A Pilot Trial of the Combination of Vemurafenib with Adoptive Cell Therapy in Patients with Metastatic Melanoma

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Abstract

Purpose: This pilot feasibility clinical trial evaluated the coadministration of vemurafenib, a small-molecule antagonist of BRAFV600E/K mutations, and tumor-infiltrating lymphocytes (TIL) for the treatment of metastatic melanoma.

Experimental Design: A metastatic tumor was resected for growth of TILs, and patients were treated with vemurafenib for 2 weeks, followed by resection of a second lesion. Patients then received a nonmyeloablative preconditioning regimen, infusion of autologous TILs, and high-dose interleukin-2 administration. Vemurafenib was restarted at the time of TIL infusion and was continued for 2 years or until disease progression. Clinical responses were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) 1.0. Metastases resected prior to and after 2 weeks of vemurafenib were compared using TCRB deep sequencing, immunohistochemistry, proliferation, and recognition of autologous tumor.

Introduction

Metastatic melanoma accounts for ~10,000 annual deaths in the United States (1). Tumor-infiltrating lymphocytes (TIL) display specificity for autologous tumor cells in vitro via tumor cell lysis and secretion of pro-inflammatory cytokines, e.g., IFNγ, following TIL and tumor cell coculture. TIL obtained from deposits of metastatic melanoma can be expanded ex vivo to large numbers and infused back into the autologous patients as adoptive T-cell therapy (2–4). The adoptive transfer of TILs with high-dose IL2 following a nonmyeloablative (NMA) chemotherapy preconditioning regimen resulted in an overall objective response rate of ~55% with ~20% complete response (CR) rate (5–12). To attempt to improve these clinical results, we sought to evaluate, in a pilot clinical trial, the safety and feasibility of adding other agents to use in combination with TIL therapy.

Results: The treatment was well tolerated and had a safety profile similar to that of TIL or vemurafenib alone. Seven of 11 patients (64%) experienced an objective clinical response, and 2 patients (18%) had a complete response for 3 years (one response is ongoing at 46 months). Proliferation and viability of infusion bag TILs and peripheral blood T cells were inhibited in vitro by research-grade vemurafenib (PLX4032) when approaching the maximum serum concentration of vemurafenib. TCRB repertoire (clonotypes numbers, clonality, and frequency) did not significantly change between pre- and post-vemurafenib lesions. Recognition of autologous tumor by T cells was similar between TILs grown from pre- and post-vemurafenib metastases.

Conclusions: Coadministration of vemurafenib and TILs was safe and feasible and generated objective clinical responses in this small pilot clinical trial.

See related commentary by Cogdill et al., p. 327

A candidate for this application was vemurafenib, a specific inhibitor of somatic mutations at the V600 codon of the BRAF oncogene (13). About half of patients with metastatic melanoma express BRAFV600E or BRAFV600K mutations, which are not present in normal tissues. These BRAF mutations result in constitutive MAP kinase signaling and uncontrolled proliferation (14), and vemurafenib directs the senescence and apoptosis of BRAFV600E/K cancer cells (15, 16). Patients with metastatic melanoma who were treated with vemurafenib experienced a 53% and 48% overall response and 6% and 1% CR rate in phase II and III clinical trials, respectively (17–20). Vemurafenib was reported to increase the density of lymphocyte infiltrates in melanoma metastases, alter the intratumor T-cell repertoire, and not significantly affect the proliferation or viability of peripheral blood T cells at concentrations of vemurafenib ≤50 μmol/L (21–23). Furthermore, tumor expression of melanocyte differentiation antigens, e.g., gp100, MART-1, tyrosinase, and TRP1/2, were reported to increase following vemurafenib treatment (21, 24). Thus, a clinical trial evaluating the combination of vemurafenib and TILs was a rational approach to evaluate therapeutic additivity or synergy with different classes of treatment modalities.

A pilot clinical trial was initiated at the Surgery Branch of the National Cancer Institute (NCI) to assess the safety and feasibility of this combination therapy. To evaluate whether vemurafenib impacted TILs, we chose to resect metastatic melanoma deposits...
Translational Relevance

One way to potentially improve adoptive T-cell therapy is to combine it with the administration of small-molecule inhibitors. Vemurafenib specifically inhibits mutated BRAF<sup>V600E/K</sup> molecules and directs cell death of BRAF<sup>V600E/K</sup>–expressing tumors. Vemurafenib has been reported to increase tumor infiltration of lymphocytes and increase expression of melanoma/melanocyte T-cell antigens. This pilot clinical trial evaluated the combination of vemurafenib with T-cell transfer. Objective clinical responses were observed in 7 of 11 patients (64%), including 2 of 11 patients (18%) with complete regressions of metastatic disease. Comparisons of metastases before and after vemurafenib treatment revealed an increased presence of tumor-infiltrating T cells but similar recognition of autologous tumor. This pilot trial showed the safety and feasibility of administering a kinase inhibitor with T-cell transfer.

Materials and Methods

Ethics

Written informed consent was granted from all study participants. This study was approved by the institutional review board (IRB) at the NCI and was registered at clinicaltrials.gov under NCT01585415.

