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LETTERS

Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells

Paul F Robbins[1], Yong-Chen Lu[1], Mona El-Gamil[1], Yong F Li[1], Colin Gross[1], Jared Gartner[2], Jimmy C Lin[3], Jamie K Teer[4,5], Paul Cliften[3], Eric Tycksen[3], Yardena Samuels[2,5] & Steven A Rosenberg[1]

Substantial regressions of metastatic lesions have been observed in up to 70% of patients with melanoma who received adoptively transferred autologous tumor-infiltrating lymphocytes (TILs) in phase 2 clinical trials[1,2]. In addition, 40% of patients treated in a recent trial experienced complete regressions of all measurable lesions for at least 5 years following TIL treatment[3]. To evaluate the potential association between the ability of TILs to mediate durable regressions and their ability to recognize potent antigens that presumably include mutated gene products, we developed a new screening approach involving mining whole-exome sequence data to identify mutated proteins expressed in patient tumors. We then synthesized and evaluated candidate mutated T cell epitopes that were identified using a major histocompatibility complex–binding algorithm[4] for recognition by TILs. Using this approach, we identified mutated antigens expressed on autologous tumor cells that were recognized by three bulk TIL lines from three individuals with melanoma that were associated with objective tumor regressions following adoptive transfer. This simplified approach for identifying mutated antigens recognized by T cells avoids the need to generate and laboriously screen cDNA libraries from tumors and may represent a generally applicable method for identifying mutated antigens expressed in a variety of tumor types.

The identification of antigens associated with tumor rejection mediated by TILs has been challenging given the diversity of these bulk lymphocyte populations and the relatively laborious nature of current antigen screening approaches. We developed a new screening method to identify mutated candidate epitopes that involves whole-exome sequencing of tumor and matched normal cell DNA to identify nonsynonymous somatic mutations. Using this method, we screened high-affinity candidate T cell epitopes for recognition by TILs that we identified *in silico* by scanning 19-amino-acid polypeptides centered on mutated residues with a peptide–major histocompatibility complex (MHC) binding algorithm[4]. We focused on identifying T cell epitopes presented in the context of HLA-A class I gene products, which were previously found to be expressed at higher levels in melanomas than HLA-B and HLA-C products[5], in three individuals with metastatic melanoma who demonstrated regression of bulky metastatic lesions after adoptive transfer of autologous TILs.

We first subjected DNA isolated from the melanoma cell line 2098 mel, which was generated from a metastatic lesion in an individual who was homozygous for the highly prevalent HLA class I allele *HLA-A*0201*, to whole-exome sequencing. We then synthesized the top 55 mutated candidate nonamer and decamer peptides identified from 2098 mel cells that were predicted to bind with high affinity to HLA-A*0201 and evaluated them for their ability to sensitize the HLA-A*0201–positive cell line T2 (ref. 6) for recognition by autologous TILs (termed here TIL 2098). The results indicated that four peptides, peptides ranked fifth, eighteenth, nineteenth and thirty-eighth in the predicted high-affinity HLA-A*02:01 binders, reproducibly stimulated the release of amounts of interferon-γ (IFN-γ) from TIL 2098 that were comparable to those released after stimulation by autologous tumor cells (10,000 pg ml^{-1} in the experiment detailed in **Fig. 1a** and **Supplementary Table 1**). The low amounts of IFN-γ that were detected in response to additional peptides depicted in **Figure 1**, as well as peptides 39–62 (detailed in **Supplementary Table 1**), were not observed in additional experiments (data not shown) and were not evaluated further. The peptides ranked 5 and 18 represent an overlapping nonamer and decamer that correspond to residues 26–34 and 26–35, respectively, of the casein kinase 1, α 1 protein (CSNK1A1), a key regulator of the Wnt–β-catenin signaling pathway[7]. A dinucleotide substitution of TA for CC at positions 80 and 81 of the wild-type (WT) *CSNK1A1* coding region resulted in a serine-to-leucine change at position 27 in the WT protein. The peptide ranked 19 is encoded by a point-mutated transcript of the growth arrest specific 7 gene (*GAS7*), which was previously identified as a mutated target of TIL 2098 using a conventional cDNA library screening approach[8], and the peptide ranked 38 corresponded

[1]Surgery Branch, National Cancer Institute, National Institutes of Health (NIH), Bethesda, Maryland, USA. [2]Cancer Genetics Branch, National Human Genome Research Institute (NHGRI), NIH, Bethesda, Maryland, USA. [3]Washington University School of Medicine, Genome Technology Access Center, Genomics and Pathology Services, St. Louis, Missouri, USA. [4]Genetic Disease Research Branch, NHGRI, NIH, Bethesda, Maryland, USA. [5]Present address: H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA, (J.K.T.) and Weizmann Institute of Science, Rehovot, Israel (Y.S.). Correspondence should be addressed to P.F.R. (paulrobbins@mail.nih.gov).

