Published in final edited form as:

Clin Cancer Res. 2017 May 15; 23(10): 2491–2505. doi:10.1158/1078-0432.CCR-16-2680.

Isolation of T-Cell Receptors Specifically Reactive with Mutated Tumor-Associated Antigens from Tumor-Infiltrating Lymphocytes Based on CD137 Expression

Maria Parkhurst, Alena Gros, Anna Pasetto, Todd Prickett, Jessica S. Crystal, Paul Robbins, and Steven A. Rosenberg

NIH/NCI Surgery Branch, Bethesda, Maryland.

Abstract

Purpose: The adoptive transfer of lymphocytes genetically modified to express tumor reactive T-cell receptors (TCR) can mediate tumor regression. Some tumor-infiltrating lymphocytes (TIL) recognize somatic mutations expressed only in the patient's tumors, and evidence suggests that clinically effective TILs target tumor-specific neoantigens. Here we attempted to isolate neoantigen-reactive TCRs as a prelude to the treatment of patients with autologous T cells genetically modified to express such TCRs.

Experimental Design: Mutations expressed by tumors were identified using whole-exome and RNA sequencing. Tandem minigene (TMG) constructs encoding 12–24 mutated gene products were synthesized, each encoding the mutated amino acid flanked by 12 amino acids of the normal protein sequence. TILs were cultured with autologous dendritic cells (DC) transfected with *in vitro* transcribed (IVT) mRNAs encoding TMGs and were evaluated for IFN γ secretion and CD137 expression. Neoantigen-reactive T cells were enriched from TILs by sorting for CD137⁺ CD8⁺ T cells and expanded *in vitro*. Dominant TCR α and β chains were identified in the enriched populations using a combination of 5' rapid amplification of cDNA ends, deep sequencing of genomic DNA, PairSeq analysis, and single-cell RT-PCR analysis. Human PBL retrovirally transduced to express the TCRs were evaluated for recognition of relevant neoantigens.

Results: We identified 27 TCRs from 6 patients that recognized 14 neoantigens expressed by autologous tumor cells.

Corresponding Author: Maria Parkhurst, NIH/NCI Surgery Branch, Bldg. CRC, Room 4-5744, 9000 Rockville Pike, Bethesda, MD 20892. Phone: 301-435-3026; Fax: 301-496-0011; Maria_Parkhurst@nih.gov.

Authors' Contributions

Conception and design: M. Parkhurst, A. Gros, S. Rosenberg

Development of methodology: M. Parkhurst, A. Gros

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Parkhurst, J. Crystal, A. Gros, A. Pasetto, T. Prickett, P. Robbins

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Parkhurst, J. Crystal, A. Gros, P. Robbins, S. Rosenberg

Writing, review, and/or revision of the manuscript: M. Parkhurst, J. Crystal, T. Prickett, P. Robbins, S. Rosenberg

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Crystal, S. Rosenberg

Study supervision: J. Crystal, S. Rosenberg

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Conclusions: This strategy provides the means to generate T cells expressing neoantigen-reactive TCRs for use in future adoptive cell transfer immunotherapy trials for patients with cancer.

Introduction

The primary goal of cancer therapy is to eliminate tumor cells without inducing toxicities in normal tissues. The adoptive transfer of normal peripheral blood lymphocytes (PBL) genetically modified by the insertion of tumor-reactive T-cell receptors (TCR) or chimeric antigen receptors (CAR) can mediate tumor regression in multiple histologies (1–7). However choosing a tumor-specific antigenic target is critical because adoptively transferred T cells reactive with epitopes presented on normal tissues, even at very low levels, can induce severe toxicities (5, 8, 9). As cancer cells contain unique somatic genetic mutations that are not present in normal tissues, it seems likely that therapies targeting such mutations might be clinically beneficial while eliminating toxicities associated with normal tissue expression. The adoptive transfer of tumor-infiltrating lymphocytes (TIL) can mediate regression of metastatic melanoma, and accumulating evidence suggests that clinically effective therapeutic TILs target tumor-specific mutations (10–12). In addition, adoptively transferred, neoantigen-reactive T cells mediated an objective partial clinical response in a patient with metastatic cholangiocarcinoma that is ongoing more than 2 years following treatment (13). To develop personalized, patient-specific gene therapy reagents, we attempted to isolate mutation-reactive TCRs that we could genetically introduce into autologous PBL.

CD137 (41BB) is a member of the TNFR family (14, 15) that functions as a costimulatory molecule to promote proliferation and survival of activated T cells (16–19). Expression of CD137 on T cells is transient and is limited to T cells that have recently been activated by TCR engagement and signaling (20). Upregulation of CD137 on recently activated T cells has been used to identify and isolate virus- and tumor-reactive T cells from peripheral blood and TILs (20–25). Here, we attempted to use CD137 upregulation on in vitro–stimulated TILs to isolate mutation-reactive T cells and subsequently isolate TCRs that mediated recognition of neoepitopes. In particular, we first screened TILs for the presence of T cells reactive with mutations identified by whole exome and RNA sequencing of the autologous patient's tumors as described previously (11, 12). We then attempted to isolate the mutation reactive T cells by stimulating them with autologous antigen-presenting cells transfected with RNA encoding the mutations and subsequently FACS sorting CD8⁺ CD137⁺ T cells. After reevaluating the reactivity of the expanded T cells, the dominant TCR α and β chains were isolated from the enriched populations and used to generate recombinant retroviruses. When these TCRs were introduced into open-repertoire PBL, many of them mediated recognition of the relevant neoepitopes. This strategy provides the means to generate tumor-reactive T cells for use in future adoptive immunotherapies.

Materials and Methods

Patients

Tumor biopsies and leukapheresis products were obtained from individuals with stage IV melanoma enrolled on a clinical protocol (03-C-0277) approved by the institutional review board (IRB) of the National Cancer Institute (NCI). All subjects had progressive disease when their samples were collected. The 7 patients evaluated in these studies (Patients 3466, 3713, 3784, 3903, 3678, 3716, and 3926) were either treatment-naïve, or had undergone prior therapies including surgery, chemotherapy, and immunotherapy (IL2, IFN α , or adoptive transfer of TCR-transduced cells). Cells from leukaphereses were prepared over a Ficoll-Hypaque gradient (LSM; ICN Biomedicals Inc.) and cryopreserved until further use. For two of the patients from whom we prospectively screened TIL for mutation-reactive T cells, we were able to establish melanoma cell lines *in vitro* (3784 and 3903). Melanoma cell lines were established from enzymatically separated tumor cells cultured in RPMI1640 medium supplemented with 10% FBS (HyClone Defined) at 37°C and 5% CO₂. Melanoma cell lines were mycoplasma negative and were authenticated on the basis of the identification of patient-specific somatic mutations and HLA molecules. For the other two patients (3716 and 3678), we were unable to establish cultured cell lines.

Whole-exome sequencing and RNA sequencing

Genomic DNA purification, library construction, exome capture of approximately 20,000 coding genes, and next-generation sequencing of fresh tumors (FrTu), early-passage cell lines, and matched normal pheresis samples were performed at Personal Genome Diagnostics as described previously (26) or in the Surgery Branch as described previously (27). RNA-seq libraries were also prepared from some tumor samples as described previously (28). Whole-exome sequencing and RNA-seq analysis of FrTu or early-passage tumor cell lines were used to identify the number of putative nonsynonymous somatic variants using previously described filters (27).

Antibodies and phenotypic characterization

The following anti-human antibodies were used for cell surface staining: CD3-AF700 (clone: UCHT1), CD4-APC-Cy7 (clone: SK3), CD8-PE-Cy7 (clone: SK1), CD137-APC (clone: 4B4-1). Antibodies were from purchased from BioLegend, Miltenyi Biotec, and BD Biosciences. Anti-PD-1 antibody was kindly provided by Linda Liu from Amplimmune (AMP-514, 1/300, PD-1 Alexa Fluor 647). Fluorochrome conjugated anti-mouse TCR β constant region antibodies (clone: H57-597, eBioscience) were used to assess TCR transduction efficiencies.

Isolation of TIL populations

Tumor-infiltrating lymphocytes (TIL) were isolated using two different methods. TILs from tumor biopsy fragments were generated as described previously (29). Alternatively, CD3⁺ CD8⁺ PD1⁺ T cells from FrTu digests were isolated and expanded as described previously (25).

Generation of autologous dendritic cells

Immature dendritic cells were generated from leukaphereses by *in vitro* differentiation of monocytes using IL4 and GM-CSF using slight modifications of a previously described method (13). Briefly, cells were thawed, resuspended in AIMV (Gibco) at a density of approximately 1×10^6 cells/cm², and incubated for 90 minutes at 37°C and 5% CO₂. Nonadherent cells were then depleted, and the remaining adherent cells were incubated with DC medium (RPMI1640, 5% human serum, 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mmol/L L-glutamine, 800 IU/mL GM-CSF, and 200 U/ml IL4). Alternatively, monocytes were isolated from leukaphereses products using anti-CD14-coated magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. CD14⁺ cells were incubated in DC media containing GM-CSF and IL4 as described above. DCs were harvested between days 4 and 7 for use in experiments.