Trial design

Eligible patients were between 18 and 66 years of age, with a life expectancy >3 months, an ECOG performance status score ≤1, and measurable metastatic melanoma that expressed BRAF<sup>V600E</sup> or BRAF<sup>V600K</sup> mutations as assessed by a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. Patients were not eligible if they had received prior vemurafenib; were receiving systemic steroid therapy; had mean QTc interval >450 ms; had <45% left ventricular ejection fraction; or were experiencing active systemic infections, coagulation disorders, active major medical illnesses of the cardiovascular, respiratory, or immune system, primary immunodeficiency, opportunistic infections, or severe immediate hypersensitivity to agents used in this study. More than 4 weeks must have elapsed since any prior systemic therapy and toxicities recovered to grade 1 or less (except for alopecia or vitiligo). More than 6 weeks must have elapsed since any antibody therapy, including anti-CTLA4 antibody. Patients with three or fewer brain metastases (<1 cm) were eligible if asymptomatic or had lesions treated with stereotactic radiosurgery and were clinically stable for 1 month after treatment or surgical resection.

Tumor and TIL coculture

Coculture assays were performed in 50/50 media: 50% AIM-V media, 45% RPMI-1640 media with l-glutamine (Gibco; Fisher Scientific), 10% heat-inactivated human AB serum (NCI Surgery Branch), and 50% AIM-V media supplemented with IL2 (PBL: 600 IU/mL; TIL: 6,000 IU/mL). Selected fragments were expanded in rapid expansion protocol with irradiated peripheral blood lymphocyte (PBL) feeder cells, 30 ng/mL OKT3 antibody (Miltenyi Biotec), and 3,000 IU/mL IL2, and then pooled and administered to patients.

In vitro proliferation and cell viability assays

Serial dilutions of PLX4032 (Fisher Scientific) were made in 50/50 media in parallel to serial dilutions of DMSO (vehicle). PBLs were stimulated with OKT3 (50 ng/mL) and IL2 (300 IU/mL) at a density of 2 × 10<sup>6</sup> cells/mL, fed 2 days later with fresh media supplemented with IL2 and assessed 3 days later. Thawed infusion bag TILs were rested overnight in 50/50 media with 3,000 IU/mL IL2. T cells were mixed with 50/50 media supplemented with IL2 (PBL: 600 IU/mL; TIL: 6,000 IU/mL) to assess the possible impact of vemurafenib on T cells.
IU/ml) and were added to an equal volume of diluted PLX4032 or DMSO. T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye according to the manufacturer’s instructions (BD Biosciences) prior to addition to the plates for proliferation assays. Baseline measurements of CFSE staining were acquired from each donor. T cells were cultured for 3 days with PLX4032 or DMSO at 37°C and stained for CD3, propidium iodide (PI), and Annexin V (viability assays only), acquired on BD FACSCanto II (BD Biosciences) and analyzed by FlowJo (v.10.0.8). Data shown for proliferation assays were gated on lymphocytes and then live T cells (CD3+ PI−). Data shown for viability assays were gated on lymphocytes.

Deep sequencing of TCRB alleles
Genomic DNA was isolated from snap frozen tumor fragments with a DNeasy kit according to the manufacturer’s instructions (Qiagen). TCRB alleles were sequenced at 200,000 reads by Adaptive Biotechnologies. Productive TCRB sequences, i.e., those that could be translated to open reading frames, were reported.

Results
Combination of adoptive T-cell therapy and vemurafenib was well tolerated
The primary objective of this clinical trial was to determine the safety and feasibility of combining vemurafenib with adoptive T-cell therapy. A total of 11 patients were enrolled, resected of a metastatic tumor, and treated on this pilot clinical trial. Lymphocytopenia, neutropenia, and thrombocytopenia were observed in all 11 patients as a direct result of the NMA chemotherapy. All patients had at least one transient and reversible grade 3 toxicity (Table 1), which included hyperbilirubinemia (n = 1), hypocalcemia (n = 1), hyperglycemia (n = 1), hypophosphatemia (n = 1), hypokalemia (n = 1), hyperkalemia (n = 1), prolonged QTc interval (n = 1), hypoxia (n = 1), an altered partial thromboplastin time (n = 1), oliguria (n = 1), rash (n = 1), thrombus (n = 1), dyspnea (n = 1), pericoronitis (n = 1), cellulitis (n = 1), increased creatinine (n = 2), febrile neutropenia (n = 4), infection (n = 5), and anemia (n = 7). Two patients had reversible grade 4 toxicities, which were increased creatinine (n = 1) and dyspnea (n = 1). The treatment cycle was well tolerated overall, and toxicity was similar to that seen with vemurafenib or standard TIL therapy alone.