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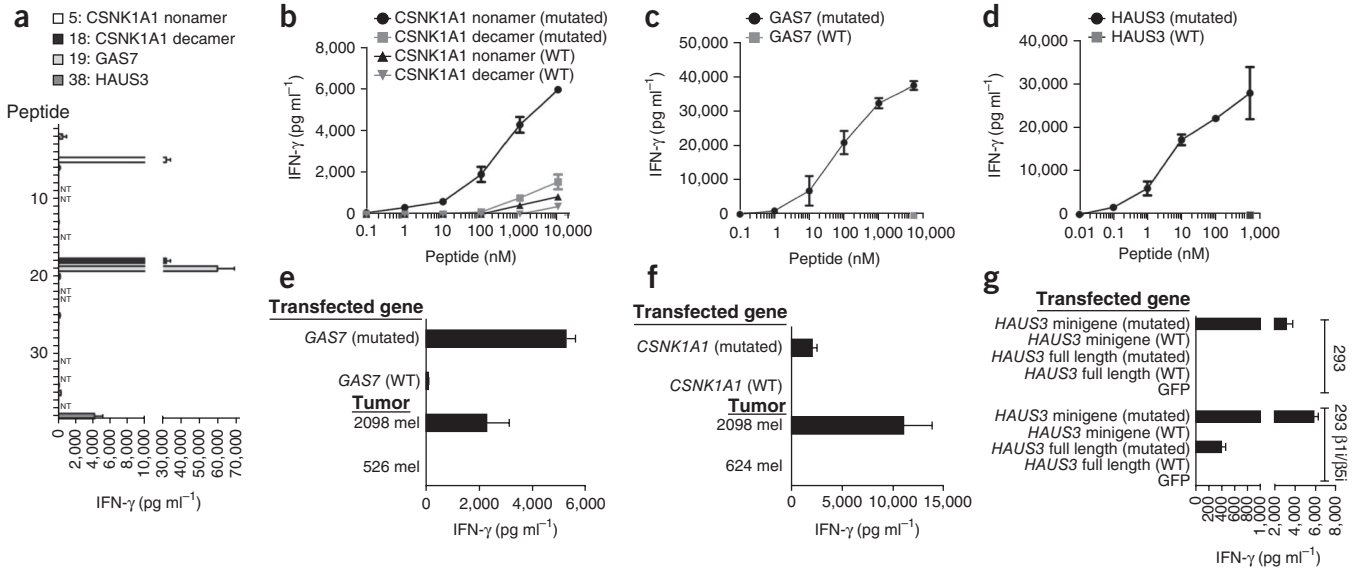

Figure 1 Response of TIL 2098 to candidate epitopes identified from autologous tumors. (**a**) A screening assay was carried out to evaluate the release of IFN-γ from TIL 2098 in an overnight coculture with peptide-pulsed T2 cells that were pulsed individually with the top 61 candidate HLA-A*0201–binding peptides (**Supplementary Table 1**) identified from 2098 mel cells, with the exception of the peptides ranked 9, 10, 14, 23, 24, 31, 33 and 37, which overlapped with the peptides ranked 1, 4, 4, 25, 12, 15, 16 and 4, respectively, and were not tested (NT). (**b**–**d**) IFN-γ release measured after overnight coculture of TIL 2098 with T2 cells that were pulsed with titered doses of the indicated mutated or WT peptides. (**e**) IFN-γ release measured after overnight coculture of TIL 2098 with COS-A2 cells expressing *HLA-A*0201* that were transiently transfected with the indicated transcripts. (**f**) IFN-γ release measured after overnight coculture of TIL 2098 with HEK293 cells that were transiently transfected with a construct encoding *HLA-*0201* plus the indicated transcripts. (**g**) IFN-γ release measured after overnight coculture of TIL 2098 with HEK293 cells (top bars) or HEK293 cells that were stably transfected with the β1i and β5i immunoproteasomal subunits (designated 293 β1i/β5i; bottom bars). The data are presented as the mean ± s.e.m. of duplicate wells.

to residues 154–162 of the HAUS augmin-like complex, subunit 3 protein (HAUS3; **Fig. 1a**), a molecule that is involved in microtubule formation within the mitotic spindle[9]. Peptide titration assays demonstrated that TIL 2098 was highly reactive with HLA-A*0201–positive target cells pulsed with the mutated CSNK1A1 nonamer, which was recognized at a minimum concentration of 1 nM (**Fig. 1b**). The substantially weaker responses detected against the mutated decamer, as well as the wild-type nonamer and decamer peptides, were only observed when target cells had been pulsed with between 100-fold and 10,000-fold higher peptide concentrations than those required for recognition of the mutated CSNK1A1 nonamer peptide, which therefore seems to represent the naturally processed epitope recognized by TIL 2098 (**Fig. 1b**). The change at position 2 in the CSNK1A1 peptides from serine to leucine, which represents an optimal residue at one of the primary anchor positions in HLA-A*0201–binding peptides[10], enhanced the predicted binding affinities of the CSNK1A1 peptides from >1 μM to ~10 nM (**Supplementary Table 1**). Similarly, TIL 2098 recognized targets pulsed with as little as 1 nM of the mutated GAS7 peptide and 0.1 nM of the mutated HAUS3 peptide and did not recognize the corresponding WT peptides (**Fig. 1c**,**d**). The change in the HAUS3 peptide was not predicted to alter the binding affinity of the mutated peptide, and the change in the GAS7 peptide was predicted to lead to a modest increase in binding affinity (from 39 nM to 12 nM), indicating that the mutated amino acids in the HAUS3 and GAS7 peptides may predominantly represent T cell contact residues.