Construction of tandem minigene constructs and *in vitro* transcription of TMG RNA

Tandem minigenes (TMG) encoding tumor-associated mutations were constructed as described previously (12, 13). Briefly, a minigene was constructed for each nonsynonymous variant identified, consisting of the mutated amino acid flanked by 12 amino acids of the wild-type protein sequence. In the case of frame-shift insertions or deletions, the frame-shifted amino acid sequence was used until the first stop codon. Twelve to 24 minigenes were strung together to generate a TMG construct. These TMG constructs were codon optimized and cloned in frame into pcRNA2SL. Linearized DNA was used for the *in vitro* transcription (IVT) of RNA using the mMessage mMachine T7 Ultra kit (Life Technologies). The full-length amino acid sequences of cancer germline antigens NY-ESO-1, MAGEA3, SSX2, and melanoma antigens gp100 and MART-1 were cloned individually into pcRNA2SL, and these constructs were used to generate IVT RNA as described above.

Transfection of DCs with IVT RNA

DCs were transfected with IVT RNA via electroporation as described previously (13). Briefly, DCs were resuspended in Opti-MEM media (Life Technologies) at $1-4 \times 10^7$ cells/mL. Two to 8 µg of IVT RNA were mixed with 50–100 µL of DCs and were electroporated with 150 V, 10 ms, and 1 pulse, using a BTX-830 square wave electroporator (Harvard Apparatus) in a 2-mm gap cuvette. Electroporated DCs were rested overnight prior to coculture.

Peptide prediction and pulsing

Candidate 8–11mers containing mutated residues that were predicted to bind to the patients' HLA-I molecules were identified using the immune epitope database (IEDB; www.iedb.org). The MHC-binding predictions were made using the IEDB analysis resource Consensus tool (30) which combines predictions from ANN also known as NetMHC (31, 32), SMM (33), and Comlib (34). Crude and HPLC-purified peptides were synthesized by GenScript or BioSynthesis.

For experiments requiring peptide pulsing, DCs were resuspended in DC media at approximately 1×10^6 cells/mL. DCs were incubated overnight at 37°C and 5% CO₂ with wild-

type or mutated 25 mers at a concentration of 10 µg/mL. Alternatively, DCs were pulsed with 1 µg/mL or with 10-fold serial dilutions starting at 10 µg/mL of minimal epitopes for approximately 1.5 hours at 37°C and 5% CO₂. Peptide-pulsed DCs were centrifuged and resuspended in 50/50 media (50% AIMV, 50% RPMI, 5% inhouse human serum) prior to cocubation with T cells in coculture assays.

Initial screening of TILs for recognition of mutated antigens

Both IFN γ enzyme-linked immunospot (ELISPOT) assay and CD137 upregulation at 20–24 hours were used to measure target cell recognition by TIL populations as described previously (35). Approximately 2e4 T cells were cocultured with approximately 3–7e4–transfected DCs in 50/50 media without exogenously added cytokines. For ELISPOT assays, raw data were plotted without subtracting background, and recognition was considered positive if more than 60 spots were observed and the number of spots exceeded twice background. Prior to processing ELISPOT assays, cells were harvested for flow cytometry detection of CD137 expression. Cells were stained with anti-CD3, anti-CD8, and anti-CD137 at 4°C, and flow cytometry acquisition was performed on Canto I or Canto II flow cytometers (BD Biosciences). Data were analyzed using FlowJo software (Treestar Inc) after gating on live cells (PI negative), single cells.

CD137⁺ T-cell sorting and in vitro expansion

For TIL populations containing mutation-reactive T cells, we attempted to isolate those T cells by FACS sorting CD8⁺ CD137⁺ cells after stimulation with autologous DCs electroporated with relevant TMG RNAs. Approximately 1–5e6 TILs were cocubated overnight with approximately 1e6 electroporated DCs in 24-well plate wells (2 mL/well). The cocultures were then stained with anti-CD3, anti-CD8, and anti-CD137 for at 4°C, and cells were washed once prior to acquisition. Live (PI-negative) CD3⁺ CD8⁺ cells were sorted using either a BD FACS Aria or BD FACS Jazz. Sorted T cells were expanded using excess irradiated (4,000 rad) allogeneic feeder cells (pool of three different donor leukapheresis samples) in 50/50 media containing 30 ng/mL anti-CD3 (OKT3) and 3,000 IU/mL IL2. Cells were typically reevaluated for recognition of relevant TMGs and peptides 2 to 3 weeks after the initial stimulation using IFN γ ELISPOT and CD137 upregulation assays as described above.

T-cell receptor sequencing and analysis

TCRs present in enriched TIL populations were identified using one or a combination of 4 different methods: 5' rapid amplification of cDNA ends (5' RACE), deep sequencing of genomic DNA, PairSeq analysis of cDNA from FrTu digests, and single-cell RT-PCR. 5' RACE was performed as described previously using degenerate constant region primers (35), and TCR PCR products were sequenced (Macrogen). TCR α and TCR β deep sequencing were carried out from genomic DNA by Adaptive Biotechnologies. Only productive TCR rearrangements were used in the calculations of TCR frequencies. PairSeq analysis of cDNA from FrTu digests was done by Adaptive Biotechnologies as described previously (36, 37). For one patient, 3678, the PairSeq analysis was not robust, and therefore, we used a single-cell RT-PCR strategy to identify productive TCR α/β pairs as described previously (37–39). Briefly, single-cell sorting for CD8⁺ cells from one of the

CD137-enriched populations from patient 3678 was performed using a modified FACSARIA instrument (BD Biosciences). TCR sequences from the sorted single cells were obtained by a series of two nested PCR reactions. PCR products were purified and sequenced by Sanger method with internally nested Ca and Cb region primers by Beckman Coulter.

Retroviral vector construction and transduction of T cells

Construction of retroviral vectors encoding TCRs identified using the methods described above was done as described previously (35). Briefly, TCR α V-J regions were linked to the mouse TCR α -constant chain, and TCR β -V-D-J regions were linked to the mouse TCR β constant (CB2) chain. Use of the mouse TCR constant regions promotes pairing of the introduced TCR (40) and also facilitates identification of positively transduced T cells by flow cytometry using an antibody specific for the mouse TCR β -constant chain (eBioscience). For TCRs from patients 3784, 3903, 3678, and 3716, the mouse-constant regions were also modified to introduce additional disulfide bonds (41), and to enhance the expression of the TCR α chain as described previously (42). The full-length TCR α and TCR β chains were cloned into pMSGV1 retroviral vectors separated by a furin SGSG P2A linker (GeneOracle or GeneScript).

Transient retroviral supernatants were generated by cotransfecting the retroviral construct encoding the TCR of interest and the envelope protein encoding plasmid RD114 into 293 GP cells (ATCC) using Lipofectamine 2000 (Invitrogen). After 48 hours, viral supernatants were harvested and diluted 1:1 with DMEM supplemented with 10% FBS. The supernatants were centrifuged at $2,000 \times g$ for 2 hours at 32°C onto nontissue culture-treated plates previously precoated overnight with $10 \mu\text{g/mL}$ of retronectin (Takara). Activated PBMCs (incubated for 48 hours in 50/50 media supplemented with 300 IU/mL IL2 and 50 ng/mL OKT3) were centrifuged onto the virally coated plates for 10 minutes at $300 \times g$. Transduced T cells were used 2–3 weeks after the initial stimulation or were cryopreserved for later use. GFP and mock-transduced T cells were used as controls in all transduction experiments.

Evaluation of mutated antigen recognition by transduced T cells

Recognition of autologous or HLA-matched DCs electroporated with relevant TMG RNAs or pulsed with relevant peptides by TCR-transduced PBL was evaluated by IFN γ secretion in coculture supernatants. When available, we also evaluated recognition of autologous melanoma cell lines, and in some cases, these cell lines were treated with IFN γ (10 ng/mL ; PeproTech) for 24 hours prior to coculture. Briefly, responder T cells ($\sim 1\text{e}5$) were coincubated with stimulator cells ($0.5\text{--}1\text{e}5$; $200 \mu\text{L}$ per 96-well plate well) approximately 20 hours at 37°C and 5% CO_2 , and the concentration of human IFN γ in coculture supernatants was measured by ELISA using commercially available reagents (Thermo Scientific). In many previous investigations, we and others have demonstrated that PBL genetically modified to express tumor antigen-reactive TCRs by means of retroviral transduction consistently secrete IFN γ , lyse relevant target cells, and bind tetramer *in vitro* (43–45). Although we evaluated recognition of mutated antigens by TIL using both IFN γ ELISPOT and CD137 upregulation, given all the previous studies demonstrating the consistent multifunctionality of TCR-transduced PBL, we believed that measuring reactivity by IFN γ secretion alone was sufficient for identifying mutation-reactive TCRs.