TIL and vemurafenib combination therapy mediated objective clinical responses
A secondary objective of this pilot clinical trial was to gain preliminary information concerning the ability of the combination therapy to mediate clinical tumor regressions in patients with metastatic melanoma. The median patient age was 41 years old (range, 22–66) and 10 of 11 (91%) patients were male (Table 1). Metastases were most commonly in the skin, lymph nodes, and lungs. Nine of 11 (82%) patients had prior therapy, including immunomodulatory agents such as high-dose IL2 or interferon. One patient (4) had prior TIL therapy. The median number of infused TILs was 9.24 × 10^9 (range, 3.39 × 10^9–1.17 × 10^10) and patients received a median 5 doses of IL2 (range, 2–7). Target lesions often decreased after 2 weeks of vemurafenib (median, 21%; range, 5%–58%). Seven of 11 patients (64%) experienced an overall objective clinical response, including 2 patients (1 and 5) with CRs (18%; Fig. 1). Patient 1 received 9.27 × 10^10 TILs and 6 doses of IL2, and his target lesion in the hilum slightly decreased in size following initial vemurafenib treatment, showed significant reduction in size after TIL therapy at 2 months, completely regressed by 26 months, and remains absent ongoing at 46 months (Fig. 1A). Patient 5 received 8.64 × 10^10 TILs and 5 doses of IL2, and multiple subcutaneous masses on the patient’s left thigh dramatically decreased after initial vemurafenib therapy. Metastases continued to shrink after TIL therapy and were completely resolved by 12 months (Fig. 1B). Three years after initial treatment, patient 5 had progressive disease from recurrent melanoma in the thigh. The initial decrease in tumors after vemurafenib did not correlate to long-term response (Table 1), which was exemplified by patient 1 who had only a 5% reduction post-vemurafenib but had a durable, complete tumor regression. We compared patient responses from this pilot trial to responses in patients with known BRAFV600 mutations who were treated during the same time interval on a separate clinical trial at the NCI Surgery Branch with TILs, IL2, and the NMA preparative regimen but did not receive vemurafenib (NCT00513604). These patients did not have a second lesion available for biopsy and, thus, were not included in the vemurafenib trial. Of these 15 patients, 9 experienced an objective clinical response (60%) and 3 had CRs (20%), which were durable and are ongoing. In the comparison of these two small pilot trials, there was no suggestion of a difference in antitumor response with the addition of vemurafenib to adoptive T-cell therapy (P = 1.0; Fisher exact test).

PLX4032 inhibited growth and decreased viability of peripheral blood T cells and infusion bag TILs in vitro only at high concentrations approaching the vemurafenib serum CMAX
Previous studies reported that PLX4032 (vemurafenib) and PLX4072 (PLX4032 progenitor) did not affect the viability or proliferation of peripheral blood T cells at concentrations up to 50 μmol/L in vitro (23, 26, 27). We performed similar testing on patients’ peripheral blood T cells to corroborate these findings and also tested the TILs used for treatment, which has not been evaluated to date. The assay stringency was increased by testing higher PLX4032 concentrations close to or above the maximum serum concentration (CMAX) of vemurafenib (125 μmol/L) when given twice per day at 960 mg (28), as was done in this trial. PBL were stimulated with OKT3 and IL2 then used to assay peripheral blood T cells and infusion bag TILs. PLX4032 inhibited growth and decreased viability of peripheral blood T cells at concentrations near the reported vemurafenib CMAX (125 μmol/L), but proliferative capacity was maintained at...
Table 1. Patient demographics, treatment, and response

<table>
<thead>
<tr>
<th>Pt</th>
<th>Age/gender</th>
<th>Sites of disease</th>
<th>Prior treatment</th>
<th>Toxicity (grade)</th>
<th>Infusion TIL number x 10^9</th>
<th>IL2 doses</th>
<th>Decrease target tumors after VEM and before TIL (%)</th>
<th>Overall response (months); greatest decrease tumor (%); PD reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30 M</td>
<td>Lung, SQ, skin</td>
<td>LND, IFN, HD IL2 (x&gt;7)</td>
<td>PTT (5) Rash (3)</td>
<td>Staphylococcus epidermidis (3)</td>
<td>92.7</td>
<td>6</td>
<td>CR (46+); 100%</td>
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<td>2</td>
<td>41 M</td>
<td>Brain, lung, liver, mediastinal LN, spleen</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td>33.9</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>22 M</td>
<td>Lung, liver, mediastinal LN, bone, muscle</td>
<td>WLE, SLNB, IFN</td>
<td>Hypoxia (5) Pericorontis (3)</td>
<td>Staphylococcus bacteremia (3)</td>
<td>92.7</td>
<td>6</td>
<td>CR (46+); 100%</td>
</tr>
<tr>
<td>4</td>
<td>24 M</td>
<td>Skin, spleen, brain, gallbladder</td>
<td>NMA arm of 1,200 TBI vs. NMA protocol at NCI (141.9 x 10^9 TIL at day 18)</td>
<td></td>
<td>Febrile neutropenia (3)</td>
<td>86.4</td>
<td>5</td>
<td>CR (36); 100%; recurrent melanoma (thigh)</td>
</tr>
<tr>
<td>5</td>
<td>53 M</td>
<td>Skin</td>
<td>WLE, SLNB, and LN resection, HD IL2 (x16)</td>
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<td>Creatinine (3) Bilirubin (3) Infection (3) Cellulitis (3)</td>
<td>86.4</td>
<td>5</td>
<td>CR (36); 100%; recurrent melanoma (thigh)</td>
</tr>
<tr>
<td>6</td>
<td>50 M</td>
<td>Skin, muscle, mesenteric LN</td>
<td>WLE, IFN, GM-CSF, HD IL2 (x23), XRT, Ipi</td>
<td>Ca^2+ (3) PO_4^- (3) Febrile neutropenia (3)</td>
<td></td>
<td>117.4</td>
<td>5</td>
<td>PR (3); 57%; new brain mets</td>
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<tr>
<td>7</td>
<td>27 M</td>
<td>Liver, pancreas, abdominal LN, RP LN, skin, lung, med LN</td>
<td>ALND, WLE, SLNB, XRT, IFN, HD IL2 (x1)</td>
<td></td>
<td>Dyspnea (3)</td>
<td>92.4</td>
<td>4</td>
<td>PR (6); 62%; new SQ mets</td>
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<tr>
<td>8</td>
<td>66 M</td>
<td>Cervical LN, muscle, med LN, skin, abdominal LN, RP LN, mesenteric LN, lung, axillary LN</td>
<td>ALND, IFN, HD IL2 (x14)</td>
<td></td>
<td></td>
<td>58.5</td>
<td>4</td>
<td>PR (5); 68%; new mesenteric met</td>
</tr>
<tr>
<td>9</td>
<td>65 M</td>
<td>Cervical LN, liver, brain</td>
<td>None</td>
<td></td>
<td></td>
<td>95.0</td>
<td>5</td>
<td>NR; 30%; new brain mets</td>
</tr>
<tr>
<td>10</td>
<td>41 F</td>
<td>Lung, adrenal, skin</td>
<td>Multiple excisions, WLE, HD IL2 (x2), Ipi</td>
<td></td>
<td></td>
<td>95.4</td>
<td>2</td>
<td>PR (7); 58%; increasing lung, cutaneous mets</td>
</tr>
<tr>
<td>11</td>
<td>51 M</td>
<td>Skin</td>
<td>WLE</td>
<td></td>
<td></td>
<td>104</td>
<td>4</td>
<td>NR; 50%; new brain mets</td>
</tr>
</tbody>
</table>