We then transfected antigen-negative HLA-A*0201–positive target cells with genes encoding mutated epitopes to evaluate their ability to be naturally processed and presented. TIL 2098 recognized *HLA-A*0201*–transduced COS-7 (COS-A2) cells transiently transfected with mutated but not WT CSNK1A1 and GAS7 constructs (**Fig. 1e**,**f**) but did not recognize HEK293 cells that were transiently transfected with *HLA-A*0201* (293-A2 cells) plus the full-length mutated *HAUS3* construct (**Fig. 1g**). TIL 2098 did, however, recognize 293-A2 cells that were transfected with a minigene construct encoding a methionine followed by the nine amino–mutated HAUS3 epitope (**Fig. 1g**), indicating that this may be an epitope that is preferentially processed by either the immunoproteasome[11,12] or recently described intermediate proteasomes that contain one or two of the three catalytic subunits of the immunoproteasome[13]. Further analysis demonstrated that TIL 2098 recognized HEK293 cells expressing the β1i and β5i immunoproteasomal subunits, which were transiently transfected with constructs encoding HLA-A*0201 plus either the full-length mutated *HAUS3* gene or the minimal mutated *HAUS3* epitope, but not constructs that encoded the corresponding WT *HAUS3* gene products (**Fig. 1g**).

We then sought to identify mutated antigens recognized by TIL 2369, which are derived from an individual with melanoma who expressed HLA-A*0101 and HLA-A*2601, by co-incubating TIL 2369 with HLA-A*0101–positive target cells that we pulsed individually with the top 53 mutated candidate HLA-A*0101–binding peptides identified from 2369 mel cells (**Supplementary Table 2**). A mutated decamer encoded by the pleckstrin homology domain containing, family M member 2 gene (*PLEKHM2*) that had the second highest affinity of the predicted HLA-A*0101–binding peptides, as well as two overlapping mutated peptides (a nonamer and a decamer) encoded by the protein phosphatase 1, regulatory subunit 3B gene (*PPP1R3B*) that were the seventeenth and twenty-third highest predicted binders of HLA-A*0101, stimulated the release of high amounts of IFN-γ from TIL 2369 (**Fig. 2a** and **Supplementary Table 2**). These amounts of IFN-γ were comparable to those stimulated by the autologous 2369 mel (approximately 21,000 pg ml^{-1} of IFN-γ in this experiment), indicating

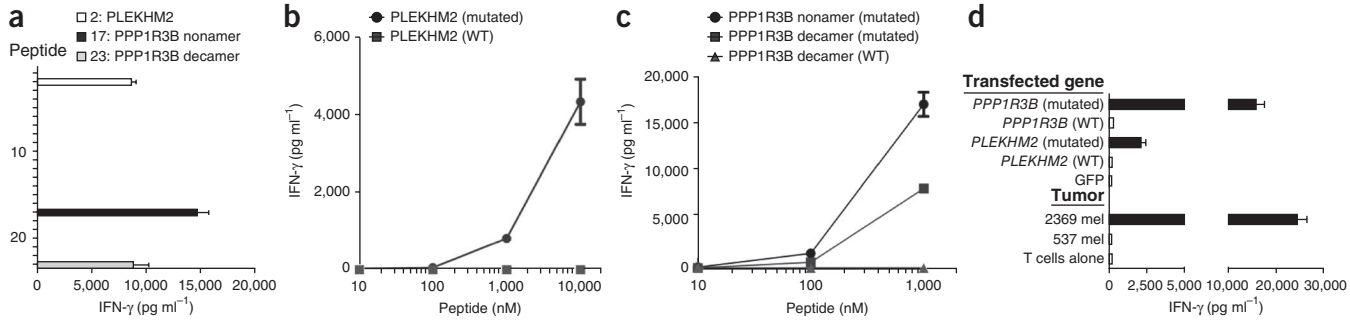

Figure 2 Response of TIL 2369 to candidate epitopes identified from autologous tumors. (**a**) A screening assay was carried out to evaluate the release of IFN-γ from TIL 2369 in an overnight coculture with peptide-pulsed HEK293 cells transduced with a recombinant retroviral expression plasmid encoding *HLA-A*01* (293-A1 cells) that were pulsed individually with the top 56 candidate HLA-A*0101–binding peptides identified from 2369 mel cells (**Supplementary Table 2**), with the exception of peptides 35, 39 and 44, which overlapped with peptides 20, 33 and 43, respectively, and were not tested. (**b**,**c**) IFN-γ release measured after overnight coculture of TIL 2369 with 293-A1 cells that were pulsed with the indicated concentrations of peptides. (**d**) IFN-γ release measured after overnight coculture with TIL 2369 from 293-A1 cells that were transiently transfected with the indicated constructs. The data are presented as the mean ± s.e.m. of duplicate wells.

that the mutated PLEKHM2 and PPP1R3B epitopes may represent dominant targets of this TIL. TIL 2369 recognized target cells pulsed with a minimum of 1.0 μM of the mutated PLEKHM2 peptide (**Fig. 2b**) and 0.1 μM of the overlapping PPP1R3B peptides (**Fig. 2c**) but failed to recognize cells pulsed with 10 μM of the corresponding WT peptides (**Fig. 2b**,**c**). TIL 2369 also recognized antigen-negative HLA-A*0101–positive cells transfected with cDNAs encoding either the mutated *PPP1R3B* or *PLEKHM2* gene products but not the corresponding WT products (**Fig. 2d**). TIL 2369 did not recognize any of the top 46 mutated candidate peptides that were predicted to bind to HLA-A*2601 with affinities ranging between 4 nM and 259 nM (**Supplementary Table 3**).

