Tumor-infiltrating BRAF\textsuperscript{V600E}-specific CD4\textsuperscript{+} T cells correlated with complete clinical response in melanoma

Joshua R. Veatch, …, William W. Kwok, Stanley R. Riddell


T cells specific for neoantigens encoded by mutated genes in cancers are increasingly recognized as mediators of tumor destruction after immune checkpoint inhibitor therapy or adoptive cell transfer. Unfortunately, most neoantigens result from random mutations and are patient specific, and some cancers contain few mutations to serve as potential antigens. We describe a patient with stage IV acral melanoma who achieved a complete response following adoptive transfer of tumor-infiltrating lymphocytes (TILs). Tumor exome sequencing surprisingly revealed fewer than 30 nonsynonymous somatic mutations, including oncogenic BRAF\textsuperscript{V600E}. Analysis of the specificity of TILs identified rare CD4\textsuperscript{+} T cells specific for BRAF\textsuperscript{V600E} and diverse CD8\textsuperscript{+} T cells reactive to nonmutated self-antigens. These specificities increased in blood after TIL transfer and persisted long-term, suggesting they contributed to the effective antitumor immune response. Gene transfer of the BRAF\textsuperscript{V600E}-specific T cell receptor (TCR) conferred recognition of class II MHC–positive cells expressing the BRAF mutation. Therapy with TCR-engineered BRAF\textsuperscript{V600E}-specific CD4\textsuperscript{+} T cells may have direct antitumor effects and augment CD8\textsuperscript{+} T cell responses to self- and/or mutated tumor antigens in patients with BRAF-mutated cancers.
Tumor-infiltrating BRAF\textsuperscript{V600E}-specific CD4\textsuperscript{+} T cells correlated with complete clinical response in melanoma

Joshua R. Veatch,\textsuperscript{1} Sylvia M. Lee,\textsuperscript{2} Matthew Fitzgibbon,\textsuperscript{3} I-Ting Chow,\textsuperscript{4} Brenda Jesernig,\textsuperscript{1} Tom Schmitt,\textsuperscript{1} Ying Ying Kong,\textsuperscript{4} Julia Kargl,\textsuperscript{1,5} A. McGarry Houghton,\textsuperscript{1} John A. Thompson,\textsuperscript{2} Martin McIntosh,\textsuperscript{3} William W. Kwok,\textsuperscript{4} and Stanley R. Riddell\textsuperscript{1}

\textsuperscript{1}Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. \textsuperscript{2}Division of Medical Oncology, University of Washington, Seattle, Washington, USA. \textsuperscript{3}Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. \textsuperscript{4}Benaroya Research Institute, Seattle, Washington, USA. \textsuperscript{5}Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Graz, Austria.

T cells specific for neoantigens encoded by mutated genes in cancers are increasingly recognized as mediators of tumor destruction after immune checkpoint inhibitor therapy or adoptive cell transfer. Unfortunately, most neoantigens result from random mutations and are patient specific, and some cancers contain few mutations to serve as potential antigens. We describe a patient with stage IV acral melanoma who achieved a complete response following adoptive transfer of tumor-infiltrating lymphocytes (TILs). Tumor exome sequencing surprisingly revealed fewer than 30 nonsynonymous somatic mutations, including oncogenic BRAF\textsuperscript{V600E}. Analysis of the specificity of TILs identified rare CD4\textsuperscript{+} T cells specific for BRAF\textsuperscript{V600E} and diverse CD8\textsuperscript{+} T cells reactive to nonmutated self-antigens. These specificities increased in blood after TIL transfer and persisted long-term, suggesting they contributed to the effective antitumor immune response. Gene transfer of the BRAF\textsuperscript{V600E}-specific T cell receptor (TCR) conferred recognition of class II MHC-positive cells expressing the BRAF mutation. Therapy with TCR-engineered BRAF\textsuperscript{V600E}-specific CD4\textsuperscript{+} T cells may have direct antitumor effects and augment CD8\textsuperscript{+} T cell responses to self- and/or mutated tumor antigens in patients with BRAF-mutated cancers.

