although neo-antigen prediction algorithms could potentially identify all the neo-antigens identified by HLA-peptidomics, predictions give hundreds, if not thousands, of potential binders without any certainty that any of them are actually presented via the HLA. Researchers must then synthesize and experimentally evaluate potentially thousands of negative peptides to identify tens of positive peptides. Therefore, the advantage of integrating bioinformatic predictions with HLA-peptidomics is the elimination of neo-antigens that may be false positives identified by bioinformatics. HLA-peptidomics, despite its lower sensitivity compared to T-cell-based detection assays, further reduces the number of peptides that need to be validated (Supplementary Figure 17).

The robustness of our neo-antigen and corresponding T-cell clone identification strategies is emphasized by the fact that our validated neo-antigen-specific T-cells kill 90% of their target melanoma cells in vitro and in vivo. We found that T-cells against our identified neo-antigens comprise the majority of T-cells within the tumor, both in frequency and in reactivity. Most strikingly, our in-depth analysis of the 12T sample detected a mirror image between the magnitude of reactivity of the combined neo-antigens and TAAs to the T-cell profile, thus...
accounting for the majority (~90%) of TIL reactivity. The remaining 10% of TILs reactivity may have been in response to as of yet uncharacterized antigens.

Most importantly, the combination of these approaches supports the existence of fewer than expected neo-antigens that elicit the response of a restricted set of identifiable neo-antigen-specific TILs. Our findings have clear implications for the future development of TIL therapy. Though the low number of identified and validated neo-antigens might be unexpected, the responses to these neo-antigens were extremely robust, emphasizing that combining strategies for neo-antigen identification, such as HLA-peptidomics, with approaches that can identify T-cell clones that react to these neo-antigens, as we demonstrate here, could significantly increase the efficacy of TIL therapy. Our finding that only a few neo-antigen-specific TILs mediate tumor rejection corroborates prior studies that have identified and clinically validated only five or fewer reactive neo-antigens per tumor, despite the tumors harboring hundreds or thousands of somatic mutations (5,17-22). This highlights that although only a few neo-antigens and corresponding T-cell clones are present in a tumor, targeting them may be sufficient for patient treatment.

The insight gathered through this analysis strengthens the notion that identification of a few targetable antigens and their corresponding TIL clones could guide personalized cancer immunotherapy. Enriching the infused T-cells used for TIL therapy for the combined, yet scant, number of reactive neo-antigens and TAAs, using MHC multimer enrichment (23), RNA vaccines (7,8,24) or other expansion protocols to select T-cell populations reactive against patient-specific antigens, will greatly personalize this approach (5,25) and potentially improve the effectiveness of the cancer therapy.

**Methods**

**Patients and cells**
All patients included in the analysis were diagnosed with metastatic melanoma. Tumor samples were received from six different patients that were treated at the University of Texas MD Anderson Cancer Center who had signed an informed consent for the collection and analysis of their tumor samples. 12T cells and TILs were collected in 2006 and established as described previously (21), with informed patient consent under a protocol approved by the NIH IRB Ethics Committee. The protocol for 6 tumor samples (from patients 42, 51, 55, 60, 86 and 92) was approved by the MD Anderson Institutional Review Board (protocol numbers 2012-0846, LAB00-063 and 2004-0069, NCT00338377). Board (protocol numbers 2012-0846, LAB00-063 and 2004-0069, NCT00338377). Synchronous metastatic tumors were resected via surgery at the same time. The metastases used for the study are described in Supplementary Table 2. An H&E was generated from OCT-embedded sections and samples were analyzed for presence of tumor by a pathologist. Tumor-positive samples were used for subsequent sequencing. The studies were performed after approval by an institutional review and board (IRB); the investigators obtained informed written consent from the subjects. All cells have been authenticated by sequencing and were tested routinely for Mycoplasma using Mycoplasma EZ-PCR test kit (#20-700-20, Biological Industries, Kibbutz Beit Ha’emek).

Production and purification of membrane HLA molecules

The tumor sample amounts ranged from 0.1 to 0.8 grams, and cell-line pellets were collected from $2 \times 10^8$ cells. Samples were processed as described previously (9). Full details are provided in the supplemental methods.