We further evaluated this approach by determining the ability of TILs isolated from a third individual with melanoma, TIL 3309, to recognize mutated candidate peptides identified from 3309 mel cells that were predicted to bind to either of the autologous class I *HLA-A* gene products expressed by this individual, *HLA-A*0101* or *HLA-A*1101*. TIL 3309 did not recognize any of the top 29 candidate HLA-A*0101–binding mutated peptides identified by exomic sequencing of the autologous melanoma (**Supplementary Table 4**); however, four of the top 46 candidate HLA-A*1101–binding mutated peptides identified in this approach, which included overlapping nonamer and decamer products of the matrilin 2 (*MATN2*) and cyclin-dependent kinase 12 (*CDK12*) genes, were recognized by TIL 3309 (**Fig. 3a** and **Supplementary Table 5**). In the same assay, autologous 3309 mel cells stimulated the release of 4,600 pg ml^{-1} of IFN-γ from TIL 3309. The MATN2 protein is a member of the von Willebrand factor A domain–containing family and may have a role in extracellular matrix formation[14], and CDK12 has been shown to regulate the expression of DNA damage response genes[15]. The mutated MATN2 nonamer and decamer had the second and fourth highest predicted binding affinities, respectively, of the candidate HLA-A*1101–binding peptides, and the mutated CDK12 nonamer and decamer had the twenty-fourth and thirty-sixth highest predicted HLA-A*1101 binding affinities (**Supplementary Table 5**). The mutated MATN2 nonamer and decamer stimulated responses at minimum concentrations of 1 nM, whereas the corresponding WT MATN2 peptides did not stimulate the release of detectable amounts of cytokine from these TILs at the highest concentration tested, 1 μM (**Fig. 3b**). TIL 3309 also recognized targets pulsed with a minimum of 1 nM and

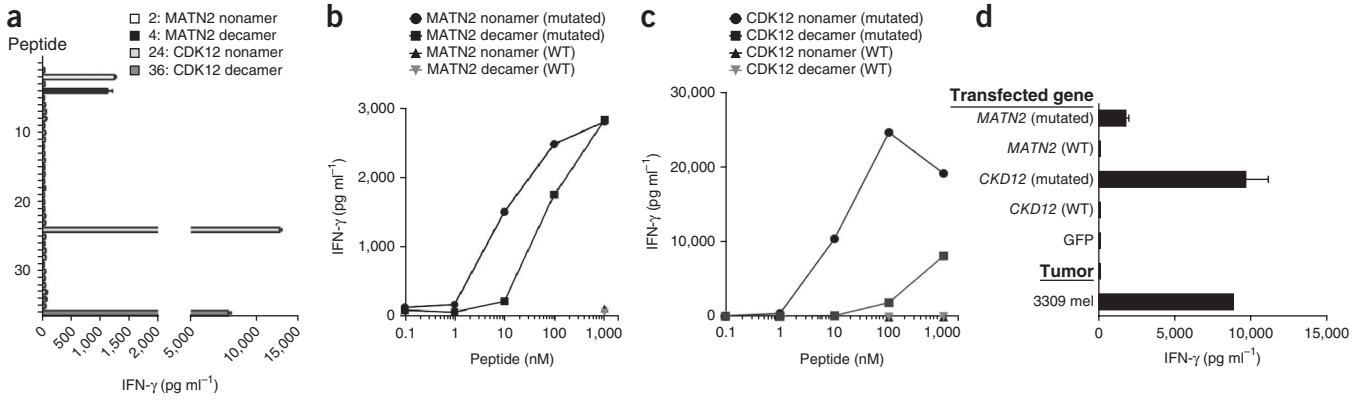

Figure 3 Response of TIL 3309 to candidate epitopes identified from autologous tumors. (**a**) A screening assay was carried out to evaluate the release of IFN-γ from TIL 3309 in an overnight coculture with COS-7 cells stably transduced with a retroviral vector expressing *HLA-A*1101* (COS-A11 cells) that were pulsed individually with the top 46 candidate HLA-A*1101–binding peptides identified from 3309 mel cells (**Supplementary Table 5**). (**b**,**c**) IFN-γ release measured after overnight coculture of TIL 3309 with COS-A11 cells that were pulsed with the indicated concentrations of peptides. (**d**) IFN-γ release measured after overnight coculture of TIL 3309 with COS-A11 cells that were transiently transfected with the indicated constructs. The data are presented as the mean ± s.e.m. of duplicate wells.