Introduction
T cells can eliminate cancer cells through recognition of peptides from nonmutated or mutated proteins bound to cell surface MHC molecules (1). T cells specific for neoantigens derived from proteins encoded by mutated genes are increasingly recognized as important mediators of antitumor immunity in patients receiving checkpoint blocking antibodies (2–5) and adoptive T cell transfer (6, 7). Neoantigens are attractive targets for T cells because they are not subject to central and peripheral tolerance mechanisms that limit the frequency and function of T cells specific for self-antigens (8). Indeed, the burden of somatic mutations in multiple tumor types correlates with response to immune checkpoint inhibitors (4, 5, 9, 10), suggesting that endogenous neoantigen-reactive T cells contribute to efficacy (11). Clinical response in patients with melanoma and cervical cancer treated with tumor-infiltrating lymphocytes (TILs) has also correlated with the presence of neoantigen-reactive T cells in the administered TIL product (6, 11, 12). Most neoantigens are random, patient specific, and heterogeneously expressed, which limits their broader utility as targets for adoptive transfer with engineered T cells in multiple patients with a particular tumor type (8). In contrast, driver mutations are actively selected, and expressed clonally and homogeneously in cancers from many patients. Unfortunately, there have been very few driver mutations described as eliciting T cell responses, perhaps as a consequence of selection based on HLA genotype (13).

The mutant BRAF kinase (BRAF\textsuperscript{V600E}) is an oncogenic driver present in 40% of melanoma, 10% of colorectal cancer, and 2% of lung cancer, and confers constitutive signaling that promotes tumor cell growth and survival. Small molecule BRAF inhibitors have impressive initial efficacy in melanoma, but resistance evolves by recruitment of alternative signaling pathways without loss of expression of BRAF\textsuperscript{V600E} protein, suggesting that BRAF inhibitor-resistant melanoma would remain susceptible to T cells specific for the BRAF mutation (14). Here we describe a CD4\textsuperscript{+} helper T cell response to BRAF\textsuperscript{V600E} in a patient with an acral melanoma containing few nonsynonymous mutations who had a sustained complete response to TIL therapy.

Results and Discussion
A 52-year-old man presented with stage IIIC, BRAF\textsuperscript{V600E} mutated melanoma originating on the left foot and was treated with wide excision, completion lymph node dissection, and adjuvant ipilimumab at 3 mg/kg every 3 weeks for 4 doses, then every 3 months for maintenance. Before completing 1 year of ipilimumab, he relapsed with 3 in-transit metastases close to his left knee, which were resected. Three months later, he developed another in-transit metastasis at his left medial thigh, which was also resected. Three more months later, he progressed with a 3-cm left iliac nodal metastasis and soft tissue nodular FDG-avid metastasis in the left thigh (Figure 1A). The iliac node was resected for whole exome sequencing and expansion of TILs, and the patient subsequently...
received TIL infusion following lymphodepleting chemotherapy. The left thigh lesion resolved, and the patient remained free of disease 32 months after therapy.

Whole exome and RNA sequencing of purified tumor cells and normal tissue identified only 29 nonsynonymous missense mutations (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI98689DS1) and no coding insertions or deletions, despite high mean coverage (more than 100× for tumor and for normal) and adequate tumor purity as measured by the variant allele frequency of 35% of the often heterozygous BRAFV600E driver mutation. This result would be an unusually low number for sun-exposed melanoma, but is consistent with reported nonsynonymous mutation burden for other acral or mucosal tumors (15).