NOTE: Eleven patients were enrolled on the vemurafenib and adoptive T-cell therapy clinical trial, and clinical data are detailed.

Abbreviations: ALND, axillary lymph node dissection; F, female; GM-CSF, granulocyte macrophage–colony stimulating factor; Hb, hemoglobin; HD IL2, high-dose interleukin-2; Ipi, ipilimumab; LN, lymph node; LND, lymph node dissection; M, male; mets, metastases; NMA, nonmyeloablative chemotherapy; NR, nonresponse; PD, progression of disease; PR, partial response; Pt, patient; PTT, partial thromboplastin time; RP LN, retroperitoneal lymph node; SLNB, sentinel lymph node biopsy; SQ, subcutaneous; TBI, total body irradiation; VEM, vemurafenib; WLE, wide local excision; XRT, radiation.
concentrations below 10 μmol/L. Some proliferation was measured in each sample, indicating that even high PLX4032 concentrations did not completely abrogate T-cell growth. Most of the T-cells were alive (Annexin V− PI−) in both TIL and PBL groups in mock-treated (Media) and DMSO conditions at concentrations near the vemurafenib Cmax or below (Fig. 2C). In contrast, the PLX4032-treated TIL had high frequencies of dying (Annexin V+ PI−) and dead (Annexin V+ PI+) cells when approaching the vemurafenib Cmax (Fig. 2C, top right). Almost all peripheral blood T cells were dead when treated with PLX4032 close to the vemurafenib Cmax (Fig. 2C, bottom right). The viability of TIL and peripheral T cells was high at PLX4032 concentration <37 μmol/L, which is consistent with previous reports in the literature (Fig. 2D; refs. 23, 26, 27). These phenomena were observed in all 11 patients and in the 4 PBL donors tested, indicating that it was not based on T-cell source (PBL or TIL), clinical response, or capacity to achieve clinical benefit. Thus, T cells infused into the bloodstream of patients receiving 960 mg of vemurafenib twice per day may have experienced decreases in viability and proliferation from the vemurafenib.

Equivalent or improved TIL fragment growth and lymphocyte infiltrate in vemurafenib-primed tumors relative to vemurafenib-naïve tumors

Another secondary objective of this study was to study the immunologic impact of vemurafenib administration on the lymphoid infiltrate in melanoma deposits. We chose to compare separate independent metastases to evaluate global effects of vemurafenib on metastases and minimize the effects of sampling the same tumor multiple times. The first tumor was resected before vemurafenib and another tumor was resected after 2 weeks of vemurafenib treatment. We were able to obtain post-vemurafenib tumors from 9 of 11 (82%) patients (Table 2). At the time of resection, the tumors were dissected into fragments (n = 24) if possible and expanded in high-dose IL2 in order to compare TIL fragment proliferation and functional responses as a function of vemurafenib administration. The frequency of fragments that expanded past the single-well stage (approximately 10⁶ cells/well) was similar between pre- and post-vemurafenib tumors. It should be noted that vemurafenib often resulted in shrinkage of each patient’s tumor, so the post-vemurafenib metastases were relatively smaller and fewer than 24 fragment cultures could be initiated for 5 of 9 patients (4, 5, 6, 7, and 11). Tumors were stained for CD3, CD4, CD8, and CD20 (B-cell marker) and evaluated by immunohistochemistry to determine the relative lymphocyte infiltrate before and after vemurafenib. Increased T-cell staining intensity, as measured by CD3 expression, was clearly observed in 6 of 8 patients evaluated (75%; 1, 5, 9, 10, and 11). Helper T cells, as measured by CD4 expression, were more frequent in 5 of 9 patients assayed (56%; 1, 4, 5, 8, and 11). Cytotoxic T cells, as measured by CD8 expression, were more frequent in 7 of 9 patients assayed (78%; 1, 4, 5, 8, 9, 10, and 11). An associated increased staining for B cells (CD20⁺) was not observed in most of these samples (patients 4, 5, 8, and 11), suggesting that the increased T-cell infiltrate was unlikely to be a result of increased lymphocytes in general. It should also be noted that one of the patients (6) who did not have increased lymphocyte infiltrate already contained high levels of T cells in the pre-vemurafenib tumor. Moreover, the two patients who experienced complete regressions of all metastatic disease (patients 1 and 5) had minimal lymphohytic infiltration prior to vemurafenib and had a large increase in the staining for CD3, CD4, and CD8 T cells in post-vemurafenib tumors. Thus, vemurafenib resulted in an increased presence of T cells in separate melanoma metastases, which may have resulted from new lymphocyte infiltration or from lymphocyte concentration due to tumor shrinkage.