Results

Validation of methods by retrospective analysis of TILs used for treatment

To evaluate the feasibility of isolating mutation reactive TCRs from TILs based on expression of CD137 after stimulation with mutated antigens, we conducted preliminary experiments using TILs from 2 patients with melanoma, 3466 and 3713. These TILs had previously been used to treat patients and had been screened for the presence of mutation-reactive T cells (28). For patient 3466, an HLA-A*0201–restricted mutated epitope in COL18A1 was identified, and for patient 3713, an HLA-A*0201–restricted mutated epitope in SRPX was found (28). Treatment TILs from patient 3466 were stimulated with autologous DCs electroporated with IVT RNA encoding TMG1 which contained the mutated COL18A1 minigene, and approximately 3% of CD8⁺ T cells upregulated expression of CD137 (Fig. 1A). Treatment TILs from patient 3713 were stimulated with autologous DCs electroporated with IVT RNA encoding TMG2 which contained the mutated SRPX minigene, and approximately 2% of CD8⁺ T cells upregulated expression of CD137 (Fig. 1B). For both of these patients, the highest 0.5% of CD3⁺ CD8⁺ CD137⁺ cells were sorted by FACS and expanded *in vitro*. The resulting T-cell populations were reevaluated for TMG recognition by overnight coculture with autologous electroporated DCs. For the enriched T-cell population from patient 3466, approximately 87% of the CD8⁺ T cells upregulated expression of CD137 in response to TMG1 (COL18A1; Fig. 1C), and for the enriched T-cell population from patient 3713, approximately 89% of the CD8⁺ T cells upregulated expression of CD137 in response to TMG2 (SRPX; Fig. 1D).

From these highly enriched populations, we identified dominant TCR α and β chains using 5' RACE. From both of these enriched populations, we identified one dominant β chain and one dominant α chain (Fig. 1E). The TCRs were cloned into MSGV1 retroviral vectors and used to transduce open-repertoire autologous PBL. The resulting T-cell populations were cocultured overnight with relevant target cells, and IFN γ in coculture supernatants was evaluated by ELISA. The TCR from the CD137-enriched TILs from patient 3466 mediated specific recognition of the HLA-A*0201 –restricted mutated COL18A1 peptide (Fig. 1F), and the TCR from the CD137-enriched TILs from patient 3713 mediated specific recognition of the HLA-A*0201–restricted mutated SRPX peptide (Fig. 1G). These results demonstrated it was feasible to isolate mutation-reactive TCRs from TILs that had been enriched for mutation-reactive T cells based on upregulation of CD137.

Prospective screening of TILs from patients for mutation-reactive T cells

TILs that have been used to treat patients with melanoma have usually been derived from multiple biopsy fragments that have been combined and have undergone a round of *in vitro* stimulation with anti-CD3 and IL2. The TCR clonotypic repertoire in these expanded TILs is often different than that in the original tumor specimen. In addition, we recently observed that CD8⁺ PD1⁺ T cells from FrTu digests are highly enriched for the presence of tumor-reactive T cells (25). Therefore, to isolate multiple mutation-reactive TCRs that we might eventually be able to use in individualized gene therapy protocols, we speculated we might want to use earlier TIL cultures from individual tumor biopsy fragments or CD8⁺ PD1⁺ T cells from FrTu digests as a source of tumor-reactive TCRs.

To evaluate the feasibility of isolating mutation-reactive TCRs from TILs without prior knowledge of any immunogenic mutations, we first screened TILs from 5 patients (3784, 3678, 3716, 3903, and 3926) for recognition of mutations identified on the basis of exome sequencing of autologous tumor cells by transducing autologous DCs with tandem minigenes (TMG), concatenated constructs encoding mutated residues with the 12 normal flanking amino acids, as previously described (12). For 3 patients (3784, 3903, and 3926), we also used CD8⁺ PD1⁺ FACS-sorted populations from FrTu digests as TIL sources. As one example, TIL screening results from patient 3784 are presented in Table 1. From 3784 TIL populations, we observed some degree of recognition of TMGs 3, 4, 5, 6, and 8; however, the frequencies of reactive T cells were generally low. We also evaluated recognition of several nonmutated shared antigens and observed specific recognition of the melanoma antigen gp100 in several TIL populations from patient 3784.

From 4 of the 5 patients, 3784, 3678, 3716, and 3903, we observed recognition of multiple TMGs by several TIL sources. Lists of the individual mutated minigenes, TMG constructs, and screening results (other than those presented in Table 1) can be found in Supplementary Tables S1–S9. For patient 3926, we did not identify any mutation-reactive T cells from TIL fragments or FrTu CD8⁺ PD1⁺ T cells.

Isolation of mutation-reactive TCRs from TILs based on CD137 expression after in vitro stimulation, FACS sorting, and expansion

To isolate mutation-reactive T cells from individual TIL fragments and/or FrTu CD8⁺ PD1⁺ T cells from patients 3784, 3678, 3716, and 3903, we FACS sorted CD8⁺ T cells with the highest expression of CD137 after overnight stimulation with immunogenic mutated TMG RNAs as described previously (27). We expanded the sorted populations *in vitro* and reevaluated recognition of mutated TMG RNAs. For populations that we successfully enriched for the presence of TMG-reactive T cells, we attempted to identify the specific mutations being recognized by evaluating recognition of synthetic peptides encoding the mutation. In addition, we attempted to identify minimal epitopes by evaluating recognition of candidate 8–11mers predicted to bind to the patients' HLA class I molecules using the immune epitope database (IEDB; www.iedb.org). A complete list of the predicted minimal epitopes for each patient is presented in Supplementary Table S5. TCR α and β chains in the enriched T-cell populations were identified by genomic DNA deep sequencing of TCR α and β chains (Adaptive Biotechnologies). In addition, for all of these patients, PairSeq analysis of cDNAs derived from T cells from FrTu digests had previously been performed, and when available, these data were used to guide the construction of TCRs (36, 37).

For some T-cell populations, one single dominant α and one single dominant β chain were identified. An example of this is presented in Fig. 2. In our original screening assay, we identified a small population of T cells in TIL fragment 6 (F6) from patient 3784 that recognized TMG5 (Table 1; Fig. 2A). From F6, we FACS sorted 756 CD8⁺ T cells that expressed high levels of CD137 (~0.1% of the CD3⁺ CD8⁺ T cells) after overnight stimulation with autologous DCs electroporated with RNA encoding TMG5 and expanded those cells *in vitro*. After 2 weeks, we reevaluated recognition of TMG5 by the sorted and expanded population (Fig. 2B). Eighty-nine percent of the CD8⁺ T cells expressed CD137

after overnight coculture indicating successful enrichment of the reactive population. We also evaluated recognition of each 25 amino acid peptide encoded by TMG5 and determined the enriched population recognized a mutated KIF16B peptide (Fig. 2C). Using the IEDB database, we narrowed the epitope to an 11 mer with high-binding affinity to HLA-B*0702 as described previously (ref. 27; Supplementary Table S5). From this enriched population, one single dominant TCR α and one single dominant TCR β chain were identified by genomic DNA deep sequencing (Fig. 2D). This TCR α/β chain pair was also identified as being present in T cells from FrTu using PairSeq analysis (Adaptive Biotechnologies), albeit at very low frequency (37). This TCR was cloned into a retroviral vector and used to transduce open-repertoire PBL, and the resulting T-cell populations were evaluated for recognition of relevant target cells. The TCR from the CD137-enriched F6 TILs from patient 3784 mediated specific recognition of the mutated KIF16B 25- and 11- amino acid peptides but not their wild-type counterparts (Fig. 2E). In addition, this TCR-mediated specific recognition of the autologous tumor cell line compared with an allogeneic HLA-mismatched melanoma cell line (Fig. 2E). Additional examples of cases in which a dominant TCR α/β chain pair-mediated recognition of mutated antigens are presented in Supplementary Figs. S1–S10.

For other enriched T-cell populations, genomic DNA deep sequencing of TCR α and β chains indicated the presence of one dominant TCR α and one dominant TCR β chain, but when we introduced that pair into PBL, it did not mediate recognition of the expected mutation. In these situations, we employed additional techniques for pairing correct α and β chains. For most cases, the PairSeq analysis of T cells from FrTu was sufficient to guide the construction of TCRs. One example of this is presented in Fig. 3. In our original screening assay, we identified a population of T cells in TIL fragment 3 (F3) from patient 3716 that recognized TMG3 (Fig. 3A). From F3, we FACS sorted CD8⁺ T cells that expressed high levels of CD137 after overnight stimulation with autologous DCs electroporated with RNA encoding TMG3 and expanded those cells *in vitro*. After 2 weeks, we reevaluated recognition of TMG3 by the sorted and expanded population (Fig. 3B). Eighty-percent of CD8⁺ T cells expressed CD137 after overnight coculture indicating successful enrichment of the reactive population. We also evaluated recognition of each 25 amino acid peptide encoded by TMG3 and determined the enriched population recognized a mutated TFDP2 peptide. From this enriched population, one dominant α and one dominant β chain were identified by genomic DNA deep sequencing (Fig. 3C), but when we transduced this TCR into PBL, it did not mediate recognition of TMG3 or the mutated TFDP2 peptide (Fig. 3D). We then used the PairSeq analysis of T cells from 3716 FrTu and identified 3 α/β pairs that were present at very low frequencies in the fresh tumor but were represented in the top 4 most frequent TCRs in the CD137-enriched population (Fig. 3C). The results of the PairSeq analysis indicated the #1 α chain was paired with the #2 β chain, the #4 α chain was paired with the #1 β chain, and the #3 α chain was paired with the #3 β chain. When we retrovirally transduced PBLs with each of these 3 different TCRs, all three mediated specific recognition of the mutated TFDP2 25 mer but not its wild-type counterpart (Fig. 3D). In addition, we identified an HLA-B*1501 restricted 9 mer (Supplementary Table S5), and all three TCRs mediated specific recognition of this mutated minimal epitope (Fig. 3D).

