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

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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^{V600E}. Analysis of the specificity of TILs identified rare CD4^{+} T cells specific for BRAF^{V600E} and diverse CD8^{+} 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^{V600E}-specific T cell receptor (TCR) conferred recognition of class II MHC–positive cells expressing the BRAF mutation. Therapy with TCR-engineered BRAF^{V600E}-specific CD4^{+} T cells may have direct antitumor effects and augment CD8^{+} 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 homogenously 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^{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^{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^{+} helper T cell response to BRAF^{V600E} in a patient with an acral melanoma containing few nonsynonymous mutations who had a sustained complete response to TIL therapy.

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Results and Discussion

A 52-year-old man presented with stage IIIC, BRAF^{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

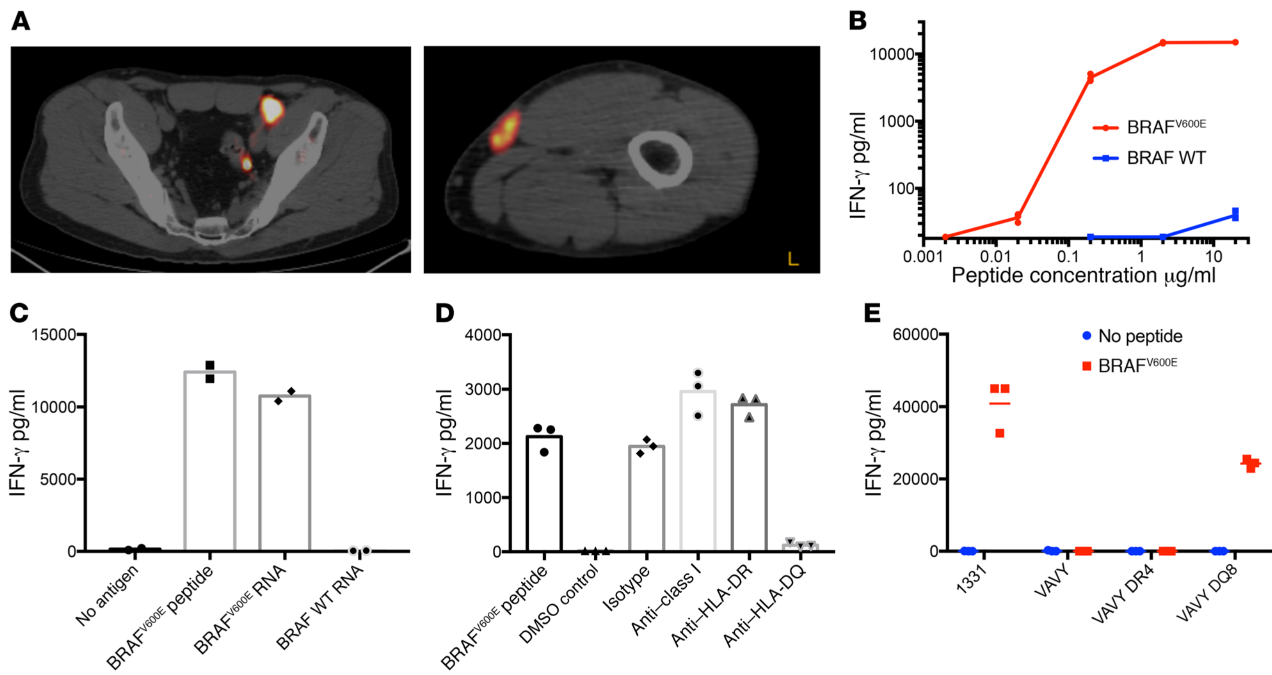

Figure 1. CD4⁺ T cells specific for BRAF^{V600E} isolated from post–TIL infusion PBMCs are restricted by HLA DQB1*03. (**A**) Positron emission tomography showing recurrent tumor in left iliac region (left) and left thigh (right). (**B**–**D**) Specificity and HLA restriction of BRAF^{V600E}-specific T cells. (**B**) IFN-γ production by a patient-derived T cell line incubated with autologous B cells pulsed with WT and mutant BRAF peptide. (**C**) Recognition of autologous B cells transfected with mRNA encoding mutant or WT BRAF sequences. (**D**) Recognition of autologous B cells pulsed with mutated BRAF peptide in the presence or absence of HLA blocking antibodies. (**E**) BRAF-specific CD4$^+$ T cell recognition of the B-LCL line 1331 (DR0404, DQA1*0301/DQB1*0302), which is matched at HLA-DQ with the patient, and the HLA-DQ-mismatched B-LCL line VAVY (DR3, DQA1*0501/DQB1*0201) prior to and after transduction with HLA-DRB1*0404 (DR4) or HLA-DQB1*0302/DQA1*0301 (DQ8). Assays were performed with and without pulsing with BRAF^{V600E} peptide. Experiments were performed with 2–3 technical replicates.