Clinical response was independent of TCR repertoire before and after vemurafenib

To evaluate whether this increased lymphocyte frequency represented a change in the T-cell repertoire, we performed TCRβ deep sequencing to measure productive TCR clonotypes in autologous tumors. Eight patients were evaluated in these assays because they had a vemurafenib-naïve lesion and a
Two post-vemurafenib lesions were resected for patient 5, and both tumors were included in analyses. Three of 8 patients (5, 7, and 8) had increased numbers of TCRB clonotypes in the pre-vemurafenib tumor relative to the post-vemurafenib lesion. Conversely, the other 5 patients (1, 6, 9, 10, and 11) had greater numbers of TCRB clonotypes in the post-vemurafenib tumor relative to the pre-vemurafenib lesion (Fig. 3A). Differences in total numbers of TCRB clonotypes were not statistically different ($P = 0.431$) between the two vemurafenib groups. The total numbers of TCRB clonotypes did not correlate with clinical outcome, which is exemplified by the 2 patients

**Figure 2.**

Proliferation and cell viability of peripheral blood T cells and infused TILs following culture with vemurafenib (PLX4032). PBL was mixed with OKT3 and IL2, and after 5 days, the actively dividing peripheral blood T cells were assayed. Infusion bag TILs, which were actively growing the rapid expansion protocol, were assayed from thawed vials of stocks frozen on the day of TIL infusion. T cells were mixed with IL2 and added to either DMSO (vehicle) or PLX4032, and then cultured for 3 days at 37°C. T cells were labeled prior to culture with CFSE to measure proliferation or were left unlabeled to assess viability following staining for Annexin V and PI to quantify frequencies of live (Annexin V$^-$ PI$^-$), dying (Annexin V$^+$ PI$^-$), or dead (Annexin V$^+$ PI$^+$) cells. Proliferation of (A) TILs or (B) peripheral blood T cells cultured with (left) OKT3 (positive control; green), media (negative control; white), DMSO (vehicle control; 111 μmol/L; blue), or PLX4032 (111 μmol/L; red) in one representative donor (patient 1; CR; 46+ months) or (right) across a range of concentrations of DMSO and PLX4032. Baseline levels of CFSE staining from day 0 of the experiments are displayed in gray histograms. C, Viability of TILs (top) or peripheral blood T cells (bottom) from one representative donor (patient 10; PR; 7 months) when cultured with media, DMSO, or PLX4032 at 111 μmol/L. D, Frequencies of live (top) or dead (bottom) cells in cultures across a range of DMSO and PLX4032 concentrations. The reported vemurafenib C$_{\text{MAX}}$ (125 μmol/L) is indicated by a dotted line on the graphs. TIL data are compiled from all patients enrolled on the trial and are displayed as mean ± standard error of the mean (SEM) ($n=11$). PBL data are compiled from patient’s PBL representing each clinical response group (1, CR; 8, PR; 10, PR; 9, NR) and are displayed as mean ± SEM ($n=4$). Data are representative of at least two independent experiments. Two-way ANOVA with Bonferroni posttests was used for statistical analysis between PLX4032 and DMSO groups. *** $P < 0.001$. mo, months.
achieving CR who had opposite trends as one had more TCRB clonotypes in the post-vemurafenib tumor relative to the pre-vemurafenib tumor (patient 1) and vice versa for the other patient (5). Clonality was measured because it normalized the productive TCRB clonotype reads for each sample and, thus, quantified TCR diversity (22, 29, 30) in each vemurafenib group. TCRB clonality increased in post-vemurafenib lesions compared with pre-vemurafenib tumors most dramatically in both patients achieving CR (1 and 5) and one patient (6) with a 3-month PR, and to a lesser extent in patients 8 (PR; 5 months) and 11 (NR; Fig. 3B). Small decreases in TCRB clonality in post-vemurafenib tumors relative to pre-vemurafenib lesions were detected in patient 7 (PR; 6 months) and patient 9 (NR), and there was no change in TCRB clonality for patient 10 (PR; 10 months). Numbers of TCRB clonotypes and TCRB clonality had similar trends in all patients except for patient 5, who had decreased TCRB clonotypes and increased clonality in post-vemurafenib tumors relative to pre-vemurafenib tumors, and patient 8, who had a log10-fold decrease in TCRB clonotypes but only a slight increase in TCRB clonality in the post-vemurafenib tumor relative to the pre-vemurafenib lesion. Overall, there was not a significant difference (P = 0.130) in TCRB clonality between pre- and post-vemurafenib tumors, and an increase or decrease in TCRB clonality could not be used to predict clinical response. Relative frequencies of unique, productive TCRB clonotypes present in both pre-vemurafenib (x-axes) and post-vemurafenib (y-axes) tumors were compared in a linear regression analysis (Fig. 3C). The line of best fit (solid line) and associated slope (m), y-intercept (b), correlation coefficient (r²), and number of shared TCRB clonotypes (n) were calculated for each patient. None of these parameters could be used to predict clinical response. Each slope was <1, suggesting that shared TCRB clonotypes were more frequent in the pre-vemurafenib tumor relative to the post-vemurafenib lesion. Autologous tumor pairs had sufficient differences in shared TCRB clonotype frequencies, as evidenced by r² values <0.5, i.e., a 1:1 frequency ratio was not observed. Hatched lines were drawn as reference to a 1:1 frequency ratio (m = 1, b = 0) on each graph. The top-ranking clonotypes present in the upper right of the graphs did not display a dominant pattern of increased or decreased frequency in pre- or post-vemurafenib tumors. Moreover, high-ranking TCRB clonotypes in one tumor were typically high ranking in the other tumor. These results suggested that vemurafenib treatment for 2 weeks did not unidirectionally alter the TCRB repertoire in independent metastases. Therefore, the increased lymphocyte infiltration following vemurafenib treatment could not be easily and directly correlated to the TCRB repertoire.