Additional examples of cases in which we employed PairSeq analysis to identify mutation-reactive TCR α/β chain pairs are presented in Supplementary Figs. S11–S13.

For one enriched T-cell population from patient 3903 reactive with TMG9, genomic DNA deep sequencing of TCR α and β chains indicated the presence of one dominant TCR α and one dominant TCR β chain, and when we introduced that pair into PBL, it mediated recognition of the TMG9 (Fig. 4A–D). However, this pair was not identified in the PairSeq analysis of FrTu from this patient. Instead, two other pairs were identified, namely the #2 α chain was found to be paired with both the #2 and #3 β chains (Fig. 4C). PBL retrovirally transduced to express the 2 α /3 β pair, but not the 2 α /2 β pair, also mediated recognition of TMG9 (Fig. 4D). Interestingly, the 2 α /1 β pair, which was not found using PairSeq analysis and would not have been predicted by pairing α/β chains by frequency, also mediated recognition of TMG9 (Fig. 4D). We previously identified the minimal epitope recognized in TMG9 to be an HLA-B*3801–restricted peptide derived from a mutation in KIAA1279 (ref. 27; Supplementary Table S5). To determine whether there were differences in the functional avidities of these TCRs, we conducted a peptide titration experiment (Fig. 4E). We observed the 2 α /3 β pair mediated recognition of lower concentrations of peptide when pulsed onto autologous DCs than the other two TCRs.

Finally, for one enriched T-cell population from patient 3678 reactive with TMG9, genomic DNA deep sequencing of TCR α and β chains indicated the presence of multiple TCR α and β chains present at less than 20% (Fig. 5A–C). In addition, when we screened the enriched population for recognition of individual peptides encoded by TMG9, it appeared as though two mutated peptides might be recognized, namely UGGT2 and XPNPEP1. These results suggested the enriched TMG9-reactive population might contain multiple TCR clonotypes. For patient 3678, the PairSeq data was not robust, so for this enriched population, single-cell RT-PCR of cDNA from individual CD8⁺ cells was conducted as described previously to identify TCR α and β chain pairs (37–39). This analysis identified 4 dominant pairs present in the enriched population at frequencies of 32%, 26%, 19%, and 11% (Fig. 5C). Interestingly, these α and β chains were also found among the 6 most frequent α and β chains identified in the genomic DNA deep sequencing analysis, but it would have been difficult to identify these pairs based on that data alone. When we retrovirally transduced PBL with each of these 4 different TCRs, all four mediated specific recognition of TMG9. Two of them recognized the mutated UGGT2 peptide, and the other two recognized the mutated XPNPEP1 peptide (Fig. 5D). In addition, we identified an HLA-A*0301–restricted 11 mer from XPNPEP1 and an HLA-A*0201–restricted 9 mer from UGGT2 (Supplementary Table S5). The two XPNPEP1-reactive TCRs mediated peptide recognition with comparable avidity as did the two UGGT2 reactive TCRs (Fig. 5D).

From the four patients on whom we performed prospective screening to identify mutation-reactive TILs, namely 3784, 3678, 3716, and 3903, we attempted to enrich mutation-reactive T cells from 31 individual TIL samples (Table 1 and Supplementary Tables S6–S9). For each of these samples, we sorted at least 50 CD137⁺ CD8⁺ T cells after stimulation with mutated TMG constructs. After *in vitro* expansion with anti-CD3 and IL2, 29 of these 31 cell populations contained more TMG reactive cells than prior to sorting. Two populations from patient 3716, namely F3 versus TMG4 and F4 versus TMG3, were not enriched for

TMG-reactive cells. As such, our success rate for enriching TMG-reactive T cells was 94%. We performed TCR sequencing on 24 of the enriched populations and identified mutation-reactive TCRs from 23 of them. One TMG3-reactive population from patient 3784 appeared to recognize mutated FLNA (data not shown), and we isolated one dominant TCR α and one dominant TCR β chain from this population. However, when we retrovirally introduced this TCR into PBMC, recognition of TMG3 and mutated FLNA was weak and inconsistent. As such, our success rate for identifying mutation reactive TCRs from enriched populations was 96%. Overall, we isolated 27 mutation-reactive TCRs from TILs from 6 patients that mediated recognition of 14 neoepitopes (Table 2).

Discussion

The adoptive transfer of TILs can mediate regression of metastatic melanoma, and accumulating evidence suggests that therapeutic TILs target tumor-specific mutations (10–12). In addition, adoptively transferred, mutation-reactive T cells appear able to mediate rejection of metastatic epithelial cancers (13). Despite these observations, some patients treated with adoptively transferred, mutation-reactive TILs do not respond to therapy. For example, patient 3716 did not respond to adoptive cell therapy despite the presence of mutation-reactive T cells in the TILs used for his treatment (Supplementary Table S9). One potential contributing factor to the lack of efficacy is that TILs used for treatments undergo extensive *in vitro* expansion and are usually highly differentiated cells with limited proliferative potential (46). Preclinical studies strongly suggest that less-differentiated T cells with more naïve phenotypes including naïve, stem cell memory, and central memory T-cell subsets are significantly more effective for treating mice with rapidly growing tumors (47–49). In the study reported here, we isolated mutation-reactive TCRs that we could genetically introduce into any autologous PBL subset to develop personalized, patient-specific gene therapy reagents.

CD137 is transiently expressed on T cells that have recently been activated by TCR engagement (20), and expression of CD137 on T cells has been used to identify and isolate virus and tumor-reactive T cells from peripheral blood and TILs (20–24). We are currently conducting a clinical trial in which patients with melanoma are being treated with adoptively transferred T cells from TILs that have been FACS sorted for CD137 prior to *in vitro* expansion (NCT02111863). As CD137 is an activation marker, it is likely that these cells have undergone multiple rounds of proliferation *in vivo* prior to their selection. We then expand them further *in vitro* to obtain sufficient numbers of cells for treatments, so that when they are adoptively transferred back to the patient, they are usually highly differentiated cells with limited proliferative potential. Therefore, in the work presented here, we used CD137 upregulation after antigen-specific stimulation to enrich mutation-reactive T cells and isolate TCRs that we could introduce into less differentiated PBLs as a treatment platform.

By enriching TILs for mutation-reactive T cells based on CD137 upregulation after *in vitro* stimulation, we successfully isolated 27 mutation-reactive TCRs from 6 patients (Table 2). For some patients, we identified TCRs reactive with multiple mutations identified through whole exome sequencing and/or RNA sequencing of the autologous patient's tumors. In

addition, for some mutations, we isolated multiple reactive TCR clonotypes, and we could select the most functionally avid TCR clonotype using peptide titration experiments (Fig. 4). Although most TCR clonotypes that recognized the same neoantigen consisted of diverse TCR α and β chains, we noted 3 of the 4 TCRs from patient 3784 that recognized a neoepitope in the SON protein used TRBV28*01 and had CDR3 regions of similar length, but each was paired with a unique α chain (Supplementary Figs. S3, S12, and S13). In addition, for patient 3903, a single α chain could be paired with two different β chains, and a very similar α chain could be paired with a third β chain, all of which mediated recognition of a mutated KIAA1279 peptide, albeit with different avidities (Fig. 4). These observations suggest that for some neoepitopes, there may be dominant TCR α or β chain usage by reactive T cells, a phenomenon that has previously been observed for nonmutated self-antigens and viral epitopes (50–52). For patient treatment, it seems likely we would want to transfer a diverse population of T cells encompassing as many mutation reactive TCRs as possible. Using the methods described here, we will likely be able to identify multiple mutation-reactive TCR clonotypes that we could introduce into the autologous patient's PBL for adoptive transfer.

For every TCR we isolated, we evaluated and observed recognition of synthetic peptides containing mutations in addition to transfected constructs. For all of the TCRs, we observed specific recognition of 25 mers containing the mutations, and for all but two of the neoantigens described here (SON and CORO7), we were able to identify 8–11 amino acid minimal epitopes. For the mutated SON antigen, we determined the restriction element to be HLA-B*0702 by evaluating recognition of COS-7 cells transfected with TMG8 and each of the patient's class I MHC molecules as described previously (27). We identified three potential minimal epitopes based on HLA-B*0702-binding algorithms, but none were recognized by our TCR-transduced T cells. We also made every overlapping 8-, 9-, 10-, and 11-amino acid peptide, but none were recognized by our TCR-transduced T cells. In addition, we reverted every mutated minigene within TMG8 to its wild-type counterpart, and when we electroporated DCs with RNAs encoding these TMGs, reactivity was only diminished when the wild-type SON was present indicating this was the correct neoantigen. It seems possible that the SON epitope presented on the surfaces of antigen-presenting cells or tumor cells may be modified during processing in some unpredictable fashion. Nonetheless, based on the aforementioned data and on the observation that the mutated 25 amino acid peptide was, whereas the wild-type counterpart was not (Supplementary Figs. S3, S12, and S13), it is clear that mutated SON was the correct neoantigen recognized by several TCRs from patient 3784. For the mutated CORO7 antigen, we determined the restriction element to be HLA-B*5101 by evaluating recognition of COS-7 cells transfected with TMG2 and each of the patient's class I MHC molecules as described previously (27). We identified three potential minimal epitopes based on HLA-B*5101-binding algorithms, but none were recognized by our TCR-transduced T cells. We did not pursue the identification of the minimal epitope further. However, based on the observation that the mutated 25 amino acid peptide was recognized, whereas the wild-type counterpart was not (Supplementary Fig. S6), it is clear that mutated CORO7 was the correct neoantigen recognized by a TCR from patient 3678.