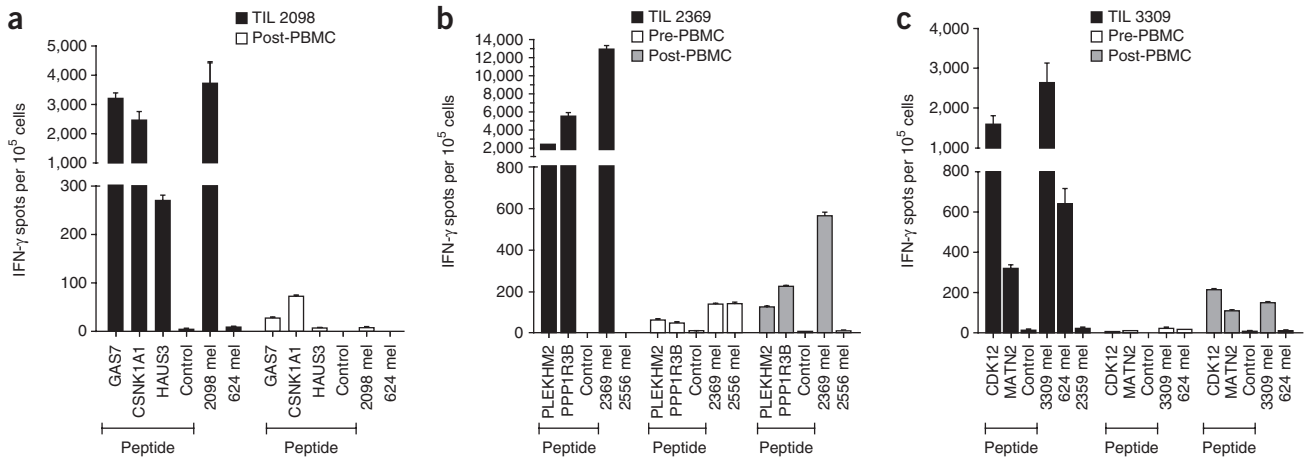

Figure 4 IFN-γ ELISPOT responses of TIL and PBMCs obtained before and after autologous TIL transfer. (**a**–**c**) IFN-γ spots per 10^5 cells of TIL 2098 from subject 1 cultured with HLA-A*0201–positive COS-7 cells (**a**), TIL 2369 from subject 2 cultured with HLA*0101–positive COS-7 cells (**b**) and TIL 3309 from subject 3 cultured with HLA*1101–positive COS-7 cells (**c**). Pre-PBMC, PBMCs obtained before transfer; post-PBMC, PBMCs obtained after transfer. Data are shown as the mean ± s.e.m. of three replicate wells per group. Control groups represented cells that were not pulsed with exogenous peptides.

100 nM of the mutated CDK12 nonamer and decamer, respectively, but did not recognize targets pulsed with 1 μM of the corresponding WT peptides (**Fig. 3c**). In addition, HLA-A*1101–positive target cells transfected with the mutated but not the corresponding WT *MATN2* and *CDK12* gene products stimulated the release of high amounts of IFN-γ from TIL 3309 (**Fig. 3d**).

We then evaluated the expression of genes encoding mutated candidate epitopes by amplifying partial cDNA transcripts from tumor cell RNA using primers that flanked the putative mutation sites. In addition, we subjected amplified transcripts to Sanger sequencing to validate the results from whole-exome sequencing. Using total RNA isolated from 2098 mel cells as a template, we successfully amplified partial cDNA transcripts that encoded 19 of the 62 candidate mutated HLA-A*0201–binding peptides identified by whole-exome sequencing of 2098 mel cells, whereas three of the amplified products seemed to exclusively encode the WT peptide and not the expected peptide variant (**Supplementary Tables 1** and **6**). We also successfully amplified transcripts encoding 27 of the top 56 candidate mutated high-affinity HLA-A*0101–binding peptides identified from 2369 mel, although one of the amplified products seemed to exclusively encode the WT rather than the expected mutated residue (**Supplementary Tables 2** and **6**). In addition, we were able to amplify transcripts encoding 28 of the 46 candidate mutated *HLA-A*1101*–binding peptides evaluated for recognition by TIL 3309 from 3309 mel cells, although one of the amplified products seemed to exclusively encode the WT and not the expected mutated residue (**Supplementary Tables 5** and **6**). Combining the peptide prediction results with gene expression analysis demonstrated that TIL 2098 and TIL 2369 recognized the mutated peptides with the highest predicted binding affinity for HLA-A*0201 and HLA-A*0101, respectively, and TIL 3309 recognized the peptide with the second highest predicted binding affinity for HLA-A*1101 among the proteins expressed by autologous tumor cells (**Supplementary Table 7**). Taken together, these results indicate that TILs may generally recognize mutated epitopes that bind with relatively high affinities to MHC class I molecules.

We then evaluated the contribution of T cells recognizing mutated targets to *in vitro* and *in vivo* antitumor responses using enzyme-linked immunosorbent spot (ELISPOT) assays on infused TIL and samples of peripheral blood mononuclear cells (PBMCs) obtained before therapy, which were only available for subjects 2 and 3, as well as samples that were obtained from all three subjects after therapy. The responses of TIL 2098 to HLA-A*0201–positive target cells that we pulsed with either the mutated GAS7 or mutated CSNK1A1 nonamer peptide were comparable to those directed against the autologous tumor, whereas we observed lower but detectable cytokine amounts in response to the mutated HAUS3 peptide (**Fig. 4a**). Although the relatively high amounts of peptide that are presumably present on peptide-pulsed target cells may represent a more effective T cell stimulus than those generated by endogenous processing, the ELISPOT response of TIL 2098 to the 2098 mel cell line was nearly 60% of that generated in response to a polyclonal activator, indicating that 2098 mel cells represent potent activators of the autologous TILs. Pretreatment samples were not available for the first subject, but the responses against the mutated GAS7 and CSNK1A1 peptides in PBMCs from this individual obtained approximately 1 month after transfer may have reflected low levels of persistence of adoptively transferred T cells recognizing these antigens (**Fig. 4a**). These results, taken together with the lack of response against *HLA*-matched melanomas (**Fig. 4a** and ref. 8), indicate that the mutated T cell epitopes identified using this approach may represent dominant targets of TIL 2098.