Pre- and post-vemurafenib TIL fragments responded similarly to autologous tumor cells

Another measure of whether there was a change in the T-cell repertoire following vemurafenib was to examine the functionality of TIL fragments grown from the metastases before and after vemurafenib treatment. Although 9 of 11 patients had post-vemurafenib resections, TIL fragments were expanded from only 8 of the 9 post-vemurafenib lesions (TILs from patient 5 were from keratinic acanthoma rather than

Table 2. Patient biopsies and lymphocyte infiltration into tumors before and after vemurafenib treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Biopsy site</th>
<th>Day</th>
<th>Tumor ID</th>
<th>(TIL infused × 10⁴)</th>
<th>#TIL fragments grown</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD20</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R-shoulder mass</td>
<td>–15</td>
<td>3745</td>
<td>92.7</td>
<td>17/24</td>
<td>1+</td>
<td>0-1</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>L-cervical LN</td>
<td>+13</td>
<td>3760</td>
<td>24/24</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
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<td>2</td>
<td>R-axilla mass</td>
<td>–46</td>
<td>3744</td>
<td>33.9</td>
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<td>n/a</td>
<td>n/a</td>
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<td>3</td>
<td>R-thoracic mass</td>
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<td>3764</td>
<td>36.3</td>
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<td></td>
<td>Abdominal and chest lesions (3)</td>
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<td>3776</td>
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<tr>
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<td>+25</td>
<td>3782</td>
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<td>3798</td>
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<td>5</td>
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<td>–18</td>
<td>3780</td>
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<td></td>
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<td>–2</td>
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<td></td>
<td>L-thigh cutaneous</td>
<td>–10</td>
<td>3790</td>
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<tr>
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<td>+20</td>
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<td>3845</td>
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<td>3896</td>
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<td>104</td>
<td>15/24</td>
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<td>2+</td>
<td>1+</td>
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NOTE: The data reported is relative to the start of vemurafenib. Infused TILs are denoted by the bolded tumor ID for each patient where the number of TIL infused (× 10⁴) is denoted to the right. TIL fragments were considered to have grown if they achieved 10⁶ cells/well in a 24-well plate. The numbers of total TIL fragments initiated are included in the denominators. Immunohistochemistry of tumors stained for CD3, CD4, CD8, and CD20 was determined by a single dedicated pathologist who was blinded to the status of vemurafenib treatment. Grading was as follows: 0 = no staining; 1 = staining <5% of tumor; 2 = staining 5%-50% of tumor; 3 = staining >50% of tumor.