The functional avidities of the TCRs we isolated varied significantly. Several TCRs mediated recognition of sub-nanomolar concentrations of their respective 8–11 amino acid minimal epitopes when pulsed onto autologous or HLA-matched DCs including one of the KIAA1279 TCRs (Fig. 4), both of the UGGT2 TCRs (Fig. 5), the GNB5 TCR (Supplementary Fig. S4), and the FBXO21 TCR (Supplementary Fig. S6). The other TCRs required higher peptide concentrations for recognition. TCRs specifically reactive with MART-1, gp100, and NY-ESO-1 that have previously been reported to mediate tumor regressions in patients have generally mediated recognition of sub-nanomolar concentrations of peptides pulsed onto T2 cells (1, 2, 53). As T2 cells are TAP-deficient, they cannot process and present peptides from endogenous proteins on their cell surfaces. Therefore, in peptide-pulsing experiments using T2 cells as APCs, there is little or no competition for the binding of exogenously loaded peptides to HLA molecules. As DCs can process and present peptides from endogenous proteins, it is not clear how T2 cells and DCs compare to each other as APCs in peptide-pulsing experiments with exogenously loaded short peptides. As such, based on the data presented here, it is not possible to compare the functional avidities of the neoantigen-reactive TCRs we isolated with TCRs recognizing nonmutated peptides that have previously been reported to mediate tumor regressions.

One significant problem we encountered in identifying mutation-reactive TCRs was pairing the correct TCR α and β chains based on genomic deep-sequencing frequencies from CD137 enriched T-cell populations. Many times, one dominant α chain and one dominant β chain were identified, and the pair constituted a mutation-reactive TCR. However, in some cases, this strategy did not work (Figs. 3 and 5), and we had to rely on data from PairSeq analysis that had previously been performed on FrTu digests or single-cell RT-PCR of the CD137-sorted cells to match reactive TCR α/β pairs. For future studies, we will likely avoid the use of individual TCR α and β chain deep sequencing and simply use single-cell RT-PCR. In particular, we will sort single CD137⁺ cells and perform RT-PCR directly. In a few rare cases, we attempted to sort for mutation-reactive CD137⁺ T cells, but the cells that proliferated *in vitro* were not appreciably enriched. For example, in the original screening assays, F3 from patient 3713 appeared to contain a significant number of TMG4-reactive T cells (Supplementary Table S9). However, when we sorted CD137⁺ CD8⁺ T cells after stimulation with TMG4, the cells that expanded *in vitro* contained a mixture of TMG3- and TMG4-reactive T cells, and the TCRs in that population overlapped with the TMG3-enriched population presented in Fig. 3. Performing single-cell RT-PCR directly on sorted CD137⁺ T cells should allow for the rapid identification of correct TCR α/β pairs from multiple clones, including those that may not expand efficiently *in vitro*.

One issue that should be addressed in terms of treating patients with individualized neoantigen-reactive TCRs is the time required to isolate them. Once a tumor has been resected from a patient, whole-exome sequencing, RNA sequencing, and data analysis to identify potential neoantigens take approximately 1 week. The synthesis of RNAs encoding TMGs containing the identified mutations, and the synthesis of mutated 25 amino acid peptides take approximately 3 weeks. Screening TILs for the presence of mutation-reactive T cells requires approximately 1 week. Single-cell sorting of CD137⁺ T cells after coculture with relevant mutations and subsequent TCR analysis by PCR and sequencing take approximately 1 week. Construction of retroviral vectors encoding potential neoantigen-

reactive TCRs requires 2–3 weeks. Retroviral supernatant production, transduction of PBL, expansion of the TCR-transduced cells, and functional evaluation take approximately 2 weeks. Collectively, using current technologies, the entire process requires 2–3 months. Individual patient characteristics will dictate whether or not this time frame is reasonable, and we are constantly considering new methods for streamlining the process.

We have previously described two different approaches for identifying tumor and/or mutation-reactive TCRs from TILs (28, 37). In one study, HLA-peptide tetramers were used to sort neoantigen-reactive T cells (28). This technique requires the use of HLA binding prediction algorithms to guide the synthesis of HLA-peptide tetramers. For many class I HLA molecules, peptide-binding prediction algorithms are reasonably accurate, and tetramers can readily be synthesized (54). However, for some class I HLA molecules and for many class II HLA molecules, peptide-binding prediction algorithms are not robust, and reagents from which tetramers can be synthesized are not available. In the work presented here, we isolated mutation-reactive T cells by sorting for CD137⁺ TIL after stimulation with autologous APCs transfected with neoantigens. This method has the significant advantage of bypassing the need to use HLA-binding prediction algorithms to guide the synthesis of HLA-peptide tetramers. In a second study, we identified and evaluated the function of the most frequent TCR clonotypes in fresh tumor digests (37). Of the 78 TCRs evaluated, 36 mediated recognition of autologous tumor cell lines, 11 were identified as recognizing tumor-associated mutations, 2 mediated recognition of nonmutated shared antigens, and we were unable to identify antigens recognized by the rest. Although this method allows for the rapid identification of TCRs from TILs, as we did not identify the specific antigens recognized by many of these TCRs, it is not clear whether we should pursue this method clinically due to concerns about potential on-target, off-tumor toxicities. The primary objective of the work presented here was to determine whether we could consistently isolate mutation-reactive TCRs from TIL. For each TCR, we identified the specific tumor-associated mutation being recognized, thus decreasing the safety concerns associated with using such TCRs in personalized gene therapy trials.

Another potential technique to enrich mutation-reactive T cells is through the use of IFN γ capture assays. IFN γ secreted by previously activated T cells is retained on the cell surface, allowing for their specific isolation and expansion (55). This assay could be used to isolate mutation-reactive T cells and TCRs after stimulation with autologous antigen-presenting cells electroporated with IVT RNAs as described here. However, we have previously identified T cells that upregulate CD137 that do not secrete IFN γ (56). Therefore, the use of IFN γ capture might miss the selection of some T cells from which mutation-reactive TCRs could be isolated.

Here we focused on the identification of class I HLA-restricted TCRs from mutation-reactive CD8⁺ T cells. However, tumor-reactive CD4⁺ T cells have been shown to play a significant role in mediating tumor regression in both animal models and patients (13, 57–59). Therefore, in the future, we will also attempt to isolate class II HLA-reactive TCRs for use in gene therapy protocols. CD137 is expressed on activated CD4⁺ T cells and has previously been used to isolate *Aspergillus fumigatus*-reactive T-helper cells for adoptive transfer (60). However, several studies have suggested that CD134 (OX40) is more robustly

expressed on activated CD4⁺ T cells than CD137 (61, 62). CD134 is transiently expressed on CD4⁺ T cells upon antigen stimulation and can be used as a marker to sort mutation-reactive T cells (35). After stimulation of TILs with DCs electroporated with IVT RNAs or pulsed with long peptides containing mutations, it seems likely we will be able to isolate mutation-reactive class II restricted TCRs from CD137- or CD134-sorted CD4⁺ T cells.