Analysis of responses in subject 2 revealed that the response of TIL 2369 to HLA-A*0101–positive cells that we pulsed with the mutated PPP1R3B nonamer was approximately 50% of that directed against autologous 2369 mel cells (**Fig. 4b**). In addition, we observed substantial responses against targets pulsed with the mutated PLEKHM2 epitope (**Fig. 4b**). TIL 2369 did not recognize allogeneic melanoma cell lines that shared expression of *HLA-A*01* (**Fig. 2d**) or *HLA-A*01* and *HLA-A*26* with 2369 mel cells (**Fig. 4b**), which, taken together with the robust response against the mutated PPP1R3B and PLEKHM2 peptides, indicated that TIL 2369 may predominantly recognize mutated gene products. Subject 2 had not responded clinically to an initial treatment with autologous TILs, which was administered in the absence of nonmyeloablative chemotherapy, although a peripheral blood sample obtained ~1 month after this treatment contained low numbers of T cells that recognized the mutated PLEKHM2

and PPP1R3B peptides, as well as autologous tumor cells. A PBMC sample collected from subject 2 following a second treatment with autologous TILs, which was administered following nonmyeloablative conditioning, was then analyzed for the presence of peptide as well as tumor-reactive T cells. The results indicated that this sample contained higher numbers of T cells recognizing the PLEKHM2 and PPP1R3B epitopes, as well as the autologous tumor 2369, than those that were observed after the first treatment. The enhanced persistence that we observed after the second treatment may have been influenced by the pretreatment regimen that this patient received prior to the second TIL transfer; however, we cannot rule out the possibility that some of the T cells observed at this time were derived from the first treatment.

An evaluation of ELISPOT responses in the third subject revealed that the response of autologous TIL 3309 to HLA-A*1101–positive target cells pulsed with the mutated CDK12 epitope was approximately 60% of that directed against autologous 3309 mel cells, whereas the response to the MATN2 peptide was approximately 10% of that directed against 3309 mel cells. TIL 3309 also strongly recognized an allogeneic melanoma, 624 mel, that shared expression of *HLA-C*07* with autologous cells (**Fig. 4c**), indicating that these T cells also probably recognized a shared epitope. Responses against the CDK12 and MATN2 peptides, as well as against autologous 3309 mel cells, were detected in the peripheral blood of this third individual 1 month after autologous TIL transfer (**Fig. 4c**), at a time when $CD8^+$ T cells comprised approximately 90% of the $CD3^+$ T cells in the peripheral blood. Reactivity against the CDK12 and MATN2 peptides, however, was undetectable in peripheral blood obtained before adoptive TIL transfer (**Fig. 4c**), and thus any increase in the percentage of $CD8^+$ T cells reactive with these epitopes reflected an increase in their absolute number.

Antigens recognized by tumor-reactive T cells have previously been identified by a variety of approaches, including screening of antigen-negative target cells transfected with individual cDNAs[16,17] or cDNA pools[18,19] generated from tumor cell lines, mass spectrometric analyses of peptides eluted from the surface of tumor cells[20] and proteomic analysis of tumor cell lysates[21]. More than 400 T cell epitopes, including over 50 mutated epitopes that were identified primarily from clonal or bulk populations of melanoma-reactive T cells, have been identified using these approaches[22]. The approach we describe here complements cumbersome cDNA library cloning methodologies, which can be influenced by factors such as the size and expression levels of transcripts that encode T cell epitopes.

A mutated tumor rejection antigen was recently identified by whole-exome sequencing of a methylcholanthrene-induced sarcoma that arose in a *Rag2*$^{-/-}$ mouse[23]. In addition, whole-exome sequencing of B16F10 mouse melanoma cells led to the identification of immunogenic mutated peptides[24]. Immunization with two of the peptides identified using this approach resulted in partial control of the *in vivo* growth of B16F10 tumor cells. These results support the potential utility of this method for identifying clinically relevant tumor antigens.