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 BRAF^{V600E} 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 BRAF^{V600E} was identified. The BRAF^{V600E}-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 BRAF^{V600E} 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 BRAF^{V600E}-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 BRAF^{V600E} genotype. One tumor cell line that expressed the HLA-DQ and upregulated expression to IFN-γ was recognized by the BRAF^{V600E}-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

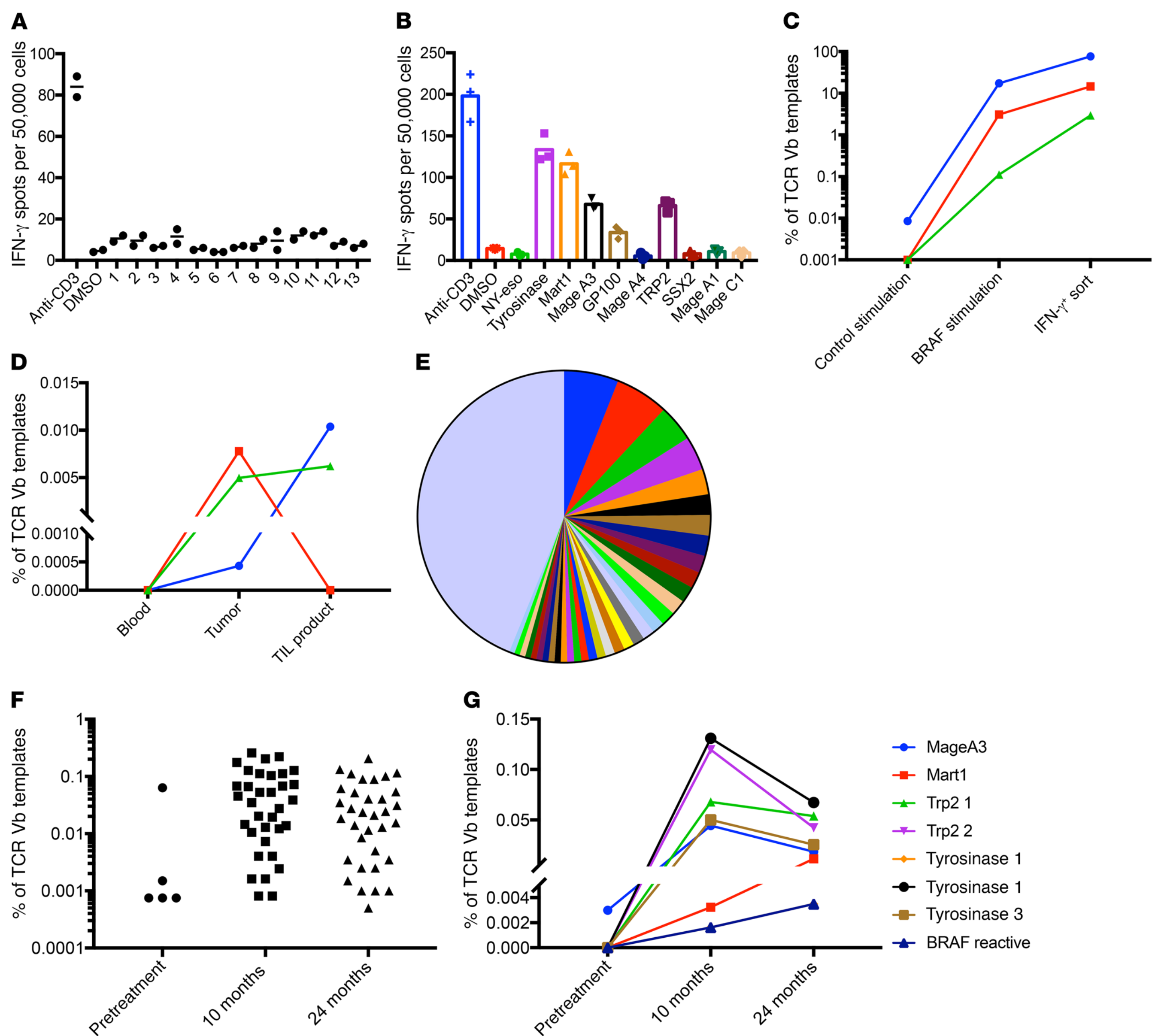

Figure 2. Specificity of CD8⁺ T cells in TILs and TCR sequencing of T cell clonotypes in blood after adoptive transfer. TILs were incubated with autologous B cells pulsed with peptide pools encompassing tumor-associated antigens from mutated antigens (**A**) and tumor-associated self-antigens (**B**), and IFN-γ release was measured by ELISPOT with 2–3 technical replicates. (**C**) Frequency of TCR Vb sequences in PBMCs after mock stimulation or $BRAF^{V600E}$ peptide stimulation, or after sorting IFN-γ–secreting cells after $BRAF^{V600E}$ peptide restimulation. CDR3 sequences: CASNEGNSGNTIYF (blue), CASGARQIPYTF(red), CASSLSAAGGGYGYTF (green). (**D**) TCR Vb clonotypes of BRAF-specific T cells were quantitated by TCRB sequencing of pretreatment blood, tumor single-cell suspension, and the TIL product infused into the patient. (**E**) TCR Vb sequences in TIL product ranked by prevalence, with the top 34 clones in colors and the remainder in gray. (**F**) Frequency of the top 34 TIL TCR Vb clonotypes from **D** in pretreatment blood and posttreatment blood obtained at 10 and 24 months. (**G**) Frequency of TCR Vb clonotypes of $CD4^+$ $BRAF^{V600E}$ and $CD8^+$ T cells specific for the specified antigens in pretreatment and posttreatment blood.

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.