Recently, we have described the isolation of mutation-reactive T cells from peripheral blood (27, 28). Although their frequencies are generally lower than in TILs, the use of peripheral blood as a source of mutation-reactive TCRs would be beneficial for patients from whom TILs cannot be isolated and/or expanded *in vitro*. In addition, recent advances have made whole-exome sequencing possible from paraffin-fixed sections of the original tumor (63), from tumor needle biopsies (64), and from tumor-derived DNA isolated from peripheral blood (65, 66). Collectively, these findings suggest it may be possible to develop adoptive cell transfer therapies using noninvasive techniques to identify tumor-specific mutations and mutation-reactive TCRs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support

This work was supported through the NIH Intramural research program.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006;314:126–29. [PubMed: 16946036]
2. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009;114:535–46. [PubMed: 19451549]
3. Kochenderfer JN, Yu Z, Frasheri D, Restifo NP, Rosenberg SA. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood* 2010;116:3875–86. [PubMed: 20631379]
4. Jensen MC, Popplewell L, Cooper LJ, DiGiusto D, Kalos M, Ostberg JR, et al. Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol Blood Marrow Transplant* 2010;16: 1245–56. [PubMed: 20304086]
5. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* 2011;19:620–26. [PubMed: 21157437]
6. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 2011;118:4817–28. [PubMed: 21849486]

7. Riddell SR, Sommermeyer D, Berger C, Liu LS, Balakrishnan A, Salter A, et al. Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition. *Cancer J* 2014;20:141–44. [PubMed: 24667960]
8. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010;18:843–51. [PubMed: 20179677]
9. Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013;36:133–51. [PubMed: 23377668]
10. Corbiere V, Chapiro J, Stroobant V, Ma W, Lurquin C, Lethe B, et al. Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res* 2011;71:1253–62. [PubMed: 21216894]
11. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* 2013;19:747–52. [PubMed: 23644516]
12. Lu YC, Yao X, Crystal JS, Li YF, El-Gamil M, Gross C, et al. Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin Cancer Res* 2014;20: 3401–10. [PubMed: 24987109]
13. Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* 2014;344:641–45. [PubMed: 24812403]
14. Kwon BS, Weissman SM. cDNA sequences of two inducible T-cell genes. *Proc Natl Acad Sci U S A* 1989;86:1963–67. [PubMed: 2784565]
15. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 2005;23:23–68. [PubMed: 15771565]
16. Wen T, Bukczynski J, Watts TH. 4–1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. *J Immunol* 2002;168:4897–906. [PubMed: 11994439]
17. Lee HW, Park SJ, Choi BK, Kim HH, Nam KO, Kwon BS. 4–1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1. *J Immunol* 2002;169:4882–88. [PubMed: 12391199]
18. Halstead ES, Mueller YM, Altman JD, Katsikis PD. *In vivo* stimulation of CD137 broadens primary antiviral CD8+ T cell responses. *Nat Immunol* 2002;3:536–41. [PubMed: 12021777]
19. Dawicki W, Watts TH. Expression and function of 4–1BB during CD4 versus CD8 T cell responses in vivo. *Eur J Immunol* 2004;34:743–51. [PubMed: 14991604]
20. Wolf M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, et al. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. *Blood* 2007;110: 201–10. [PubMed: 17371945]
21. Ye Q, Song DG, Poussin M, Yamamoto T, Best A, Li C, et al. CD137 accurately identifies and enriches for naturally occurring tumor-reactive T cells in tumor. *Clin Cancer Res* 2014;20:44–55. [PubMed: 24045181]
22. Watanabe K, Suzuki S, Kamei M, Toji S, Kawase T, Takahashi T, et al. CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy. *Int J Hematol* 2008;88: 311–20. [PubMed: 18677654]
23. Zandvliet ML, van LE, Jedema I, Kruithof S, Kester MG, Guchelaar HJ, et al. Simultaneous isolation of CD8(+) and CD4(+) T cells specific for multiple viruses for broad antiviral immune reconstitution after allogeneic stem cell transplantation. *J Immunother* 2011;34:307–19. [PubMed: 21389867]
24. Wolf M, Kuball J, Eyrich M, Schlegel PG, Greenberg PD. Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. *Cytometry A* 2008;73:1043–49. [PubMed: 18561198]

25. Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. *J Clin Invest* 2014;124:2246–59. [PubMed: 24667641]
26. Jones S, Wang TL, Shih I, Mao TL, Nakayama K, Roden R, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science* 2010;330:228–31. [PubMed: 20826764]
27. Gros A, Parkhurst MR, Tran E, Pasetto A, Robbins PF, Ilyas S, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. *Nat Med* 2016;22:433–8. [PubMed: 26901407]
28. Cohen CJ, Gartner JJ, Horovitz-Fried M, Shamalov K, Trebska-McGowan K, Bliskovsky VV, et al. Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes. *J Clin Invest* 2015;125:3981–91. [PubMed: 26389673]
29. Jin J, Sabatino M, Somerville R, Wilson JR, Dudley ME, Stronck DF, et al. Simplified method of the growth of human tumor infiltrating lymphocytes in gas-permeable flasks to numbers needed for patient treatment. *J Immunother* 2012;35:283–92. [PubMed: 22421946]
30. Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, et al. Immune epitope database analysis resource. *Nucleic Acids Res* 2012;40: W525–W30. [PubMed: 22610854]
31. Nielsen M, Lundegaard C, Wornig P, Hvid CS, Lamberth K, Buus S, et al. Improved prediction of MHC class I and class II epitopes using a novel Gibbs sampling approach. *Bioinformatics* 2004;20:1388–97. [PubMed: 14962912]
32. Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. *Nucleic Acids Res* 2008;36:W509–W12. [PubMed: 18463140]
33. Peters B, Sette A. Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. *BMC Bioinformatics* 2005;6:132. [PubMed: 15927070]
34. Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, et al. Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. *Immunome Res* 2008;4:2. [PubMed: 18221540]
35. Tran E, Ahmadzadeh M, Lu YC, Gros A, Turcotte S, Robbins PF, et al. Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science* 2015;350:1387–90. [PubMed: 26516200]
36. Howie B, Sherwood AM, Berkebile AD, Berka J, Emerson RO, Williamson DW, et al. High-throughput pairing of T cell receptor alpha and beta sequences. *Sci Transl Med* 2015;7:301ra131.
37. Pasetto A, Alena G, Robbins PF, Deniger DC, Prickett TD, Matus-Nicodemus R, et al. Tumor- and neoantigen-reactive T-cell receptors can be identified based on their frequency in fresh tumor. *Cancer Immunol Res* 2016;4:734–43. [PubMed: 27354337]
38. Dossinger G, Bunse M, Bet J, Albrecht J, Paszkiewicz PJ, Weissbrich B, et al. MHC multimer-guided and cell culture-independent isolation of functional T cell receptors from single cells facilitates TCR identification for immunotherapy. *PLoS One* 2013;8:e61384. [PubMed: 23637823]
39. Kobayashi E, Mizukoshi E, Kishi H, Ozawa T, Hamana H, Nagai T, et al. A new cloning and expression system yields and validates TCRs from blood lymphocytes of patients with cancer within 10 days. *Nat Med* 2013;19: 1542–46. [PubMed: 24121927]
40. Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA, Morgan RA. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res* 2006;66:8878–86. [PubMed: 16951205]
41. Cohen CJ, Li YF, El-Gamil M, Robbins PF, Rosenberg SA, Morgan RA. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. *Cancer Res* 2007;67:3898–903. [PubMed: 17440104]
42. Haga-Friedman A, Horovitz-Fried M, Cohen CJ. Incorporation of transmembrane hydrophobic mutations in the TCR enhance its surface expression and T cell functional avidity. *J Immunol* 2012;188: 5538–46. [PubMed: 22544927]
43. Chinnasamy N, Wargo JA, Yu Z, Rao M, Frankel TL, Riley JP, et al. A TCR targeting the HLA-A*0201-restricted epitope of MAGE-A3 recognizes multiple epitopes of the MAGE-A antigen superfamily in several types of cancer. *J Immunol* 2011;186:685–96. [PubMed: 21149604]

44. Johnson LA, Heemskerk B, Powell DJ Jr, Cohen CJ, Morgan RA, Dudley ME, et al. Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. *J Immunol* 2006;177: 6548–59. [PubMed: 17056587]
45. Morgan RA, Dudley ME, Yu YY, Zheng Z, Robbins PF, Theoret MR, et al. High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. *J Immunol* 2003;171:3287–95. [PubMed: 12960359]
46. Gattinoni L, Klebanoff CA, Restifo NP. Paths to stemness: building the ultimate antitumor T cell. *Nat Rev Cancer* 2012;12:671–84. [PubMed: 22996603]
47. Hinrichs CS, Borman ZA, Cassard L, Gattinoni L, Spolski R, Yu Z, et al. Adoptively transferred effector cells derived from naive rather than central memory CD8⁺ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A* 2009;106:17469–74. [PubMed: 19805141]
48. Klebanoff CA, Gattinoni L, Palmer DC, Muranski P, Ji Y, Hinrichs CS, et al. Determinants of successful CD8⁺-T-cell adoptive immunotherapy for large established tumors in mice. *Clin Cancer Res* 2011;17:5343–52. [PubMed: 21737507]
49. Hinrichs CS, Borman ZA, Gattinoni L, Yu Z, Burns WR, Huang J, et al. Human effector CD8⁺ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood* 2011;117:808–14. [PubMed: 20971955]
50. Trautmann L, Labarriere N, Jotereau F, Karanikas V, Gervois N, Connerotte T, et al. Dominant TCR V alpha usage by virus and tumor-reactive T cells with wide affinity ranges for their specific antigens. *Eur J Immunol* 2002;32:3181–90. [PubMed: 12555663]
51. Mantovani S, Palermo B, Garbelli S, Campanelli R, Robustelli Della Cuna G, Gennari R, et al. Dominant TCR-alpha requirements for a self antigen recognition in humans. *J Immunol* 2002;169:6253–60. [PubMed: 12444131]
52. Li D, Gao G, Li Z, Sun W, Li X, Chen N, et al. Profiling the T-cell receptor repertoire of patient with pleural tuberculosis by high-throughput sequencing. *Immunol Lett* 2014;162:170–80. [PubMed: 25173045]
53. Robbins PF, Kassim SH, Tran TL, Crystal JS, Morgan RA, Feldman SA, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin Cancer Res* 2015;21:1019–27. [PubMed: 25538264]
54. Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ, et al. Design and use of conditional MHC class I ligands. *Nat Med* 2006;12: 246–51. [PubMed: 16462803]
55. Becker C, Pohla H, Frankenberger B, Schuler T, Assenmacher M, Schendel DJ, et al. Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. *Nat Med* 2001;7:1159–62. [PubMed: 11590442]
56. Turcotte S, Gros A, Tran E, Lee CC, Wunderlich JR, Robbins PF, et al. Tumorreactive CD8⁺ T cells in metastatic gastrointestinal cancer refractory to chemotherapy. *Clin Cancer Res* 2014;20:331–43. [PubMed: 24218514]
57. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, et al. Tumorspecific Th17-polarized cells eradicate large established melanoma. *Blood* 2008;112:362–73. [PubMed: 18354038]
58. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, et al. Treatment of metastatic melanoma with autologous CD4⁺ T cells against NY-ESO-1. *N Engl J Med* 2008;358:2698–703. [PubMed: 18565862]
59. Kreiter S, Vormehr M, van de Roemer N, Diken M, Lower M, Diekmann J, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* 2015;520:692–96. [PubMed: 25901682]
60. Bacher P, Jochheim-Richter A, Mockel-Tenbrink N, Kniemeyer O, Wingenfeld E, Alex R, et al. Clinical-scale isolation of the total *Aspergillus fumigatus*-reactive T-helper cell repertoire for adoptive transfer. *Cytotherapy* 2015;17:1396–405. [PubMed: 26188965]
61. Dawicki W, Bertram EM, Sharpe AH, Watts TH. 4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses. *J Immunol* 2004;173:5944–51. [PubMed: 15528328]