We have begun to evaluate the potential correlation between reactivity to mutated antigens and clinical response to adoptive TIL transfer. Here three therapeutic TILs contained dominant populations of T cells reactive with mutated epitopes that persisted in the peripheral blood for at least 1 month after adoptive transfer. Reactivity to mutated antigens appears to be of high importance, as only a relatively small portion of T cells present in bulk TIL populations from 34 individuals with melanoma appeared in a recent study to recognize melanocyte differentiation antigens and cancer germline antigens[25]. The apparent dominance of responses against mutated antigens, particularly those observed in subjects 1 and 2, coupled with the persistence of T cells reactive with these epitopes in peripheral blood following adoptive transfer suggests that T cells present in these TILs that recognize mutated epitopes may have played a part in the tumor regressions observed in these patients. Whole-exome sequencing was also carried out on tumors isolated from two additional patients who did not respond to autologous TIL transfer (data not shown). Using the approach described above, we identified a single mutated epitope targeted by one of the two additional TIL types that we evaluated. In summary, whole-exome sequencing of tumor cell DNA, followed by the use of MHC binding algorithms, has led to the identification of eight mutated epitopes recognized by four of the five melanoma TILs we have evaluated to date. This relatively simple and rapid genomic approach should provide the means to further explore the relationship between the clinical efficacy of adoptively transferred melanoma-reactive TILs and their ability to recognize mutated T cell epitopes. In addition, mutated antigens identified using this approach can potentially be used to carry out *in vitro* sensitization of cells from patient peripheral blood that can be further expanded *in vitro* for use in patient adoptive transfer protocols, as well as providing potential cancer vaccine targets. Whole-exome sequencing approaches may also provide an opportunity to extend these therapeutic approaches to a variety of additional tumor types.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.F.R. designed and developed the experimental screening system, analyzed data and drafted the manuscript. Y.-C.L. and M.E.-G. performed experiments evaluating TIL responses against candidate mutated peptides and analyzed results. Y.F.L. cloned and sequenced gene products encoding candidate epitopes identified by exomic sequencing and analyzed results. J.K.T., C.G., E.T., J.C.L. and P.C. carried out bioinformatic analyses. J.G. provided advice on exomic sequencing, prepared samples for sequencing and carried out validation studies using Sanger sequencing. Y.S. provided advice on sequencing of DNA isolated from tumor and normal cells and assisted with data analysis. S.A.R. helped design the studies and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Subject 1, a 53-year-old female with extensive refractory metastatic melanoma to the lung and lower extremity, received autologous TILs (TIL 2098) in 2003 and underwent a complete regression of all metastatic melanoma that was sustained until her death from unrelated ovarian cancer 6 years later. Subject 2, a 32-year-old male with metastatic melanoma to the brain, liver and periportal lymph nodes refractory to previous treatment, received autologous TIL (TIL 2369) in 2005 and experienced a complete cancer regression that is still ongoing 6.5 years later. Subject 3, a 27-year-old female with metastatic melanoma to the brain, lung, thigh and popliteal fossa, received autologous TILs (TIL 3309) in August 2009 and had a nearly complete regression of all lesions but then developed recurrent thigh and chest wall lesions 6 months after therapy. All patients were enrolled in a clinical trial and signed an informed consent form that had been approved by the Institutional Review Board of the US National Cancer Institute.

Whole-exome sequencing. Genomic DNA purification, library construction, exome capture of approximately 20,000 coding genes and next-generation sequencing of tumor and normal samples were performed at Personal Genome Diagnostics (Baltimore, MD). Bioinformatic analyses were carried out by Personal Genome Diagnostics and the Genome Technology Access Center, Genomics and Pathology Services of the Washington University School of Medicine. In brief, genomic DNA from tumor and normal samples was fragmented and used for Illumina TruSeq library construction (Illumina, San Diego, CA). Exonic regions were captured in solution using the Agilent SureSelect 50 Mb kit (version 3) according to the manufacturer's instructions (Agilent, Santa Clara, CA). Paired-end sequencing, resulting in 100 bases from each end of each fragment, was performed using a HiSeq 2000 Genome Analyzer (Illumina, San Diego, CA). Sequence data were mapped to the reference human genome sequence, and sequence alterations were determined by comparison of over 50 million bases of tumor and normal DNA. Over 8 billion bases of sequence data were obtained for each sample, and a high fraction of the bases were from the captured coding regions. Over 43 million bases of target DNA were analyzed in the tumor and normal samples, and an average of 42–51 reads were obtained at each base in the normal and tumor DNA samples. The tags were aligned to the human genome reference sequence (hg18) using the Eland algorithm of the CASAVA 1.6 software (Illumina, San Diego, CA). The chastity filter of the BaseCall software of Illumina was used to select sequence reads for subsequent analyses. The ELANDv2 algorithm of the CASAVA 1.6 software was then applied to identify point mutations and small insertions and deletions. Known polymorphisms recorded in dbSNP were removed from the analysis. Potential somatic mutations were filtered and visually inspected as described previously[26]. The DNA isolated from the 2098 mel, 2369 mel and 3309 mel cell lines had 264, 574 and 278 combined nonsynonymous single-nucleotide and dinucleotide substitutions, respectively. Between 80 and 90% of the nonsynonymous point mutations identified in the 2098 mel, 2369 mel and 3309 mel tumor cell lines represented C/G to T/A transitions, consistent with the role of ultraviolet light in generating the majority of mutations found in nonacral melanomas[27,28].

Analysis of gene expression. Oligonucleotide primers were designed to amplify fragments of gene products ranging between ~100 and ~600 nucleotides encompassing the mutated epitopes in 2098 mel, 2369 mel and 3309 mel cells (**Supplementary Table 6**). These primers sets were used to carry out RT-PCR, as previously described[19], from approximately 1 μg of total RNA that was isolated from the appropriate tumor cell lines using RNeasy (Qiagen Inc., Valencia, CA). Amplified cDNA transcripts were either directly sequenced after purification of the RT-PCR products using the PureLink PCR Purification Kit (Life Technologies, Grand Island, NY) or cloned into the pCDNA3.1 TOPO TA plasmid vector (Life Technologies) and sequenced using the T7 oligonucleotide primer 5′-TAATACGACTCACTATAGGG-3′.