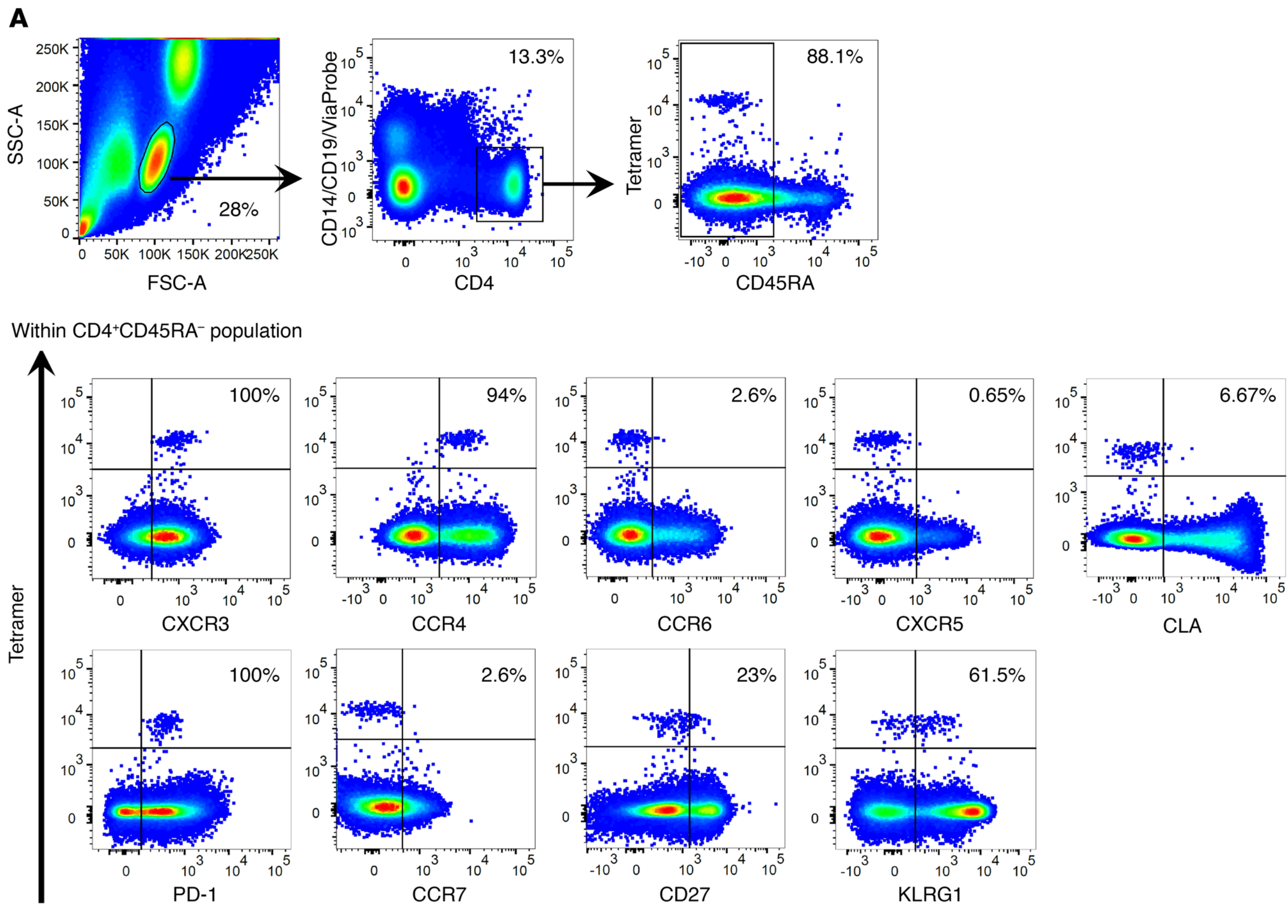

Figure 3. Phenotypic analysis for BRAF-specific T cells following TIL treatment. (**A**) A dump channel was used to exclude monocytes (CD14+), B cells (CD19+), and dead cells (ViaProbe) from lymphocytes. Viable CD4+ T cells were plotted against tetramer and CD45RA. CD45RA– memory cells (88.1% of CD4+) that were tetramer-positive and -negative were next plotted against the surface markers indicated in the figure. Numbers indicate the percentage of cells in the gated regions or the percentage of tetramer-positive cells for each marker. (**B**) Intracellular cytokine staining revealed that activated (CD154+) BRAF-specific T cells secreted IFN-γ, IL-4, TNF-α, and IL-21.

We utilized deep sequencing to identify T cell receptor (TCR) gene usage in BRAF^{V600E}-specific CD4+ T cells and CD8+ T cells. Three TCR Vb clonotypes showed marked expansion after stimulation of posttreatment PBMCs with BRAF^{V600E} peptide, and these 3 sequences were further enriched after restimulation and IFN-γ capture (Figure 2C and Supplemental Table 5), suggesting each of these 3 clonotypes was specific for the BRAF^{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,

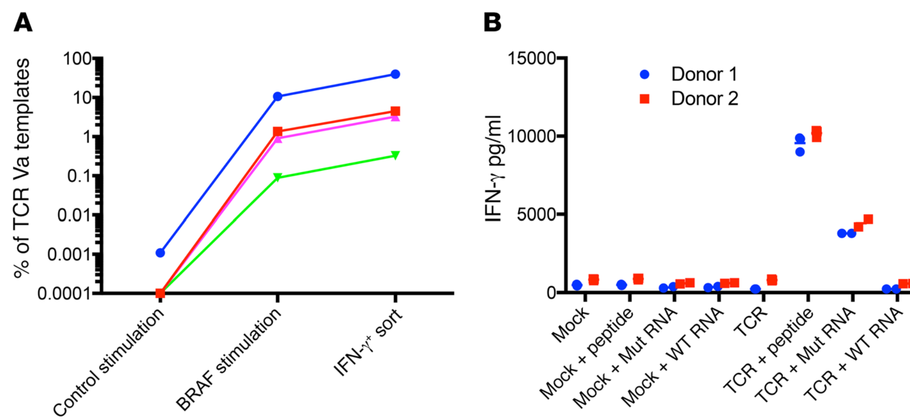

Figure 4. A synthetic TCR derived from the dominant Va and Vb sequences recognizes cells expressing BRAF^{V600E}. (**A**) Frequency of TCRB Va sequences in PBMCs after mock stimulation or BRAF^{V600E} stimulation, or after sorting of IFN-γ–secreting cells after BRAF^{V600E} peptide restimulation. CDR3 sequences: CAVRRGNNDMRF (blue), CIVRAYSGYSTLTF (red), CAVITLNNNAGNMLTF (purple), CAVTSNAGKSTF (green). (**B**) IFN-γ production by CD4$^+$ T cells from 2 normal donors transduced with a synthetic TCR construct and incubated with an HLA-DQB1*0302 B cell line 1331 pulsed with BRAF^{V600E} peptide or transfected with mRNA encoding mutant (Mut) or WT BRAF sequences, with 2 technical replicates.