62. Taraban VY, Rowley TF, O'Brien L, Chan HT, Haswell LE, Green MH, et al. Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses. *Eur J Immunol* 2002;32: 3617–27. [PubMed: 12516549]
63. Van Allen EM, Wagle N, Stojanov P, Perrin DL, Cibulskis K, Marlow S, et al. Whole-exome sequencing and clinical interpretation of formalin-fixed, paraffin-embedded tumor samples to guide precision cancer medicine. *Nat Med* 2014;20:682–88. [PubMed: 24836576]
64. Lee HB, Joung JG, Kim J, Lee KM, Ryu HS, Lee HO, et al. The use of FNA samples for whole-exome sequencing and detection of somatic mutations in breast cancer surgical specimens. *Cancer Cytopathol* 2015;123: 669–77. [PubMed: 26265110]
65. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 2013;497:108–12. [PubMed: 23563269]
66. Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nat Biotechnol* 2014;32:479–84. [PubMed: 24752078]

Translational Relevance

Somatic mutations in tumor cells can be recognized by tumor-infiltrating lymphocytes (TIL), and these appear to be the target antigens that result in cancer regression following adoptive cell transfer (ACT) with TIL. We are currently screening patients in our ACT protocols for the presence of mutation reactive T cells and are selecting T-cell populations for treatment based on this information. However, many patients who have received large numbers of mutation-reactive T cells have not responded to therapy, perhaps because the adoptively transferred cells are highly differentiated with little proliferative potential. To overcome this problem, we would like to treat patients with autologous lymphocytes with less differentiated phenotypes that have been genetically modified to express mutation-reactive T-cell receptors (TCR). Here we report a strategy for isolating such TCRs from patients with melanoma that can readily be adapted to patients with other more common epithelial cancers.

Figure 1. Isolation of mutation reactive TCRs from TILs based on CD137 expression after *in vitro* stimulation. **A** and **B**, Treatment TIL from patients 3466 (**A**) and 3713 (**B**) were cocultured overnight with autologous DCs electroporated with IVT RNAs encoding TMG constructs previously identified as being recognized: TMG1 (containing mutated COL18A1) for patient 3466 and TMG2 (containing mutated SRPX) for patient 3713. CD3⁺ CD8⁺ CD137⁺ cells were sorted by FACS and expanded *in vitro*. **C** and **D**, The resulting T-cell populations were cocultured overnight with autologous DCs electroporated with IVT RNAs encoding the

TMGs, and CD137 expression on CD3⁺CD8⁺ T cells was evaluated by FACS. **E**, TCR sequences in cDNA from the enriched populations were determined by 5' RACE. **F** and **G**, TCRs were cloned into MSGV1 retroviral vectors and used to transduce autologous PBL. Transduction efficiencies were measured by staining cells with an anti-murine TCR β -constant region antibody. As both the mutated COL18A1 and SRPX epitopes were previously identified as being HLA-A* 0201 restricted, the transduced T-cell populations were evaluated for recognition of peptide-pulsed T2 cells based on IFN γ secretion.

Figure 2.

Isolation of a single mutated KIF16B-reactive TCR from a tumor biopsy fragment from patient 3784. **A**, TIL fragment F6 from patient 3784 was cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG5, and recognition was evaluated on the basis of IFN γ ELISPOT (■) and CD137 expression by FACS (▨). **B**, CD3⁺ CD8⁺ CD137⁺ cells were sorted by FACS and expanded *in vitro*, and the resulting T-cell population was again cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG5. Recognition was again evaluated on the basis of IFN γ ELISPOT and CD137 expression. **C**, Recognition of individual 25 amino acid peptides encoded by TMG5

by the enriched T-cell population was evaluated on the basis of IFN γ ELISPOT and CD137 expression after overnight coculture with autologous peptide-pulsed DCs (10 mg/mL pulsed for ~20 hours prior to coculture). **D**, TCR α and β chain sequences from the enriched population were determined by genomic DNA deep sequencing (Adaptive Biotechnologies), and frequencies (%) of productively rearranged sequences were calculated. **E**, The dominant TCR was cloned into an MSGV1 retroviral vector and used to transduce PBLs. Transduction efficiency was measured by staining cells with an anti-murine TCR β constant region antibody. Recognition of the mutated KIF16B 25 mer as well as a shorter 11 mer predicted to bind to HLA-B*0702 was evaluated by TCR-transduced T cells based on IFN γ secretion after overnight coculture with peptide pulsed autologous or HLA-matched allogeneic DCs (10 mg/mL pulsed for ~20 hours prior to coculture for the 25 mer; 10^{-6} – 10^{-10} μ g/mL pulsed for ~1.5 hours prior to coculture for the 11 mer). Recognition of IFN γ treated (10 ng/mL 24 hours prior to coculture) autologous and allogeneic melanoma cells lines was also evaluated on the basis of IFN γ secretion after overnight coculture.

Figure 3.

Isolation of multiple mutated TFDP2- reactive TCRs from a tumor biopsy fragment from patient 3716. **A**, TIL fragment F3 from patient 3716 was cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG3, and recognition was evaluated on the basis of IFN γ ELISPOT and CD137 expression. **B**, CD3 $^{+}$ CD8 $^{+}$ CD137 $^{+}$ cells were sorted by FACS and expanded *in vitro*, and the resulting T-cell population was again cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG3. Recognition was again evaluated on the basis of IFN γ ELISPOT and CD137

expression. **C**, TCR α and β chain sequences in genomic DNA from the enriched populations were determined by deep sequencing, and frequencies (%) of productively rearranged sequences were calculated. These were then compared to TCR α/β pair sequences identified in cDNA from FrTu digests using PairSeq analysis. The dominant TCR α/β chain pair identified by deep sequencing (TCR 1) was cloned into an MSGV1 retroviral vector as were the three TCR α/β chain pairs identified via PairSeq analysis (indicated by *). **D**, Retroviral vectors were used to transduce PBL, and transduction efficiencies were measured by staining cells with an anti-murine TCR β constant region antibody. Recognition of TMG3 and a mutated TFDP2 25 amino acid peptide encoded by TMG3 were evaluated by the TCR- transduced T cells based on IFN γ secretion. In addition, we identified a minimal 9 amino acid HLA-B*1501- restricted epitope from TFDP2 encoded by TMG3. To evaluate the functional avidities of the reactive TCRs, recognition of titrated amounts of this minimal epitope pulsed onto HLA-matched DCs (~1.5 hours prior to coculture) was evaluated on the basis of IFN γ secretion after overnight coculture.

Figure 4. Isolation of multiple mutated KIAA1279 reactive TCRs from a tumor biopsy from patient 3903. **A**, TIL fragment F1 from patient 3903 was cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG9, and recognition was evaluated on the basis of IFN γ ELISPOT and CD137 expression. **B**, CD3 $^{+}$ CD8 $^{+}$ CD137 $^{+}$ cells were sorted by FACS and expanded *in vitro*, and the resulting T-cell population was again cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG9. Recognition was again evaluated based on IFN γ ELISPOT and CD137 expression. **C**, TCR sequences in

genomic DNA from the enriched populations were determined by deep sequencing, and frequencies (%) of productively rearranged sequences were calculated. These were then compared with TCR α/γ pair sequences identified in cDNA from FrTu digests using PairSeq analysis. Five different TCR α/γ chain pairs were cloned into MSGV1 retroviral vectors, including 2 identified using PairSeq analysis (indicated by *). **D**, Retroviral vectors were used to transduce PBL, and transduction efficiencies were measured by staining cells with an anti-murine TCR γ constant region antibody. Recognition of TMG9 was evaluated by the TCR-transduced T cells based on IFN γ secretion after overnight coculture with electroporated autologous DCs. **E**, We identified a minimal 8 amino acid HLA-B*3801 restricted epitope from KIAA1279 encoded by TMG9. To evaluate the functional avidities of the reactive TCRs, recognition of titrated amounts of this minimal epitope pulsed onto autologous DCs (~1.5 hours prior to coculture) was evaluated on the basis of IFN γ secretion after overnight coculture.