To identify potential neoantigen-reactive T cells that may have contributed to antitumor efficacy of TIL therapy, we stimulated peripheral blood mononuclear cells (PBMCs) obtained from the patient after TIL therapy with a pool of overlapping peptides flanking each of the 20 mutations with the highest variant allele frequency and/or with evidence of RNA expression (Supplemental Table 1). No CD8+ T cell responses to candidate neoantigens were detected; however, a CD4+ T cell response specific for 20-mer peptides encompassing BRAFV600E was identified. The BRAFV600E-reactive T cells were purified by IFN-γ capture and shown to recognize autologous B cells pulsed with mutant but not WT BRAF peptide, confirming specificity for the mutant peptide (Figure 1B). To determine whether BRAFV600E is processed and presented by class II MHC+ antigen-presenting cells (APCs), we transfected autologous B cells with mRNA encoding WT or mutant BRAF sequences targeted to the endosome. The T cells recognized B cells expressing mutant but not WT BRAF (Figure 1C). Recognition was blocked by anti–HLA-DQ but not anti–class I or anti–HLA-DR, identifying HLA-DQ as the likely restricting allele (Figure 1D). The patient’s class II MHC haplotypes were HLA-DRB1*04, HLA-DQB1*0302/HLA-DRB1*09, and HLA-DQB1*0303. Analysis of multiple B cell lines of known genotype suggested restriction by HLA-DQB1*03 paired with HLA-DQA1*03, with weak recognition of DQB1*0301 and stronger recognition of DQB1*0302 and DQB1*0303 (Supplemental Table 2 and Supplemental Figure 1). B-lymphoblastoid cell lines (B-LCLs) transfected with HLA-DQA1*0301 and DQB1*0302 cDNAs but not the HLA-DRB1*04 cDNA were recognized by BRAFV600E-specific CD4+ T cells when pulsed with the mutant peptide, confirming the HLA restriction (Figure 1D). The T cells recognized B cells expressing mutant but not WT BRAF (Figure 1C). Recognition was blocked by anti–HLA-DQ but not anti–class I or anti–HLA-DR, identifying HLA-DQ as the likely restricting allele (Figure 1D). The patient’s class II MHC haplotypes were HLA-DRB1*04, HLA-DQB1*0302/HLA-DRB1*09, and HLA-DQB1*0303. Analysis of multiple B cell lines of known genotype suggested restriction by HLA-DQB1*03 paired with HLA-DQA1*03, with weak recognition of DQB1*0301 and stronger recognition of DQB1*0302 and DQB1*0303 (Supplemental Table 2 and Supplemental Figure 1). B-lymphoblastoid cell lines (B-LCLs) transfected with HLA-DQA1*0301 and DQB1*0302 cDNAs but not the HLA-DRB1*04 cDNA were recognized by BRAFV600E-specific CD4+ T cells when pulsed with the mutant peptide, confirming the HLA restriction (Figure 1E). We tested recognition of 3 melanoma cell lines with an HLA-DQB1*0302 and BRAFV600E genotype. One tumor cell line that expressed the HLA-DQ and upregulated expression to IFN-γ was recognized by the BRAFV600E-specific CD4+ T cells, suggesting the epitope can be presented directly by some tumor cells (Supplemental Figure 2).