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 BRAF-specific 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 BRAF^{V600E}-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/BRAF^{V600E} tetramer by showing that 24 of 26 clones isolated by tetramer sorting released IFN-γ after rechallenge with the peptide. BRAF^{V600E}-specific CD4$^+$ T cells showed an effector memory phenotype (CD45RA$^-$CCR7$^-$CD27$^-$KLRG1$^+$) and expressed low levels of PD-1 (Figure 3A). The majority of BRAF^{V600E}-specific cells expressed CXCR3 and CCR4. A fraction of the cells also expressed the skin-homing marker cutaneous lymphocyte-associated antigen (CLA). BRAF^{V600E} 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 BRAF^{V600E}-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 BRAF^{V600E}-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 BRAF^{V600E}-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 TCR 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 BRAF^{V600E} 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 BRAF^{V600E} 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 BRAF^{V600E}-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 BRAF^{V600E}-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 BRAF^{V600E}-specific CD4$^+$ T cells and cotransferred CD8$^+$ T cells specific for self-antigens. The BRAF^{V600E}-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 $BRAF^{V600E}$-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).

Author contributions

JRV, SML, MF, IC, TS, WK, and SRR designed experiments. JRV, IC, BJ, YYK, and JK performed experiments. JRV, MF, IC, TS, AMH, MM, WWK, and SRR analyzed the data. SML, TS, and JAT provided materials, and JRV, SML, MF, IC, JAT, WWK, and SRR wrote the manuscript.

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