Figure 5.

Isolation of multiple mutated XPNPEP1- and UGGT2-reactive TCRs from a tumor biopsy fragment from patient 3678. **A**, TIL fragment F4 from patient 3678 was cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG9, and recognition was evaluated on the basis of IFN γ ELISPOT and CD137 expression. **B**, CD3⁺ CD8⁺ CD137⁺ cells were sorted by FACS and expanded *in vitro*, and the resulting T-cell population was again cocultured overnight with autologous DCs electroporated with IVT RNA encoding TMG9. Recognition was again evaluated on the basis of IFN γ ELISPOT and CD137 expression. **C**, TCR sequences in genomic DNA from the enriched populations were determined by deep sequencing, and frequencies (%) of productively rearranged sequences were calculated. In addition, single CD8⁺ T cells from the enriched population were sorted, and RT-PCR was conducted to identify TCR α and γ chain pairs. Four different TCR α / γ

chain pairs were cloned into MSGV1 retroviral vectors based on single-cell RT-PCR analysis (indicated by *). **D**, These retroviruses were used to transduce PBL, and transduction efficiencies were measured by staining cells with an anti-murine TCR β constant region antibody. Recognition of TMG9 and two mutated 25 amino acid peptides encoded by TMG9, UGGT2 and XPNPEP1, were evaluated by the TCR-transduced T cells based on IFN γ secretion after overnight coculture with peptide pulsed autologous DCs. In addition, we identified an HLA-A*0301-restricted 11 mer from XPNPEP1 and an HLA-A*0201 restricted 9 mer from UGGT2, both of which were encoded by TMG9. To evaluate the functional avidities of the reactive TCRs, recognition of titrated amounts of these minimal epitopes pulsed onto HLA-matched DCs (~1.5 hours prior to coculture) was evaluated on the basis of IFN γ secretion after overnight coculture.

Table 1.

Screening of patient 3784 sorted populations from fresh tumor digest and TIL fragments for recognition of mutated tandem minigenes (TMGs)

FrTu 3784										TIL 3784											
CD8+			CD8+ PD1−			CD8+ PD1+			Rxl		F2			F4			F5			F6	
ELISPOTS ^a	41BB+ ^b		ELISPOTS	41BB+		ELISPOTS	41BB+		ELISPOTS	41BB+	ELISPOTS	41BB+		ELISPOTS	41BB+	ELISPOTS	41BB+		ELISPOTS	41BB+	
No target	0	0.0	0	0.0	0	0	0.1	0	0.1	9	1.1	3	0.5	0	0.2	2	0.2	0.2			
DC + control TMG	1	0.1	4	0.2	2	0.4	39	0.1	0.1	5	1.7	6	1.9	6	0.4	25	0.4	0.4			
DC + TMG1	0	0.1	1	0.2	0	0.5	1	0.0	0.0	5	1.8	35	1.6	4	0.3	14	0.3	0.3			
DC + TMG2	26	0.2	1	0.2	4	0.3	14	0.1	0.1	5	2.1	100	1.6	1	0.2	11	0.4	0.4			
DC + TMG3	6	0.1	0	0.0		72 ^c	108	0.2	0.2	131	2.8	213	0.9	52	0.5	268	0.4	0.4			
DC + TMG4	2	0.1	1	0.2	9	0.5	53	0.2	0.2	10	1.2	43	0.9			46	0.2	0.2			
DC + TMG5	4	0.1	3	0.2		125	162	0.4	0.4	27	2.3	21	1.1			123	0.7	0.7			
																36	0.2	0.2			
DC + TMG6	0	0.1	2	0.1	2	0.5	128	0.1	0.1	31	2.0	76	0.8	22	0.4	113	0.6	0.6			
DC + TMG7	1	0.0	0	0.2	3	0.2	13	0.0	0.0	4	1.4	15	0.8	7	0.3	26	0.0	0.0			
DC + TMG8	7	0.1	2	0.1		210 ^{d,e}	404	2.4	2.4		265	291	3.1			250	1.7	7.6			
DC + TMG9	0	0.1	1	0.0	3	0.5	24	0.1	0.1	31	1.7	229	1.1	51	0.4	58	0.4	0.4			
DC + MART	4	0.2	3	0.0	30	0.6	8	0.2	0.2	10	0.8	52	0.9	6	0.2	52	0.3	0.3			
DC + gp100	12	0.1	3	0.1		171	235	0.7	0.7	287	1.5	407	2.5	233	1.5	56	0.6	0.6			
DC + tyrosinase	4	0.1	4	0.1	0	0.2	16	0.0	0.0	7	1.0	18	1.0	2	0.2	9	0.3	0.3			
DC + MAGE-A3	0	0.1	1	0.1	3	0.5	29	0.1	0.1	1	1.2	8	0.8	7	0.4	37	0.7	0.7			
DC + NY-ESO-1	1	0.1	2	0.2	3	0.1	34	0.2	0.2	3	1.3	7	0.9	2	0.2	6	0.5	0.5			
DC + SSX2	1	0.0	1	0.1	3	0.3	30	0.1	0.1	3	0.6	22	1.0	3	0.2	5	0.2	0.2			
OKT3	497	70.4	478	68.3	466	35.7	558	87.2	87.2	550	60.3	561	64.4	535	43.5	535	63.7	63.7			

^aIFNγ ELISPOTS (per 2e4 T cells).

^b% 41BB + cells (gated on CD3⁺ CD8⁺ cells).

^cUnderlined values indicate >60 ELISPOTS or >0.5% 41BB+ and >2× background with control TMG and no target.

^dhighlighted cells indicate positive results in both ELISPOT and 41BB assays.

Circled cells indicate populations from which we sorted and expanded T cells for further analysis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2.
Tumor-associated mutation-reactive T-cell receptors identified from patients with melanoma

Patient	TMG ^a	Mutated antigen	TIL Sources ^b	# of independent TCRs and methods used to identify them	Data presentation
3466	1	COL18A1	Rx TIL	1 (5' RACE)	Fig. 1
3713	2	SRPX	Rx TIL	1 (5' RACE)	Fig. 1
3903	9	KIAA1279	FrTu CD8+ PD1+/F1	3 (TCR deep seq and PairSeq)	Fig. 4
3903	3	KIAA1967	FrTu CD8+ PD1+	1 (TCR deep seq)	Supplementary Fig. S10
3903	8	PHKA1	FrTu CD8+ PD1+	1 (TCR deep seq and PairSeq)	Supplementary Fig. S1
3784	5	KIF16B	FrTu CD8+ PD1+	1 (TCR deep seq and PairSeq)	Supplementary Fig. S11
3784	5	KIF16B	F5	1 (TCR deep seq and PairSeq)	Supplementary Fig. S2
3784	5	KIF16B	F6	1 (TCR deep seq and PairSeq)	Fig. 2
3784	8	SON	FrTu CD8+ PD1+	2 (TCR deep seq and PairSeq)	Supplementary Fig. S12
3784	8	SON	FrTu CD8+ PD1+/F2/F6/F5	1 (TCR deep seq and PairSeq)	Supplementary Fig. S3
3784	8	SON	F5	1 (TCR deep seq and PairSeq)	Supplementary Fig. S13
3784	4	GNB5	F5	1 (TCR deep seq and PairSeq)	Supplementary Fig. S4
3678	3	FBXO21	F1/F3/F4	1 (TCR deep seq)	Supplementary Fig. S5
3678	2	CORO7	F1/F2	1 (TCR deep seq)	Supplementary Fig. S6
3678	7	RECQL5	F3	1 (TCR deep seq)	Supplementary Fig. S7
3678	7	RECQL5	F6	1 (TCR deep seq)	Supplementary Fig. S8
3678	9	UGGT2	F4	2 (TCR deep seq and SC RT-PCR)	Fig. 5
3678	9	XPNPEP1	F4/F6	2 (TCR deep seq and SC RT-PCR)	Fig. 5
3716	3	TFDP2	F3	3 (TCR deep seq and PairSeq)	Fig. 3
3716	3	TFDP2	PF2	1 (TCR deep seq and PairSeq)	Supplementary Fig. S9

^aTMG: Tandem minigene construct against which reactivity was identified.

^bThree different types of TIL products were used as sources for the identification of mutation reactive TCRs: Rx TIL refers to *in vitro* expanded TIL that had been used to treat the patient; TIL denoted with F or PF refers to TIL from fragments from tumor biopsies that had been expanded *in vitro* with IL2; and FrTu CD8⁺ PD1⁺ refers to TIL from fresh tumor digests that had been FACS sorted for CD8⁺ PD1⁺ T cells and expanded *in vitro* in the presence of irradiated feeders, OKT3, and IL2.