Tumor-specific CD4+ T cells can have antitumor activity through direct cell killing and cytokine release (16, 17), but a major role is to support the development and function of CD8+ T cells by licensing APCs for efficient antigen presentation and producing cytokines (18, 19). Although we did not identify CD8+ T cell responses to neoantigens in blood, these cells were the prevalent...
subset in TILs (Supplemental Table 3). Moreover, the majority of IFN-γ produced by stimulation of multiple independent TIL cultures with autologous tumor was blocked by an HLA class I blocking antibody (Supplemental Table 4). We evaluated TIL responses to neoantigens but did not observe IFN-γ production when TILs were cultured with autologous B cells pulsed with pools of peptides that included the 20 nonsynonymous mutations screened previously (Figure 2A) or to autologous B cells transfected with tandem RNA minigenes encoding the entire set of 29 potential nonsynonymous mutations (Supplemental Figure 3). Any neoantigen-specific CD4+ T cells present in the TIL product were below the level of detection of this assay. However, IFN-γ was produced after coculture with B cells pulsed with peptides from lineage-restricted self-antigens (tyrosinase, Mart-1, TRP2) and a cancer testes antigen (Mage A3), which are known targets of T cells in melanoma (ref. 20 and Figure 2B). Consistent with these results, CD8+ T cells in TILs produced IFN-γ in response to B cells transfected with minigenes encoding tyrosinase and Mart 1 (Supplemental Figure 3). These data demonstrated that in the patient’s TILs, CD8+ T cells responded to multiple self-antigens, but not to any of the putative neoantigens.
We utilized deep sequencing to identify T cell receptor (TCR) gene usage in BRAF\textsuperscript{V600E}-specific CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells. Three TCR Vb clonotypes showed marked expansion after stimulation of posttreatment PBMCs with BRAF\textsuperscript{V600E} peptide, and these 3 sequences were further enriched after restimulation and IFN-\(\gamma\) capture (Figure 2C and Supplemental Table 5), suggesting each of these 3 clonotypes was specific for the BRAF\textsuperscript{V600E} antigen. TCR Vb sequencing of tumor, TILs, and PBMCs obtained prior to TIL infusion identified all 3 TCR Vb clones in the tumor, and 2 of 3 in TILs, albeit at relatively low frequency. All 3 TCR Vb sequences were below the level of detection in pretreatment PBMCs, indicating enrichment at the tumor site (Figure 2D and Supplemental Table 5). Analysis of TCR sequences in the TILs identified 34 Vb sequences that collectively made up more than 50% of the TIL product (Figure 2E and Supplemental Table 5). Only 5 of these 34 clones were detected in the blood prior to TIL infusion,
with 4 at very low frequency (Figure 2F and Supplemental Table 5). We assessed TCR gene usage of the CD8+ T cells that recognized each of the 4 lineage-specific or C/T antigens using IFN-γ capture to sort these cells from TILs. We identified 7 different Vb sequences that were highly enriched in the IFN-γ–captured T cells, and these clonotypes represented 4.7% of the T cells in the TIL product (Supplemental Table 5). All 7 of these clonotypes and 1 of the CD4+ T cells specific for these clonotypes were detected in blood obtained 10 and 24 months after TIL infusion, demonstrating that TIL therapy resulted in sustained augmentation of T cell responses to tumor antigens (Figure 2, F and G, and Supplemental Table 5).

We next characterized the phenotype of circulating BRAFV600E–specific CD4+ T cells from post-treatment blood samples using DQA1:0301/DQB1:0302 tetramers loaded with the mutant BRAF peptide (GDFGLATEKSRWSGS) for direct ex vivo staining, and isolated tetramer-positive T cells for cloning. We confirmed specificity of the DQA1:0301/DQB1:0302/BRAFV600E tetramer by showing that 24 of 26 clones isolated by tetramer sorting released IFN-γ after rechallenge with the peptide. BRAFV600E–specific CD4+ T cells showed an effector memory phenotype (CD45RA−CCR7−) and expressed low levels of PD-1 (Figure 3A). The majority of BRAFV600E–specific cells expressed CXCR3 and CCR4. A fraction of the cells also expressed the skin-homing marker cutaneous lymphocyte-associated antigen (CLA). BRAFV600E peptide–activated cells produced IFN-γ, TNF-α, IL-4, and IL-21 (Figure 3B), sometimes in combination (data not shown). Taken together, these data suggest that circulating BRAF-specific CD4+ T cells after TIL infusion have a mixed Th1/Th2 phenotype, consistent with an established memory cellular immune response to mutated BRAF in melanoma.