Prediction of neo-antigens

The NetMHCpan (14,15) algorithm version 3.0 was used to predict the possible neo-antigens from the WES data (Supplementary Tables 1 and 14). The residues surrounding the amino acids resulting from nonsynonymous mutations were scanned to identify candidate 8-14-mer peptides that were predicted to bind with high affinity (strong binders, %Rank $\leq 0.5$) or low affinity (weak binders, 0.5
≤ %Rank ≤ 2) to the cells’ HLA-I alleles. For samples 12T and 51A, we sorted the peptides according to the RNA expression level of the genes and selected the most highly expressed genes for peptide screens (RNA sequencing data is found in Supplementary Table 15 and selected genes are described in Supplementary Table 10). Crude synthetic 25-mer peptides with the mutation in the middle were used to screen point mutations, and overlapping 25-mers (12 amino acids overlap) were used to screen frame shifts. All possible peptides that were predicted to derive from the 25-mer peptides that showed reactivity by the IFN-γ release assay were tested to identify the reactive minimal epitopes (Supplementary Table 10).

**In vivo mice assays**

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).

Additional details regarding patients and cells, production and purification of membrane HLA molecules, identification of eluted HLA peptides, identification of TAAs, analysis of T-cell reactivity by IFN-γ release assay, flow cytometry analysis, TCR sequencing, fluorescence-based *in vitro* killing assay, *in vitro* live cell imaging, *in vivo* rejection assay, live two-photon microscopy of melanoma tumors, calculation of neo-antigen and TAA frequencies in TILs and sequencing of mutations from cDNA are provided in the Supplementary Methods.

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Author Contributions

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Figure 1: Tumor antigen discovery pipeline. Whole-exome sequencing (WES) of 15 melanoma tumor samples derived from six patients and one melanoma cell line was performed in parallel to HLA-peptideome analysis of the cells' HLA-I and HLA-II repertoires. Integrating the WES data with the human proteome database in the mass spectrometry analysis allowed us to reveal the neo-antigens and TAAs presented by the patients' tumor cells. In parallel we applied a neo-antigen prediction pipeline followed by a long peptide screen (NT, not tested). TILs isolated from the tumor were sequenced to identify their TCR sequences and reveal any high similarity between the different metastases from the same patient. Neo-antigens and TAAs were tested for their reactivity to TILs and the ability of TILs to target the melanoma cells was characterized using in vitro and in vivo imaging. Neo-antigen-specific clones were isolated using tetramers and sequenced to identify their TCR sequence and TIL reactivity was also derived from their prevalence in the bulk TILs.

Figure 2: High similarity in the HLA peptides and TCR repertoires of metastases from the same patient. (A-B) Similarity in the heat maps of HLA-I (A) and HLA-II (B) peptides from the different tumor metastases. Color code indicates the Jaccard index. The highest similarity was observed between metastases from the same patient and between patients with shared HLA alleles. (C-D) The similarity between the presented peptides is observed not only in terms of their identity but also in terms of their intensity. The log2 of the peptides intensities were plotted for HLA-I (C) and HLA-II (D) peptides. Unique peptides for each sample were given a constant value of 15. Peptides derived from TAAs are marked in red. Pearson correlations are indicated in red. (E) Similarity heat maps of the TCR amino acid sequences identified in the various tumor metastases. Color code indicates the Jaccard index. (F) The frequencies of the various TCRs in the different metastases from the same patient. Since at lower frequencies we found many different TCRs with same frequency in both metastases, we color coded the number of TCRs represented by each dot. Pearson correlation is indicated in red. (G-H) The frequency of the nucleotide sequences of the two most convergent amino acids TCRß sequences. In patient 51 (G) and 92 (H), one of the most convergent sequences was detected as most
convergent in both metastases. Each nucleotide sequence is represented by layer, with the overlapping sequences presented in the same color in both metastases.