Abbreviations: Bx, biopsy; L, left; LN, lymph node; n/a, not available; R, right; SQ, subcutaneous.
melanoma) and only 3 of the 8 patients with post-vemurafenib TILs had autologous tumor available for testing. Post-vemurafenib lesions were small, presumably because vemurafenib mediated transient tumor regression, which inhibited our capability to test more patients’ TILs. We were also restricted to testing against the pre-vemurafenib tumors because the post-vemurafenib tumors did not yield enough cells from the enzymatic digestion for in vitro analysis. Thus, fragments...
available for evaluation in patients 8, 9, and 11 were cocultured with autologous pre-vemurafenib single-cell tumor digests [fresh tumor (FrTu)] overnight in the presence of either MHC class I–blocking antibody (clone W6/32; blue bars) or blocking antibody vehicle (PBS; red bars), and secretion of IFNγ was assessed by ELISA (Fig. 4). Media (T cells only; open bars) and OKT3 (agonistic anti-CD3 antibody; black bars) were negative and positive controls for cocultures, respectively. A fragment was considered reactive if IFNγ > 200 pg/mL and reached statistical significance compared with both no target (media) and MHC class I–blocking antibody with > 50% inhibition of the unblocked response. Infusion bag TILs (Rx1) for patient 8 responded to the autologous tumor, which was inhibited by MHC class I–blocking antibody (Fig. 4A, left). This patient had 2 of 7 (29%) reactive pre-vemurafenib (FrTu#3885) fragments and 0 of 7 reactive post-vemurafenib (FrTu#3918) fragments (Fig. 4A). Infusion bag TILs for patient 9 were reactive to autologous tumor but secreted the least IFNγ of the infused products tested (Fig. 4B, left). Two of 4 (50%) pre-vemurafenib (FrTu#3922) fragments and 15 of 24 (63%) post-vemurafenib (FrTu#3938) fragments were reactive to autologous tumor for patient 9 (Fig. 4B). Patient 11 also had infusion bag TILs with significant autologous tumor recognition (Fig. 4C, left). Seven of 23 (30%) pre-vemurafenib (FrTu#3957) fragments and 0 of 14 post-vemurafenib (FrTu#3964) fragments demonstrated significant reactivity to autologous tumor (Fig. 4C). In this small pilot sample, there was no consistent change in autologous tumor reactivity of TILs obtained before or after vemurafenib administration. In sum, it appeared that vemurafenib added no significant benefit to adoptive T-cell therapy, as

Figure 4. Tumor responses of TIL fragments from tumors before or after in vivo vemurafenib sensitization. Results shown for patient 8 (A), patient 9 (B), and patient 11 (C). A tumor was resected for growth of TIL fragments and generation of the infused TIL product, and this sample was designated the pre-vemurafenib (pre-VEM) sample. Another tumor was resected after 2 weeks of vemurafenib treatment, but prior to TIL infusion, and TIL fragments were grown for comparison as the post-vemurafenib (post-VEM) sample. Cryopreserved TIL fragments (pre- and post-vemurafenib) were thawed and cocultured with autologous pre-vemurafenib single-cell tumor digestion at a 1:1 ratio overnight at 37°C. Tumor cells were preincubated with pan-MHC class I–blocking antibody (W6/32) or vehicle (PBS) prior to coculture to assess CD8-specific responses to the tumors. Target only (“Media” on x-axes) was included to quantify the baseline secretion of IFNγ into the cocultures by TIL in the autologous tumor digest. Supernatants from cocultures were evaluated for IFNγ secretion by ELISA. T cells only (media; open bars) was a negative control, and OKT3 (agonistic CD3 antibody; black bars) was a positive control for TIL samples. Infused TIL (Rx1) was included for reference to clinical response. Fragments are indicated by an “F,” and “PF” represents a pool of multiple fragments. Results are pooled from two independent experiments, and data are mean ± standard error of the mean (SEM) (n = 4 technical replicates). Dotted line represents 200 pg/mL IFNγ, which was the cutoff for calling a fragment reactive. Student two-tailed t tests were performed for statistical analysis between no target (media) and tumor digest (“above red bars) or between tumor digest treated with PBS (mock; vehicle) or pan-MHC class I–blocking antibody (“above blue bars). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
evidenced by (i) similar clinical responses to adoptive T-cell therapy alone, (ii) inhibition of proliferation and increased cell death of both TILs and peripheral blood T cells at concentrations near the C_{MAX} of vemurafenib at therapeutic doses, (iii) lack of consistent changes in TCRB repertoire in tumors before and after in vivo vemurafenib treatment, and (iv) similar antitumor reactivity of TILs either naive or primed with vemurafenib.

Discussion

This pilot clinical trial evaluated the impact of targeted therapy combined with adoptive T-cell therapy for patients with metastatic melanoma. The primary endpoint of the study was to evaluate the safety and feasibility of combining TIL therapy with vemurafenib. The combination treatment was well tolerated overall, and toxicity was consistent with either agent as monotherapy. No unexpected, serious adverse events were observed. Additivity of toxicity may have occurred but was not a limiting factor in treatment and/or objective clinical response. Seven of 11 patients achieved clinical response, and 2 patients had complete regressions of metastatic melanoma—one for 36 months, and the other is ongoing after 46 months (Fig. 1). When compared with patients with BRAF_{V600E/R} mutations who were treated with a similar TIL therapy without vemurafenib during the same time interval, the combination of vemurafenib and TIL was similar to TIL therapy. The numbers of patients used for these analyses (TIL alone, n = 15; TIL and vemurafenib, n = 11) were not powered to detect small differences. A larger, randomized clinical trial powered to detect differences between these two cohorts could further evaluate differences in response rates.