Durable remissions in melanoma after adoptive transfer of self-antigen-reactive CD8+ T cells alone are exceedingly rare (21–23). The specificity of T cells responsible for tumor eradication after polyclonal TIL therapy is difficult to define precisely, but it is tempting to speculate that the BRAFV600E–specific CD4+ T cells may have provided direct antitumor effects and aided the induction, persistence, and function of self-antigen-reactive CD8+ T cells against a tumor that contained few neoantigens. The HLA-DQA1*03/DQB1*03-restricting allele for the BRAFV600E–specific CD4+ T cells is present in 29% of individuals in the International Histocompatibility Working Group database (Effie Petersdorf, International Histocompatibility Working Group in Hematopoietic Cell Transplantation, personal communication), and isolation of the BRAFV600E–specific TCR genes from this patient would facilitate adoptive therapy for patients with BRAF mutant tumors with TCR-engineered T cells to test these hypotheses. TCR Va sequencing on samples with varying levels of BRAF-reactive clones identified 4 TCR Va sequences that correlated in frequency with the 3 TCRB Vb sequences (Figure 4A). A synthetic TCR comprising the dominant Va and Vb sequences was constructed and expressed in CD4+ T cells from 2 healthy donors and conferred specificity to cells expressing BRAFV600E but not WT BRAF sequences (Figure 4B).

Immunotherapies that elicit or augment T cell responses to shared neoantigens derived from driver mutations are especially attractive because they allow treatment of multiple patients and should reduce antigen-negative escape variants. Most studies have focused on neoantigen-reactive CD8+ T cells; however, recent work in murine models has highlighted the importance of local and systemic CD4+ T cells in tumor rejection (24, 25). Indeed, the adoptive transfer of CD4+ T cells specific for a non-driver neoantigen induced a clinical response in a single patient with cholangiocarcinoma (26). Although BRAFV600E is common in melanoma, and present in some thyroid, colon, and lung cancers, CD8+ or CD4+ T cells specific for this and other driver mutations are rarely identified (27, 28). One prior study isolated BRAFV600E–specific CD4+ T cells after repetitive peptide stimulation of PBMCs, but a relationship to tumor localization or regression was not established (28). Our data identify BRAFV600E–specific CD4+ T cells restricted by a common class II MHC molecule enriched at the tumor site in a patient who achieved a durable remission after adoptive therapy with TILs and long-term persistence of BRAFV600E–specific CD4+ T cells and cotransferred CD8+ T cells specific for self-antigens. The BRAFV600E–specific TCR isolated in our study provides a reagent for future stud-
ies of adoptive cell therapy with TCR-transgenic CD4+ T cells in patients with BRAFV600E-positive tumors that express HLA-DQA1*03/DQB1*03, alone or in combination with CD8+ T cells specific for self-antigens. This approach may determine the potential for direct antitumor effects and for augmenting CD8+ T cell responses to other tumor-associated antigens by targeting a driver mutation with CD4+ T cells.

Methods
Detailed methods are described in Supplemental Methods.

Statistics. ELISA assays were performed in technical duplicate or triplicate to allow qualitative measurement of large differences, and the mean of the technical replicates is presented in the figures along with the individual data points.

Study approval. The patient was enrolled under FDA Investigational New Drug (IND) approval and a clinical protocol approved by the Institutional Review Board of Fred Hutchinson Cancer Research Center (FHCRC 2643; NCT01807182).

Acknowledgments
JRV was supported by NIH grants T32 T32CA009515 and K12 CA076930-16A1 and a contribution from the Lembersky family. MF, JRV, and SRR were supported by a gift from the Bezos family. JK was supported by European Commission grant EU-FP7-PEOPLE-2012-IOF 331255. MM was supported by NIH grant SU01CA176270-02. Thanks go to Margot Pont for helpful advice on this project.

Address correspondence to: Joshua R. Veatch, 1100 Fairview Ave N, Mailstop D3-100, Seattle, Washington 98109, USA. Phone: 206.667.5108; Email: jveatch@fredhutch.org.