**Figure 3: Neo-antigen specific T-cells show more reactivity, killing ability and proliferation in vitro and in vivo.** (A) IFN-γ release measured after overnight co-culture of the TILs with EBV transformed B-cells that were pulsed with 10μM of the peptides that were identified in the HLA peptidomics analysis. (B) IFN-γ release measured after overnight co-culture of the TILs with EBV transformed B-cells that were pulsed with 10μM of the 25mer peptides. For the reactive 25mers we also checked the reactivity of each minimal epitope that was predicted to bind the patients’ HLA alleles. (C) A fluorescence-based in vitro assay comparing the killing of 12T melanoma cells by autologous bulk 12TILs, enriched neo-antigen TILs population or the rest of the non-neo-antigenic TILs population with an increasing effector:target (E:T) ratio. (D) Bulk TIL were stained with NCAPH2, MED15, GPNMB and CDK4 tetramers to evaluate the percentage of the different populations is the bulk TIL. (E) 12T melanoma cells were co-cultured with 12TILs for 24 hours, and later were stained with anti-4-1BB antibody and the two tetramers against the neo-antigens. The percentage of reactive and non-reactive T-cells for each neo-antigen, neo-antigen-negative and bulk TIL are indicated. The MFI of the 4-1BB staining was calculated for the reactive T-cells in each population. (F) Flow cytometry analysis of the different antigen populations in the 12TILs before and after injection to NSG mice with 12T melanoma cells tumor. The first panel shows the percentage of the 12TILs and irrelevant 108TILs in the T-cells mixture. The last three panels are gated only to the violet positive cells (only 12TILs) and show the percentage of T-cells in each neo-antigen or tetramer-negative population that proliferate. The images are representative for three replicates.

**Figure 4: Ten most frequent TCRs in the bulk 12TILs comprise >99% of the TIL population, and the neo-antigens are shown to be the most dominant clones**. Bulk TILs and five different populations isolated from two different sorting experiments were analyzed using TCR sequencing to identify neo-antigen-specific clones and reactive and non-reactive T-cell populations. (A) Frequency of the top-10 abundant amino acid sequences found in the bulk TILs across the different samples and the second most abundant sequence from NCAPH2. As shown in the bars, these eleven TCRs comprise 90.75-99.94% of
the productive rearrangements in all the samples. (B) The table indicates the eleven TCR sequences and their frequencies in the different samples. Clones 1 and 11 are against NCAPH2 and clones 3 and 4 are against MED15. Clone 2 is found in the tetramer negative population and mostly negative to 4-1BB, therefore probably represent a clone that expended in the tumor but its antigen was down-presented in the tumor cells as part of immune-editing process.
Figure 1
Figure 2

A - HLA class I
B - HLA class II
C - Intensity 92B class I vs 92A class I
D - Intensity 92A class II vs 92A class II
E - Frequency in 92B vs 92A
F - Frequency in 92B vs 92A
G - Frequency in 51A
H - Frequency in 92A vs 92B

Figure 2
**Image 1**

Cytotoxic T cell activity against tetrameric complexes of Neo-antigens. (A) Various Neo-antigens were tested with TILs from patients with different cancer types.

B) Minimal epitopes reactive with 25mer reactivity.

**Image 2**

Expression of IFN-γ in TILs infected with Neo-antigens. (B) IFN-γ expression was assessed in TILs infected with different Neo-antigens.

**Image 3**

**Figure 3**

Quantitative analysis of IFN-γ expression in TILs infected with Neo-antigens. (C) The expression of IFN-γ was quantified using flow cytometry.

**Image 4**

**Figure 4**

Expression of tetramers in TILs infected with Neo-antigens. (D) Tetramer expression was evaluated in TILs infected with different Neo-antigens.

**Image 5**

Pre- and post-injection analysis of Neo-antigens. (E) The expression of Neo-antigens was compared before and after injection in TILs.

**Image 6**

**Figure 5**

Expression of Neo-antigens in TILs. (F) The expression of Neo-antigens was quantified in TILs using flow cytometry.

---

*Authors:*

[Names of authors]

*Institution:*

[Institution name]

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*Supplementary information:*

[Additional information or references]

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[Acknowledgments if applicable]
### Figure 4

**A**

![Bar chart showing amino acid sequences and their distributions across different clones.](image)

**B**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Amino acid sequence</th>
<th>Bulk</th>
<th>NCAPH2</th>
<th>MED15</th>
<th>Tertramer negative</th>
<th>4-1BB positive</th>
<th>4-1BB negative</th>
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Shelly Kalaora, Yochai Wolf, Tali Feferman, et al.

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