Future clinical trials could also use post-vemurafenib TILs for therapy. We chose vemurafenib-naïve tumors to generate TIL therapy, because it was unknown how vemurafenib would impact TIL in the adoptive T-cell therapy setting. A post-vemurafenib TIL strategy could have particular importance for patients who are ineligible for adoptive T-cell therapy due to heavy disease burden. Vemurafenib would debulk their existing tumors, bridge them to surgery for TIL harvest, and make them eligible for adoptive T-cell therapy. Vemurafenib could be administered before, during, and after T-cell transfer similarly to our clinical trial with the expectation of a similar safety profile. A murine model demonstrated that halting vemurafenib after adoptive T-cell transfer abrogated the antitumor response of pmel-1 T cells toward vemurafenib-primed melanoma (31), suggesting that continuing vemurafenib following T-cell transfer is warranted. Post-vemurafenib TILs were able to recognize pre-vemurafenib tumor cells in vitro, albeit without significant improvement in tumor reactivity compared with pre-vemurafenib TILs (Fig. 4). We did not recover enough tumor cells to coculture TILs with autologous pretreatment tumor cells treated in vitro with or without vemurafenib. Future experiments could generate TIL fragments in the presence or absence of vemurafenib ex vivo and evaluate their autologous tumor recognition as a function of vemurafenib. Given that pre- and post-vemurafenib TILs were also similar in TIL fragment growth (Table 2) and TCRB repertoire (Fig. 3), it is plausible that post-vemurafenib TILs could be used for treatment in a manner comparable with conventional TIL therapy.

Some of the early benefits of vemurafenib on TILs may have been limited by our trial design. We observed higher staining for T cells infiltrating tumors after vemurafenib (Table 2), corroborating previous studies (21, 32), but the NMA chemotherapy preparative regimen likely eliminated these T cells prior to adoptive T-cell therapy. When melanoma lesions were serially sampled during vemurafenib treatment, the frequency of shared TCRB clonotypes increased (22). In our study, two independent tumors were compared by TCRB deep sequencing to evaluate global changes in the TCR repertoire as a function of vemurafenib. If the same TCRB clonotype was present in both tumors and was highly frequent in one tumor, then it was typically highly prevalent in the other tumor as well (Fig. 3C). Thus, selection of a pre- or post-vemurafenib tumor for generation of TIL treatment would not generally yield higher frequencies of the top shared TCRB clonotypes. These data also suggest that there were common antigens expressed by both tumors that were targets of shared TCRB clonotypes. Expression of melanocyte differentiation antigens, which are common antigens recognized by melanoma TILs, increased after treatment with vemurafenib and led to enhanced tumor recognition by TCR-transduced human T cells and murine pmel-1 T cells (23, 32). Similar increases in antigen expression were likely maintained after the NMA regimen and could have served as enhanced antigenic targets for infused TILs. It is possible that some of the TCRB clonotypes lost during the NMA regimen made it into the infusion bag TILs given the high similarity between pre- and post-vemurafenib tumors in shared TCRB clonotypes. If so, then an improvement in their clinical benefit is plausible given that these T cells were removed from the immunosuppressive tumor microenvironment and given in large quantities to the patient.

We established that vemurafenib (PLX4032) had a negative impact on the viability and proliferation of peripheral blood T cells and treatment TILs at concentrations approaching the vemurafenib serum C_{MAX} of 125 μmol/L (Fig. 2). Our results were unexpected given that studies performed prior to the onset of this pilot clinical trial established that peripheral blood T cells were unharmed by PLX4032 and PLX4072 (early-stage vemurafenib), but these experiments only evaluated concentrations ≤50 μmol/L (23, 27). Our data recapitulate the reported data at these lower concentrations but add new information in regard to the impact of PLX4032 on T cells at higher therapeutic levels. This is an important consideration for future vemurafenib clinical trials, because emerging immunity could be compromised by vemurafenib at sufficient concentrations. Upcoming clinical trials could monitor serum vemurafenib levels and adjust the vemurafenib dosing such that antitumor effects are maintained and damage to the peripheral and intratumoral T-cell repertoires is minimized. In adoptive T-cell therapy trials, vemurafenib administration could be stopped for a few days after T-cell transfer to allow for TIL trafficking out of the bloodstream into the tumor microenvironment. Vemurafenib could then be started or restarted to achieve therapeutic additivity or synergy in the absence of deleterious effects on T cells at high vemurafenib serum concentrations.

It may be worthwhile to focus future trials on combining other agents with TIL or adding more agents to the combination of vemurafenib and TIL. Resistance to vemurafenib can occur through downstream activation of MEK, and combinations of MEK inhibitors, e.g., trametinib or cobimetinib, with vemurafenib have demonstrated improved clinical response, albeit
with few durable CRs (33–37). Dabrafenib, another BRAFV600E inhibitor, has been given in combination with trametinib with objective clinical activity, but the combination affected function of peripheral blood T cells (38–40). MEK inhibitors alone were shown to inhibit T cells, which may limit enthusiasm for combination of T-cell therapy with both MEK- and BRAFV600E K-targeted therapy (21, 41). T-cell immunotherapy could also be combined directly with checkpoint inhibitor-neutralizing antibodies, e.g., pembrolizumab or nivolumab (anti–PD-1), atezolizumab (anti–PD-L1), or ipilimumab (anti–CTLA4), either with or without kinase inhibitors. The antibody would release inhibition of infused TILs as they encountered the negative regulatory molecule in the tumor microenvironment (42). This trial established that targeted therapy and adoptive T-cell therapy can be safely administered to patients and resulted in objective clinical responses, which opens opportunities for testing other agents or combinations in clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.C. Deniger, M.L.M. Kwong, M.E. Dudley, S.A. Rosenberg


References


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Drew C. Deniger, Mei Li M. Kwong, Anna Pasetto, et al.


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