Peptides. The residues surrounding the amino acids resulting from nonsynonymous mutations were scanned to identify candidate nonamer and decamer peptides that were predicted to bind with high affinity to individual HLA class I alleles using the NetMHCPan2.4 binding algorithm[4]. Peptides were obtained from PiProteomics (Huntsville, AL) or Peptide 2.0 (Chantilly, VA). Mutated peptides identified by this approach were then purified to greater than 95% homogeneity by HPLC and re-evaluated to confirm their ability to stimulate T cell responses.

Analysis of T cell responses. TIL 2098, TIL 2369 and TIL 3309 were expanded from fresh tumor digests as previously described[29,30]. The reactivity of TIL 2098 was evaluated by incubating HLA-A*02:01–positive T2 cells with antigen-processing defects that allow for the efficient loading of exogenous peptides[31] with candidate HLA-A*02:01–binding peptides at a concentration of 10 μM for 2 h at 37 °C followed by two washing steps. Endogenous processing of the epitopes recognized by TIL 2098 was evaluated in HEK293 cells that were co-transfected with 200 ng of a recombinant pCDNA3.1 plasmid (Life Technologies, Grand Island, NY) encoding candidate epitopes and 50 ng of a recombinant pCDNA3.1 plasmid encoding HLA-A*02:01, and the amounts of soluble IFN-γ released from the TILs cultured overnight with peptide-pulsed target cells, transfectants or tumor cells were measured, as previously described[19]. Evaluation of the responses of TIL 2098 to the WT and mutated GAS7 and C4orf15 constructs was carried out in HEK293 cells expressing the β1i and β5i immunoproteasomal subunits. The HLA-A*01:01–positive tumor cell line 537 mel, which is not recognized by TIL 2369, and the HLA-A*02:01–positive tumor cell line 624 mel, which is not recognized by TIL 2098, were the negative controls for T cell reactivity.

Evaluation of the peptide reactivity of TIL 2369 was carried out by pulsing 293-A1 cells with candidate *HLA-A*01:01*–binding peptides at a concentration of 10 μM for 2 h at 37 °C followed by two washing steps. To determine whether the epitopes identified as targets of TIL 2369 were endogenously processed and presented, 293-A1 target cells were transiently transfected with either the genes encoding the mutated candidates or the corresponding WT genes. Evaluation of the reactivity of TIL 3309 was carried out by pulsing COS-7 cells that had been stably transduced with retroviral constructs encoding either *HLA-A*0101* or *HLA-A*1101* with candidate HLA-A*01– or HLA-A*11–binding peptides at a concentration of 10 μM for 2 h at 37 °C followed by two washing steps. To determine whether the epitopes identified as targets of TIL 3309 were endogenously processed and presented, COS-A11 target cells were transiently transfected with either the genes encoding the mutated candidates or the corresponding WT genes, and the amounts of soluble IFN-γ released from TILs cultured overnight with target cells were measured as previously described[19]. The responses directed against tumor cell lines and peptide-pulsed target cells were quantified in an IFN-γ ELISPOT assay using 96-well PVDF-membrane filter plates (EMD Millipore, Billerica, MA) coated with 15 μg/ml of the monoclonal anti–IFN-γ antibody 1D1K (Mabtech, Inc., Cincinnati, OH). Bound cytokine was detected using 1 μg/ml of the biotinylated anti–IFN-γ antibody 7-B6-1 (Mabtech). ELISPOT assays were carried out by thawing T cells overnight in media lacking exogenous cytokines followed by culturing with tumor cells or COS-7 cells expressing *HLA-A*0201*, *HLA-A*0101* or *HLA-A*1101* that were pulsed with candidate peptides for 2 h at 37 °C. Cells were also incubated in parallel overnight in complete medium containing 50 ng/ml PMA plus 1 μM ionomycin (PMA/I) to provide an estimate of the maximum number of IFN-γ–secreting cells in these populations. The numbers of IFN-γ ELISPOT responses per 10^5 T cells generated in responses to PMA/I were as follows: subject 1 TIL 2098: 7,300, subject 1 PBMCs after transfer: 4,700; subject 2 TIL 2369: 21,800, subject 2 PBMCs before transfer: 21,800, subject 2 PBMCs after transfer: 11,000; subject 3 TIL 3309: 7,700, subject 3 PBMCs before transfer: 12,500, subject 3 PBMCs after transfer: 26,300. TIL 2098 were plated at 2,000 cells per well, TIL 2369 and 3309 were plated at 1,000 cells per well and PBMCs were plated at 100,000 cells per well. All of the groups stimulated with PMA/I were plated at 1,000 cells per well.

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Corrigendum: Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells

Paul F Robbins, Yong-Chen Lu, Mona El-Gamil, Yong F Li, Colin Gross, Jared Gartner, Jimmy C Lin, Jamie K Teer, Paul Cliften, Eric Tycksen, Yardena Samuels & Steven A Rosenberg

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In the version of this article initially published online, the second sentence of the abstract stated, "In addition, 40% of patients treated in a recent trial experienced complete regressions of all measurable lesions lasting between 5 and 9 years after treatment[3]." The correct statement should read, "In addition, 40% of patients treated in a recent trial experienced complete regressions of all measurable lesions for at least 5 years following TIL treatment[3]." The error has been corrected for the print, PDF and HTML versions of this article.